

Morphological, pathogenic and genetic diversity of *Botrytis cinerea* Pers. in blackberry cultivations in Colombia

Liliana Isaza¹, Yenni Paola Zuluaga² y Marta Leonor Marulanda³

Abstract—The Andean blackberry (*Rubus glaucus* Benth) is one of the most important fruits with the greatest commercial projection in Colombia. The factors that most affect its production are the attack of diseases and the scarce information about its control. One of the most limiting diseases of the crop is the gray mold, produced by the fungus *Botrytis cinerea*. This research presents the diversity of *B. cinerea* isolates from fields of andean blackberry in Colombia, based on the morphological, pathogenic and genetic characteristics allowing broadening the knowledge of the phytopathogen for subsequent management measures. Andean blackberry fruits were collected with characteristic symptoms of gray mold in farms located in eight producing areas of Colombian Andean region. A total of 50 samples were analyzed finding two types of growth, micelial and sclerocial, differences in the layout and size of the sclerotia, as well as differences in the daily growth of the mycelium. Regarding genetic characterization with microsatellite markers, it was shown that genetic diversity is concentrated within populations and that there is a tendency to group by geographical origin. With the pathogenicity tests, the two most pathogenic isolates were selected and it was found that, although the four possible genotypes were found with the transposable elements (Vacuma, Transposa, Boty, Flipper), none of them presented high resistance to the fungicide fenhexamide.

Index terms: Gray mold, *Boty*, *Flipper*, fungal resistance.

Diversidade morfológica, patogênica e genética de *Botrytis cinerea* Pers. em lavouras de amora-preta var. Castilla na Colômbia

Resumo—A amora-preta (*Rubus glaucus* Benth) é uma das frutas mais importantes e com a maior projeção comercial da Colômbia. Os fatores que mais afetam sua produção são o ataque de doenças e a pouca informação sobre o seu controle. Uma das doenças mais limitantes no cultivo é o mofo cinzento, produzido pelo fungo *Botrytis cinerea*. Esta pesquisa apresenta a diversidade de isolados de *B. cinerea* presentes nas culturas de amora-preta na Colômbia, com base em características morfológicas, patogênicas e genéticas, permitindo ampliar o conhecimento do fitopatôgeno para adotar medidas de manejo subsequentes. Frutos de amora-preta com sintomas característicos de mofo-cinza foram coletados em fazendas localizadas em oito departamentos produtores de amora-preta da região andina colombiana. Um total de 50 amostras foram analisadas, encontrando-se dois tipos de crescimento, micelial e esclerodial, diferenças na disposição e no tamanho dos escleródios, bem como diferenças no crescimento diário do micélio. Com relação à caracterização genética com marcadores microsatélites, demonstrou-se que a diversidade genética está concentrada no interior das populações e que há uma tendência de agrupar-se por origem geográfica. Com os testes de patogenicidade, os dois isolados mais patogênicos foram selecionados e ficou evidente que, embora os quatro genótipos possíveis tenham sido encontrados com os elementos transponíveis (Vacuma, Transposa, Boty, Flipper), nenhum deles apresentou alta resistência ao fungicida fenhexamida.

Termos para indexação: Mofo cinzento, Boty, Flipper, resistência a fungos.

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Introduction

The Andean blackberry (*Rubus glaucus*, Benth) is one of the most prominent fruits with the eminent commercial production in Colombia. It is cultivated by small and medium farmers, and is one of the primary sources of income, rural employment, and agro-industrial alternatives (BOTERO and FRANCO, 2007). The cultivation of the Andean blackberry (also known as the Mora de Castilla) has increased considerably in recent years with the increase in the national demand for the fruit for both fresh and agribusiness consumption, as well as for export. It is rapidly growing into a productive alternative, which is generating income for the producers (LA-ROTTA, 2001). Despite its recognized importance in generating income, this crop has seen little technological development. Its quality and productivity are highly variable, mainly due to the lack of recognized varieties and planting materials with genetic and phytosanitary qualities.

The attacks of diseases with scarce information about their control serve as the main factor that affects the production of this crop. One of the most limiting diseases of the crop is gray mold, which is caused by the fungus, *Botrytis cinerea* (DASHWOOD AND FOX, 1998; MOLINA et al. 2004). This disease is considered to be one of the most common and serious diseases caused by the genus *Rubus*. It affects another 200 crops of agricultural importance (MOLINA et al. 2004; ZHOU et al. 2014).

Tamayo and Peláez (2000) have reported production losses between 50% and 76% in blackberry fruit in Colombia due to this etiological agent. Recent studies have identified the existence of multiple cryptic species that are identified by a single species name (FEKETE et al. 2012). Thus, *Botrytis cinerea* is considered to be a complex system of species, instead of a single variable organism in which two sympatric groups have been identified by multiple gene genealogies (FOURNIER et al. 2005). These factors have created considerable genetic variation within the population, thereby affecting the phenotypic characteristics and reproduction modes and also causing an increase in the adaptability and structure of the populations (GIRAUD et al. 1997; PANDE et al. 2010). However, these groups exhibit marked differential characteristics that can be used as phenotypic markers. Recent studies have concluded that *B. cinerea* Group I is resistant to the fungicide Fenhexamid and has a characteristic volume of asexual spores (FEKETE et al. 2012), whereas *B. cinerea* Group II has reported sensitivity to Fenhexamid (ISENEGGER et al. 2008).

Additionally, the species that make up the complex *B. cinerea* system have revealed the presence of two transposons, *Boty* and *Flipper*, in their genomes. This, in turn, creates another mode of taxonomic grouping and increases the variability in species (PANDE et al. 2006),

thereby making it possible to distinguish the groups, *B. cinerea* variety *Transposa* (*Bct*), which has one or both of the two transposable elements, and the group, *B. cinerea* variety *Vacuma* (*Bcv*), which does not have either of the elements (ZHOU et al. 2014). As a result, these groups have displayed a remarkable geographical distribution pattern since the isolation of species associated with the *Boty* transposon has been reported mainly in Europe and Australia, whereas the isolation of species associated with the *Flipper* transposon has only been detected in South Eastern Europe (VERCESI et al. 2014). In addition, Beever and Weeds (2004) state that there are no species, which belong to Group I, that have been detected in Australia and Southern Asia; however, there are two cryptic species present in Europe and America. Therefore, a clear relationship is observed between Group I of *B. cinerea* and the content of transposable elements.

These factors create the need for studies whose aim is to measure and characterize the genetic diversity of local species of *B. cinerea*. In addition, these studies need to define the best management strategies for this phytopathogen (KUMARI et al. 2014). Although, it has been concluded that the application of fungicides does not induce transposon distribution patterns (VERCESI et al. 2014); however, the excessive application of these products mediates the occurrence of resistance phenomena and increased pathogenicity in fungi such as *B. cinerea* (ALVAREZ et al. 2017; GEEP et al. 2012).

Although there have been numerous studies of the morphological, pathogenic, and genetic diversities of *Botrytis cinerea* in different parts of the world (FEKETE et al. 2012; ZHOU et al. 2014; VERCESI et al. 2016), there are no reports that indicate the current status of this phytopathogen in Colombia associated with Andean blackberry crops. The purpose of this study is to show the diversity of *B. cinerea* isolates present in the Andean blackberry crops in Colombia on the basis of morphological, pathogenic, and genetic characteristics which allow for increased awareness of the phytopathogen. As a result, measures would be identified regarding its control.

Materials and Methods

Plant material

Andean blackberry fruit with the characteristic symptoms of gray mold (Figure 1) were collected from the farms located in eight blackberry-producing departments of the Colombian Andean region. In total, 50 samples were obtained and labeled according to their place of origin (Table 1). The farms visited were located at an altitude of 1,800–2,200 m, with an average temperature of 16°C and annual rainfall exceeding 2,000 mm.



Figure 1. Gray mold produced by *Botrytis cinerea* in blackberries.

Pathogen isolation

The infected fruit was taken to the Plant Biotechnology Laboratory of the Universidad Tecnológica de Pereira. Small slices of the diseased tissue were acquired from the fruits that had the greatest sporulation, and four fragments of the tissue were placed in the petri dishes with potato dextrose agar (PDA) and incubated at 23°C in the dark. All samples were streak seeded until the sporulation of the fungus was achieved. Finally, the monosporic cultures of each of the isolates were carried out following the methodology proposed by CASTELLANOS et al. (2011) with slight modifications.

Identification and morphological characterization

The isolates were identified on the basis of their phenotypic characteristics, such as appearance and coloration of the mycelium along with the microscopic observations of conidiophores and conidia, which corresponded to the previously reported descriptions (CROUS et al. 2009; ELLIS, 1971) in the case of *Botrytis cinerea*. The isolates, previously visualized by optical microscopy, were observed by using a FEI brand scanning electron microscope of Quanta 250 model (Thermo Fisher Scientific).

The *in vitro* growth rate was determined by transferring a disc of fungal material with a defined diameter (5 mm). Thereafter, ten replications of each of the isolates were prepared in which daily mycelium growth (horizontal and vertical) was recorded. These measurements were carried out until the total surface of the petri dish (92 mm) was covered by the fungus. The average growth rates of each of the growths were determined for the purpose of statistical analyses.

To define the type of mycelium, three aspects were defined after 20 days of fungus growth: sparse mycelium (0), erumpent mycelium (1), and mycelium with mass growth (2) (MARTÍNEZ et al. 2003).

The distribution of the sclerotia of each of the isolates was observed on the 20th day after inoculation in a PDA medium. A total of ten petri dishes were selected for each isolation. These petri dishes were classified based on the types of distribution suggested by (TANOVIĆ et al. 2009), with some modifications: (E1) sclerotia only on the edge, (E2) sclerotia arranged radially, (E3) large and irregularly distributed sclerotia, (E4) numerous small and scattered sclerotia, and (E5) without sclerotia. In terms of size, for each isolation, five petri dishes were selected and ten sclerotia from each one were chosen at random. Each of them were measured, and the data were subsequently averaged for the respective statistical analyses.

On the 20th day after seeding, the mycelium colors of each of the isolates were evaluated. For this purpose, the RAL color chart (SANHUEZA, 2012) was employed as a reference, and a number was assigned to each isolate according to its color and intensity.

Genetic characterization

DNA extraction

The mycelium from each of the isolates was extracted after 20 days of growth in the PDA medium by using the commercial Plant DNeasy Mini Kit (QIAGEN) after following the manufacturer's instructions.

Sample characterization with microsatellite (SSR) markers

Genetic characterization with microsatellite molecular markers was performed via PCR amplification of DNA samples from each of the isolates. As previously reported by Fournier et al. (2002), nine microsatellite primers were used for this purpose.

The amplification reactions were prepared in a final volume of 12.5 µL with 0.3 µM of each of the primers, 15 µM of each dNTP, 1 X of reaction buffer (10 mM of Tris HCl, 50 mM of KCl), 1 U of Taq polymerase, and 2 mM of MgCl₂ and 10 ng/µL of DNA. A 32-cycle touchdown PCR program was performed with denaturation at 95°C

for 60 seconds; pairing for 60s with a decrease of 1°C in every two cycles from 64°C to 59°C, 10 cycles at 58°C, and 10 cycles at 57°C; elongation at 72°C for 60 seconds; and a final extension at 72°C for 5 min. The separation of the amplified products was carried out by electrophoresis in denaturing gels of 6% polyacrylamide. These amplified products were subsequently stained with silver nitrate following the protocol set out by Benbouza et al. (2006).

Evaluation of the pathogenicity of *B. cinerea* isolates

The pathogenicity test was performed by inoculating the healthy ripe Andean blackberries for the determination of the latency period and incubation of the fungus in each of the isolates. A concentration of 1.2×10^6 ml⁻¹ conidia was used for these tests, and a suspension of fungus spores was prepared in sterile and distilled water. Healthy fruits were disinfected by using 70% alcohol for 2 minutes. Thereafter, the fruits were rinsed consecutively for three times with sterile and distilled water. The fruits were then submerged in 1% hypochlorite and again rinsed consecutively for three times with sterile and distilled water. As a control, some fruits were inoculated with sterile water. All fruits were punctured with a sterile needle and, then, sprinkled with the fungus suspension. The sprinkled fruits were kept in humid chambers at the room temperature, with observations taken every 24 hours. Subsequently, a rating was given to each fruit following a severity scale of 1–4, until the fruit was totally affected. The score of 1 was synonymous with a healthy fruit and 4 was synonymous to the fruit with the presence of mycelium greater than 75% of its area.

Each of the fungi belonging to each sampling area was inoculated. For each test, five repetitions were performed for each five fruits. Each of the fruits was subsequently evaluated with the aforementioned scale, and, finally, the two most pathogenic isolates were selected.

Determination of the presence/absence of *Boty* and *Flipper* transposons in *B. cinerea* isolates

The presence of associated transposable elements (*Boty* and *Flipper*) was evaluated by using primers LTR98 (5'-AGCCTGTAGAATCACCAACG-3') and LTR728 (5'-CGGTATTTCTGGTTGGCA-3) for *Boty* (FERRADA et al. 2016), and F300 (5'-GCACAAAACCTACAGAAGA-3') and F1550 (5'-ATTCGTTTCTTGGACTGTA-3) for *Flipper* (MA AND MICHAILIDES, 2005). The reaction conditions consisted of a final volume of 12.5 µL (0.4 µM of each of the primers, 0.2 mM of each dNTP, 1 X of reaction buffer (10 mM of Tris HCl, 50 mM of KCl), 1 U of Taq polymerase, 2 mM MgCl₂, and 10 ng/µL of DNA) and the touchdown temperature profile that is described above. The purified products were visualized on a 2% agarose gel.

Determination of the susceptibility of *B. cinerea* isolates to Fenhexamid

For the antifungal test, the individual isolates were seeded in the PDA agar seven days before the test until sporulation. This determination was developed in a 96-well plate with successive dilutions, after following the methodology previously proposed by (MENDES et al. 2016; TROSKIE et al. 2012), with slight modifications.

To perform this task, 200 µL of Fenhexamid was placed in the first column at a concentration of 150 ppm. Then, 200 µL of sterile YMG broth was poured into the rest of the plate (columns 2–12). Subsequently, the microdilution of the samples was continued. For this process, 100 µL of the sample column was taken and, then, deposited in column 2. It was again stirred, and 100 µL was taken from this column for deposition in the next column. The process was repeated sequentially until column 10 was filled completely; the excess volume was discarded. Finally, the growth and sterility controls were arranged in the last two columns (11 and 12), respectively. Thus, the concentrations to be evaluated were between 50 µg mL⁻¹ and 0.0076 µg mL⁻¹ by considering the serial dilutions with a dilution factor of 3. The optical density was measured immediately after inoculation (zero time) at 595 nm in a Thermo MultiSkan GO microplate reader. The plate was incubated at 25°C for 48 hours, and the optical density was measured again.

Growth control is the value from which the percentage of inhibition exerted by Fenhexamid is calculated.

Statistical analysis

All morphological and pathogenic characterization analyses were performed by using the Past software (Paleontological Statistics Software Package for Education and Data Analysis) (HAMMER et al. 2001) and InfoStat, which complied with the normality assumptions. Allelic genotyping was obtained by analyzing the polymorphic bands with the GenAlex 6 program (PEAKALL AND SMOUSE, 2006), which estimated the measures of diversity, variability and genetic distance. The cluster analysis was performed by using the UPGMA method, and a dendrogram was generated by using the Dice coefficient with the Past statistical package (HAMMER et al. 2001).

In the Fenhexamid susceptibility tests, the logarithm of the Concentration versus Percentage of Inhibition was plotted based on the obtained data and means of a linear regression. In addition, the value of x was determined in order to make the dependent variable equal to 50 (50% inhibition). Finally, the EC₅₀ was calculated on the basis of the logarithmic transformation performed on the concentration.

Results and discussion

In total, 50 isolates were identified as *B. cinerea* based on the macroscopic and microscopic phenotypic characteristics, such as conidiophores and hyaline conidia, long and abundant gray mycelium, branched conidiophores with rounded apical cells in clusters, colorless or gray, unicellular and with the presence of ovoid conidia. Conidiophores and conidia clusters resembled a bunch of grapes, which was described in previous studies (AGRIOS, 2005; CROUS, 2009; ELLIS, 1971) (Figure 2). Table 1 presents the collection sites and a summary of the morphological, molecular, and pathogenic characteristics.

B. cinerea is a species, which shows a great variability in its morphology, sporulation intensity, mycelial growth, enzymatic production, fungicide resistance, along with great genetic diversity (CHARDONNET et al. 2000; LEROUX et al. 2004).

Morphological characterization

In total, 58.7% of the isolates reported sparse mycelium, 39.1% of them reported erumpent mycelium, and 2.2% showed mass growth (Table 1) (Figure 3).

It was possible to observe the three types of mycelial appearance described by Martínez et al. (2003) in the 50 isolates, which correspond to short, aerial, and cottony mycelium. Xie et al. (2016) along with other authors have reported only two types of appearances: woolly and mass mycelial in bean and mustard crops in China. Other authors, such as Nuñez et al. (2013), have only reported a cottony appearance in *B. cinerea* isolates in the cucumber crops from Mexico.

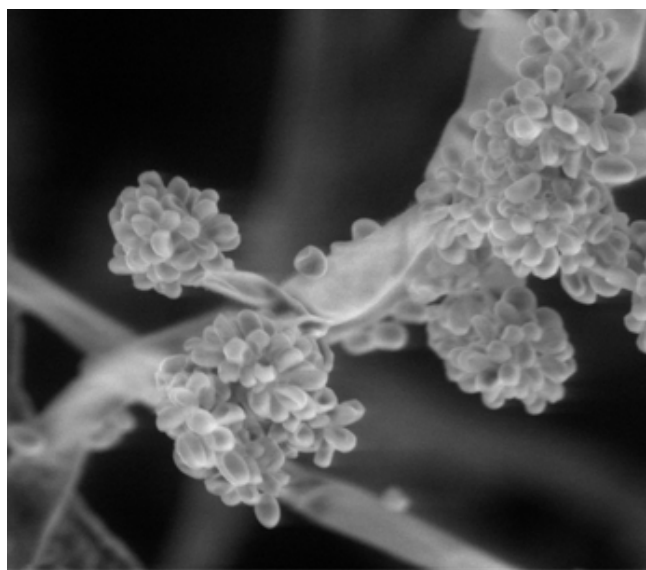


Figure 2. Branched conidiophores with rounded apical cells in clusters of *Botrytis cinerea*. Image seen with a scanning electron microscope



Figure 3. Type of mycelium growth observed in the isolates of *Botrytis cinerea*. A. Sparse mycelium; B. Erumpent mycelium; C. Mass growth

Table 1. Identification and the collection place of the *B. cinerea* isolates.

Isolates	Department	Georeference	Sclerotia distribution ^a	Type of mycelium ^b	Transposable elements ^c	EC50 $\mu\text{g/mL}^d$
RQB1	Risaralda/Quinchía1	W 75° 45' 23,6" N 5°21' 12,2" 5°21'	E2	0	Vacuma	<0.1 HysS
RQB2	Risaralda/Quinchía2	W 75° 45' 25,2" N 5° 21' 14,7"	E4	0	Vacuma	<0.1 HydS
RQB3	Risaralda/Quinchía3	W 75° 45' 25,2" N 5° 21' 12,4"	E5	1	Transposa	0.15 HydR3
RGB1	Risaralda/Guática1	W 75° 47' 20,7" N 5° 17' 26,9"	E3	0	Vacuma	<0.1 HydS
RGB2	Risaralda/Guática2	W 75° 47' 25,9" N 5° 17' 36,3"	E3	0	Vacuma	<0.1 HydS
RGB3	Risaralda/Guática3	W 75° 47' 27,1" N 5° 19' 07,0"	E4	0	Transposa	<0.1 HydS
RBUB1	Risaralda/Belén de Umbria1	W 75° 54' 43,1" N 5° 11' 10,6"	E3	1	Flipper	<0.1 HydS
RBUB2	Risaralda/Belén de Umbria2	W 75° 54' 55,4" N 5° 11' 05,3"	E3	0	Vacuma	<0.1 HydS
RSRB1	Risaralda/Santa Rosa1	W 75° 55' 15,7" N 5° 11' 13,0"	E4	1	Transposa	<0.1 HydS
RSRB2	Risaralda/Santa Rosa2	W 75° 33' 39,5" N 4° 53' 19,64"	E5	2	Vacuma	0.13 HydR3-
RSRB3	Risaralda/Santa Rosa3	W 75° 33' 40,8" N 4° 53' 15,5"	E3	0	Vacuma	<0.1 HydS
RAB1	Risaralda/Apía1	W 75° 33' 51,3" N 4° 53' 11,9"	E5	1	Transposa	0.2 HydR3-
RAB2	Risaralda/Apía2	W 75° 59' 12,6" N 4° 08' 0,01"	E4	1	Vacuma	0.2 HydR3-
RAB3	Risaralda/Apía3	W 75° 59' 19,6" N 5° 07' 59,5"	E4	0	Vacuma	<0.1 HydS
RSB1	Risaralda/Santuario1	W 75° 59' 16,9" N 5° 0,08' 02,3"	E1	1	Vacuma	0.2 HydR3-
RSB3	Risaralda/Santuario2	W 75° 59' 29,7" N 5° 0,6' 33,7"	E3	0	Vacuma	<0.1 HydS
QGB1	Quindío/Génova1	W 75° 59' 45,7" N 5° 0,6' 37,2"	E3	0	Vacuma	0.2 HydR3-
QGB2	Quindío/Génova2	W 75° 46' 06,6" N 4° 16' 43,2"	E3	1	Vacuma	0.15 HydR3-
QFB1	Quindío/Filandia1	W 75° 46' 04,9" N 4° 16' 40,0"	E1	1	Flipper	<0.1 HydS
QFB2	Quindío/Filandia2	W 75° 37' 05,4" N 4° 41' 08,8"	E3	0	Vacuma	<0.1 HydS
QCB1	Quindío/Córdoba1	W 75° 37' 01,8" N 4° 41' 12,11"	E3	0	Vacuma	<0.1 HydS
QCB2	Quindío/Córdoba2	W 75° 39' 18,1" N 4° 24' 0,08"	E3	1	Vacuma	<0.1 HydS
CVB1	Caldas/Villamaría1	W 75° 39' 13,9" N 4° 24' 04,2"	E2	1	Flipper	<0.1 HydS
CVB2	Caldas/Villamaría2	W 75° 29' 39,1" N 4° 58' 00,1"	E1	1	Flipper	<0.1 HydS
CAB1	Caldas/Aguadas1	W 75° 29' 46,0" N 4° 57' 26,8"	E5	1	Transposa	1.3 HydR3-
CAB2	Caldas/Aguadas2	W 75° 27' 27,3" N 5° 34' 19,1"	E5	1	Transposa	0.13 HydR3-
CAB3	Caldas/Aguadas3	W 75° 27' 30,8" N 5° 35' 00,0"	E5	1	Vacuma	0.12 HydR3-
CPB1	Caldas/Pácora1	W 75° 27' 18,0" N 5° 35' 21,8"	E3	0	Vacuma	ND
CPB2	Caldas/Pácora2	W 75° 27' 48,8" N 5° 30' 32,5"	E3	1	Vacuma	<0.1 HydS
CUSB1	Cundinamarca/Silvania1	ND	E3	0	Vacuma	<0.1 HydS

CUSB2	Cundinamarca/Silvania2	W 74° 19' 32,6" N 4° 24' 21,0"	E3	0	Vacuma	<0.1 HydS
CUSB3	Cundinamarca/Silvania3	W 74° 19' 44,5" N 4° 25' 01,3"	E3	0	Boty	<0.1 HydS
CUSBB3	Cundinamarca/San Bernardo3	W 74° 19' 51,3" N 4° 25' 40,3"	E3	0	Vacuma	<0.1 HydS
CUFB1	Cundinamarca/Fusagasugá1	W 74° 23' 0,72" N 4° 08' 58,3"	E3	1	Vacuma	ND
CUFB2	Cundinamarca/Fusagasugá2	W 74° 21' 20,7" N 4° 17' 05,2"	E4	0	Vacuma	<0.1 HydS
CUFB3	Cundinamarca/Fusagasugá3	W 74° 21' 26,0" N 4° 15' 42,1"	E4	0	Vacuma	<0.1 HydS
SPB1	Santander/Pie de cuesta1	W 74° 21' 04,2" N 4° 14' 35,6"	E4	1	Vacuma	0.12 HydR3-
SPB3	Santander/Pie de cuesta3	W 72° 58' 50,1" N 7° 00' 31,6"	E4	0	Vacuma	<0.1 HydS
TCB1	Tolima/Cajamarca1	W 72° 59' 46,8" N 6° 59' 06,3"	E3	0	Flipper	<0.1 HydS
TCB2	Tolima/Cajamarca2	W 75° 30' 03,0" N 4° 22' 13,4"	E3	0	Vacuma	<0.1 HydS
TCB3	Tolima/Cajamarca3	W 75° 29' 50,4" N 4° 22' 29,9"	E3	0	Vacuma	0.2 HydR3-
ACB1	Antioquia/La ceja1	W 75° 29' 26,4" N 4° 22' 29,9"	E4	0	Flipper	0.5 HydR3-
AEB1	Antioquia/Envigado1	W 75° 26' 44,7" N 5° 58' 53,1"	E5	1	Transposa	<0.1 HydS
AEB2	Antioquia/Envigado2	W 75° 29' 26,3" N 6° 10' 56,0"	E1	1	Transposa	<0.1 HydS
AEB3	Antioquia/Envigado3	W 75° 29' 28,3" N 6° 10' 31,8"	E3	0	Transposa	<0.1 HydS
AGB1	Antioquia/Guarne1	W 75° 29' 27,8" N 6° 10' 32,1"	E3	1	Flipper	<0.1 HydS
AGB2	Antioquia/Guarne2	ND	E4	1	Flipper	<0.1 HydS
AGB3	Antioquia/Guarne3	W 75° 24' 49,7" N 6° 16' 35,4"	E3	0	Flipper	<0.1 HydS
VGB1	Valle del Cauca/Ginebra1	W 75° 24' 59,1" N 6° 16' 37,5"	E4	0	Vacuma	<0.1 HydS
VGB2	Valle del Cauca/Ginebra2	W 76° 12' 15,1" N 3° 16' 50,8"	E5	1	Flipper	0.14 HydR3-

^aE1 = sclerotia only on the edge, E2 = sclerotia arranged radially, E3 = large and irregularly distributed sclerotia, E4 = numerous and scattered small sclerotia and E5 = no sclerotia.

^b0 = sparse mycelium, 1 = erumpent mycelium and 2 = mycelium with mass growth

^cTransposa = strain with the two Boty and Flipper transposable elements present, Vacuma = strain that lacks the two transposable elements, Flipper = strain with only the Flipper transposable element, and Boty = strain with only the Boty transposable element.

^dValues of $EC_{50} < 0.1$ correspond to strains susceptible to Fenhexamid (HydS) and values of $EC_{50} > 0.1$ and < 3 correspond to strains moderately resistant to Fenhexamid (HydR3).

Statistically significant differences were observed in the growth of the fungus cultured in PDA agar. The isolates from Guarne (Antioquia) were the reported to be the fastest growing; their growth was registered on the third day. Their horizontal and vertical growths were reported to have average values of 74.4 mm and 73.7 mm, respectively, whereas the QCB1 isolates grew the slowest, with average values of 23 mm and 24.7 mm (horizontal and vertical). Tanović et al. (2009) reported growth rates

from the third day ranging from 7.3 mm to 26.7 mm/day by using the same culture medium. Other authors, such as Chardonnet et al. (2000) and Mirzaei et al. (2008), also reported heterogeneities in the growth rates of *B. cinerea*. We also observed that the second and third days reported the highest growth rates.

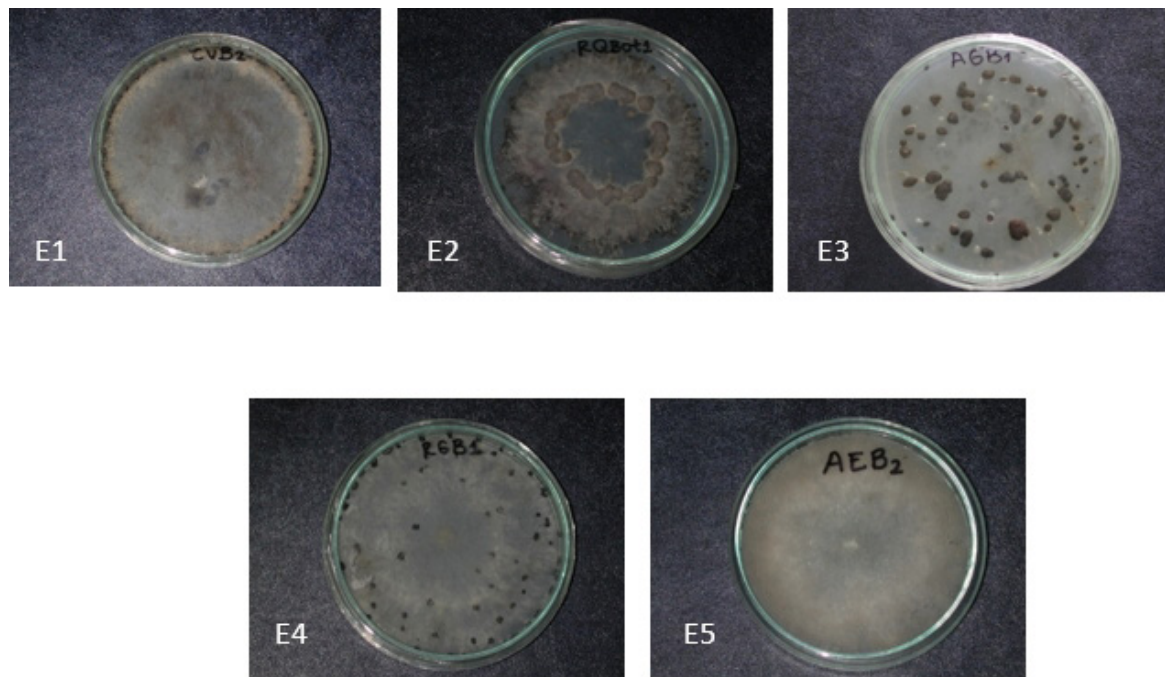


Figure 4. The distribution of sclerotia observed in the *Botrytis cinerea* isolates

Regarding the distribution of sclerotia, 43% of the isolates showed randomly distributed large sclerotia (E3), 22% showed randomly distributed small sclerotia (E4), and the same percentage (22%) of isolates did not show sclerotia (E5). 7% of the isolates had an E1 classification corresponding to sclerotia, which was arranged only at the edge of the petri dish. On the contrary, 3% of the studied isolates showed radially distributed sclerotia (E2) (Figure 4).

Martínez et al. (2003) reported the distributions of random sclerotia in circles or on the edge of the petri dish, whereas Tanović et al. (2009) referred to mycelial and sclerotial growths, which depended on the absence or presence of sclerotia. These researchers reported that 38.7% of the isolates lack sclerotia. However, in our investigation, 22% of the isolates corresponded to category E5 (no sclerotia). Sclerotia are considered to be the most important structures for fungal survival (*Botrytis* spp). All *Botrytis* species form sclerotia, which can vary in size and shape depending on the host and environmental conditions. The production, size, and shape of sclerotia in a natural substrate is extremely fluctuating unlike a culture medium. Due to this reason, some isolates of the fungus do not form sclerotia, whereas others abundantly produce sclerotia (AGRIOS, 1988).

Regarding the size of the sclerotia, we find that for a value of $p=0.0001$, the ANOVA analysis suggests that the hypothesis of equality of means between the isolates is rejected. According to Fisher's LSD test, there are statistically significant differences between the different groups; the isolates of CUSBB3, RQB1, QGB2, RSB3, TCB3, and QGB1 have shown sclerotia of larger sizes. The RAB2 isolate showed the smallest sclerotia with an

average of 1.5×1.53 mm and the largest with the value of 5.93×4.58 mm were found in the CUSBB3 isolate.

Using the RAL color chart as a reference, the colors shown in all of the isolates are gray and brown in different shades, with gray tones being the most predominant. All of the isolates showed shades described by *B. cinerea*, which is initially whitish. Later, different shades of gray and brown were also shown (KHAZAELI et al. 2010; XIE et al. 2016).

Genetic characterization

Analysis of microsatellite molecular markers

The genetic characterization of the *B. cinerea* isolates performed with nine pairs of primers obtained an amplification of 12 loci. The population with the highest number of alleles was Risaralda (8.083), whereas Santander showed the lowest number of alleles. The expected heterozygosity (H_e) was also higher in Risaralda (0.808), followed by the Caldas population (0.764). The lowest heterozygosities were registered in the departments of Santander and Valle (Table 2).

The populations of *B. cinerea* have remarkable levels of genetic diversity. Rajaguru and Shaw (2010) analyzed nine microsatellite loci in the strains of *B. cinerea* isolated from four plant genera in Southern England and concluded that the isolated strains from strawberries were very different from the isolated blackberry strains. However, both hosts belong to the rosacea family, and their mechanisms of infection and fruit ripening are similar, which could suggest that disease cycles in the separate hosts in the same region may be weakly linked. By using the same microsatellites, Karchani et al. (2008) concluded that the population of this fungus in Tunisia reproduces

mainly through sexual reproduction. In this investigation, only one host (the Andean blackberry) was studied and the form of reproduction was not contemplated.

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Table 2. Parameters of genetic diversity in *Botrytis cinerea* isolates.

Parameter	Risaralda	Quindío	Caldas/ Villamaría	Cundimamarca	Santander	Tolima	Antioquia	Valle
Number of alleles (Na)	8,083 ± 0,645	4,583± 0,336	5,333± 0,284	5,250± 0,479	2,917± 0,260	3,500± 0,337	5,333± 0,333	3,000± 0,246
Number of effective alleles (Ne)	5,708± 0,514	3,464± 0,194	4,368± 0,233	4,028± 0,316	1,011± 0,261	3,168± 0,289	3,795± 0,294	2,856± 0,263
Expected heterozygosity	0,808± 0,017	0,702± 0,016	0,764± 0,011	0,734± 0,021	0,615± 0,032	0,653± 0,032	0,716± 0,025	0,615± 0,036

The ANOVA analysis (Table 3) allows for the identification of the greatest variability that exists within each population with 84% variation and, to a lesser extent, between populations (16%). By using nine microsatellite markers, Isenegger et al. (2008) studied the genetic diversity of *B. cinerea* in Bangladesh and concluded, as in this research, that genetic diversity is concentrated within populations and there is a little difference between populations in different regions.

In the case of Colombia, there are two significant geographical barriers (the bifurcation of the Andes mountain range) that separate the sampling sites, which would allow us to infer that the geographical factor did not impose a specific level of structuring in *B. cinerea*, with larger barriers perhaps necessary to observe the differentiation between populations. Isenegger et al. (2008) found that genetic differentiation between the

regions of Southern Asia and Australia has indicated that geographical barriers and possible events of founder or bottleneck effects have played a significant role in the genetic differentiation between the *B. cinerea* populations of these regions. This could have implications for disease management strategies based on the host resistance between these regions (PANDE et al. 2006). Walker (2016) stated that, in microsatellite studies, indexes of differentiation or analysis, the hosts are recognized as the most powerful structuring factor for the populations of *Botrytis* sp, rather than geography. In that sense, populations collected from the same host in distant regions may be more genetically similar than the populations collected from different hosts within the same region.

Table 3. ANOVA analysis of Colombian *Botrytis cinerea* isolates.

Molecular variance	Degrees of freedom	Sum of squares	Mean Squares	Statistical variation	Percentage
Between populations	7	123,586	17,655	1,592	16%
Within populations	42	351,134	8,360	8,360	84%
Total	49	474,720		9,952	100%

For the nine microsatellites used in this study, it was found that the number of different alleles (N_a) ranged from four to seven (Table 3). Rasiukevičiūtė et al. (2018) reported between 9 and 23 alleles by using four markers (Bc2, Bc6, Bc7, and Bc10) with an average of 13.25 alleles per marker. Fournier and Giraud (2008) found between 3 and 6 alleles per marker as observed in this investigation. The heterozygosities observed for loci Bc1, Bc2, Bc4-1, and Bc10-1 provided values of 1, thereby indicating that all the isolates collected were heterozygous for these markers.

Walker (2016) reported that a single allele of 86-bp in the Bc6 locus serves as a diagnostic tool for determining *B. cinerea* Group I. In this study, none of the isolates were presented with this allele; therefore, we can infer that all of the samples belong to *B. cinerea* Group II.

The main coordinate analysis (Figure 5) shows a tendency for the isolates to be grouped by the geographical area. A large group conforms to the majority of the coffee axis isolates (Caldas, Quindío, and Risaralda), whereas another group conforms to the isolates from Santander, Antioquia, and Tolima. In addition, a small group presents the place where most of the Cundinamarca isolates are found.

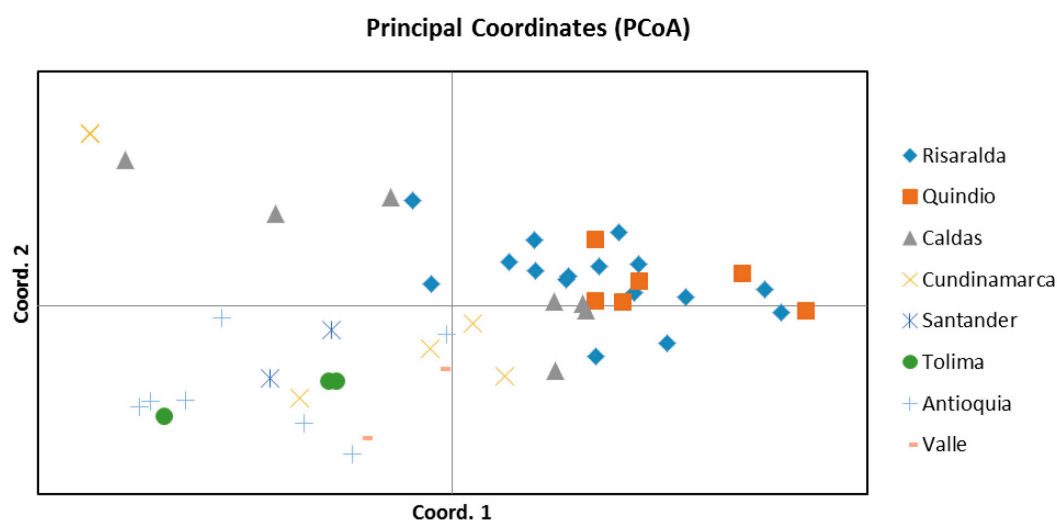


Figure 5. Analysis of the main coordinates of *Botrytis cinerea* isolates

Evaluation of the pathogenicity of *B. cinerea* isolates

The results of the pathogenicity tests show us that a value of $p=0.0001$ in the ANOVA suggests that there are statistically significant differences between the isolates, with RQB3 and CUSB3 being the most pathogenic. On the fourth day of inoculation, both isolates reached Level 4 on the severity scale. Similar results were obtained by Núñez et al. (2013), who conducted tests on cucumber fruits that showed the symptoms of dark gray rot and abundant fungus sporulation on the sixth day after inoculation of the fruits. On the contrary, Li et al. (2016) reported that symptoms of the disease were apparent from the third day of the inoculation of *Botrytis cinerea* in beans.

The RQB3 isolation belongs to the *Transposa* genotype and has a mycelium with erumpent growth and no sclerotia-forming capacity, whereas CUSB3 is the only one of the isolates found in this research that has a *Boty* genotype. It has erumpent mycelial growth and has large and irregularly distributed sclerotia. With this observation, we can show that there are no direct relationships between morphological and pathogenic characteristics. *B. cinerea* shows a high degree of diversity, thereby making it the species where it is difficult

to establish relationships between pathogenicity and other characteristics (REBORDINOS et al. 2000).

Determination of the presence/absence of *Boty* and *Flipper* transposons in *B. cinerea* isolates

Fungi possess a variety of extrachromosomal genetic elements such as mitochondrial chromosomes, plasmids, and transposable elements (ROSEWICH AND KISTLER, 2000). One of the reasons for the variations in population dynamics in *B. cinerea* is the presence of mobile elements called transposons (ELAD et al. 2007). Based on the presence or absence of the *Boty* and *Flipper* transposable elements, we were able to ascertain that there were four possible types of genotypes in the population of *Botrytis cinerea*. *Transposa* (with the presence of the two elements), *Vacuma* (without the presence of these elements), *Boty* or *Flipper* (only one of the two elements present). Therefore, 58% of the studied isolates belong to *Vacuma*, 22% to *Flipper*, 18% to *Transposa*, and only 2% correspond to *Boty*. Li et al. (2016) reported that all of the isolates studied in China belong to the *Transposa* genotype, whereas Isenegger et al. (2008) reported the types of transposons for Bangladesh: 69% were only *Flipper*, 24% *Transposa*, 2% were only *Boty*, and 1%

Vacuma. For India/Nepal, 72% were *Boty*, 22% were *Flipper*, 6% were *Transposa*, and 0% were *Vacuma*. Kumari et al. (2014) reported the following distribution frequencies in India: 49% *Boty*, 23% *Transposa*, 15% *Flipper*, and 13% *Vacuma*. These authors also determined that the distribution of transposable elements have different characteristics, by considering the hosts and geographical location. For example, in Northern India, there are four types of genotypes, whereas in the center of Nepal, they only found *Boty* genotypes. As for the hosts, the 33% of chrysanthemum isolates had *Transposa* and *Vacuma*, whereas 17% of chrysanthemum isolates had *Boty* and *Flipper*. In lentils, the *Flipper* and *Transposa* genotypes were not found. All strawberry isolates belonged to the *Boty* molecular type, whereas in roses, the *Transposa* isolate was found most often, followed by *Boty* and *Flipper*, with the absence of *Vacuma*. Studies with transposable elements have shown that the *Transposa* genotype predominates in the populations of grapes, strawberries, and tomatoes, whereas *Vacuma* dominates in the populations of kiwi (fruit) and apples (ESTERIO et al. 2011; JOHNSTON et al. 2013; MARTINEZ et al. 2005; SAMUEL et al. 2012). However, there is presently no clear explanation for these observations (WALKER, 2016)

This investigation, which was carried out in the area known as the Colombian Andean region, found four types of genotypes in a single host – the Andean blackberry or the Mora de Castilla. This result is of the utmost importance for the adequate management of the crops. By considering the studies carried out in vineyards and on other crops, the *Transposa* genotype is characterized as being more stable and homogeneous and presents with a more parasitic character than the *Vacuma* genotype. *Vacuma* is less stable and has a more saprophytic character. In Chile, it has been detected in plants that are not subjected to high fungicide pressure. On the contrary, *Boty* is present in a smaller proportion than the first two, but it occurs in plants subjected to higher fungicide pressure (ESTERIO et al. 2007). In addition, it has been determined that *Botrytis* genotypes would have a differential sensitivity response to some botryticides. For example, only the *Transposa* and *Flipper* isolates have some level of resistance to Iprodione (dicarboximide), whereas *Vacuma* and *Boty* have always been sensitive to this fungicide (GIRAUD et al. 1999; ESTERIO, 2005). In relation to anilinopyrimidines (Pyrimethanil, Cyprodinil) and carboxamides (Boscalid), partial results do not yet indicate a marked differential effect toward a certain genotype (ESTERIO et al. 2006).

Determination of the susceptibility of *B. cinerea* isolates to Fenhexamid

Fenhexamid is a widely used fungicide globally for the control of *Botrytis*. It is a sterol biosynthesis inhibitor

used in the control of gray mold disease. Studies have reported that gray mold disease is caused by a complex of two sympatric species: *Botrytis* Group II (*Botrytis cinerea sensus stricto*) and *Botrytis* Group I (*Botrytis pseudocinerea*) (FOURNIER, et al. 2005). Suty et al. (1999) reported that the latter metabolizes Fenhexamid more quickly than *Botrytis cinerea*. The natural resistance of fungus to Fenhexamid is associated with mutations in the *erg27* gene that codes for the enzyme 3-keto reductase (ALBERTINI AND LEROUX, 2004; FILLINGER et al. 2008). The fungus' resistance to Fenhexamid has been classified into categories: HydS (Fenhexamid sensitive) HydR1, HydR2 and HydR3 (resistant to Fenhexamid) (ALBERTINI AND LEROUX, 2004; LEROUX, 2004). HydR3 was subdivided into HydR3 + and HydR3- by Fillinger et al. (2008). One of the ways to evaluate this classification is by taking into account the EC_{50} of germination and tube elongation and mycelial growth *in vitro*. With our methodology, EC_{50} values allowed us to classify the isolates as HydS with $EC_{50} < 0.1 \mu\text{g/mL}$ and HydR3- isolates with EC_{50} between > 0.1 and $< 3 \mu\text{g/mL}$ (FILLINGER, et al. 2008).

In total, 68% of the isolates studied are susceptible to Fenhexamid (HydS) and 34% have a moderate resistance to the fungicide (HydR3-) (Table 1). In the first group (HydS), we found 20 isolates of the *Vacuma* genotype, 8 *Flipper*, 5 *Transposa*, and the only *Boty* genotype was reported in this investigation. In the isolates with intermediate resistance to Fenhexamid (HydR3-), we found eight of the *Vacuma* type, four of *Transposa* type, and two of *Flipper*. Albertini and Leroux (2004) reported that only the *Vacuma* and *Transposa* genotypes were susceptible isolates (HydS), and only *Transposa* genotypes (HydR2 and HydR3) were resistant isolates. As already mentioned, there are different characteristics, such as hosts, geographical position, seasonal periods and submission to fungicides, that are related to the transposable elements (ISENEGGER et al. 2008; ESTERIO et al. 2007; LI et al. 2016). In addition, it is important to bear in mind that this study was carried out in a tropical area, where optimal conditions for the survival and reproduction of fungi are present due to temperature stability, permanent water availability, the presence of wind, and so on, which can greatly affect the variability of these types of microorganisms.

Conclusion

In this investigation, it was possible to show the great morphological, genetic, and pathogenic diversities that *B. cinerea* depicts in the Andean blackberry crops in Colombia. This high degree of diversity makes it difficult to establish relationships between the characteristics that allow the management strategies to be established. However, it can be determined that high resistance to

the fungicide Fenhexamid is not present in the studied isolates. Although the four possible genotypes found had transposable elements, the two types of growth (mycelial and sclerotial) had different sclerotia layouts and size, as well as differentiated mycelium growths. From a microsatellite genetic point of view, it was possible to establish that genetic diversity is concentrated within populations and that there is a little difference between the populations of different regions. In addition, there is a tendency for isolates to be grouped by geographical origin, especially in the two large coffee axis groups: Risaralda, Quindío and Caldas, and Santander, Antioquia and Tolima.

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