The entomopathogenic fungi *Lecanicillium sabanerum* sp. nov. a natural control agent of *Parthenolecanium* sp. (Hemiptera: Coccidae) in Bogotá, Colombia

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Abstract

Parthenolecanium sp. is a scale insect that causes a candelabrum-like symptom in branches and a progressive terminal defoliation in trees of *Ficus soatensis* var *bogotensis*, an ornamental urban tree from Bogotá. Traditional pesticides were forbidden due to possible negative effects to human health, fauna and flora of the location. Natural epizootics of entomopathogenic fungi were observed in a residential zone of Bogota. Six districts were sampled to identify the entomopathogenic fungi. Environmental conditions were recorded from each sample site. Morphological characterization was developed for mycosed insects and isolates. Six nuclear loci were amplified: small subunit ribosomal RNA nu-rSSU, large subunit ribosomal RNA nu-rLSU, translation elongation factor 1-alpha-like (*tef1*), RNA polymerase I (B) subunit (Rpb1), RNA polymerase II (B) subunit (Rpb2) and internal transcribed spacer 1 5.8S ribosomal (*nrITS*). A phylogeny using Maximum Likelihood was performed for *nrITS* amplified sequences to identify the fungal specie, and a phylogenetic tree was constructed to place fungus in the Cordycipitaceae family. *Lecanicillium sabanerum* sp. nov. was proposed as a natural control agent of *Parthenolecanium* sp. and was placed in Cordycipitaceae family with bootstraps of 95 and 100 according to *nrITS* tree and phylogenetic tree, respectively. The influence of particulate matter concentration and other characteristics observed in the field on the incidence of entomopathogenic fungi in the city was discussed.

Key words: *natural control, entomopathogenic fungi,* Parthenolecanium, Ficus soatensis*,* Lecanicillium.

1. Introduction

Ficus soatensis var *bogotensis* is a major ornamental tree in Bogotá that has the ability to tie to the land, provides habitat to birds and offers shadow due to its size (Weisner, 2009). In addition, like others members of Moraceae family, this tree has great ecological importance

because its strong relation with wasps that have in this kind of ficus a unique host (Cardona et al. 2007). Ficus tree was chosen as emblematic tree of the city in 1997 (Weisner, 2009), which was replaced by Walnut Tree (*Juglans neotropica Diels*) in 2002 (Concejo de Bogotá Distrito Capital, 2002). *Ficus soatensis* var *bogotensis* is in the arborization program of Bogota (Alcaldía Mayor de Bogotá Distrito Capital, 2011) and its population is about 100.000 individuals.

In the last two years, Ficus population has been affected by the scale insect *Parthenolecanium* sp. (Hemiptera: Coccidae). These coccids cause a candelabrum-like symptom in branches and produce a massive defoliation until the tree dies (Fig. 1.). Coccids are important pathogens of higher plants due to their feeding habits and have been reported as pest of ornamental plants (Peronti et al., 2001; Van Driesche et al., 2010). Scale insects are sap sucking hemipterans that may inject toxins, transmit viruses and attract ants to plants (Kondo et al., 2008). Scale population can exceed its density when a tree is weak due to, for example, its environmental conditions (Fig. 1.). In this point, infestation becomes a problem, disease worsens and tree is even susceptible to other pests (Hanson and Miller, 1984). Morphological features of these arthropods allow them surviving in unfavorable environmental conditions (Granara de Willink and Claps, 2003). Female coccids secrete waxes that protect them and their eggs by filtrating toxic particles from their environment (Foldi and Pearce, 1985; Gullan and Kosztarab, 1997). These protective compounds are produced in glands of the epidermis of scale insect (Zhang et al., 2012). Parthenogenesis is another characteristic that allocates an increasing in population density due the exclusive production of females. Additionally, the dissemination of the disease increases when males are produced due to their capacity to fly (Gullan and Kosztarab, 1997; Nur, 1971).

The use of fungi as bioinsecticides has been developed in the last 20 years. Strains of species like *Beauveria bassiana*, *Metarhizium anisoplae, Paecilomyces fumosoroseus* and *Lecanicillium lecanni* have been used in biological control with success (Vega and Blackwell, 2004). All of them are grouped in Cordycipitaceae and Hypocreaceae families, and they are associated with larvae and pupae of insects of the orders Hemiptera, Lepidoptera and Coleoptera (Sung et al., 2007). Epizootics of entomopathogenic fungi have been seen on *Parthenolecanium* population in the district of Barrios Unidos about last year. Members of Hypocreaceae family have been documented as pathogens of scale insects (Chaverri

Fig. 1. Candelabrum-like symptom in branches of Ficus tree, *Parthenolecanium* sp. (scale insects), epizootics of entomopathogenic fungi in scale insects. (Left to right).

et al., 2008). These microorganisms could be an alternative to control these coccids in the Ficus population (Fig. 1). The aim of this research is the isolation and identification of entomopathogenic fungi specie that is a natural control agent of *Parthenolecanium* sp. in Bogotá. In addition, we aim to determine the environmental conditions that favor behavior of the fungus as biological control agent.

2. Materials and Methods

2.1. Field collection

La Candelaria, Santa Fé, Teusaquillo, Chapinero, Usaquén and Barrios Unidos were the districts selected for sampling the entomopathogenic fungi in Bogotá (Colombia). The weather conditions of Bogotá were an average temperature of 14 °C, an average annual precipitation of 1013 mm and a relative humidity of 72 %. Monthly precipitation (PMPLL) were maximum in months of march-may and october-december (Secretaría Distrital de Ambiente, 2011).

2.2. Collecting of entomopathogenic fungi

Scale insects with epizootics of entomopathogenic fungi were collected from each Ficus sampled that presented symptoms of the disease described above. Samples were characterized according to their size, color, height in the tree, position relative to sun light and environmental conditions. Also number of diseased trees and number of trees with presence of entomopathogenic fungi were recorded per sample site. Presence of epiphytic organisms was described per tree. Environmental conditions were determined according

to maps of particulate matter (PM) with aerodynamic diameter < 10 µm diameter (PM_{10}), relative humidity and precipitations of the city (Álvarez et al., 2008). Samples were taken to the laboratory for morphological and molecular characterization. Branches of approximately 10 cm with mycosed coccid were collected for conservation of different disease stages in silica gel.

2.3. Isolation of entomopathogenic fungi

Isolations were performed from collected insects with epizootics of fungi. Cadavers were attached with vaseline to the lid of petri dishes to induce expulsion of conidia or ascospores on culture media (Nielsen et al., 2003). Potato dextrose agar (PDA) (Scharlau), malt extract agar (MEA) (Scharlau) and czapek's dox (CZD) (Scharlau) were the media used for isolation and maintenance of strains. Samples of all strains were stored in glass flasks containing 4 mL of distilled water at environmental conditions (Diogo et al., 2005).

2.4. *Morphological characterization*

Morphology of mycosed insects were described according to body layers of scale insect (Zhang et al., 2012). Fungal features were determined treating samples with lactophenol cotton blue (Thomas et al., 1991) and Congo red solution (KOH 3%, Congo red 10% and fuscidic acid 10%) (Slifkin and Cumbie, 1988). The morphology of internal mycelium, phialides and conidia were measured and compared with published description of different entomopathogenic fungi. Description of the colony after 10 days of incubation in PDA, MEA and CZD was made in room laboratory conditions. Characteristics as color, size, consistency, elevation and folding were recorded. Color of fungi were described according to assigned color code patches (Watrud et al., 2006). Fungi were cultured in PDA at environmental conditions to prepare slides for both light microscope and scanning electron microscope (SEM). SEM was performed by freezing circular cuts of 0.5 mm of diameter. Lengths and widths of phialides and conidia of each strain isolated were measured.

2.5. Molecular characterization

Total genomic DNA was extracted from collected fungi by the DNA extraction protocol for basidiomycetes with some modifications (Gardes and Bruns, 1993; Góes-Neto et al., 2005). Total genomic DNA was isolated from cultures grown in SDY liquid media (20 g L^{-1} glucose, 20 g L^{-1} yeast extract, 10 g L^{-1} casein protein) by using an adaptation of a DNA extraction protocol for basidiomycetes decay fungi (Jasalavich et al., 2000). DNA concentration was read in Spectrophotometer ND-1000 (Nanodrop ©) and was adjusted to a

Tabla 1. List of primers

Genes	Primers	Primer sequences (5' to 3')	References
nrITS	ITS1f	CTTGGTCATTTAGAGGAAGTAA	Gardes and Bruns, 1993
	ITS4	GGAAGTAAAAGTCGTAACAAGG	Gardes and Bruns, 1993
nrSSU	NS1	GTAGTCATATGCTTGTCTC	White et al., 1990
	NS4	CTTCCGTCAATTCCTTTAAG	White et al., 1990
nrLSU	LR05	GTACCCGCTGAACTFAAGC	Vilgalys and Sun, 1994
	LR5	ATCCTGAGGGAAACTTC	Vilgalys and Sun, 1994
nrTEF	983 TEF	GCYCCYGGHCAYCGTGAYTTYAT	Castlebury et al., 2004
	2218 TEFr	ATGACACCRACRGCRACRGTYTG	Castlebury et al., 2004
RPB1	CRP1	CC WGGYTTYATCAAGAARGT	Castlebury et al., 2004
	RPB1C	CCNGCDATNTCRTTRTCCATRTA	Castlebury et al., 2004
RPB2	fRPB2-5F	GAYGAYMGWGATCAYTTYGG	Liu et al. 1999
	RPB2-7cR	CCCATRGCTTGTYYRCCCAT	Liu et al. 1999

final concentration of 100 ηg μL^{-1} with a refraction index (260/280) approximately of 2.00. The nuclear gene region of internal transcribed spacer (*nrITS*) 1-5.8S-ITS2 of the rDNA was amplified and sequenced to identify fungi and to define a relation with close groups. Five nuclear gene regions were amplified and sequenced to place it in reported phylogeny for entomopathogenic fungi (Sung et al., 2007). These regions were from small subunit (*nrSSU*) ribosomal RNA gene, large subunit (*nrLSU*) ribosomal RNA gene, the elongation factor 1-α (*tef1*), RNA polymerase I (B) subunit (Rpb1) and RNA polymerase II (B) subunit (Rpb2). Each polymerase chain reaction (PCR) consisted of 2 µL of DNA extracted added to 23µL PCR mix (0.5µL of each primers 100nmol (Table 1), 2.5 µL of 10x Taq buffer + $(NH_4)_2SO_4$ (Fermentas), 2.0 µL of $MgCl_2$ 25mM (Fermentas), 0.5 µL dNTPs 5 µM, and 0.2 µL of Taq DNA Polymerase (recombinant) 5 µ μL^{-1} (Fermentas)) was performed. PCR for SSU amplification was carried out using the following program: 95 °C for 5 min (predenaturation), 35 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, with a final extension at 72 °C for 10 min. PCR for *nrITS*, *tef1,* Rpb1 and Rpb2 amplification was carried out using the fallowing program: 94°C for 3 min (denaturation), 10 cycles at 94°C for 30 s, 55 °C for 1 min, 72 °C for 2 min, 35 cycles at 94 °C for 30 s, 50 °C for 1 min, 72 °C for 2 min, with a final extension at 72 °C for 3 min. DNAs amplified were sequenced by Sanger sequencing method. Forward and reverse nucleotide sequences were assembled and cleaned with Geneious 5.1.2v Program (Drummond et al., 2010). The amplified sequences were compared with a DNA sequence database in the

Tabla 2. Taxa used in Maximum Likelihood tree for *nrITS*.

Specie	Voucher Info.	nrITS
Akanthomyces pistilariformis	TS 772	
Cordyceps coccidioperitheciata	N.H.J. 6709	JN049865
Cordyceps confragosa	CBS 101247	JN049836
Cordyceps tuberculata	OSC 111002	JN049830
Isaria farinosa	CBS 111113	AY624181
Lecanicillium attenuatum	KYK 0324	AB378513

GenBank using a similarity search program of BLAST (Johnson et al., 2008) in NCBI portal.

2.6. Phylogenetic analysis

Obtained and retrieved sequences were aligned in MUSCLE software (Robert C., 2004) on CIPRES Science Gateway portal (Miller et al., 2009). *tef1* sequences were aligned in MAFFT server (Katoh et al., 2009) on EMBL-EBI Web Services. Alignment editing was performed in BioEdit Sequence Alignment Editor Program (Hall, 1999). Ambiguously aligned regions were excluded from phylogenetic analysis and gaps were treated as missing data. Maximum Likelihood (ML) analysis was carried out for ITS nuclear gene region sequences (Table 2). Phylogenetic tree based in ML analysis was constructed for concatenated sequences for other genes (Table 3). ML was performed using RAxML-VI-HPC v2.0 (Stamatakis, 2006; Stamatakis et al., 2008) on CIPRES Science Gateway portal (Miller et al., 2009). Phylogenetic tree was read and edit with Fig Tree program (Rambaut).

3. Taxonomy

Lecanicillium sabanerum is proposed as new species, which is pathogen of *Parthenolecanium* sp. in Bogota. Morphological characterization of collected specimens and isolated strains combined with phylogenetic analyses of molecular data revealed that fungi do not match with *L. lecanii*, *L. attenuatum* and other related species.

Lecanicillium sabanerum J. S. Chiriví-Salomón, Sanjuan, T. & Restrepo, S., sp. nov.

Colonies on PDA and MEA reaching 1.3-1.8 cm diam. in 10 days at laboratory conditions, cottony with exudate drops, yellowish white (PY), with orange (O) to deep yellow (Y) reverse. Colonies on CZA reaching 1.4-1.7cm diam. In 10 days at laboratory conditions, cottony with low relief and folding, yellowish white (PY), with orange (O) reverse (Fig. 2). Phialides short, 13-19 x 1.0-2.0 x 0.5-1.0 µm, tapering to a narrow tip form, more or less erect conidiophores. Ellipsoidal conidia, 3.5-4.5 x 1.5-2.0 µm, formed in mucilaginous heads, 9.0-20 µm of diameter (Fig. 3).. On the host cottony, pale yellow (PY), solitary epizootics or grouped in clusters, 0.5-1.5 mm diameter. Phialides 13-19 µm x 1.0-2.0 µm x 0.5-1.0 µm, tapering to a narrow tip form. Ellipsoidal conidia 3.5-4.5 µm x

Tabla 3. Taxa used in multilocus phylogenetic analysis.

Specie	Voucher Info.	GenBank Accesion Number				
		nrSSU	*nrLSU*	*tef1*	Rpb1	Rpb2
Akanthomyces pistilariformis	TS 772					
Aphysiostrome stercorarium	ATCC 62321 T	AF543769	AF543792	AF543782	AY489633	EF469103
Aschersonia badia	BCC 8105	DQ522573	DQ518752	DQ522317	DQ522363	DQ522411
Balansia epichloë	A.E.G. 96-15a	EF468949		EF468743	EF468851	EF468908
Beauveria caledonica	ARSEF 2567 T	AF339570	AF339520	EF469057	EF469086	
Bionechtria cf. *aureofulva*	G.J.S. 71-328	DQ862044	DQ862027	DQ862029		DQ862013
Bionectria ochroleuca	CBS 114056	AY489684	AY489716	AY489611		DQ522415
Claviceps purpurea	GAM 12885	AF543765	AF543789	AF543778	AY489648	DQ522417
Cordyceps (cf.) *coccidioperitheciata*	N.H.J. 5112	EU369109	EU369043	EU369026	EU369066	
Cordyceps bifusispora	EFCC 8260	EF468953	EF468807	EF468747	EF468855	EF468910
Cordyceps cardinalis	CBS 113412 AUT	AY184974	AY184963	EF469059	EF469088	EF469106
Cordyceps cf. *ochraceostromata*	ARSEF 5691	EF468964	EF468819	EF468759	EF468867	EF468921
Cordyceps cf. *pruinosa*	N.H.J. 10627	EF468967	EF468822	EF468763	EF468870	
Cordyceps cf. *pruinosa*	N.H.J. 10684	EF468968	EF468823	EF468761	EF468871	
Cordyceps cf. *takaomontana*	N.H.J. 12623	EF468984	EF468838	EF468778	EF468884	EF468932
Cordyceps confragosa	CBS 101247	AF339604	AF339555	DQ522359	DQ522407	DQ522466
Cordyceps gunii	OSC 76404	AF339572	AF339522	AY489616	AY489650	DQ522426
Cordyceps kyusyuensis	EFCC 5886	EF468960	EF468813	EF468754	EF468863	EF468917
Cordyceps militaris	OSC 93623	AY184977	AY184966	DQ522332	DQ522377	AY545732
Cordyceps scarabaeicola	ARSEF 5689	AF339574	AF339524	DQ522335	DQ522380	DQ522431
Cordyceps takaomontana		AB044631	AB044637			
Cordyceps tuberculata	OSC 111002	DQ522553	DQ518767	DQ522338	DQ522384	DQ522435
Cosmospora coccinea	CBS 114050	AY489702	AY489734	AY489629	AY489667	DQ522438
Elaphocordyceps capitata	OSC 71233	AY489689	AY489721	AY489615	AY489649	DQ522421

Specie	Voucher Info.	GenBank Accesion Number				
		nrSSU	*nrLSU*	*tef1*	Rpb1	Rpb2
Elaphocordyceps fracta	OSC 110990	DQ522545	DQ518759	DQ522328	DQ522373	DQ522425
Elaphocordyceps japonica	OSC 110991	DQ522547	DQ518761	DQ522330	DQ522375	DQ522428
Elaphocordyceps ophioglossoides	OSC 106405	AY489691	AY489723	AY489618	AY489652	DQ522429
Elaphocordyceps subsessills	OSC 71235	EF469124	EF469077		EF469090	EF469108
Engyodontium aranearum	CBS 309.85	AF339576	AF339526	DQ522341	DQ522387	DQ522439
Epichloë typhina	ATCC 56429	U32405	U17396	AF543777	AY489653	DQ522440
Glomerella cingulata	CBS 114054	AF543762	AF543786	AF543773	AY489659	DQ522441
Haptocillium sinense	CBS 567.95 T	AF339594	AF339545	DQ522343	DQ522389	DQ522443
Hirsutella sp.	N.H.J. 12525	EF469125	EF469078	EF469063	EF469092	EF469111
Hydropisphaera erubescens	ATCC 36093	AY545722	AY545726	DQ522344	DQ522390	AY545731
Hypocrea lutea	ATCC 208838	AF543768	AF543791	AF543781	AY489662	DQ522446
Hypocrella schizostachyl	BCC 14123	DQ522557	DQ518771	DQ522346	DQ522392	DQ522447
Hypocrella sp.	G.J.S. 89-104	U32409	U47832	DQ522347	DQ522393	DQ522448
Hypomyces polyporinus	ATCC 76479	AF543771	AF543793	AF543784	AY489663	
Isaria farinosa	OSC 111005	DQ522558	DQ518772	DQ522348	DQ522394	
Isaria farinosa	OSC 111006	EF469127	EF469080	EF469065	EF469094	
Isaria tenuipes	OSC 111007	DQ522559	DQ518773	DQ522349	DQ522395	DQ522449
Lecanicillium antillanum	CBS 350.85 T	AF339585	AF339536	DQ522350	DQ522396	DQ522450
Lecanicillium aranearum	CBS 726.73a	AF339586	AF339537	EF468781	EF468887	EF468934
Lecanicillium attenuatum	CBS 402.78	AF339614	AF339565	EF468782	EF468888	EF468935
Lecanicillium fusisporum	CBS 164.70 T	AF339598	AF339549	EF468783	EF468889	
Lecanicillium psaliotae	CBS 101270	EF469128	EF469081	EF469066	EF469095	EF469113
Leuconectria clusiae	ATCC 22228 T	AY489700	AY489732	AY489627	AY489664	EF469114
Mariannaea pruinosa	ARSEF 5413 AUT	AY184979	AY184968	DQ522351	DQ522397	DQ522451

Specie	Voucher Info.	GenBank Accesion Number				
		nrSSU	*nrLSU*	*tef1*	Rpb1	Rpb2
Metarhizium anisopliae	ARSEF 3145	AF339579	AF339530	AF543774	DQ522399	DQ522453
Metarhizium chlamydosporia	CBS 101244 AUT	DQ522544	DQ518758	DQ522327	DQ522372	DQ522424
Metarhizium flavoviride	ARSEF 2037 T	AF339580	AF339531	DQ522353	DQ522400	DQ522454
Metarhizium tail	ARSEF 5714	AF543763	AF543787	AF543775	DQ522383	DQ522434
Nomuraea typicola	CBS 744.73	EF468987	EF468841	EF468786	EF468892	
Ophiocordyceps acicularis	OSC 110987	EF468950	EF468805	EF468744	EF468852	
Ophiocordyceps agriotidis	ARSEF 5692	DQ522540	DQ518754	DQ522322	DQ522368	DQ522418
Ophiocordyceps brunneipunctata	OSC 128576 AUT	DQ522542	DQ518756	DQ522324	DQ522369	DQ522420
Ophiocordyceps entomorrhiza	KEW 53484	EF468954	EF468809	EF468749	EF468857	EF468911
Ophiocordyceps gracilis	EFCC 8572	EF468956	EF468811	EF468751	EF468859	EF468912
Ophiocordyceps heteropoda	EFCC 10125	EF468957	EF468812	EF468752	EF468860	EF468914
Ophiocordyceps irangiensis	OSC 128579	EF469123	EF469076	EF469060	EF469089	EF469107
Ophiocordyceps melolonthae	OSC 110993	DQ522548	DQ518762	DQ522331	DQ522376	
Ophiocordyceps nigrella	EFCC 9247	EF468963	EF468818	EF468758	EF468866	EF468920
Ophiocordyceps nutans	OSC 110994	DQ522549	DQ518763	DQ522333	DQ522378	
Ophiocordyceps sinensis	EFCC 7287	EF468971	EF468827	EF468767	EF468874	EF468924
Ophiocordyceps sobolifera	KEW 78842	EF468972	EF468828		EF468875	EF468925
Ophiocordyceps sphecocephala	OSC 110998	DQ522551	DQ518765	DQ522336	DQ522381	DQ522432
Ophiocordyceps tricentri		AB027330	AB027376			
Ophiocordyceps unilateralis	OSC 128574	DQ522554	DQ518768	DQ522339	DQ522385	DQ522436
Ophiocordyceps variabilis	OSC 111003	EF468985	EF468839	EF468779	EF468885	EF468933
Ophionectria trichospora	CBS 109876	AF543766	AF543790	AF543779	AY489669	DQ522457
Paecilomyces lilacinus	CBS 284.36 T	AY624189	AY624227	EF468792	EF468898	EF468941
Paecilomyces lilacinus	CBS 431.87	AY624188	EF468844	EF468791	EF468897	EF468940

Specie	Voucher Info.	GenBank Accesion Number				
		nrSSU	*nrLSU*	*tef1*	Rpb1	Rpb2
Phytocordyceps ninchukispora	E.G.S. 38.165 AUT	EF468991	EF468846	EF468795	EF468900	
Phytocordyceps ninchukispora	E.G.S. 38.166 AUT	EF468992	EF468847	EF468794	EF468901	
Pochonia bulbillosa	CBS 145.70 T	AF339591	AF339542	EF468796	EF468902	EF468943
Roumegueriella rufula	G.J.S. 91-164	EF469129	EF469082	EF469070	EF469099	EF469116
Schimizuomyces paradoxus	EFCC 6279	EF469131	EF469084	EF469071	EF469100	EF469117
Simplicillium lamellicola	CBS 116.25 T	AF339601	AF339552	DQ522356	DQ522404	DQ522462
Simplicillium lanasoniveum	CBS 704.86	AF339602	AF339553	DQ522358	DQ522406	DQ522464
Sphaerostilbella berkeleyana	CBS 102308	AF543770	U00756	AF543783	AY489671	DQ522465
Tolypocladium parasiticum	ARSEF 3436 AUT	EF468993	EF468848	EF468799	EF468904	EF468945
Torrubiella luteorostrata	N.H.J. 11343	EF468995	EF468850	EF468801	EF468906	
Torrubiella wallacei	CBS 101237 T	AY184978	AY184967	EF469073	EF469102	EF469119
Verticillium epiphytum	CBS 384.81	AF339596	AF339547	DQ522361	DQ522409	DQ522469
Verticillium incurvum	CBS 460.88 T	AF339600	AF339551	DQ522362	DQ522410	DQ522470

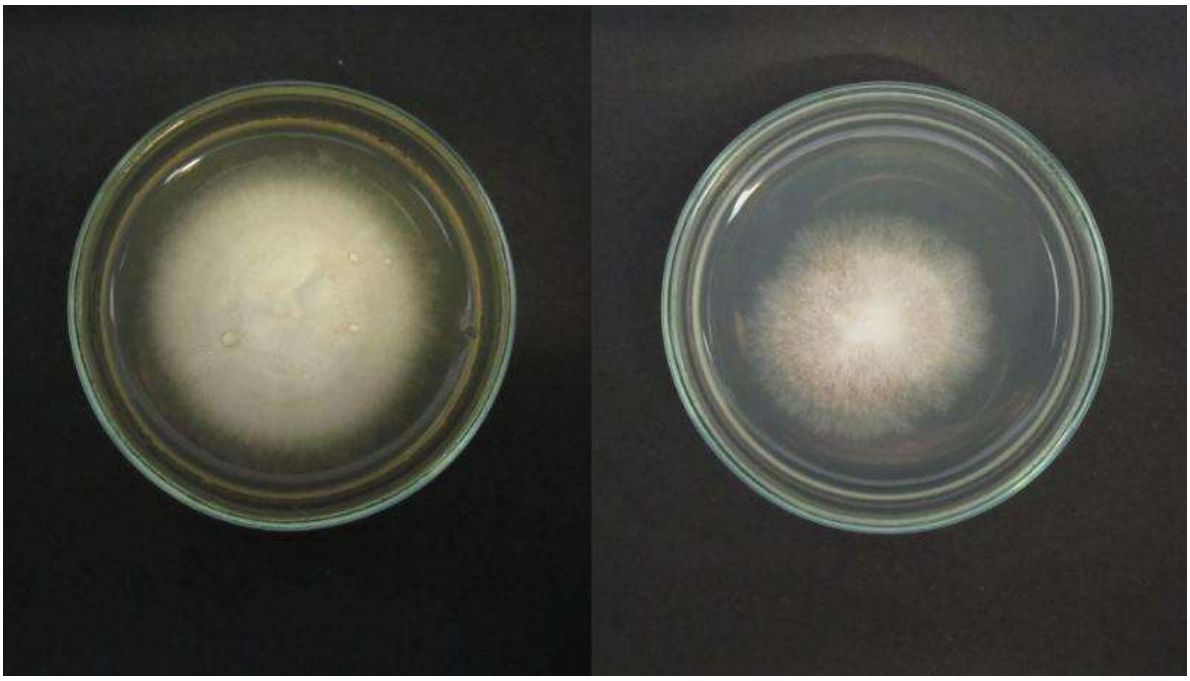

Fig. 2. *Lecanicillium sabanerum* colonies on PDA and CZA (Left to right)*.*

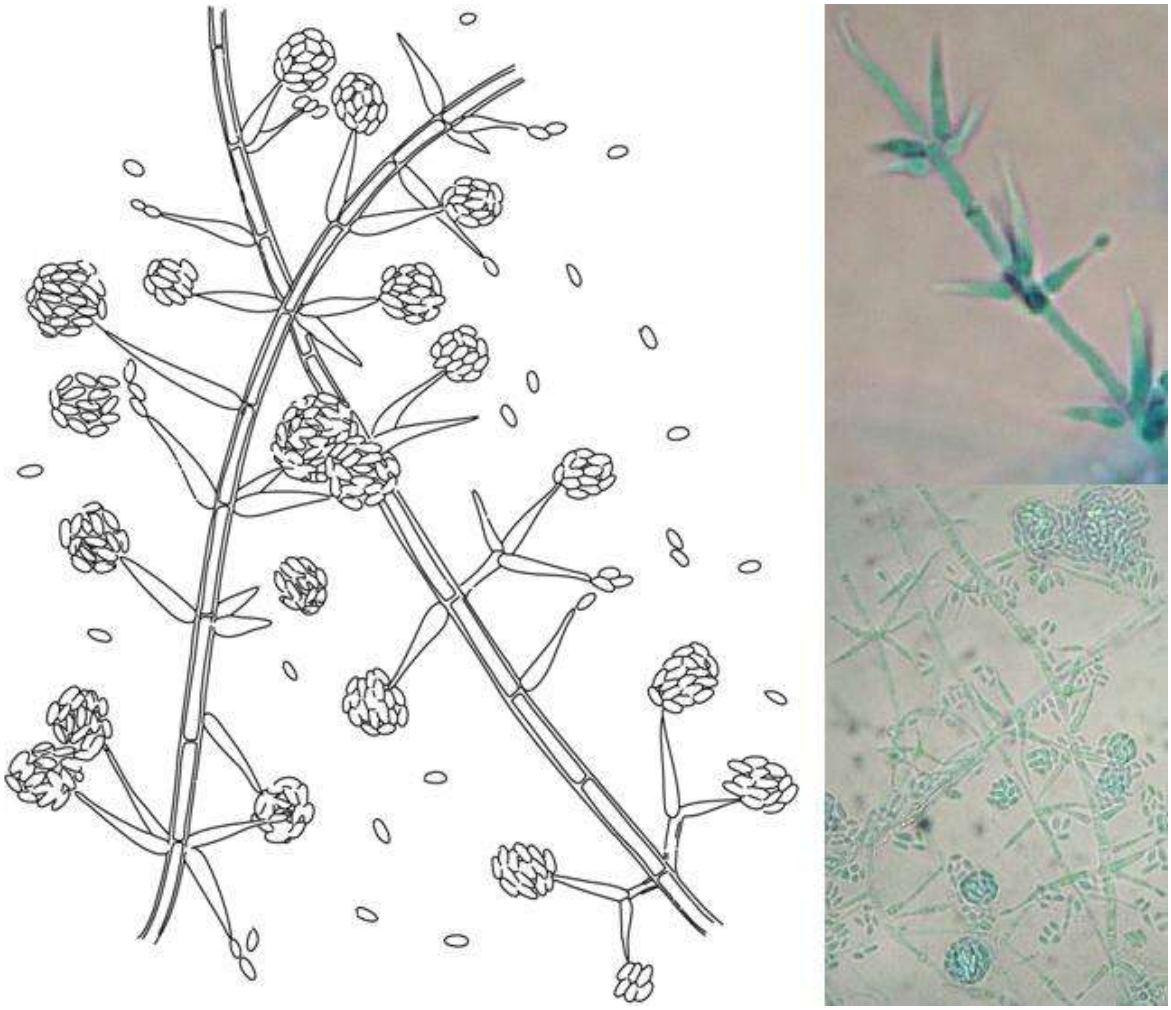

Fig. 3. Microscopy of *Lecanicillium sabanerum* in lactophenol cotton blue.

1.5-2.0 µm. Three layers of infection on scale insect. First layer: phialides and conidia on epicuticle of scale insect, prosenchymal mycelia on exocuticle of scale insect. Second layer: prosenchymal mycelia on endocuticle and formation zone of scale insect. Third layer: crossing hyphae, surrounding mycelia of wax-secreting glands and fat bodies on haemocoele of scale insect. (Fig. 4). Temperature optimum 16-20 °C.

Known distribution. 2.560 m.a.s., Bogota, Cundinamarca, Colombia.

Etimology. *Lecani-*, referring to the major component, *-cillium*, suffix taken from *Verticillium*, *sabanerum*, referring to location of the tree host of *Parthenolecanium* sp., *Ficus soatensis* var. *bogotensis*.

Commentary. *L. sabanerum* presents frequently a pale yellow (PY) color and cottony mycelia on scale insects. But there are specimens with flat white (FW) and grayish tan (LT) colors, corresponding to young and old infections, respectively. Also, cottony texture is found in young infections while tough texture in old ones. Fungi are collected most frequently below young branches of the tree where sunlight is limited. Epizootics of *L. sabanerum* is found commonly in periods of highly humid and rainfalls. Old colonies on PDA and MEA performed a powdery texture. Exudate drops are produced in first subcultures of fungi after isolation. Colonies on CZA described above can perform another morphotype: without folding.

4. Results

From 220 Ficus trees sampled, 190 individuals were found with symptoms or signs of the disease (candelabrum-like symptom, presence of insects in trunk, branches or leafs or, instead both, presence of protective waxes of coccids). Noteworthy those most healthy trees had mosses and lichens in their surface, especially in lower areas of tree. On the other hand, entomopathogenic fungi were found infecting coccids in 44 Ficus trees from diseased trees. Ficus in AK 7 CL 28 address presented a natural control by fungi, but this zone was operated by botanical garden workers. Intervention consisted of introducing grafts of branches with entomopathogenic fungi. Fungi were collected principally from dark areas of the plant (lower leaf surface, lower little branches surface, and cracks in branches and trunk) where sunlight is limited. Entomopathogenic fungi were found in an upper area of two trees from AK 68 CL 49A, but area is below a pedestrian bridge and sunlight is limited almost most of the day. Fungi were found in different stages of infection with various sizes (maximum of 0.8 cm and minimum of 0.4 cm) and there are generally grouped in clusters. Dataset of environmental conditions of sampled areas, the number of diseased trees and the

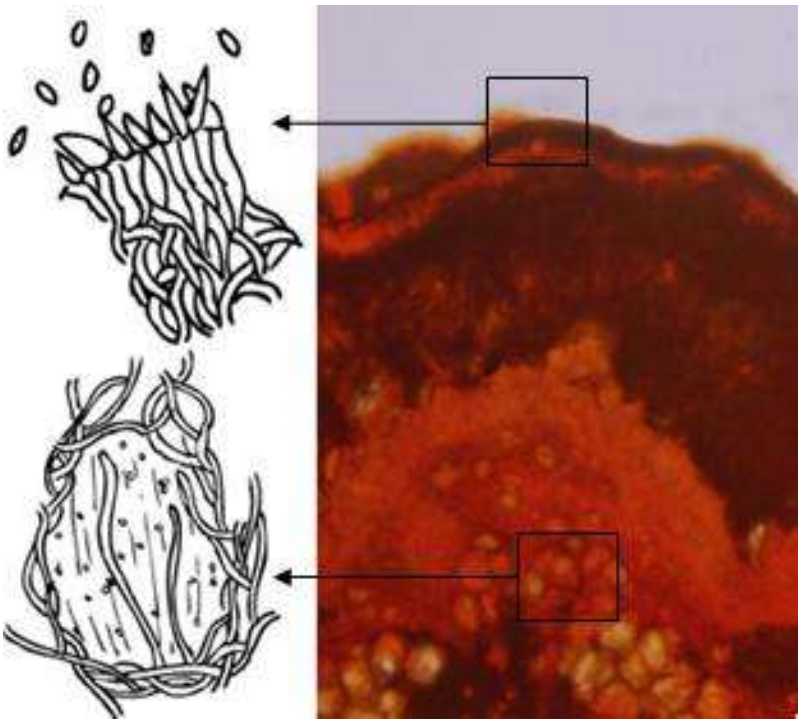

Fig. 4. Microscopy of epizootic of *Lecanicillium sabanerum* on scale insect in Congo red solution.

number of trees with epizootics of fungi infecting coccids were shown in Table 4. This information was overlapped and compared with humidity, particulate matter and precipitation maps (Fig. 5-7).

Sequences showed high similarity with *Lecanicillium lecanii* (also named *Verticillium lecanii*) (Sung et al., 2001; Zare et al., 2000), *Torrubiella* sp. (Chaverri et al., 2005) and *Cordyceps confragosa* (Spatafora et al., 2007), according to BLAST. The *nrITS* region of fungi did match *L. lecanii* with an average maximum identity of 99%. ML tree for *nrITS* is well supported with bootstrap values above 95 (Fig. 8). Families in the multilocus phylogenetic tree were well supported and they were organized in agreement with previous results (Sung et al., 2007). Bionectriaceae family contained 4 taxa and showed a bootstrap support of 100. Nectriaceae family was well supported with a bootstrap value of 99 and included 3 taxa. Hypocreaceae family, *Simplicillium* clade and Cordycipitaceae family were separated from Ophiocordycipitaceae family and sister groups with a boostrap of 99. Similarly, Cordycipitaceae family achieved a separation with a bootstrap of 100 from both Hypocreaceae family and *Simplicillium* clade. Cordycipitaceae family included 36 taxa. *L. sabanerum* was placed in *Cordyceps* s.s. clade in Cordycipitaceae family and *Cordyceps confragosa* was set as sister specie of *L. sabanerum* (Fig. 9).

L. sabanerum is placed as a new specie of entomopathogenic fungi because its morphological

Table 4. Data collected from sampling

Neighborhood	Address	Number of sampled trees	Number of diseased trees	Number of trees with epizootics of fungi	Isolates of entomopathogenic fungi	PM_{10} concentration (µg/m3)	Humidity	Precipitations (mm)
Barrios Unidos	AK 24 CL 62	3	3	0	0	40-50	Semidry zone	900-1000
	AK 15 CL 71A	1	1	0	0	60-70	Semidry zone	900-1000
	KR 41A CL 62	3	3	0	0	50-60	Semidry zone	900-1000
	KR 59 CL 70A	1	1	0	0	40-50	Semidry zone	900-1000
	KR 31 CL 89	5	5	0	0	40-50	Semidry zone	900-1000
	TV 24 DG 83	3	3	0	0	60-70	Subwet zone	900-1000
	KR 19 CL 65A	1	1	0	0	60-70	Semidry zone	900-1000
	TV 44A CL 95A	2	2	0	0	60-70	Semidry zone	900-1000
	KR 34 CL 72	3	3	0	0	50-60	Semidry zone	900-1000
	AK 68 AC 63	21	19	8	8	60-70	Subwet zone	900-1000
Teusaquillo	TV 43 DG 40	1	1	1	0	60-70	Semidry zone	900-1000
	KR 13 CL 39	7	7	0	0	70-80	Wet zone	1000-1100
	KR 39 CL 25B	7	5	0	0	70-80	Semidry zone	900-1000
	KR 51 CL 28	6	6	0	0	70-80	Semidry zone	900-1000
	KR 13 CL 52	9	7	0	0	60-70	Wet zone	900-1000
	TV 35B DG 40	12	10	10	7	60-70	Semidry zone	900-1000
	KR 18A CL 38	1	1	1	0	60-70	Wet zone	900-1000
	AC 42 TV 16A	2	2	0	0	60-70	Subwet zone	900-1000
	AK 68 CL 49A	35	35	17	11	60-70	Semidry zone	800-900
	AK 68 AC 55	3	3	3	2	60-70	Semidry zone	800-900

Neighborhood	Address	Number of sampled trees	Number of diseased trees	Presence of entomopathogenic fungi	Isolates of entomopathogenic fungi	PM_{10} concentration (µg/m3)	Humidity	Precipitations (mm)
Chapinero	AK 11 CL 87	2	1	0	0	60-70	Subwet zone	1000-1100
	AK 7 CL 72	5	0	0	0	70-80	Subwet zone	1000-1100
	KR 21 CL 87	4	3	0	0	60-70	Subwet zone	900-1000
	AK 7 CL 43	2	2	0	0	60-70	Wet zone	1000-1100
	AK 7 CL 87	10	10	0	0	60-70	Subwet zone	900-1000
	KR 7 CL 79	6	6	0	0	70-80	Subwet zone	900-1000
	K3B CL 64	1	0	0	0	70-80	Subwet zone	1000-1100
La Candelaria	AK 7 CL 28	17	17	4	11	70-80	Wet zone	1000-1100
y Sante Fe	KR 9 CL 11	5	2	0	0	70-80	Wet zone	1000-1100
	KR 13A CL 19	6	6	0	0	70-80	Wet zone	1000-1100
	KR 6A CL 16	14	6	0	0	70-80	Wet zone	1100-1200
	KR 3E CL 7	2	2	0	0	70-80	Wet zone	1100-1200
	KR 10 CL 13	2	2	0	0	70-80	Wet zone	1100-1200
	KR 13 AC 13	3	3	0	0	70-80	Wet zone	1000-1100
	KR 6 CL 34	4	4	0	0	70-80	Subwet zone	1000-1100
	KR 12 CL 15	2	2	0	0	70-80	Wet zone	1100-1200
Usaquén	KR 7 CL 117	5	5	0	0	40-50	Semidry zone	800-900
	KR 6A CL 114	3	1	0	0	40-50	Semidry zone	800-900
	TV 2 CL114	1	0	0	0	40-50	Dry zone	700-800

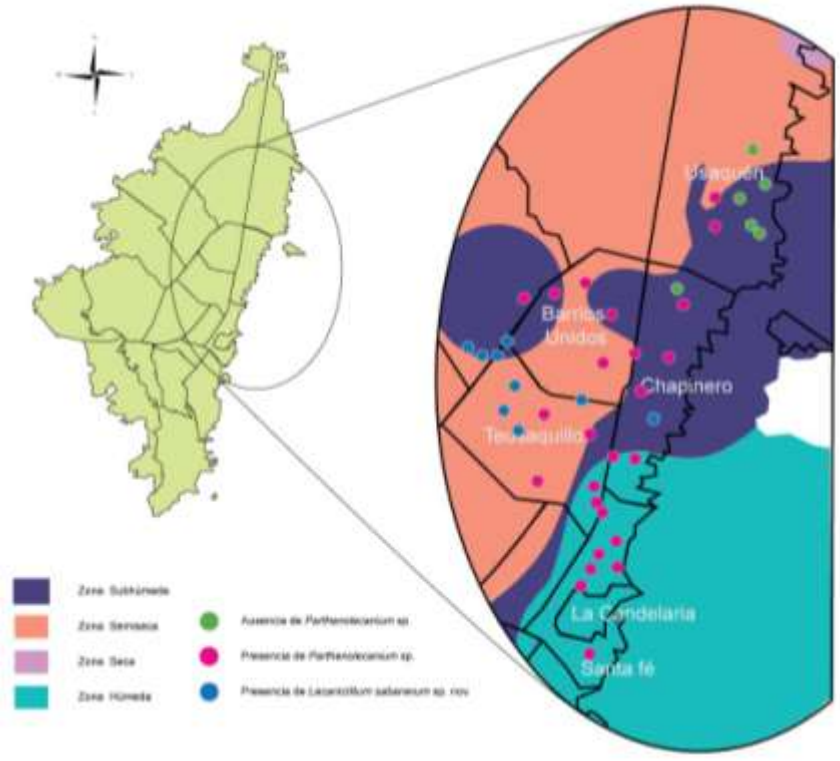

Fig. 5. Humidity map and collections sites. *Parthenolecanium* sp. absence (green circles), *Parthenolecanium* sp. presence (pink circles), *Lecanicillium sabanerum* presence (blue circles), subhumid zone (dark purple), semidry zone (orange), dry zone (purple), humid zone (cyano).

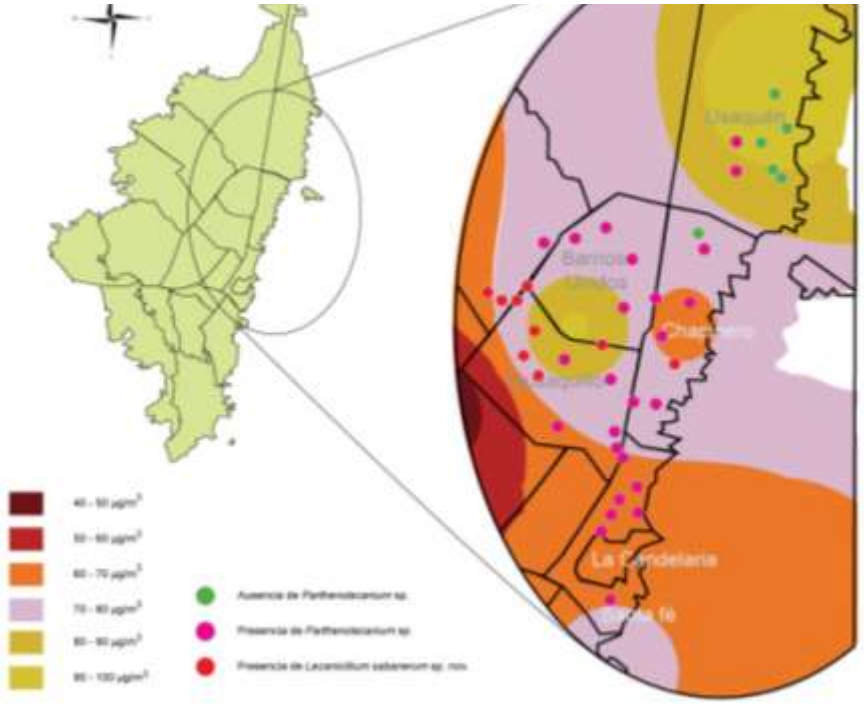

Fig. 6. PM_{10} map and collections sites. *Parthenolecanium* sp. absence (green circles), *Parthenolecanium* sp. presence (pink circles), *Lecanicillium sabanerum* presence (red circles), 40 - 50 µg/m^3 (dark red), 50 – 60 µg/m^3 (red), 60 – 70 µg/m^3 (orange), 70 – 80 µg/m^3 (purple), 80 – 90 µg/m^3 (dark ochre), 90 – 100 µg/m^3 (ochre).

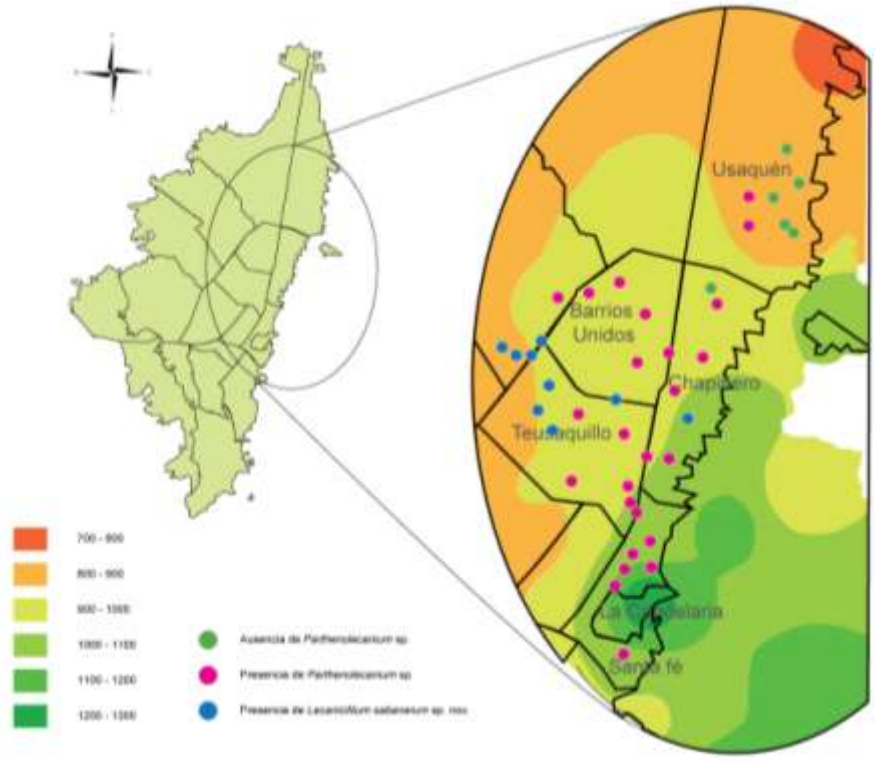

Fig. 7. Precipitations map and collections sites. *Parthenolecanium* sp. absence (green circles), *Parthenolecanium* sp. presence (pink circles), *Lecanicillium sabanerum* presence (blue circles), 700 – 800 (dark orange), 800 – 900 (orange), 900 – 1000 (ochre), 1000 – 1100 (green faint), 1100 – 1200 (green), 1200 – 1300 (dark green).

characteristics and phylogenetic position. The SEM showed mycelium and conidiophores with phialides reported above and conidia organized in mucilaginous heads (Fig. 10). *L. sabanerum* was compared with *L. lecanii* because its highly similarity reported in BLAST. These two species differ in several microscopic and cultural characteristics. Phialides of *L. sabanerum* were longer and thicker than *L. lecanii* (11-20(-30) µm x 1.3-1.8 µm). Ellipsoidal conidia of *L. sabanerum* were considerably larger than *L. lecanii* (2.5-3.5(-4.2) µm x 1-1.5 µm). *L. sabanerum* colonies were smaller than *L. lecanii* (1.5-2.0 cm), also texture of both fungi was similar, *L. sabanerum* performed several morphotypes according to age and culture media. On the other hand, *L. lecanii* teleomorph (*C. confragosa*) was phylogenetic compared with *L. sabanerum* and a separation between these species was shown.

5. Discussion

L. lecanii is an entomopathogenic fungi that is reported as anamorph of *Torrubiella* genus in phylogenetic analyses for *nrITS* sequences (Gams et al., 2000). Nevertheless, recent rigorous phylogenetic analyses connected *L. lecanii* with the teleomorph of *Cordyceps confragosa* (Sung et al., 2007). On the other hand,

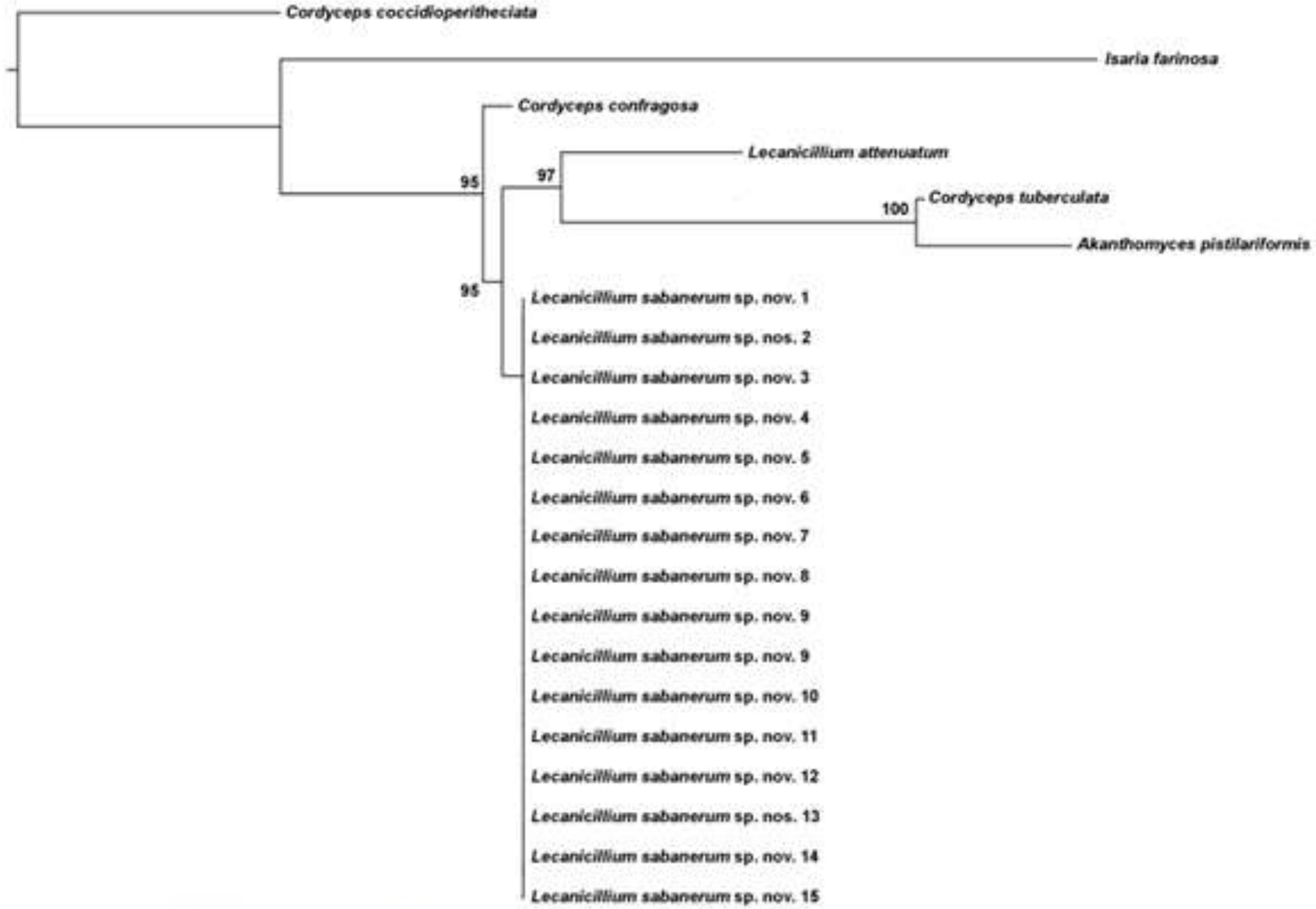

Fig. 8. Maximum Likelihood performed for *nrITS* gen.

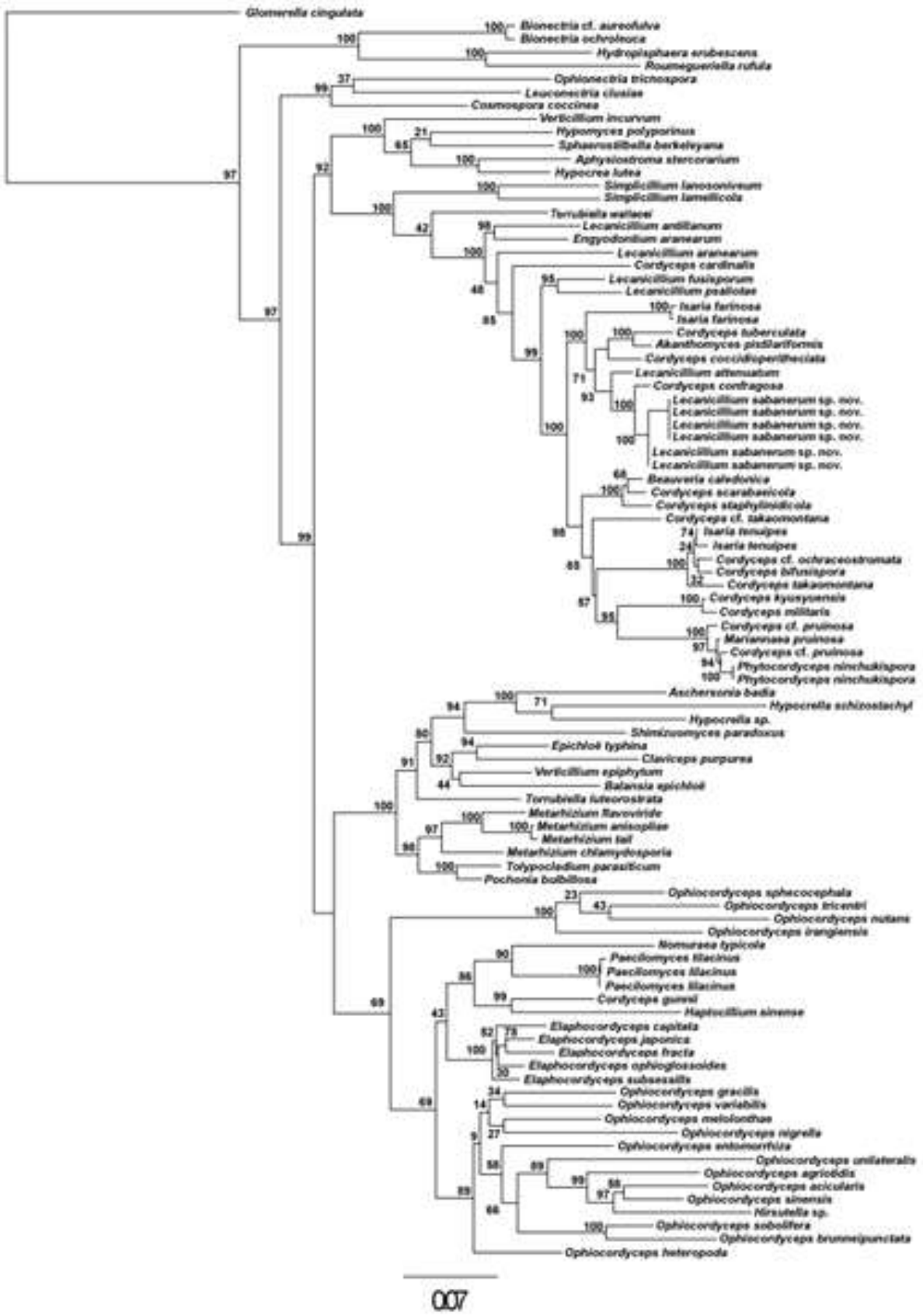

Fig. 9. Multilocus phylogenetic tree

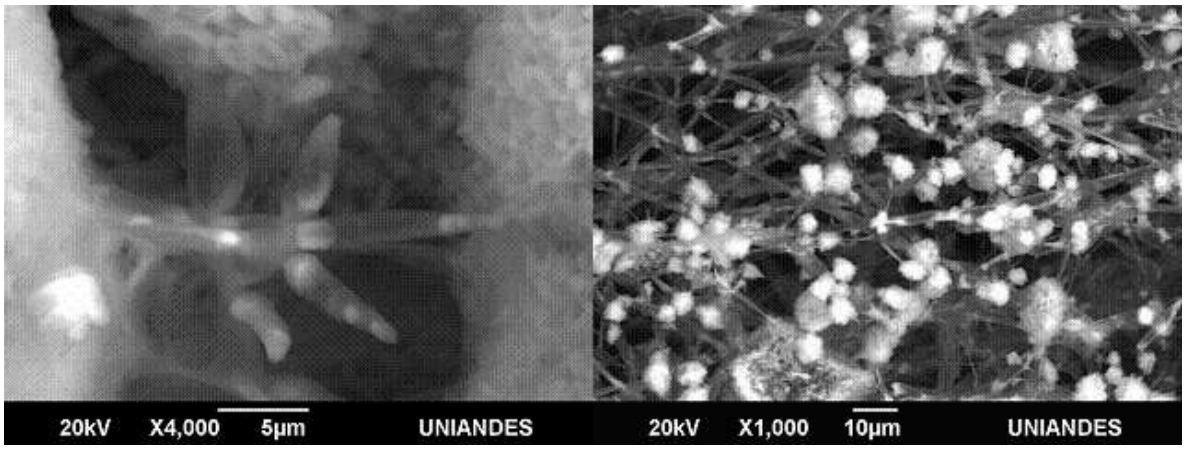

Fig. 10. Scanning Electron Microscopy (SEM) for *Lecanicillium sabanerum*

the *nrITS* tree presented in this study indicated that the entomopathogenic fungus characterized belong to singular specie, and it is separated from other hemipteran pathogens. *C. confragosa* was considered in our analysis, and our fungi resulted distinct from this specie with a bootstrap of 95. *L. attenuatum*, *Cordyceps tuberculta* and *Akanthomyces pistilariformis* were different with a bootstrap of 95 and their hosts are lepidopteran (Dennis, 1995; Sung et al. 2007; Zare and Gams, 2001), a very dissimilar insect order of Hemiptera. In agreement with morphological analysis discussed above, *Lecanicillium sabanerum* is proposed as a new species and is a natural control agent of *Parthenolecanium* sp. Multilocus phylogenetic tree showed that *L. sabanerum* is placed in *Cordyceps* s.s. clade in Cordycipiticeae family, according with the proposed phylogenetic tree of Sung and collaborators (Sung et al., 2007). In this multilocus analysis, *L. sabanerum* individuals resulted in two clades with a bootstrap of 100. Their short length and established relations in *nrITS* tree indicates that collected and isolated fungi are grouped in singular specie. Otherwise, *C. confragosa* and *L. sabanerum* were grouped in a clade with a phylogenetic difference of 100 of bootstrap. As stated above, *C. confragosa* and *L. sabanerum* represent different species. It is notorious that these fungi were closely related, which fits with the obtained high similarity with *L. lecanii* in BLAST.

Preliminary morphological identifications showed that these fungi could belong to the *Lecanicillium* genus, anamorph of *Cordyceps* s.s., which has been described as pathogens of some spiders and members of Hemiptera order (Evans and Hywel-Jones, 1997). Species of this genus have been used as biological control agents, like *Lecanicillium attenuatum* in green peach aphids (Hemiptera: Aphidoideae) (Kim et al., 2008) and *Lecanicillium lecanii* in brown soft

scales (Hemiptera: Coccidae) (Liu et al., 2011), respectively. Coincidentally, isolates of entomopathogenic fungi of *Parthenolecanium* sp. showed morphological characteristics that placed it in *Lecanicillium* genus. This genus is recognized by its colony characteristics on PDA and MEA. Additionally, morphology and arrangement of its conidiophores, phialides and conidia are important features to consider in this genus. Characters of isolated fungi are comparable with *L. lecanii* (also commercially named *Verticillium lecanii*), *L. longisporum* and *L. nodulosum*. Nevertheless, morphology of isolated fungi present more similarities with *L. lecanii* than the other *Lecanicillium* spp. i.e. Diameter of growth for isolates is within growing range of *L. lecanii* at 20°C, colonies aspect are equal (excluding that color reverse of isolates in some cases changing to orange), phialides are short and present a tapering to tips, the arrangement of ellipsoidal conidia is in mucilaginous heads at tips of the phialides (Fig. 5 and Fig. 7). On the other hand, the main character that differentiates these fungi is conidia size, because conidia of isolated fungi are bigger than *L. lecanii* conidia (Zare and Gams, 2001). SEM photographs showed the same characteristics that features observed in light microscopy. Conidia organized in mucilaginous heads were clearly noted at the tips of the phialides proving a similarity with *L. lecanii* in conidia arrangement (Fig. 6).

The presence of exudate drops could be related to C:N ratio of culture media (Hutwimmer et al., 2010) but it has been suggested an enzymatic activity with ecological role by fungi, according to studies with *L. lecanii* (Rocha-Pino et al., 2011), *Metharhyzium anisopliae* (Hutwimmer et al., 2010) and Ophiocordyceps sinensis (Kuo et al., 2005), among other species. *L. lecanii* is induced to produce hydrophobins and chitinases in submerged culture and solid-substrate culture (Rocha-Pino et al., 2011). It has been shown that *M. anisopliae* secretes droplets of exudates that contain dextruxins, enzymes with insecticidal properties (Hutwimmer et al., 2010). *O. sinensis*, a Lepidoptera larvae entomopathogenic, exudates compounds with anti-tumour and anti-bacterial activity, likely for competition with other organisms (Kuo et al., 2005). In accordance with these studies and the wide range of metabolites that these fungi can produce, a chemical analysis of exudates from *L. sabanerum* sp. nov. is recommended to consider for future works. Moreover, there is reported that *Lecanicillium* fungi infect scale insects by penetrating the integument with mechanical force and secreting extracellular enzymes (Liu et al., 2011).

The emergence of these fungi seemed to be related with La Niña phenomenon, a period of highly humidity of weather. But there is no relation between both humidity and precipitations with the disease of Ficus or presence of epizootics of *L. sabanerum* sp. nov. (Table 4, Fig. 5 and Fig. 7). Probably moisture condition inside trees is the determinant factor of incidence of entomopathogenic fungi. Water is retained in branches and leafs creating a moisture microhabitat, due to precipitations and humidity from La Niña phenomenon. On the other hand, presence of epiphytic organism and particulate material concentration seem to be important factors to consider. First, coccids were absent when moss and/or lichen covered the cortex of trees, because they may generate a physical barrier by limiting the space where scale insects feed. Second, mycosed coccids were only observed in areas where concentration of particulate material was below 70-80 μg m^{-3}, excluding collected material from AK 7 CL 28 where Botanical Garden workers intervened with grafts. The PM impacts in the health of plants by injuring the leaf surface, decreasing the photosynthetic rate and causing uptake deficiencies in the rhizosfere (Prajapati, 2012). In this sense, fluctuations caused by PM could be affect the physical-chemical condition and microbial communities of soil that help to plant in disease suppression (van Bruggen et al., 2006). Also morphology and reproduction of some fungi can be affected by air pollutants (Estrabou et al., 2004). Although PM_{10} concentration in the district of Usaquén is low, the absent of natural control evidence could be explain due dissemination of fungi. Studies indicate that fungi from the genus *Lecanicillium* notoriously increase its persistence when is dispersed by thirds agents (Down et al., 2009). Moreover, cleaning routes of Bogotá are made by different cleaning companies per district. Pruning is realized and cut branches are transported by trucks. Vegetal material and debris are incinerated (pers. com. Vargas, G. from Unidad Administrativa Especial de Servicios Públicos, 2012). Transportation may not be suitable because it resembles helping to the dissemination of coccids. Additionally, proliferation of fungi seems to be affected by pruning.

6. Conclusions

Morphological characteristics of collected fungi place them in the *Lecanicillium* genus, reported pathogens of hemipteran insects. Fungi belong to a single species, with a bootstrap that separate them from brother species (*Akanthomyces aculeatus, Cordyceps tuberculta, Lecanicillium attenuatum, Cordyceps confragosa, Isaria farinosa and Cordyceps cocciodioperitheciata*). Multilocus phylogenetic tree indicate that the fungus is placed in

Cordyceps s.s. clade (Cordycipitaceae family). *Lecanicillium sabanerum* sp. nov. is proposed as natural control agent of *Parthenolecanium* sp. and it has a great potential as biological control agent.

PM seems to be a determinant factor in *L. sabanerum* sp. nov. incidence. Although both precipitations and humidity are not definitive factors, both shadow and humidity can generate propitious microhabitats for fungal growing and eventuality control of scale insects on a tree. Presence of moss and/or lichen likely may avoid those scale insects to feed from Ficus Tree. Morphology and physiology of coccids, and cultural practices ease their dissemination on Ficus population. Cultural practices seem to be affecting the *L. sabanerum* proliferation too.

An epidemiological study of diseased caused by scale insects is recommended for setting a correct cleaning program in Bogota city. A suitable cleaning program will avoid a rapid dissemination of disease and permit a better control with entomopathogenic fungi. This cleaning program could include a pruning that provides microhabitats of shadow and humidity to maintaining recycling and persistence of *L. sabanerum*. On the other hand, pathogenicity test of isolated strains is important to guaranteeing a good biological control. Finally, a chemical analysis of produced exudates is recommended to determine the metabolic content, which may be advantageous for the industry.

7. Acknowledgements

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TITULO DEL TRABAJO DE GRADO:

The entomopathogenic fungi Lecanicillium sabanerum sp. nov. a natural control agent of Parthenolecanium sp. (Hemiptera: Coccidae) in Bogotá, Colombia

DESCRIPCIÓN FÍSICA

Número de páginas: 28

Ilustraciones: 10

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*RESUMEN DEL TRABAJO DE GRADO:

Parthenolecanium sp. is a scale insect that causes a candelabrum-like symptom in branches and a progressive terminal defoliation in trees of Ficus soatensis var bogotensis, an ornamental urban tree from Bogotá. Traditional pesticides were forbidden due to possible negative effects to human health, fauna and flora of the location. Natural epizootics of entomopathogenic fungi were observed in a residential zone of Bogota. Six districts were sampled to identify the entomopathogenic fungi. Environmental conditions were recorded from each sample site. Morphological characterization was developed for mycosed insects and isolates. Six nuclear loci were amplified: small subunit ribosomal RNA nu-rSSU, large subunit ribosomal RNA nu-rLSU, translation elongation factor 1-alpha-like (tef1), RNA polymerase I (B) subunit (Rpb1), RNA polymerase II (B) subunit (Rpb2) and internal transcribed spacer 1 5.8S ribosomal (nrITS). A phylogeny using Maximum Likelihood was performed for nrITS amplified sequences to identify the fungal specie, and a phylogenetic tree was constructed to place fungus in the Cordycipitaceae family. Lecanicillium sabanerum sp. nov. was proposed as a natural control agent of Parthenolecanium sp. and was placed in Cordycipitaceae family with bootstraps of 95 and 100 according to nrITS tree and phylogenetic tree, respectively. The influence of particulate matter concentration and other characteristics observed in the field on the incidence of entomopathogenic fungi in the city was discussed.

OBJETIVOS DEL TRABAJO DE GRADO:

The aim of this research is the isolation and identification of entomopathogenic fungi specie that is a natural control agent of Parthenolecanium sp. in Bogotá. In addition, we aim to determine the environmental conditions that favor behavior of the fungus as biological control agent.

METODOLOGÍA DEL TRABAJO DE GRADO:

Six districts were sampled to identify the entomopathogenic fungi. Environmental conditions were recorded from each sample site. Morphological characterization was developed for mycosed insects and isolates. Six nuclear loci were amplified: small subunit ribosomal RNA nu-rSSU, large subunit ribosomal RNA nu-rLSU, translation elongation factor 1-alpha-like (tef1), RNA polymerase I (B) subunit (Rpb1), RNA polymerase II (B) subunit (Rpb2) and internal transcribed spacer 1 5.8S ribosomal (nrITS). A phylogeny using Maximum Likelihood was performed for nrITS amplified sequences to identify the fungal specie, and a phylogenetic tree was constructed to place fungus in the Cordycipitaceae family.

CONCLUSIONES DEL TRABAJO DE GRADO:

Morphological characteristics of collected fungi place them in the Lecanicillium genus, reported pathogens of hemipteran insects. Fungi belong to a single species, with a bootstrap that separate them from brother species (Akanthomyces aculeatus, Cordyceps tuberculta, Lecanicillium attenuatum, Cordyceps confragosa, Isaria farinosa and Cordyceps cocciodioperitheciata). Multilocus phylogenetic tree indicate that the fungus is placed in
Cordyceps s.s. clade (Cordycipitaceae family). Lecanicillium sabanerum sp. nov. is proposed as natural control agent of Parthenolecanium sp. and it has a great potential as biological control agent.

PM seems to be a determinant factor in L. sabanerum sp. nov. incidence. Although both precipitations and humidity are not definitive factors, both shadow and humidity can generate propitious microhabitats for fungal growing and eventuality control of scale insects on a tree. Presence of moss and/or lichen likely may avoid those scale insects to feed from Ficus Tree. Morphology and physiology of coccids, and cultural practices ease their dissemination on Ficus population. Cultural practices seem to be affecting the L. sabanerum proliferation too.

An epidemiological study of diseased caused by scale insects is recommended for setting a correct cleaning program in Bogota city. A suitable cleaning program will avoid a rapid dissemination of disease and permit a better control with entomopathogenic fungi. This cleaning program could include a pruning that provides microhabitats of shadow and humidity to maintaining recycling and persistence of L. sabanerum. On the other hand, pathogenicity test of isolated strains is important to guaranteeing a good biological control. Finally, a chemical analysis of produced exudates is recommended to determine the metabolic content, which may be advantageous for the industry.

*PALABRAS CLAVES (TEMAS) DEL TRABAJO DE GRADO:

Natural control, Entomopathogenic fungi, Parthenolecanium, Ficus soatensis, Lecanicillium.

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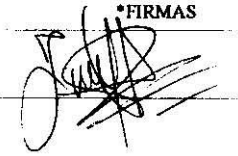
Juan Sebastián Chiriví Salomón	

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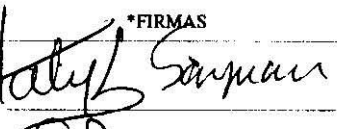
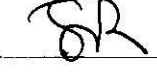
Tatiana Ibeth Sanjuan Giraldo	
SILVIA RESTREPO	

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