Characterization of *Phytophthora nicotianae* isolates from tobacco plants (*Nicotiana tabacum*) in Colombia

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ABSTRACT

The black shank disease caused by *Phytophthora nicotianae* causes losses in tobacco crops up 100%. In Colombia, *P. nicotianae* populations are poorly known causing wrong diagnostics and erratic management. Amplification of the *Ypt1* gene and morphological characteristics of colonies, sporangia, chlamydospores and hyphae were used to identify *P. nicotianae* isolates. Races were identified according to the reaction induced by each isolate on the differential tobacco varieties Hicks, L8, KY 14 x L8 and NC 1071. As results, 71 isolates of *P. nicotianae* were identified and classified by races. Colonies of *P. nicotianae* were of white color, cottony and fluffy texture with smooth, non-swollen hyphae; spherical papillae with an average of 1.26 ìm and non-papillated and intercalary chlamydospores of medium size of 1.02 ìm that are typical characteristics of *P. nicotianae*. A species-specific PCR-amplified band of 389 bp was detected in all isolates tested. The presence of races 0,1 and 3 of *P. nicotianae* were determined in the Colombian departments of Huila and Santander. To the best of our knowledge, this is the first report of physiological races 0,1 and 3 of *P. nicotianae* in Colombia. Results are of relevance for disease management and tobacco breeding.

Keywords: chlamydospores; black shank; oomycetes; races 0, 1, 3; sporangia; *YpT1* gene.

INTRODUCTION

The black shank disease of tobacco is caused by the oomycete plant pathogen *Phytophthora nicotianae* van Breda de Haan. This disease is the most limiting phytosanitary problem in more than 120 countries where tobacco is grown including Colombia (Abad, 2008; Panabières *et al*., 2016; Gallup *et al*., 2018). *P. nicotianae* infects tobacco roots, stems and leaves during any stage of plant development, causing symptoms such as root and stem necrosis, chlorosis, stunting, leaf necrosis, wilt and finally plant death (Csinos, 2005; Lamour, 2013). Black shank disease develops faster under high temperature and humidity, conditions which are frequent in the growing areas of the departments of Huila and Santander where 66% of tobacco is produced in Colombia. Losses may reach 100% under favourable conditions for pathogen development, even with cultivars reported as highly resistant to *P. nicotianae* as was observed in Colombia in the Department of Huila with cv. K346 (Wilkinson *et al*., 2003).

P. nicotianae has a wide host range with more than 255 plant genera in 90 families and causes large losses in a number of crops around the world (Panabières *et al*., 2016). Some extent of host specialization has been detected in isolates collected from *Citrus*, *Solanum* and *Nicotiana* plants. In addition, isolates collected from nurseries usually show high heterozygosity and relative equilibria between A1 and A2 mating types (Biasi *et al*., 2016). Host resistance and crop rotation are the most economic measures to control black shank, but they are not always effective (Shew, 1987; Johnson *et al*., 2002). The primary reason for the difficulty in controlling *P. nicotianae* is the production of resistant chlamydospores and oospores that allow the pathogen survival under unfavorable conditions, as well as efficient dissemination in soil water, irrigation water and hydroponic solutions through mobile zoospores.

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The ability of *P. nicotianae* populations to infect tobacco cultivars with different resistance genes defines five physiological races (i.e., 0, 1, 2, 3 and 4) (Sullivan *et al*., 2005a,b; Gallup & Shew, 2006; Sullivan *et al*., 2010). The predominant physiological races, 0 and 1, are widely distributed throughout China, the United States and other major tobacco growing countries (Apple, 1962; Li, 2015). Previous studies using tobacco cultivars with moderate or high levels of resistance found that race 0 has higher virulence and ecological fitness levels than race 1, in fact, more RxLR effector genes were found in the genome of race 0 than in that of race 1 (Liu *et al*., 2016), suggesting that the difference in virulence between the two races is affected by additional genetic factors (Sullivan *et al*., 2005a,b). In addition, several authors have reported high variability in virulence and in the physiological races of *P. nicotianae*: 0, 1, 2, 3 and 4, in different places and countries around the world (Apple, 1962; Csinos & Bertrand, 1994; Van Jaarsveld *et al*., 2002; Sullivan *et al*., 2005a,b; Gallup & Shew, 2010). *P. nicotianae* is heterothallic requiring mating types A1 and A2 to produce oospores (Panabières *et al*., 2016). Most research on sexual variability has been performed under laboratory controlled conditions, thereby the potential of genetic adaptation to new hosts and fungicide applications derived from sexual recombination on field crops is mostly unknown (Panabières *et al*. 2016; Gallup *et al*., 2018).

Wild *Nicotiana* species *N. longiflora* (gene *phl*) and *N. plumbaginifolia* (gene *php*) are completely resistant to Race 0, *N. nesophila* is resistant to Race 3 (McIntyre & Taylor, 1978) and *N. plumbaginifolia* shows resistance to Race 4 but susceptibility to race 1 (Csinos & Bertrand, 1994; Gallup & Shew, 2010). Evidence suggests that tobacco plants that have the gene *Php* are resistant to Race 3 (Gallup & Shew, 2010; Gallup *et al*., 2018). Tobacco cv. Delcrest 202 (MK 95 x MK165) exhibits a single dominant gene with resistance to Race 2 (Mclntyre & Taylor, 1978; Van Jaarsveld *et al*., 2002). Cultivars from USA are resistant to Race 0 and susceptible to Race 1; when those cultivars are grown continuously, Race 1 is selected and becomes prevalent in tobacco crops (Sullivan *et al*., 2005a). Cv. Fla 301 is partially resistant to races 0 and 1, therefore is an option when both races of *P. nicotianae* are present (Apple, 1967; Lucas, 1975; Carlson *et al*., 1997; Csinos, 2005). Race 4 is able to overcome single-gene resistance conferred by the *Phl* gene from *N. longiflora* but not the *Php* gene from *N. plumbaginifolia* (Gutiérrez & Mila, 2007). In Colombia, most seeds used for commercial crops are imported from USA, where they are improved using cv Fla 301 (Johnson *et al*., 2002; Csinos, 2005). However, cv. Fla 301 may not be viable in tropical places with high levels of disease pressure (Csinos, 2005).

To apply appropriate control methods for plant diseases and to establish successful breeding programs, it is crucial to accurately identify the causal agent and to characterize populations investigating prevalent races to deploy appropriate cultivars. *P. nicotianae* has been identified using morphological characters such as sporangium, hyphae, growth pattern in culture media and other parameters (Erwin & Ribeiro, 1996; Abad, 2008; Gallup & Shew, 2010). Additionally, molecular markers based on the Internal Transcribed Spacer (ITS) and other regions, have been successfully used to discriminate *P. nicotianae* from other *Phytophthora* species (Meng & Wang, 2010; Monday *et al*., 2010). However, it has been not possible to differentiate *P. nicotianae* races using morphological characteristics or molecular markers (Mclntyre & Taylor, 1978; Abad, 2008). To date, physiological races of *P. nicotianae* are still differentiated using differential cultivars (Sullivan *et al*., 2005a,b; Gutiérrez & Mila, 2007).

To the best of our knowledge, in Colombia information about *P. nicotianae* populations and races affecting tobacco crops is mostly unknown and in most cases limited to internal reports in private companies. Therefore, accurate pathogen identification and characterization of prevalent races is very important for a sustainable tobacco industry in Colombia. The present research had as objective the morphological, pathogenic, immunological and molecular characterization of *P. nicotianae* populations present in Colombian tobacco growing areas, located mainly in the Departments of Santander and Huila.

MATERIALS AND METHODS

Isolates

Isolates were obtained using diseased tissues of tobacco plants. A piece of plant tissues taken from the edge of the lesion including green healthy and diseased tissue was placed in sterile oat-agar media culture. Petri dishes were incubated in laboratory conditions and transferred to fresh media (oat- agar) every two months; in addition, agar plugs containing mycelia were transferred every year to sterile tap water and incubated at room temperature.

Immunological and Molecular identification of isolates

ELISA test (Agdia® kit) was used to confirm the genus *Phytophthora* following the manufacturer instructions. Mycelia of purified isolates grown for 15 days in oat-agar media were macerated. Two wells of the plate for ELISA test were used as replicates per each isolate tested. Two wells without mycelia were used as negative controls. As positive control, mycelium from an identified isolate of *P.*

nicotianae kindly donated by Dr. Csinos from Department of Plant Pathology of University of Georgia, USA, was used, in addition to the standards available in the kit. A well with solution of strong yellow color was considered positive for *Phytophthora* and a well containing transparent solution was considered negative.

DNA extraction

Isolates were grown in 100 mL of sterile liquid pea-sucrose broth for 10 days (pea 100 g/L, sucrose 10 g/L, distilled water up to 1L), supplemented with 60 µl of ampicillin (20mg/L) and incubated at 24 °C ± 2°C in darkness without shaking for ten days. Mycelium was vacuum-filtered and freeze-dried for 24 h at room temperature; then, was macerated in a mortar with a pestle with liquid nitrogen until a fine powder was obtained and stored at -20 °C for further use.

DNA was extracted following the method described by Álvarez *et al*. (2004). 600 µL of extraction buffer SDS (SDS 1%, NaCL 1.4M, EDTA 20mM and Tris-HCl pH 8.0 100 mM) were added to mycelia (100-150µL) powder and were incubated at 65°C for 30 min; then 200µL of ammonium acetate7.5M were added and incubated at room temperature for 10 min, followed by centrifugation; supernatant was recovered and mixed with 500µL of chloroform : isoamyl alcohol (24:1); then nucleic acids were precipitated by adding 500µL of isopropyl alcohol and RNA eliminated with RNase (10mg/mL) incubated for 30 min at 37°C. Finally, nucleic acids were precipitated by centrifugation. DNA was dissolved in 60 µL of buffer Tris-EDTA (Tris – HCL 10 mM, EDTA 1 mM; pH 8.0). DNA was analyzed by agarose (0.8%) gel electrophoresis in Buffer TBE1x, at 80v. Images were taken under UV using a Biometra BioDoc equipment (Biometra GmbH, Goettingen Germany). DNA was quantified by fluorometry (Qubit®, Invitrogen Corporation), and diluted at a final concentration of 10 ng/µL, for PCR amplification.

PCR amplification of regions of the Ypt1 gene

Primers *Pn1* (5'GACTTTGTAAGTGCCACCATAC3') and *Pn2* (5'CTCAGCTCTTTTCCTTGGATCT3') were used for specific amplification of *Ypt*1 gene (Meng & Wang, 2010), in a reaction containing: Taq buffer 1X (Tris HCl 100mM pH 8, $MgCl_2$ 2.5 mM and KCl 500 mM), 0.1 mM of each of dNTPs, 0.2ì M of each primer, 2.5 mM of $MgCl_2$, 1.2ng/µL of DNA, 0.05 U/µL of DNA *Taq* polimerase (Fermentas) and HPLC-ultrapure water to complete a final volume of 25 µL. PCR amplification was performed in a thermalcycler equipment Biometra TPersonal (Biometra GmbH, Goettingen Germany) using the following program: initial denaturation at 94°C for 5 min, followed by 35 cycles of 30 s at 94°C, 30 s at 64°C, 30 s at 72°C and a final extension at 72°C for 10 min. Amplified products (6µL) were separated by agarose (1%) gel electrophoresis in TBE buffer 1x, at 5 V/cm for 1 h, stained with SYBRSafe (3µL for 100mL of agarose), and visualized under UV and photographed using an equipment Biometra BioDoc (Biometra GmbH, Goettingen Germany). Bands in the gel electrophoresis were compared with a molecular marker (1Kb, Gibco®), to verify the size of the amplified product.

Pathogenicity tests

Two pathogenicity tests were performed. The first was made to fulfill the Koch postulates to verify that isolates were tha causal agents of observed symptoms. The second was made to determine *P. nicotianae* races present in the collected isolates.

Koch postulates and disease severity quantification were performed for 71 isolates of *P. nicotianae*. Isolates were inoculated on tobacco plants cv. Hicks of 50 days old, which is considered highly susceptible to all known races of *P. nicotianae* (Bowman & Sisson, 2000; Xiao *et al*., 2013). Tests were performed following the methods described by (Csinos, 2005) with a minor modification consistent in an incubation temperature of 23-25°C. Each isolate was inoculated in stems and roots; in stems, basal leaves were removed and an agar disk with the isolate was applied on the insertion zone of the removed leaf. A humid cotton speck was placed on the isolate and covered with parafilm tape (Parafilm MR) to avoid desiccation. Roots were inoculated with 5mL of a solution with zoopores at a concentration of 10^6 zoospores/mL.

A severity scale from 0 to 10 was used to measure the pathogenicity index (Sullivan *et al*., 2005a). Disease development was recorded daily from the third day after inoculation (dai) and until 21 dai. A value of 10 in the scale was assigned to plants that died three dai; a value of 8 was for plants dead 5 dai; a value of 6 for plants dead 7dai; 4 was for plants dead 14 dai; 2 for plants dead at 21 dai and 0 was for plants without symptom development at 21 dai. A completely randomized experimental design with three replicates per treatment, was applied, i.e., three plants inoculated per each isolate for a total of 210 plants inoculated for 71 isolates. A plant of cv. Hicks, which is considered highly susceptible to all known races of *P. nicotianae*, of 50 days old was considered as an experimental unit (Xiao *et al*. 2013, Bowman & Sisson 2000).

Disease severity was determined as the sum of pathogenicity indexes divided by the total number of plants inoculated with each isolate (Equation 1):

Equation 1

$$Severity = \frac{\sum_{i=1}^{n} n_i}{N} \quad (1)$$

Where,

N: total number of plants inoculated with each isolate

n: pathogenicity index for each plant inoculated

Determination of physiological races of isolates

71 isolates from Colombian and a reference isolate from USA, were tested using the methodology proposed by Gallup & Shew (2010) and Gutiérrez & Mila (2007), but using tobacco differentials Hicks, KY 14xL8, L8 and NC 1071, which harbor different types of resistance to *P. nicotianae* (Table 1). Tobacco differentials were kindly donated by Dr. Ramses Lewis from North Carolina State University, USA. Each isolate of *P. nicotianae* was inoculated on the four tobacco differentials mentioned above. Thirty plants of each cultivar were inoculated with each isolate with two replicates and a non-inoculated control of each cultivar tested, for a total of 60 plants of each cultivar per each isolate.

Plant distribution and maintenance

Plants of each differential were sown in plastic boxes of 12 wells (Corning Incorporated®). Ten seed were placed in each well. Sterile distilled water was added between wells to keep moisture. Plastic boxes were kept in humid chambers incubated at room temperature (~23°C) and 13 hours of light. Plants were fertilized twice per week with 0,5 mL of a solution with 200ppm of N-P-K (20-10-20). A completely randomized experimental design with six replicates, was used. Each plastic box was considered as an experimental unit. Tobacco cultivars Hicks, NC1071, L8 and KY 14 x L8, were used to test *P. nicotianae* isolates (Table 1).

Preparation of inocula and plant inoculation

Leaf disks of 5mm of diameter of tobacco plants cv. Hicks were surface sterilized in sodium hypochlorite (5,25%) for 3 min, followed by three rinses in sterile distilled water. Sterile disks were placed in circular form in Petri dishes containing oat-agar medium. A plug of agar with *P. nicotianae* mycelia from a culture grown for 15 days was placed in the center of each Petri dish containing leaf disks. Petri dishes were incubated at 23ºC ± 2°C in complete darkness for 10 days.

Tobacco seedlings were inoculated 25 days after seeds were sown, by placing a leaf disk of tobacco cv. Hicks infected with *P. nicotianae* in each well. Two microplates of 12 wells each were inoculated and another microplate of 12 wells was used as a non-inoculated control. Microplates inoculated with each isolate were incubated in a humid chamber at 27ºC with 13 hours of light and 11 hours of darkness for 14 days.

Evaluation of results

Incidence was measured 14 days considering a plant infected by *P. nicotianae* as the one showing total chlorosis, wilt, dark roots, necrosis and death. A plant genotype has been considered as susceptible/positive to *P. nicotianae* if more than 5% of a total of 60 inoculated plants were infected, otherwise has been considered negative (Gutiérrez & Mila, 2007). Races were determined following the dichotomic response (negative or positive) method. Race 0 is positive in cv. Hicks but negative in cv. NC 1071, KY 14 x L8 and L8. Race 1 is positive for cv. Hicks, NC 1071, KY 14 x L8 and L8. Race 3 is positive for cv. Hicks, KY 14 x L8 and L8, but negative for cv. NC 1071 (Sullivan *et al*., 2005a,b; Gallup & Shew, 2010; Gallup *et al*., 2018).

Morphological characterization of P. nicotianae

Isolates of *P. nicotianae* grown for 15 days in oat-agar media culture were used for morphological characterization of sporangia (shape, size, presence/absence of papillae, pore opening and length of the pedicel), sporangiophore (shape), hyphae (shape, growth type), chlamydospores (presence, position, size) and colony in culture media (growth, color) (Appiah *et al*., 2003; Abad, 2008). Measurements were performed in the light microscope at 100X (Nikon). Three copies of each isolate were measured, considering one petri dish as an experimental unit. Normal distribution of residuals was determined by Shapiro-Wilks and Kolmogorov-Smirnov tests and homoscedasticity was determined using the Levene test ($P < 0.05$). Analysis of variance (ANOVA) followed by the Tukey test ($P < 0.05$), were performed to determine significant differences between isolates. Non-parametric data were analyzed using the Kruskall-Wallis test ($P < 0.05$) and *post-hoc* Dunn test.

RESULTS

Identification of P. nicotianae

ELISA test results were positive for *Phytophthora* spp. in all isolates tested. Primers Pn1 and Pn2 amplified the

Table 1: Differential cultivars of tobacco used for determination of physiological races of *P. nicotianae*

Cultivar	Type of tobacco	Resistance gene	Type of resistance
NC 1071b	FLUE CURED	Php	Complete resistance to Race 0. No resistance to Race 1.
HICKSa	FLUE CURED	No Php	susceptible to all known races
L8b	BURLEY	Phl (homozygous)	Complete resistance to Race 0. Low resistance to Race 1.
KY 14 x L8a	BURLEY	Phl (heterozygous)	No resistance to Race 0. Complete resistance to Race 1.

a: commercial cultivar; b: improved line.

expected 389 bp DNA fragment for 100% of isolates, which confirmed positive identification of isolates of tobacco from Colombia and the USA as *Phytophthora nicotianae* (Figure 1).

Pathogenicity of P. nicotianae isolates

Seedlings showed a rapid response to inoculation with the pathogen on stems and roots. The faster appearance of symptoms was recorded at 24 hours and continue up to three dai. Most *P. nicotianae* isolates (86%) showed a PI of 10 according to the severity scale of disease used, showing typical wilt, necrosis in the basal part of the stem and death of seedlings (Figure 2, Table 2). At 7 dai, plants showed from 10 to 30% of mortality and at 14 days, most plants were dead.

Identification of physiological races of P. nicotianae

Results evidenced that races R0 (43.66%) and R1 (33.8%) showed the highest incidence for all isolates collected from Colombian Departments. Race 3 (incidence of 11.27%) was identified only in the Departments of Santander and Huila (Table 3). Race 1 was present in 55.56% of isolates from Huila and in 28.57% of isolates from Santander growing areas. Race 0 was present in 16.67% of isolates from Huila and in 45.24% of isolates from Santander (Table 2 and 3). Race 3 was identified in 16.67% of isolates from Huila and in 11.9% of isolates from Santander. Sixty three isolates of *P. nicotianae* were accurately identified as race R0, R1 or R3. For remaining eight isolates it was not possible to determine a known Physiological race and were classified as IND (Not determined) (Table 2 and 3). Seven days after the inoculation, symptoms such as chlorosis, plant wilt, dark radicular system, general necrosis and death of the seedling, typical of plant reaction to races, were observed (Figure 3). Cv. NC 1071 showed complete resistance to R0, cv. KY 14 x L8 and L8 showed low resistance to both races, allowing a clear identification of R0 and R3 according to what was reported previously (Gutiérrez and Mila, 2007; Gallup & Shew, 2010; Gallup *et al*., 2018). Isolate No. 61 – CPN 02 used as a reference, kindly donated by Dr. Alex Csinos from University of Georgia, USA and certified as R0, reproduced the typical expected reactions supporting its previous classification. To the best of our knowledge, it is the first time that R3 is identified in Colombian tobacco fields. Significant differences in the pathogenicity index between the races of *P. nicotianae* (P = 0.05) were identified with Race 3 showing the highest PI value (Figure 4).

Morphological Characterization of P. nicotianae

Isolates of *P. nicotianae* from tobacco growing areas of Santander and Huila, presented very similar morphological characteristics with no significant differences between Races. Table 4 shows relevant micro-morphometric results for each race such as sporangia, chlamydospores and hyphae shape, type of sporangiophore and differences on growth rate in culture media (Figure 5).

Culture medium: White colony color predominate on different media culture, mostly with fluffy and cottony texture, best growth temperature was 23 ± 1 °C, which is

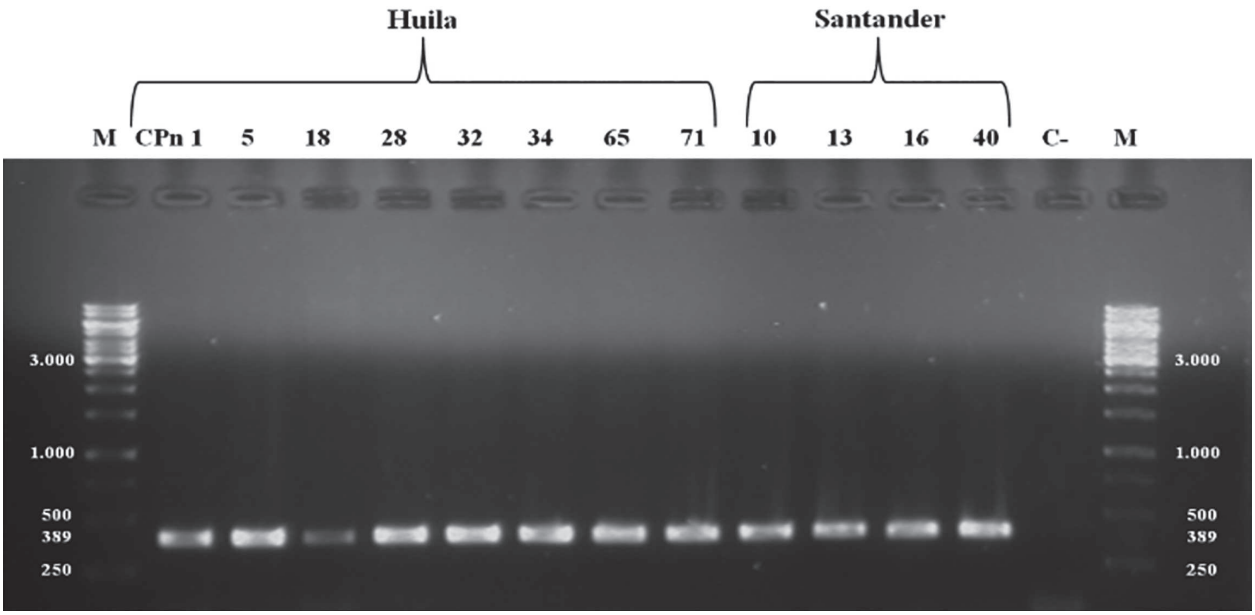

Figure 1: Molecular identification of *P. nicotianae* based on the *Ypt1* gene. M: 1 Kb DNA molecular weight marker. Wells 2-9: isolates CPn 1 to 71 from the Department of Huila, Colombia. Wells 10-13: isolates CPn 10 to 40 from the Department of Santander, Colombia C-: negative control.

within the range reported for the species (Abad, 2008). **Hyphae:** irregular in relation to the angle of insertion and thickness, mostly smooth, showing some curves or spirals with thin and coenocytic walls (Figure 5), characteristics that correspond to those reported for *P. nicotianae* (Lucas, 1975; Erwin & Ribeiro, 1996; Álvarez *et al*., 2007; Meng *et al*., 2014). A large percentage of hyphae had a non-bloated growth rate with an insertion angle less than 90° as reported before (Hall, 1993; Erwin & Ribeiro, 1996) (Table 4, Figure 5), but in contrast to observations reported by Meng *et al*. (2014), who described sporadic presence of swollen hyphae. **Sporangiophores:** generally unbranched, indeterminate growth and branch out sympodially, in agreement to what was described previously (Waterhouse, 1963) (Figure 5). **Sporangia:** mostly (Figure 5), as reported (Waterhouse, 1963; Hall, 1993; Meng *et al*., 2014). Sporangia varied slightly in shape and size, with spherical and pyriform shape as the most abundant (Figure 5), with an average size of 37.15 x 28 ìm (L x W) and a ratio of 1.26 (Figure 5 and 6), which was within the range reported for the species (Stamps *et al*., 1990; Hall, 1993; Abad, 2008).

In most isolates sporangia were papillated with very short or no pedicel present, few sporangia were semi-papillated and showed medium or long pedicels, with size within the ranges described by Stamps *et al*. (1990) (Figure 5). Statistical differences in sporangia size were identified between different races of isolates; Races 0, 1 and isolates for which a race was not possible to be determined (IND isolates) had higher size ratio (L x W) than isolates of R3 (Figure 6A). All sporangia measurements were within the range reported for *P. nicotianae*. In addition, isolates showed statistical differences in sporangia size according to their geographic origin; isolates from Huila showed smaller sporangia compared with those from Santander. Evenmore, smallest sporangia were measured in the group of isolates from Huila for which a race was not determined (IND) (Figure 6B). **Chlamydospores:** spherical in shape and mostly intercalary, with size (LxW) 30.33 x 28.69 ìm and a ratio of 1.023 on average (Figure 5 and 6A), terminal chlamydospores were also found (Figure 5). Chlamydospores from isolates in race 1 were significantly smaller than chlamydospores from isolates in race 0, 3 and IND (Figure 6A). Similarly as found for sporangia size, isolates from Huila for which it was not possible to determine a race (IND-HUI) exhibited chlamydospores smaller than isolates from other Departments and races (Figure 6B). As found for sporangia, all chlamydopore measurements were within the range reported for *P. nicotianae*. All isolates grew at 23 ± 1ºC, a temperature within the optimum range reported for *P. nicotianae* (Erwin & Ribeiro, 1996; Gallegly & Hong, 2008).

DISCUSSION

One of the first and most important steps for a prompt and appropriate integrated disease management program is the early and accurate identification of the causal agent. In the present research we identified 71 isolates causing the black shank disease in tobacco crops in the tobacco growing areas of Colombia, by immunological-based ELISA test, DNA amplification of specific fragments and morphology. We used the ELISA test for identification at the genus level and PCR-amplified DNA to the species level, because detection of pathogens using PCR is at least 10 times more sensitive than ELISA and ELISA test allows the identification only up to genus level (Peteira *et al*., 2008). In addition to *P. nicotianae*, other species of oomycetes have been reported causing diseases in tobacco such as *Phytophthora* spp. and *Pythium* spp. causing damping-off, *P. glovera* causing yellow stunt, (Abad *et al*., 2011), *Peronospora tabacina* causing blue mold or downy mildew (Derevnina *et al*., 2015) and others, that may be confused between them, highlighting the importance of correct identification for appropriate disease management. Even though, morphological parameters are important and valid for genus identification in *Phytophthora* and related oomycetes, it is laborious and requires high levels of experience. Molecular identification is nowadays an important tool

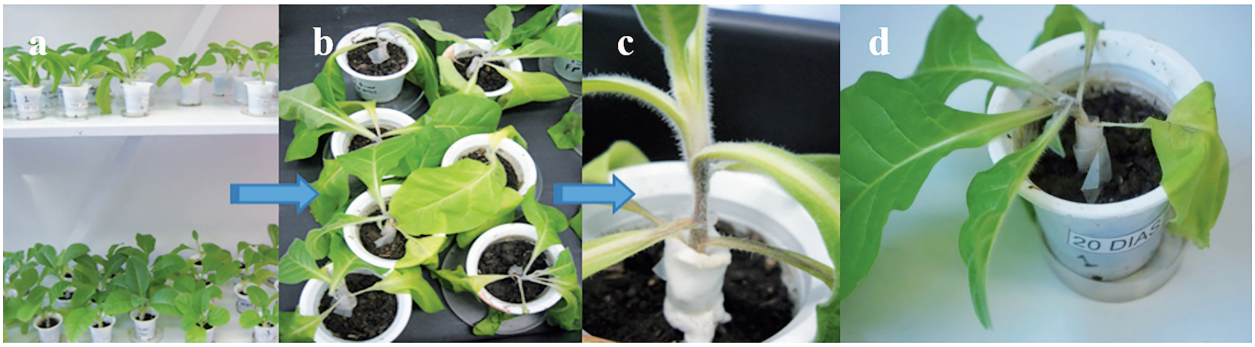

Figure 2: Tobacco seedlings cv. Hicks showing disease symptoms by *P. nicotianae* three days after inoculation: a) inoculated tobacco seedling under laboratory conditions, b) tobacco seedlings showing epinasty symptoms, c) stem necrosis, d) stem necrosis and epinasty symptoms.

Table 2: Number of isolates of *P. nicotianae*, codes and locations of the tobacco fields in Colombia sampled, pathogenicity index (IP) values, detection of races in differential tobacco hosts and percentages of each race by location

Number	Isolate code	Region	Location	Sidewalk	PI/Isolate	Race	Ratio proportion by area (%)				TOTAL
							R0	R1	R3	RU	
1	CPN 01	Huila	Altamira	Llano de la Virgen	10	R1	16,67	55,56	16,67	11,11	100
2	CPN 03		Altamira	Llano de la Virgen	3,3	R0					
3	CPN 05		Altamira	Llano de la Virgen	10	R0					
4	CPN 18		Campoalegre	Rio Frio	2,9	RU					
5	CPN 34		Campoalegre	Bajo Piravante	10	R1					
6	CPN 36		Campoalegre	Bajo Piravante	3,3	R1					
7	CPN 38		Campoalegre	Bajo Piravante	10	R1					
8	CPN 65		Campoalegre	Piravante	10	R1					
9	CPN 67		Campoalegre	Piravante	5,7	R1					
10	CPN 69		Algeciras	Lagunilla	10	R0					
11	CPN 71		Algeciras	Lagunilla	10	R3					
12	CPN 73		Algeciras	Lagunilla	10	RU					
13	CPN 74		Algeciras	Lagunilla	10	R1					
14	CPN 28		Garzon	Alto Sertenejo	10	R1					
15	CPN 76		Garzón	Alto Zartenejo	10	R3					
16	CPN 32		Garzón	(lote: El Campamento)	10	R1					
17	CPN 75		Rivera	Riverita	10	R3					
18	CPN 78		El Agrado	La Escalerita	10	R1					
19	CPN 04	Santander	Capitanejo	Junta	10	RU					
20	CPN 23		Capitanejo	La Parada	10	R1					
21	CPN 40		Capitanejo	La Loma del Negro	10	R1					
22	CPN 55		Capitanejo	Junta	10	R1					
23	CPN 60		Capitanejo	N.A.	10	R3					
24	CPN 27		Capitanejo (Miranda)	Popagá	9	R1					
25	CPN 10		Villanueva	N.A.	10	R1					
26	CPN 37		Villanueva	El Hato	10	R0					
27	CPN 41		Villanueva	Centro	10	R1					
28	CPN 42		Villanueva	Hato viejo	10	R3					
29	CPN 43		Villanueva	Macaregua Hato	10	R0					
30	CPN 49		Villanueva	Trigo	10	R0					
31	CPN 50		Los Santos	Macaregua	10	R3					
32	CPN 68		Los Santos	Hato viejo	10	R0					
33	CPN 70		Los Santos	Trigo	3,3	R0					
34	CPN 79		Los Santos	Centro	10	R0					
35	CPN 80		Los Santos	Centro	10	R0					
36	CPN 15		Los Santos	Delicias	10	RU					

Continua...

Continuação

Table 2: Number of isolates of *P. nicotianae*, codes and locations of the tobacco fields in Colombia sampled, pathogenicity index (IP) values, detection of races in differential tobacco hosts and percentages of each race by location

Number	Isolate code	Region	Location	Sidewalk	PI/Isolate	Race	Ratio proportion by area (%)				TOTAL
							R0	R1	R3	RU	
37	CPN 17		Los Santos	Delicias	10	R0					
38	CPN 19		Los Santos	Delicias	10	R0	45,24	28,57	11,9	14,29	100
39	CPN 29		Los Santos	Espinal	10	R1					
40	CPN 31		Los Santos	Lanadas	10	R1					
41	CPN 33		Los Santos	Delicias	10	R1					
42	CPN 35		Barichara	N.A.	10	R0					
43	CPN 45		Barichara	La Purnia	10	RU					
44	CPN 46		Barichara	Garvanzal	10	R1					
45	CPN 47		Barichara	Delicias	5,7	R0					
46	CPN 62		Barichara	Garbanzal	10	R0					
47	CPN 66		Barichara	Garvanzal	10	R0					
48	CPN 51		Cabrera	Caraquitas	10	R1					
49	CPN 52		Cabrera	Higueras	10	R3					
50	CPN 54		Pie de cuesta	Higueras	10	RU					
51	CPN 56		Enciso	Higueras	8	R0					
52	CPN 59		San Gil	Paramito	10	R0					
53	CPN 61		Jordan	Paramito	10	R0					
54	CPN 44		Curiti	Cuchillas	10	R1					
55	CPN 72		Los Santos	Ojo de Agua	10	R0					
56	CPN 16		Los Santos	Guatiguará	10	R0					
57	CPN 21		Los Santos	Mosgua	10	RU					
58	CPN 48		Los Santos	Flora	10	R0					
59	CPN 63		Los Santos	Pomaroso	10	R3					
60	CPN 13		Los Santos	La Peña	10	RU					
61	CPN 02	Carolina Norte USA	N.A.	N.A.	10	R0					
62	CPN 06	Tolima	Espinal	Dindalito	10	R0					
63	CPN 08	Tolima	Espinal	Dindalito	10	R0					
64	CPN 12	Tolima	Espinal	Saman	6,7	R1					
65	CPN 14	Tolima	Espinal	Saman	10	R1					
66	CPN 09	Boyaca	Covarachia	Soldado Bravo	3,3	R0	81,82	18,18	0	0	100
67	CPN 11	Boyaca	Covarachia	Soldado Bravo	10	R0					
68	CPN 07	Valle	N.A.	N.A.	10	R0					
69	CPN 53	Valle	Candelaria	N.A.	10	R0					
70	CPN 58	Valle	N.A.	N.A.	10	R0					
71	CPN 25	Quindio	Calarca	N.A.	10	R0					

RU= Unknown race. NA= not known. PI= Pathogenicity Index

that may be combined with morphological data for a more accurate identification of *Phytophthora* species and contributes to perform a more efficient management disease strategy for tobacco and other crops (Peteira *et al.*, 2008).

P. nicotianae pathogenicity depends on the environment and the cultivar affected and is very important to quantify disease development as a tool for tobacco breeding programs. The pathogenicity index (PI) is a measure that reflects the magnitude and speed of disease development (Apple, 1957), being useful for genotype selection in tobacco fields. In the present research, *P. nicotianae* isolates induced symptoms as early as 24 hours after inoculation. Other authors had reported first disease symptoms 18 hours, post inoculation of tobacco plants (Meng & Wang, 2010), suggesting short incubation periods of *P. nicotianae* populations on cultivated genotypes of tobacco. In addition, most isolates tested here showed high PI values and cause plant death at 14 dai, indicating high aggressiveness and pathogen populations well adapted to the tobacco cultivars commonly grown in Colombia (Panabières *et al.*, 2016). This finding is very important because implies that much more effort should be done in basic and applied research to support the tobacco breeding programs looking for resistant varieties to *P. nicotianae*.

Physiological races have been extensively used for characterization of plant pathogen populations (Flor, 1971). Pathogen races are determined according to the plant reaction when a given genotype of the plant host recognizes or not a given strain of the pathogen. When the plant recognizes the pathogen, the response leads to the hypersensitive response, a form of a programmed cell death similar to apoptosis in mammals (Pitsili *et al.*, 2020). This reaction usually happens in a gene-for-gene interaction manner (Flor, 1971). Characterization of

Table 3: Incidence and number of isolates of each race found in each Department of Colombia

Race / Department	Santander	Huila	Other Departments	Total	Incidence (%)
R0	19	3	9	31	43.66
R1	12	10	2	24	33.8
R3	5	3	0	8	11.27
IND	6	2	0	8	11.27
Total number of isolates / Department	42	18	11	71	100

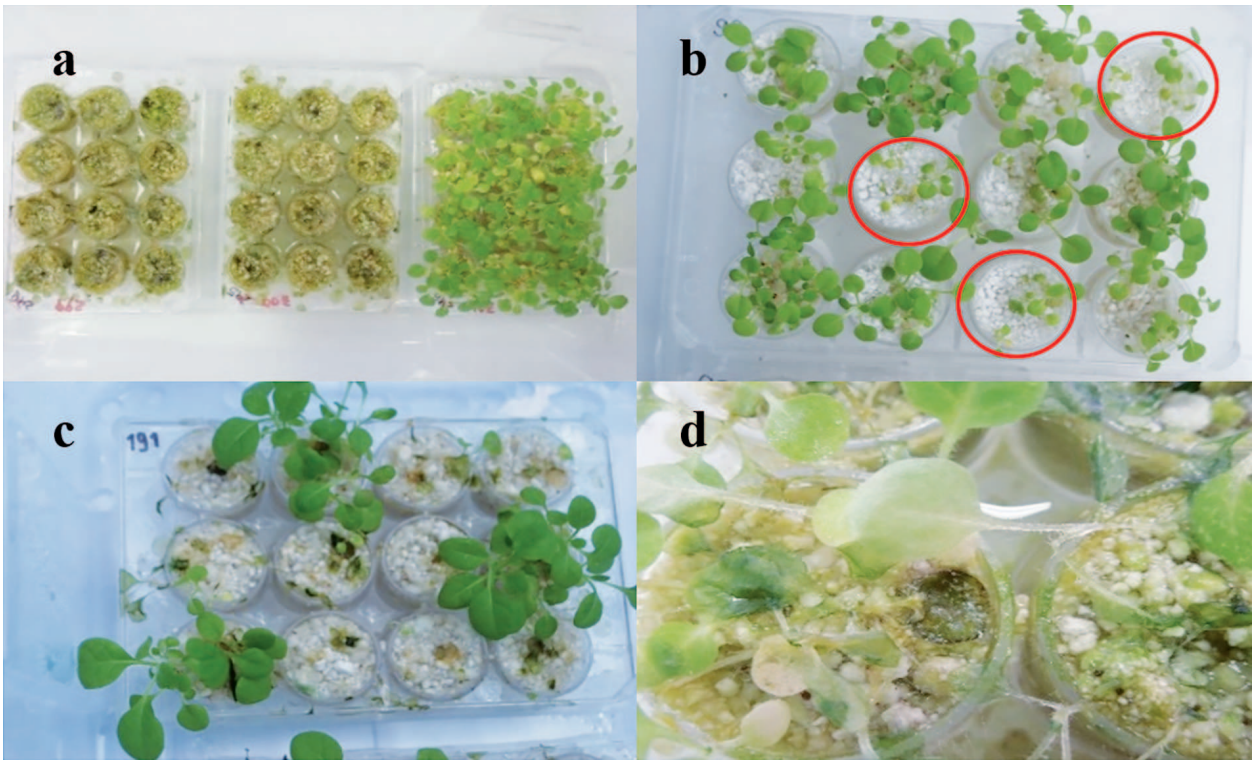

Figure 3: Tobacco differential host assay for identification of physiological races of *P. nicotianae*. Plants showing symptoms 14 days after inoculation: a) plant showing R1 race response b) plant showing R0 response, c) plants showing R3 race response, d) seedlings showing wilting, chlorosis, necrosis and death.

pathogen populations is useful to deploy commercial cultivars with genes conferring resistance to prevalent races of the pathogen in field crops. Here, we identified Races 0, 1 and 3 of *P. nicotianae*. It is the first time that race 3 is detected in Colombia, with incidences of 17% in the Department of Huila and 12% in Santander. Interestingly, for a group of isolates (ND) it was not possible to determine a given race. Considering controls and replicates if errors in the methods used are ruled out, it is reasonable to speculate that those isolates were not recognized by plant differentials used in the present research. Hence, genetic variants in pathogen populations of Colombia may exist, suggesting the need of further research on *P. nicotianae* diversity studies involving advanced methods such as multi-loci genotyping or even whole genome sequence analyses. Sexual recombination by crossing the A1 and A2 mating types maybe a source of new variants, although it is important to highlight that in asexual reproduction of filamentous microorganisms there are other sources of generation of genetic variation such as parasexuality, hyphal anastomosis, heterokaryon formation and others. Evenmore, the relative frequency of A1 and A2 isolates in *P. nicotianae* populations is often biased in nature, suggesting that the role of sexual

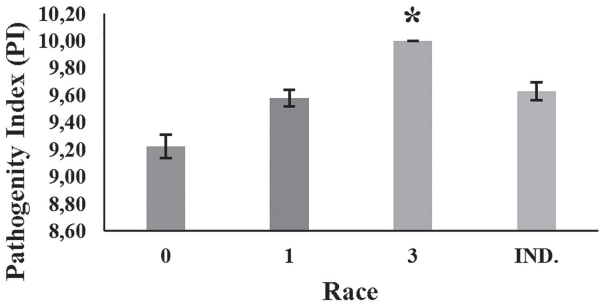

Figure 4: Pathogenicity index of *P. nicotianae* races isolates in Colombia. Asterisk indicates significant differences between races by Kruskall- Wallis test ($p < 0.05$), PI: Pathogenicity index. IND: race not-determined.

Table 4: Morphological characteristics of *P. nicotianae* Races 0, 1, 3 and the group of isolates with undetermined race

Morphological Features	Characteristic Description	Race			
		Race 0	Race 1	Race 3	RU
COLONY PATTERN OBSERVED	cottony mycelium (cm), fluffy mycelium (fm), rosette (rm), radiate (ram), and clustered (clum)	cm 44,19%; fm 33,56%.	cm 37,40%; fm 31,58%	fm 32,12%; cm 19,06%	fm 51,01%; cm 21,05%
COLONY COLORS	white, yellow	white 87,5%	white 81,97%	white 75,45%	white 82,18%
SPORANGIOPHORE FORM	branched-Unbranched	Unbranched 64,79%	Unbranched 56,20%	Unbranched 71,48%	Unbranched 64,79%
SPORANGIA SHAPE	spherical-Ovoid-Ellipsoid-Pyriform Clavate	spherical 54,04%; Pyriform 34,50%	spherical 60,65%; Pyriform 29,65%	Spherical 49,81%; Pyriform 36,82%	spherical 53,03%; Pyriform34,41%
SPORANGIA PAPILLAE	papillate (size ≥3.5 µm)-semipapillate (size < 3.5 µm)	Papillate 81,16%	Papillate 77,90%	Papillate 77,26 %	Papillate 83,40%
PEDICLE LENGTH	short (< 5 µm) -medium (5-20 µm)-long (> 20µm)-ND	short 28,87%; ND 24,47%	ND 29,55%; short 29,15%	short 26,71%; ND 25,27%	ND 29,55%; short 29,15%
SPORANGIA DIMENSIONS	Length / width (l / w ratio µm)	1,27 ± 0,036	1,24 ± 0,016	1,26 ± 0,051	1,28 ± 0,029
SPORANGIA EXIT PORE	narrow-Medium-wide-ND	ND 83,80%	ND 74,03%	ND 76,17%	ND 83,80
HYPHAL FORM	smooth-curve-spiral	smooth 72,76%	smooth 77,84%	smooth 73,76%	smooth 80,83%
FORMATION OF HYPHAL SWELLINGS	swellings / Non swellings / angle > 90° / angle < 90°	non swellings 69,7%; angle < 90° (34,8%)	non swellings 70,9%; angle < 90° (31.3%)	non swellings 73,4%; angle < 90° (30,7%)	non swellings 69%; angle < 90° (34,3%)
FORMATION OF CHLAMYDOSPORES	formed-absent	formed 98%	formed 93%	formed 92%	formed 96%
CHLAMYDOSPORES POSITION	intercalary and terminal	intercalary 70%	intercalary 77%	intercalary 70%	intercalary 59%
CHLAMYDOSPORES DIMENSIONS	Length / width (l / b ratio µm)	1,0280 ± 0,013	1,0254 ± 0,023	1,0118 ± 0,040	1,0271 ± 0,025
Number Isolated Totals Observed / Race		568	516	277	247

RU= Unknown race. ND= character non determined due to either loss, lack of information or not present in the isolate. Colony pattern was determined in oat grain-agar culture medium.

reproduction may be less important than generally considered, and that the threat of emergence of new virulences generated by oospore production may be overestimate (Panabières *et al*., 2016). In the tree pathogen *P. ramorum*, structural variants (SVs) arising from somatic mutations considerable contribute to genetic variation within the pathogen population and parallel overlap of SVs with genes involved in pathogenicity such as RXLRs have the potential to change the course of an epidemic (Yuzon *et al*., 2020). In oomycetes such as *P. nicotianae*, the RXLRs proteins have been closely related with race structure since RXLRs genes encode for avirulence proteins recognized by R resistance proteins in the host plant, highlighting the importance of asexual reproduction in the pathogen race structure. However, sexual reproduction by mating types is important in pathogen populations. Since in the present research the mating type of isolates was not determined, it would be worth of further research to identify which mating types reported for *P. nicotianae* are present in Colombia and if sexual reproduction eventually has incidence in the pathogen population diversity and virulence observed.

High incidence in the Department of Santander of Races 1 (28.57%) and 3 (11.9%) (Table 2 and 3), may be due to continuous use of commercial varieties with complete, high or partial resistance to Race 0, exerting selection pressure that results in an increase in frequency of other pathogen races not recognized by current grown varieties (Csinos, 2005). Similar results have been observed in a number of pathosystems including *Nicotiana* spp. - *P. nicotianae* (Sullivan *et al*., 2010). Host specific lineages have been reported for *P. nicotianae*, evidence suggests that isolates may be more aggressive in their host of origin than in other plant hosts and clonality prevails in pathogen populations suggesting asexual dispersion (Mammella *et al*., 2013; Biasi *et al*., 2016; Chowdappa *et al*., 2016). Lamour *et al*. (2003) evaluated isolates from different geographic areas and observed a similar compatibility between them and the host, a characteristic maybe due to asexual reproduction of *P. nicotianae*. This suggested that asexual reproduction perhaps is the most frequent type in the tropics and may play an important role in pathogen dissemination and epidemics, which must be confirmed in Colombian populations of *P. nicotianae* not only from tobacco but from other plant hosts. As found in USA, most isolates collected from Colombia were highly aggressive independently of the geographical region of origin, which may indicate high adaptation to most commercial cultivars grown (Sullivan *et al*., 2010), which prompt for research on cultivars with novel genes for

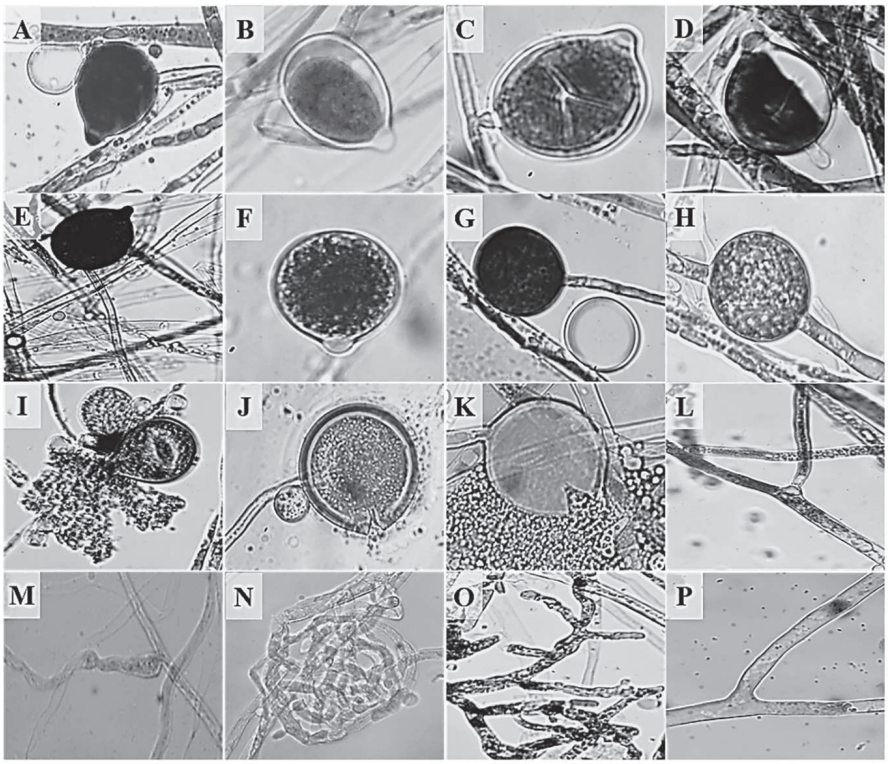

Figure 5: Morphological characteristics of *P. nicotianae* isolates from Colombia: Sporangia shape: A) pyriform, B) obpyriform, F) spherical. Pedicle length: C) short, D) medium, E) long, F) ND. chlamydospore position: G) terminal, H) intercalary. Sporangia exit pore: I) narrow, J) medium, K) wide. Hypha form: L) smooth, M) curled, N) spiral. Formation of hyphal swellings: O) swellings, P) non-swellings.

resistance to *P. nicotianae* as part of an integrated black shank disease management (Sullivan *et al*., 2005a,b; Nifong *et al*., 2011; McCorkle *et al*., 2013).

Micro-morphometric analyses have been successfully applied for the characterization of *Phytophthora* spp. populations including *P. nicotianae* (Hall, 1993; Appiah *et al*., 2003). Authors have shown morphological characteristics that may be used for *P. nicotianae* differentiation. Hall (1993) and Abad (2008) reported variation in sporangia size in different isolates of *P. nicotianae* and Gallegly & Hong (2008) considered sporangia size as a relevant characteristic for accurate identification of *Phytophthora* species. However and in contrast, Erwin & Ribeiro (1996) pointed out that sporangia size perhaps is not relevant, since it varies with growth conditions of each isolate. In our work, sporangia size was significantly different between isolates for which a race was not possible to be determined (ND), compared to a group composed of isolates of races 0, 1 and 3, and in addition, isolates from Huila were smaller than those from Santander, suggesting variation in the Colombian population of *P. nicotianae*. However, all measurements were found to be within the range reported for *P. nicotianae*. As in our work only isolates from tobacco were analyzed, it would be important to broaden research including isolates from other hosts and geographical regions for a better understanding of ecology, genetics and dynamics of the *P. nicotianae* populations. Sporangia identified here were non-caducous, characteristic that easily differentiates *P. nicotianae* from *P. palmivora*, which has caducous sporangia. Chamydospore measurements agreed with those reported previously (Hall, 1993; Gallegly & Hong, 2008; Meng *et al*., 2014), but interestingly isolates of race 1 exhibited smaller chlamydospores than isolates of race 0, that together with sporangia size, results indicate morphometric differences between races. *P. nicotianae* mycelia have been described as arachnoid and tuffy as characteristic and sometimes enough for identification of this species. However, Hall (1993) stands out that isolates may vary according to environmental conditions and the growth media used for cultures. Hence, for accurate characterization of

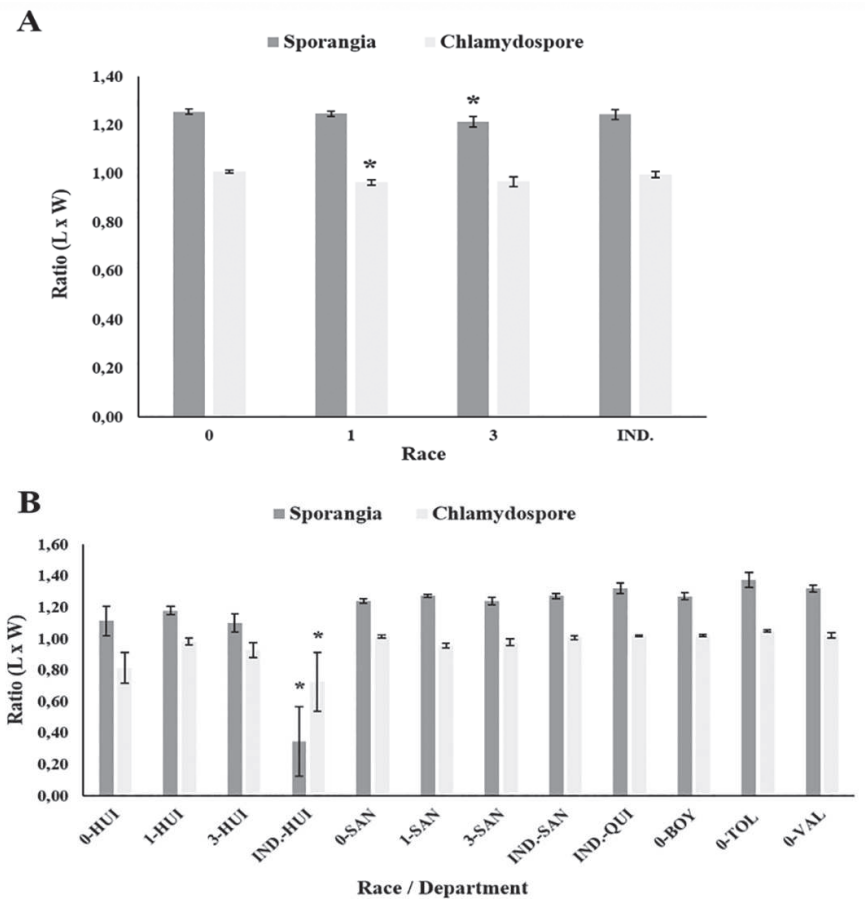

Figure 6: Relationship between sporangia and chlamydospore size ratio (L x W), races and Department of collection of *P. nicotianae* isolates. A) Graph showing relationship between sporangia and chlamydospore size ratio (L x W) with races of *P. nicotianae*. B) Interaction between sporangia and chlamydospore size ratio (L x W) with races and Department of collection of *P. nicotianae* isolates. Asterisks represent significant differences between races determined by the Kruskall- Wallis test ($p < 0.05$). IND = race not determined, HUI: Huila, SAN: Santander, QUI: Quindío, BOY: Boyacá, TOL: Tolima, VAL: Valle.

Phytophthora spp. populations is more appropriate the use of combined morphological and molecular methods (Meng & Wang, 2010; Li *et al*., 2015; Liu *et al*., 2016).

CONCLUSIONS

Morphological, pathogenic and molecular characterization of *P. nicotianae* populations in Colombia allowed the identification of 3 races of highly aggressive isolates and a group for which a known race was not possible to be determined. It is the first time that race 3 is reported in Colombia.

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