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Artículo científico

Diversity of fungal communities inhabiting symptomatic leaves of Cape gooseberry (*Physalis peruviana*) in the Ecuadorian Andes

Diversidad de comunidades fúngicas que habitan en tejido foliar sintomático de la uvilla *Physalis peruviana* en los Andes Ecuatorianos

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ABSTRACT.- We used microbial culture specific methods to assess the occurrence and diversity of fungal communities inhabiting symptomatic foliar tissue of Cape gooseberry (*Physalis peruviana*) at four different field plots in the northern Ecuadorian Andes. We categorized the isolated fungi based on their morphological characteristics and subsequently identified them through rDNA sequencing of the ITS region. We recovered 75 ascomycetous isolates which represented 13 genera, revealing a total of 20 taxa. We found most isolates recovered from necrotic and straw-colored tissues to belong to the genus *Alternaria.* Other fungal taxa frequently found were *Epicoccum, Diaporthe*, and *Xylaria.* To our knowledge, this is the first study to report the mycobiota associated to *P. peruviana* plants showing disease symptoms, including 2 new genera of fungi for the first time in Ecuador. Our results are expected to provide useful information for future assessments of biological control on tropical commercial.

KEYWORDS: Ascomycota, rDNA, ITS, plant disease, uvilla

RESUMEN.- Utilizamos métodos cultivo-dependientes para evaluar la ocurrencia y diversidad de comunidades fúngicas que habitan en tejido foliar sintomático de uvilla (*Physalis peruviana)* en cuatro localidades del norte de los Andes ecuatorianos. Categorizamos los aislados basándonos en las características morfológicas de los cultivos obtenidos *in vitro*, los cuales identificamos al secuenciar la región de ADNr ITS. En total, recuperamos un total de 75 hongos ascomycetos que comprendían 13 géneros y 20 taxa. Encontramos que la mayoría de hongos recuperados de tejidos necróticos o cloróticos pertenecían al género *Alternaria*. Otros taxa que encontramos con frecuencia fueron *Epicoccum, Diaporthe* y *Xylaria*. A nuestro conocimiento, este es el primer estudio en reportar la micobiota asociada a plantas de *P. peruviana* que presenten hojas con síntomas de enfermedad, incluyendo 2 géneros de hongos nuevos para el Ecuador. Esperamos que nuestros resultados provean información útil para futuros estudios sobre control biológico aplicado a cultivos comerciales en los trópicos.

PALABRAS CLAVE: Ascomycota, ADNr, ITS, patología vegetal, uvilla

INTRODUCTION

Cape gooseberry (*Physalis peruviana* L.) is a biannual shrub native from the Andean highlands of northern South America that can grow up to 1 m high (Legge 1974; Fischer 2000). In recent years, Cape gooseberries have gained increasing attention in the markets of South and Central America, due to the plant's high tolerance to a broad variety of environments (Ramadan and Moersel 2003; Puente

et al. 2011). Given the variety of antioxidant types and high sugar content (Novoa et al. 2006), this crop is of great economic importance for industrial purposes worldwide (Morton 1987; Rehm and Espig 1991; McCain 1993; Ramadan and Moersel 2003; Mazorra 2006), as it has also been widely used for its medicinal properties to treat cancer and diabetes (see review in Puente et al. 2011).

In Ecuador, *P. peruviana*, localy known as *uvilla,* is grown in the northern region between 2 000 – 3 000 m.a.s.l., where moderately cold temperatures prevail (Puente et al. 2011; Fischer et al. 2014). The production of *P. peruviana* has increased considerably in recent years across Ecuador due to its growing export demands to European countries (Muñoz 2003; Altamirano 2010; Fischer et al. 2014). However, a wide array of selective pressures including biotic factors (herbivory, parasites) and abiotic stress (low nutrient availability, drought) may result in major production losses by inducing yellowing or necrosis in photosynthetic tissues (Douanla-Meli et al. 2013). As a result, several toxic pesticides (e.g. herbicides and fungicides) have been used in this region as agricultural practices to prevent plant diseases (Rodríguez-Amézquita et al. 2010). Yet, even though the main biological constraint to Cape gooseberry are fungi of the genera *Cladosporium, Phoma, Alternaria, Botrytis* and *Colletotrichum* (Angulo 2005; Fischer and Miranda 2012; De La-Rotta 2014), little is known on the mycobiota inhabiting symptomatic leaves of *P. peruviana,* which could provide useful information and assist crop management.

Studies based solely on morphology have failed to be conclusive at taxonomic species level for fungi associated to *P. peruviana* (Crozier et al. 2006), while fungal isolates often fail to form fruiting structures in culture (Gazis and Chaverri 2010). Thus, considering that leaves, as ecological niches, might influence the diversity and composition of fungal symbionts (Kriel et al. 2000), the present study is aimed at understanding the community structure and diversity of fungi isolated from symptomatic leaves of Cape gooseberry in the Andean highlands of northern Ecuador.

MATERIALS AND METHODS

Sample collection.- We collected unhealthy leaf samples showing necrosis symptoms or straw-colored tissues in plantations of *Physalis peruviana* during June 2015 at four different sites of the Pichincha Province in the northern Ecuadorian Andes: La Merced (00º17.635´ S, 078º24.158´ W, 2 611 m.a.s.l.), Virgen de Lourdes (00º17.311´ S, 078º24.384´ W, 2 628 m.a.s.l.), Tumbaco (00º13.539´ S, 078º23.979 W, 2 450 m.a.s.l.), and Yaruquí (00º11.596´ S, 078º19.710´ W, 2 579 m.a.s.l.). We randomly selected 14 mature *P. peruviana* (2 to 7 plants per site > 0.5 m in height) and then transported 75 leaf samples (5 x 5 mm) to the laboratory in sterile plastic bags. We stored the leaves for 2 d at -10 °C before the isolation of fungi.

Isolation of fungi.- To isolate fungi, we followed a modified protocol from Crous et al. (2009). In order to induce sporulation, we surface-disinfected the samples through immersion in 70 % ethanol for 1 min and 3 % sodium hipochlorite for 5 min. We rinsed the samples with sterile distilled water three times before transferring them to Petri dishes (90 mm) that contained a sterile Potato Dextrose Agar (PDA, Difco Laboratories, Detroit, MI) supplemented with streptomycin to suppress bacterial proliferation. We incubated the plates at 25 °C with a 12-h photoperiod and checked them regularly for fungal growth for 7 to 15 days. After fungal growth was visible, we subcultured different colonies on new Petri dishes with the same medium for further purification and identification (Crozier et al. 2006). We categorized and divided filamentous fungi isolates based on the morphology of the colonies.

DNA extraction.- We collected about 10 mg of fresh fungal tissue using a sterile scalpel and extracted genomic DNA using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI) in accordance with the manufacturer's instructions. To each tissue sample, we added 600 µl of nuclear lisis solution and vortexed the resulting solution for 15 min at 65 °C, followed by 15 min of water immersion at 37 °C. Then, we added 3 µl of RNAase solution and incubated the resulting solution at room temperature for 5 min, before we mixed it with a protein precipitation solution. We vortexed the samples for 15 s and centrifuged the protein precipitation solution at 13 000–16 000 g for 3 min. We transferred the supernatant liquid into a 1,5 mL fresh tube mixed with 600 µl ethanol at room temperature and centrifuged the columns at 13 000–16 000 g for 1 min, before we discarded the flow-through. Finally, we washed the samples as *per* manufacturer's protocol and we quantified the purified DNA concentration with a Qubit® 2.0 Quantitation Starter Kit (Invitrogen, USA) following instructions provided by the manufacturer and stored the tubes at -20 ºC.

PCR amplification.- We used PCR to amplify primer pairs of the Internal Transcribed Spacers (ITS), ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′), which are rDNA genes commonly used for fungal barcoding (White et al. 1990). We performed PCR using GoTaq Green Master mix (Promega, Madison, WI) according to manufacturer's instructions. We carried out reactions in 25 µl volume samples as follows: first initial denaturation at 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final elongation at 72 °C for 10 min in a mixture of 22 µl GoTaq® Green Master Mix, 10 µM of primers and 2 µl of template. We analyzed PCR products by electrophoresis on a 2,5 % (w/v) agarose gel containing Sybr Safe (Invitrogen, Mulgrave, Australia) and visualized them under UV light. We purified the samples using the Wizard SV Gel and PCR Clean-up System (Promega). Reaction mixtures were sequenced by a private biotechnology company (Macrogen, Seoul, South Korea).

Fungal identification.- To identify the fungal isolates based on ITS, we compared nucleotide sequences against GenBank's database using the basic local alignment search tool (BLASTn) with the National Center for Biotechnology Information (NCBI, Bethesda, MD) database. Whenever possible, we defined fungi species considered in this study using an ITS similarity threshold of 97 % to sequences deposited in GenBank (Nilsson et al. 2008). If the ITS sequence was not discriminant at a species level, we assigned the isolates to the closest taxonomic unit. We deposited all gene sequences retrieved in this work in the GenBank database under the accession numbers: MF434350.1–MF435178.1. Fungal classification follows MycoBank (http://www.mycobank.org) database.

Statistical analysis.- We measured fungal occurrence considering both, the colonization frequency (CF) and the relative frequency of isolation (RF). We calculated CF as the total number of segments colonized by a given fungus divided by the total number of segments in that sample, expressed as percentage. We determined RF as the total number of segments colonized by a given taxon divided by the total number of taxa. We calculated fungal species dominance, diversity, and richness using Simpson's, Shannon's and Margalef's indices, respectively. We included singlets (occurrence of ≤ 2 isolates) for all analyses as they are likely to be keystone species of symptomatic foliar tissues (Gazis and Chaverri 2010). We calculated all diversity indices with PAST, version 1.9 (Paleontological Statistics) software (Hammer et al. 2001), in accordance to the following formulas:

1. Simpson's diversity (1-D) = $1-\Sigma(pi)^2$
2. Shannon-Wiener index (H') = $-\Sigma p_i (\log_2 p_i)$
3. Margalef's (D^{mg}) = (S-1) / ln N

where, pi is the proportion of colonization frequency of a given taxon, S is the total number of taxa isolated, and N is the number of species recorded.

RESULTS

Identification by ITS rDNA sequencing showed that all fungal isolates recovered in this study were Ascomycota, placed in seven orders: Pleosporales, Xylariales, Capnodiales, Glomerellales, Dothideales, Diaporthales and Hypocreales. Most isolates belonged to the Pleosporales (40 %) and Xylariales (20 %) orders. BLAST analysis revealed 13 different genera associated to symptomatic tissues of *P. peruviana*, while we were not able to identify the genus of two isolates and limited their categorization to the order level (Table 1). From a total of 20 identified taxa, the most frequently recovered fungal genera were *Alternaria*, *Epicoccum* and *Diaporthe* (Table 2), while 20 % of the isolates occurred as doubletons (occurrence of 2 isolates) and 50 % occurred only once. Fungal communities showed a low overall isolation rate of 5 ± 7.50 %, while diversity indices displayed high values for species dominance (1-D = 0,85), diversity (H' = 3,33), and richness (D^{mg} = 4,40).

DISCUSSION

To date, almost all species in the plant kingdom are known to host bacteria or fungi within the intra and inter-cellular spaces of their vegetative tissues (Petrini 1996; Arnold and Lutzoni 2007; Mondal et al. 2007; Hyde and Soytong 2008, Ghimire et al. 2011, Rocha et al. 2011, Douanla-Meli et al. 2013). Despite the fact that there is no apparent harm caused by these microorganisms on the plant's health, many fungal species have been reported to enter the plant trough stomata or wounds (Andersen and Walker 1985; Schulz and Boyle 2006; Sieber 2007), while some species may act as latent pathogens during the host's senescence (Photita et al. 2004; Promputtha et al. 2007; Rodríguez and Redman 2008; Prihastuti et al. 2009).

Tabla 1. ITS-based identification of fungal isolates recovered from symptomatic leaves of P*hysalis peruviana* in the Ecuadorian Andes.

Proposed fungal taxon	[a] GenBank Accesion No.	QC (%)	Id (%)	Sequence length (bp)	[b] Top BLAST search results (GenBank accession No.)	Reference
Alternaria sp.	MF435050.1	100	100	568	KM215624.1	Raja et al. 2015
Bipolaris cynodontis	MF435062.1	100	100	541	KJ909767.1	Manamgoda et al. 2014
Cercospora dubia	MF435168.1	99	100	537	KX287277.1	Videira et al. 2016
Colletotrichum boninense	MF435150.1	100	98.99	592	JX258799.1	Weir et al. 2012
Colletotrichum gloeosporioides	MF435164.1	100	100	571	KM257026.1	Waculicz-Andrade et al. 2017
Diaporthe helianthi	MF435054.1	98	97.73	579	AJ312356.1	Rekab et al. 2004
Diaporthe sp.	MF435071.1	100	99.65	572	KC339218.1	Panno et al. 2013
Didymella glomerata	MF435166.1	100	99.81	538	AY183371.1	Catal 2002
Dothideales sp	MF435051.1	99	99.64	553	HQ607988.1	Rodrigues et al. 2011
Epicoccum nigrum	MF435053.1	100	100	544	KX869965.1	Kernaghan et al. 2017
Fusarium equiseti	MF435058.1	100	100	547	KR094440.1	Kaur et al. 2016
Fusarium venenatum	MF435064.1	100	100	523	KP295496.1	Stefańczyk et al. 2016
Nigrospora oryzae	MF435092.1	100	100	550	EU272503.1	Miles et al. 2012
Periconia byssoides	MF435088.1	97	99.82	577	MK370654.1	Herrmann et al. 2019
Phoma sp.	MF435059.1	100	99.26	538	JN207257.1	Loro et al. 2012
Pleosporales sp.	MF435097.1	100	99.62	533	HQ631051.1	Shrestha et al. 2011
Stagonosporopsis cucurbitacearum	MF435112.1	100	100	552	GU045304.1	Ling et al. 2010
Xylaria multiplex	MF435085.1	99	99.66	588	KP133436.1	Thomas et al. 2016
Xylaria sp.	MF435158.1	100	98.45	576	FJ799949.1	Van Bael et al. 2009
Xylaria venosula	MF435102.1	97	99.65	587	EF026149.1	Hsieh et al. 2010

[a] All sequences were deposited in the NCBI GenBank (Accession No. column). QC: query cover, Id: identity. [b]

Sequences available in GenBank used for comparisons with sequences obtained in this study via BLAST software.

Tabla 2. Total number of isolates, isolation rate (%) and relative frequency of ascomycetous fungi recovered from symptomatic foliar tissues of *Physalis peruviana* in the Ecuadorian Andes.

Taxa	No. Isolates	Isolation rate (%)	Relative frequency
Capnodiales			
Cercospora malayensis	1	0.775	0.0244
Diaporthales			
Diaporthe helianthi	3	2.326	0.0732
Diaporthe sp.	11	8.527	0.2683
Dothideales			
Dothideales sp.	1	0.775	0.0244
Glomerellales			
Colletotrichum boninense	1	0.775	0.0244
Colletotrichum gloeosporioides	1	0.775	0.0244
Hypocreales			
Fusarium equiseti	2	1.550	0.0488
Fusarium venenatum	1	0.775	0.0244
Pleosporales			
Alternaria sp.	22	17.054	0.5366
Bipolaris cynodontis	2	1.550	0.0488
Didymella glomerata	2	1.550	0.0488
Epicoccum nigrum	15	11.628	0.3659
Periconia byssoides	1	0.775	0.0244
Phoma sp.	2	1.550	0.0488
Pleosporales *sp.*	1	0.775	0.0244
Stagonosporopsis cucurbitacearum	1	0.775	0.0244
Xylariales			
Nigrospora oryzae	3	2.326	0.7320
Xylaria multiplex	3	2.326	0.0732
Xylaria sp.	1	0.775	0.0244
Xylaria venosula	1	0.775	0.0244

In the present study, we used ITS sequencing to identify fungal communities inhabiting symptomatic leaves of *P. peruviana*. Our results are consistent with previous studies that have also shown a dominance by Ascomycota members within fungal assemblages of tropical plants (Arnold and Lutzoni 2007; Gonzaga et al. 2015). Previous studies on fungal assemblages of commercial crops have reported *Alternaria, Colletotrichum, Epicoccum, Fusarium, Nigrospora, Phoma,* and *Xylaria* taxa inhabiting foliar tissues (Crous et al. 1995; Gazis and Chaverri 2010; Parsa et al. 2016). However, most studies have focused on isolating fungal endophytes inhabiting asymptomatic aerial organs, while very few of them have investigated the fungal species from symptomatic tissues (Maher et al. 2012; Bruez et al. 2014; Dávila et al. 2018). This work constitutes the first report of mycobiota on Cape gooseberry, from which *Alternaria, Bipolaris, Cercospora, Colletotrichum, Didymella, Epicoccum, Fusarium, Nigrospora, Phoma* and *Xylaria* taxa have been previously reported in Ecuador (Evans and Reeder 2000; Pacin et al. 2003; Ramírez et al. 2006; Thomas et al. 2008; Cornejo-Espinoza 2014; Moya-Maldonado 2016; Dávila et al. 2018), whereas *Periconia* and *Stagonosporopsis* represent new records for the mycoflora of continental Ecuador and are reported for the first time in *P. peruviana* within its center of origin.

The overall colonization of fungal species screened in the present study is low when compared to that reported in asymptomatic tissues of other plant species (Arnold and Lutzoni 2007; Sun et al. 2008). However, the diversity and richness of species is

similar to other studies of fungal communities inhabiting symptomatic tissues in tropical crops (Dávila et al. 2018). Furthermore, the most dominant genera isolated in the current study were *Alternaria* and *Epicoccum*, which coincides with other fungal communities from tropical angiosperms (Parsa et al. 2016). Within the fungal isolates obtained here, we detected common Cape gooseberry pathogens belonging to the genera *Alternaria, Fusarium, Colletotrichum, Phoma* and *Cercospora* (Rao and Subramonian 1976; Zapata et al. 2002; Angulo 2005; Fischer and Miranda 2012; De La-Rotta 2014) although only the *Alternaria* genus was isolated considerably in this study. As we did not conduct Koch's postulates for the isolates obtained in this study, our results do not imply that the isolated taxa are pathogenic.

The great diversity of fungal species reported in this study is expected to provide novel information for the crop management and biological control on the Ecuadorian Cape gooseberry, when dealing with necrosis symptoms in leaves. The current inventory of fungal species in *P. peruviana* suggests potential sources of culturable secondary metabolites isolated from decaying foliar tissues (Paparu et al. 2008), which may help to establish healthier agricultural practices, while it also contributes to the ecological understanding of specificity patterns shown by fungal communities. Further research of fungal interactions in Cape gooseberry is needed to comprehend the role that the most abundant species isolated in this study play within the fungal assemblages of symptomatic leaf tissues.

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