Colegio de Postgraduados

Molecular identification of fungal isolates from different tissues samples of Blueberry (*Vaccinium* sp.) in Baja California

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ABSTRACT

Objective: Molecular identification of fungal isolates presents in fruits and leaf samples of *Vaccinium* with pathogenic or agro-industrial potential.

Design/methodology/approach: Plant material (fruits and leaves) was collected in blueberry commercial plantations of San Quintin, Baja California, México. The samples were placed in humid chambers for fungal growth and then in culture plates with Potato-Dextrose-Agar alone or with lactic acid for purification. The resulting fungal isolates were cultured in liquid media, the total DNA was extracted and quantified, afterwards the ITS region was amplified by PCR, the fragments were purified and sequenced. Finally, the resulting sequences were compared in the NCBI database with the BLAST algorithm, the phylogenetic reconstruction was performed with the MEGA (v.10.0) software.

Results: A total of 22 isolates from *Vaccinium* were obtained from leaves and fruits. These isolates showed high identity percentages (96-100 %) with *Botrytis*, *Didymella*, *Phoma*, *Alternaria* and *Cladosporium* genera. The fruit isolates were closely related with *B. cinerea* Group I, whereas the leaf samples grouped with other complexes such as the *C. cladosporoides*, *A. muriae*, *Dydimella bervipilosa* and *Phoma*.

Limitations on study/implications: The use of the ITS region provides only a partial characterization in some types of fungi, the use of other molecular markers are required to fully characterize some isolates.

Findings/conclusions: The molecular characterization of the fungal isolates showed that most of the genera were saprophytes with phytopathogenic members reported. The reported genera could have an impact in post-harvest due fruit spoilage or by the presence of cytotoxic compounds. The presence of fungal genera (*Cladosporium*) with reported potential antagonistic and growth promoting capabilities was identified.

Keywords: Agrobiotechnology, Bioinformatics, Molecular characterization, Phylogeny, Phytopathogens, *Vaccinium*.

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INTRODUCTION

The state of Baja California, located at Mexico northwest, is among the main producers of several fruit crops, such as the blueberry (*Vaccinium* sp). This crop is produced in the coast region and in recent years has shown a steady increase in the production surface,

with incomes of 1,417 million pesos in 2022 being the exportation the main destiny of this product (Figueroa *et al.*, 2010, SIAP, 2023, SADER, 2023).

One of the main factors that impacts severely the crop production is the presence of pathogens, which in turn causes yield reductions and quality losses. In dependence of the causal agent the losses that can be generated would oscillate from 30-50% for *Vaccinium* (Figueroa *et al.*, 2010; Mondragón *et al.*, 2013). The control of phytopathogens is performed by the use of chemical agents, which have a high economic cost, could present low specificity, environmental risk and, depending on the type of chemical, his use in fruits for exportation could be restricted due its residuality [Altieri *et al.*, 2004; Zadehdabag *et al.*, 2010; Samaniego-Gámez *et al.*, 2012).

Also, the chemical management of phytopathogens alters the composition of the microorganisms present, both in the plant and in the environment, due the reduction of the population of potential suppressive agents for some types of pathogens (Weller *et al.*, 2002; Lugtenber *et al.*, 2009). Recent studies have shown that microorganisms influence several aspects plant processes such as health, development, nutrition and production (Hartmann *et al.*, 2009; Philippot *et al.*, 2009; Caro-Quintero *et al.*, 2015; Samaniego-Gámez *et al.*, 2017). Previous studies in different species of berries have shown that the microbial communities are strongly dependent of the culture conditions and several agents for the growth promotion could be isolated (Salhi *et al.*, 2022; Thimmappa *et al.*, 2023).

The isolation and molecular study of microorganisms allows an effective management of isolated phytopathogenic genera, and also allows the identification of those that may be agents of biocontrol, growth promotion and agro-industrial interest. In the present study, the molecular identification of several fungal isolates in different blueberry tissues was performed, in order to identify fungal pathogens and fungi with agro-industrial potential.

MATERIALS AND METHODS

Collection of samples from Blueberries (*Vaccinum* sp.) plantations

Sample collection was performed during November-February, 2022 with growers of San Quintin, Baja California. During the collection, leaf and fruits samples were taken from plants with symptoms from pathogens, as well as from asymptomatic plants. Both types of samples were placed individually in closed labeled plastic bags and placed in a cooler (4 °C) for subsequent analysis in the UABC Physiology and Postharvest Management laboratory.

Processing of blueberry samples

The superficial sample disinfection was made according to the Samaniego-Gámez and Cervantes-Diaz, (2012). The samples were placed in humid chambers (20±2 °C) in photoperiod (12 h) for 7 d, with daily observations. The growths were cultured in Potato-Dextrose-Agar (PDA) medium and PDA with lactic acid (0.1% v/v) in Petri dishes by central stinging. The plates were sealed with Parafilm (Sigma-Aldrich) and incubated (20±1 °C, in darkness) for 6 d with daily observations. The resulting fungal growths were purified by the monosporic culture method on PDA plates (Li *et al.*, 2012).

Molecular identification of fungal isolates

The cultured fungal isolates were sectioned with a sterile scalpel and placed in Yeast Extract-Peptone-Glucose broth, 10 d (25±2 °C), the culture media was filtered and the samples were stored in tubes (−20 °C) until further use. Total DNA was extracted with the protocol of Al-Sammarrai and Schmidt, (2020). DNA was observed in 1% (w v^{-1}) agarose gels, subsequently the samples were treated with RNase (Ambion) and quantified with a spectrophotometer.

The amplification of the inter-transcribed spacer (ITS) was performed using the ITS1(TCCGTAGGTGAACCTGCGG) and ITS4(TCCTCCGCTTATTGATATGC) in a reaction mixture that consisted in: 250 nM of each primer, 1.5 mM of $MgCl_2$, 1.5 U of Taq-Polymerase in a final volume of 20 μL. The reaction mixture was placed in a thermocycler (BioRad) with the following conditions: 95 °C (3 min), 30 cycles of 95 °C (1 min), 55 °C (1 min) and 72 °C (1.5 min) with a final extension of 72 °C (10 min).

Bioinformatic analyses and processing

The amplification products (420-825 bp) were visualized in agarose gels (1% w v^{-1}), the amplicons were purified with the Wizard SV PCR Clean-Up System (Promega) according to the manufacturer's instructions. The purified fragments were sequenced in Macrogen (Korea).

The obtained sequences were introduced in the GenBank for his identification using the BLASTn algorithm, with the Megablast option, the sequence search was made using the "ITS Fungi for Type-Material" database.

The BLAST search results were retrieved in fasta format and introduced in the MEGA (v10.0) software for the phylogenetic analyses. The fungal sequences were aligned with the CLUSTALW algorithm, the phylogenetic reconstruction was made with the Maximum Parsimony (MP) model with the following parameters: BooStrap=1000 replicates, Number of Parsimony tres=10 and the option Tree Bisection Reconnection. The Tree Length (TL), Consistency Index (CI), Retention Index (RI) and Composite Index (Comp-Indx) were also calculated.

RESULTS AND DISCUSION

In the blueberry producing areas located in San Quintin, Baja California it was observed symptoms associated with *Bortytis* sp., the symptoms developed under the ideal conditions for the pathogen (low temperature and high humidity). The symptoms observed where: mummified brown-colored fruits (Figure 1B), leaf with necrotic spots (Figure 1C) and brown-colored flowers with reduced size, which agrees with the reported symptoms for *Botrytis* in berries (Figueroa *et al.*, 2010; Mondragón-Flores *et al.*, 2012).

A total of 22 isolates from *Vaccinium* were obtained from leaves and fruits, the asymptomatic samples did not show the presence of fungal colonies related to the macroscopic characteristics associated with *Botrytis*.

The fruit isolates showed at 72 h of growth the formation of mycelium with a dark-grey coloration in the front of the plate (Figure 2A) and a light-grey coloration at the back of the plate (Figure 2B).

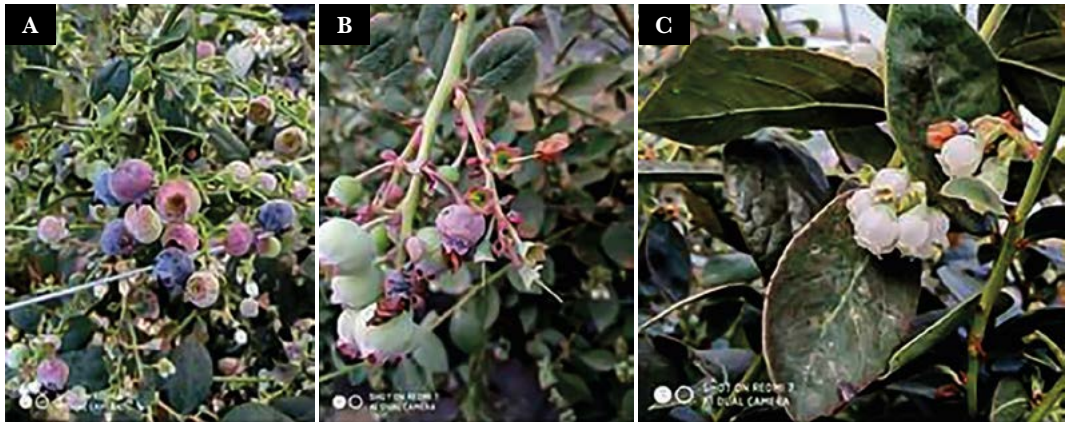

Figure 1. Observed symptomatology in Blueberries (*Vaccinium*) in San Quintín, Baja California. A) Asymptomatic plant, B) Brown-colored fruits and mummification; C) Necrosis and leaf yellowing and brown-spotted flowers.

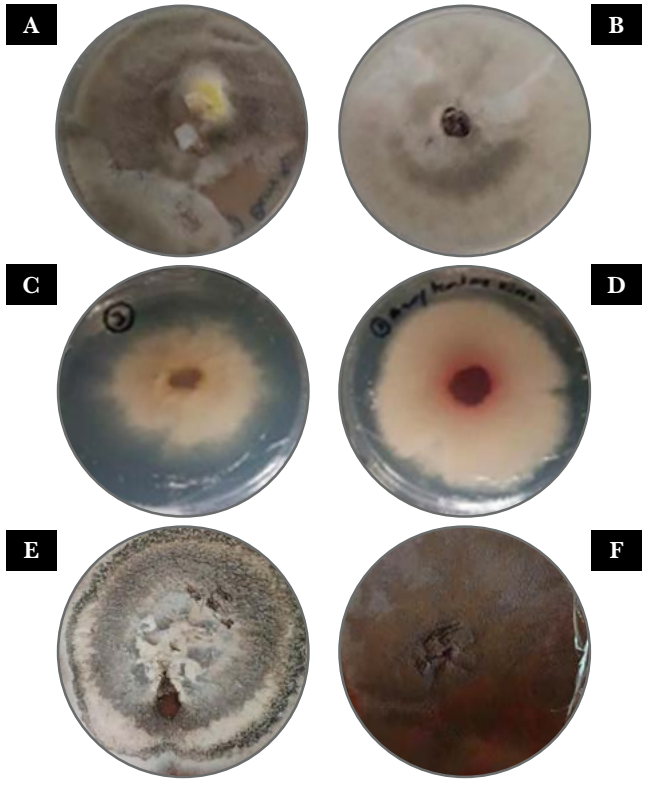

Figure 2. Fungal isolates from *Vaccinum* sp. with 72 h of incubation. A) Gray mycelial growth (front), B) Gray mycelial growth (back), C) White mycelium (front) from leaf tissue, D) White colored mycelium with a pink halo (back), E) Leaf isolate with olive-green mycelium with white borders, F) Green-gray mycelial growth from leaf samples.

Leaf samples showed white-colored mycelium at 72 h, with irregular borders at the front of the plate (Figure 2C) and a pale-pink coloring at the back of the plate (Figure 2D). Other isolates from leaves formed woolly mycelium, with regular borders, a green-olive coloration at the front of the plate y white-colored borders (Figure 2E).

The isolates were sequenced and showed high identity percentages with the *Botrytis*, *Didymella*, *Phoma*, *Alternaria* and *Cladosporium* genera (Table 1).

The gray isolates from fruit samples showed identity percentages from 99.6-100% with different strains of *Botrytis cinerea*. The white isolates from leaves, showed 99.6-99.8% identity with Phoma and 97.1-99.1% identity with two species of *Didymella* (*D. keratinophila*, *D. brevipilosa*). In this sense, the olive-green isolates showed high percentages of identity with two *Cladosporium* species: *C. ramonetellum* (99%) and *C. cladosporoides* (96-99.2%).

The phylogenetic analyses showed that all the fruit isolates of this study grouped close to *Botrytis* sp. EX2019-m34 (Figure 3A). Also, they formed a paraphyletic group with *B. cinerea* and *B. californica* (Figure 3B).

The presence of *Botrytis* in *Vaccinium* fruits have been reported previously, this genus affects more than 200 plant species and is considered an important postharvest pathogen in several types of berries [Samaniego-Gámez *et al.*, 2012; Saito *et al.*, 2016; Terrones-Salgado *et al.*, 2019; Garay-Serrano *et al.*, 2021; Esterio *et al.*, 2020; Saito *et al.*, 2020; Amed and Abed, 2023). The use of molecular markers, such as the ITS region, have been used previously for the characterization of the *Botrytis* complex, which has been difficult to characterize because its high morphological variability (Esterio *et al.*, 2020; Saito *et al.*, 2020; Amed and Abed, 2023).

In the present study the use of the ITS region grouped members of the *B. cinerea* Group I (*B. cinerea*, *B. californica*, *B. pelargoni*, *B. pseudocinerea*) in a single clade as reported previously (Esterio *et al.*, 2020; Saito *et al.*, 2020). The *Botrytis* isolates obtained in the present study did not form a group with members of *B. cinerea* Group I. The use of additional markers would be necessary in order to fully characterize the isolates from the present study. The use of additional molecular markers has been previously reported in *Botrytis* with a substantial improvement in the allocation of isolates in the different *Botrytis* complexes (Saito *et al.*, 2016; Terrones-Salgado *et al.*, 2019; Garay-Serrano *et al.*, 2021; Delong *et al.*, 2020).

Table 1. BLAST search results of sequences from fungal isolates in fruits and leaves of *Vaccinum* in the NCBI database.

Description	Accession	Identity (%)	E-value
Botrytis cinerea	MH665643.1 OR544948.1 OR544951.1 OR237160.1	99.6-100	0.0
Botrytis sp.	MT912776.1	99.6	0.0
Phoma sp.	MT420629.1 MT912578.1	99.8-99.6	0.0
Didymella keratinophila	NR_158275.1	99.1	0.0
Didymella brevipilosa	NR_158236.1	97.1	0.0
Alternaria destruens	NR_137143.1	99.4-99.2	0.0
Cladosporium ramontellum	MZ301265.1	99.2	0.0
Cladosporium cladosporoides	OP006753.1 LC325159.1 OP006753.1	96.4-99.2	0.0

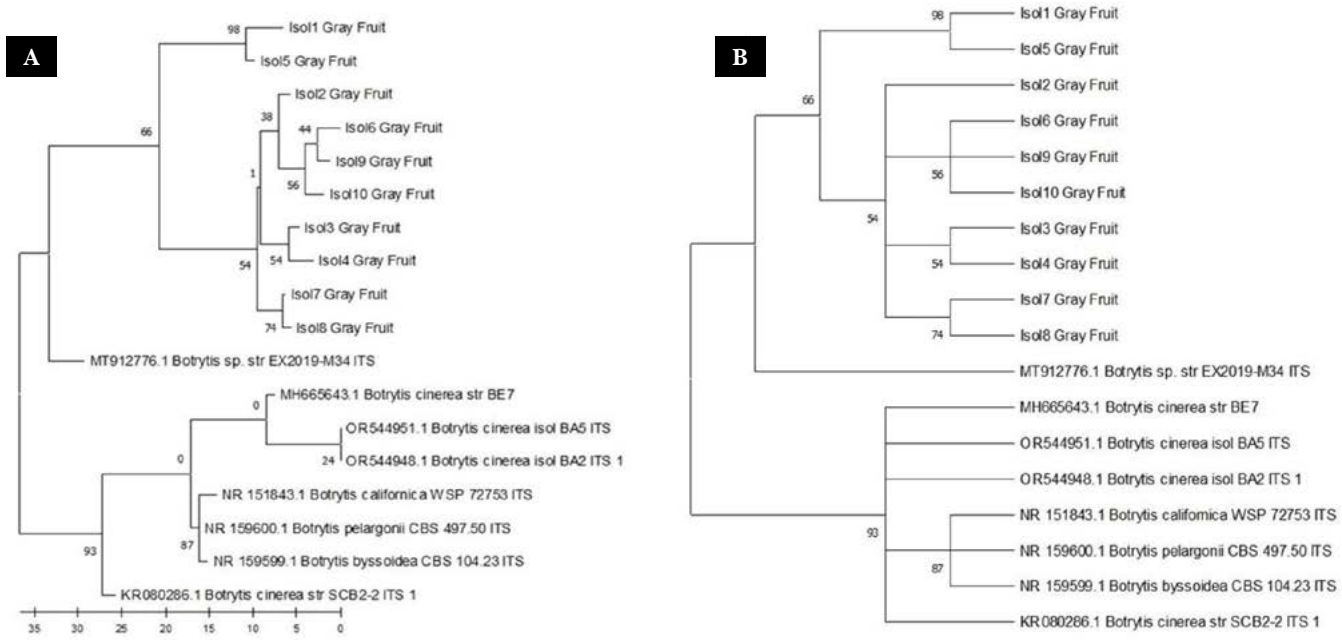

Figure 3. Phylogenetic generated by Maximum Parsimony of fungal isolates from Vaccinium fruits. A) Tree in terms of the number of changes in the entire sequence, B) Condensed tree with a minimum branch support cut-off=50%. TL=116, CI=0.876289, RI=0.897436, Comp-Indx=0.804598 (0.786413).

White fungal isolates with pink halos (Figure 4A) formed two monophyletic groups with the two strains of *Phoma* sp. (isolates 1 and 2) and with *Didymella brevipilosa* (isolates 3 and 4). The gray isolates formed a monophyletic group with *Alternaria murispora* (Figure 4B). Olive green mycelial isolates from *Vaccinium* leaves, clustered closely with *Cladosporium cladosporoides* complex (Figure 4C).

It was observed that most of the isolated genera in leaves (*Phoma*, *Didymella*, *Alternaria*, *Cladosporium*) have been listed in previous studies as saprophytes with phytopathogenic potential (Woundenberg *et al.*, 2009; Aveskamp *et al.*, 2010; Bennett *et al.*, 2018, Derviş *et al.*, 2024). The presence of this type of fungi it could be associated to the presence of necrotic spots in the collected samples.

The presence of *Phoma* and *Didymella* (*Didymellaceae*) were identified in isolates with similar morphology, previous studies considers the *Dydimellaceae* as a polyphyletic family, with members which have a high morphological and molecular variability making his identification complicated, it's also been reported to find Phoma and Dydimella in complexes (Aveskamp *et al.*, 2010; Bennett *et al.*, 2018, Derviş *et al.*, 2024), a similar behavior was observed in the present study.

Didymella and *Phoma* can colonize different surfaces, such as: water, soil and other inorganic substrates, thus being considered as cosmopolites (Stranska *et al.*, 2022; Luo *et al.*, 2024; Magaña-Dueñas *et al.*, 2021). Regarding the phytopathogenicity of these two genera, previous reports considers that *Phoma* is an opportunistic pathogen, with a wide host range and can cause respiratory and dermal symptoms in humans (Bennett *et al.*, 2018; Lugauskas *et al.*, 2006). In the case of *Didymella*, two species (*D. glomerata* and *D.*

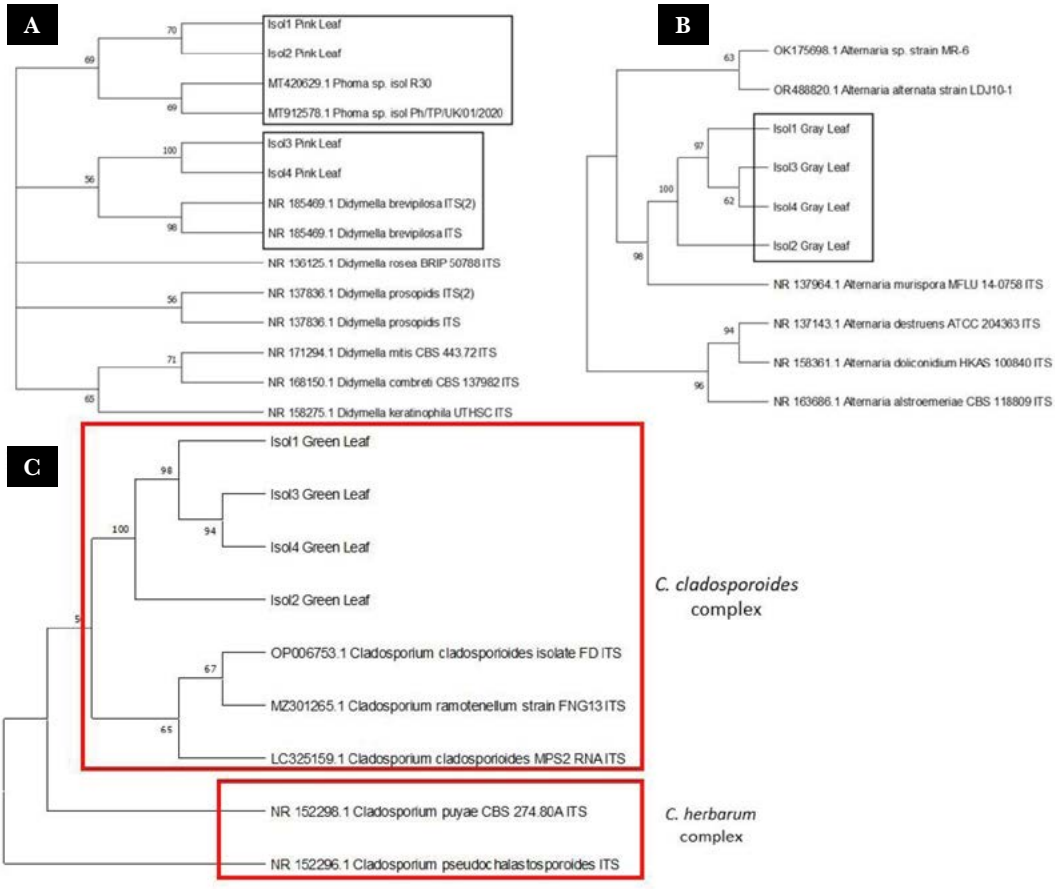

Figure 4. Phylogeny generated by MP from fungal isolates of *Vaccinum* leaves, condensed trees with a minimum branch support cut-off=50%. A) White fungal isolates. TL=391. CI=0.985591, RI= 0.985915 and Comp-Indx=0.973308 (971709). B) Gray fungal isolates, TL=419, CI=0.990291, RI =0.996507, Comp-Indx=0.986993 (0.986832). C) Green fungal isolates. TL=383, CI=0.979042, RI = 0.991716, Comp-Indx=0.973591 (0.970932).

aplanata) have been reported as causal agents of necrotic leaf spots in blackberries (*Rubus fruticosus*) (Woundenberg *et al.*, 2009; Derviş *et al.*, 2024), which agrees with the symptoms of the collected samples.

Previous studies performed in berries mentions that the presence of *Alternaria* and *Cladosporium* as cosmopolite species and are part of the fungi associated with fruit spoilage (Torres *et al.*, 2017; Raynaldo *et al.*, 2024; Sinkevičienė *et al.*, 2023; Woundenberg *et al.*, 2013; Ariyawansa *et al.*, 2015; El-Dawy *et al.*, 2021; Iturrieta-González *et al.*, 2021). In the present study the presence of these two genera were observed in leaf isolates with the presence of necrotic spots, which also has been reported previously (Ariyawansa *et al.*, 2015; El-Dawy *et al.*, 2021; Iturrieta-González *et al.*, 2021; Martin-Feliux *et al.*, 2017).

The *Cladosporium* genera has been divided in three complexes: *C. cladosporoides*, *C. herbarum* and *C. sphaerospermum* (Iturrieta-González *et al.*, 2021; Martin-Feliux *et al.*, 2017; Sandoval-Denis *et al.*, 2016), in the present study the molecular characterization of the isolates formed a monophyletic group related to *C. cladosporoides* complex.

The presence of *Phoma*, *Cladosporium* and *Alternaria* genera are important because different species of the aforementioned genera are able to generate cytotoxic compounds such as: gliotoxin, tenuazonic acid and prenylated alkaloids (Bennett *et al*., 2018; Stranska *et al*., 2022; Klapec *et al*., 2022), which pose a risk for the food safety and the commercialization of these products in the international markets. On the other hand, previous studies were able to identify *Cladosporium* strains with antagonistic activity against phytopathogens and as plant growth promoters through the nutrient solubilization (Torres *et al*., 2017; Raynaldo *et al*., 2024; Zalewska *et al*., 2022; Patriarca *et al*., 2019; Mantzoukas *et al*., 2023; Räut *et al*., 2021).

CONCLUSIONS

In the present study isolates from leaf and fruit samples from *Vaccinium* were identified by the ITS region sequencing, where differences in the fungal composition were observed in dependence of the type of tissue sample. In this sense, several isolates with similar morphology could be associated, through his molecular identity in different genera. Most of the isolates were associated with saprophytic genera such as: *Phoma*, *Didymella*, *Alternaria* and *Botrytis*, where phytopathogenic members have been reported. These genera have been reported to have an impact in postharvest due fruit spoilage or by the presence of cytotoxic compounds that may pose a food-safety risk.

Fungal isolates identified as members of the *C. cladosporoides* complex were obtained, previous reports have found *Cladosporium* strains with antagonistic capabilities against pathogens and as plant growth promoters. Further research should be focused in the use of additional molecular markers in order to elucidate more accurately the fungal isolates. In this sense, the antagonistic and the growth promoting capability of certain isolates should be determined

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