UNIVERSIDAD NACIONAL DE COLOMBIA

Construction of pathogenic, biologic and genetic bases of the Colombian populations of *Rhizoctonia solani* AG-3 necessary for the development of management strategies of stem canker and black scurf diseases of potato

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Bogotá, Colombia

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This work is dedicated to my grandfather for being the light of my life, for his love and care, he gave me the way to be a good human being and to Ursula for being a guide in a difficult moment.

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Abstract

Rhizoctonia solani AG-3 Kühn (teleomorph: *Thanatephorus cucumeris* (Frank) Donk) is a main soil-borne pathogen on potato crops around the world. Actually this pathogen is recognized as important in Colombia; however there is no information about the pathogen and the disease. This research was conducted using biological and molecular tests to know the Anastomosis Groups (AG) associated with symptoms in the main producing potato areas in Colombia. Additionally the genetic variability of the isolates and the distribution of variability along the main potato producing areas in Colombia as well as the response of the pathogen to temperatures and fungicides and its aggressiveness on potato plants were tested. The samples were collected from symptomatic stems of potato on naturally infested fields and then were processed in laboratory. The isolates were subject of different analysis. The AG-3 of *R. solani* was the most common associated to symptoms in fields. The isolates from different geographical populations were highly variable and the variability was not structured geographically. All the populations are genetically similar showing the effect of gene flow in the genetic structure of the populations. The evaluation of the response of the pathogen to two temperatures, the sensitivity to the fungicide thifluzamide and the aggressiveness on different organs of the potato plants showed that the isolates of all the geographical localities vary in the response to the factors evaluated. The levels of black scurf and cankers were low and variable among isolates in the cultivar of potato evaluated.

Key words: Soil-borne fungi, Biological diversity**,** Population structure, Evolution, Adaptability.

Resumen

Rhizoctonia solani AG-3 Kühn (teleomorph: *Thanatephorus cucumeris* (Frank) Donk) es un patógeno del suelo importante en cultivos de papa a nivel mundial. Aunque este patógeno es importante en Colombia actualmente no existe información acerca del patógeno y la enfermedad. Esta investigación se realizo utilizando pruebas moleculares y biológicas con el fin de conocer los Grupos de Anastomosis (GA) asociados a síntomas en las principales regiones productoras de papa en Colombia. Adicionalmente la variabilidad genética y su distribución geográfica, así como la respuesta del patógeno a dos temperaturas, a un fungicida y su agresividad sobre plantas de papa fueron evaluadas. Las muestras fueron colectadas en tallos de plantas sintomáticas de papa en campos naturalmente infestados, posteriormente fueron procesadas en el laboratorio y los aislamientos obtenidos se sometieron a diferentes análisis. El GA-3 de *R. solani* fue el comúnmente asociado a los síntomas en campo. Los aislamientos colectados en diferentes regiones geográficas fueron variables y la variabilidad no está asociada a regiones geográficas, las poblaciones son genéticamente similares mostrando el efecto del flujo de genes en la estructura genética de la población. La evaluación de la respuesta del patógeno a dos temperaturas, la sensibilidad al fungicida thifluzamide y la agresividad en diferentes órganos de las plantas de papa mostraron que los aislamientos de todas las localidades geográficas varían en la respuesta los factores evaluados. Los niveles de costra negra y de chancros variaron entre aislamientos sobre el cultivar de papa evaluado.

Palabras clave: Hongos del Suelo, Diversidad Biológica, Estructura de la Población, Evolución, Adaptabilidad

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Introduction

Rhizoctonia solani Kühn Anastomosis Group 3 (AG-3) (teleomorph: *Thanatephorus cucumeris* (Frank) Donk) is an important soil-borne pathogen of potato (Banville *et al.*, 1996). *R. solani*–AG-3 affects plants on different growth stages. Early in the season the fungus causes necrosis in emerging sprouts and the killing of the sprout tip; later in the season dark brown cankers are formed at the base of the stems, for that reason the disease is named "stem canker" (Banville *et al.*, 1996; Jeger *et al.*, 1996; Simons and Gilligan, 1997). At the end of the crop cycle, sclerotia are formed on the tubers surface, especially after vine death; in this state the disease is called black scurf. Cankers formed on the stem base cause growth delay as secondary symptom due to the diminishing of water and minerals uptake by the plant. The infection on stolons has great influence on the number and size of tubers. Additionally, severe infections cause deformation and cracking. Sclerotia affect tuber quality reducing their marketability and are fundamental for the survival of the pathogen on the fields, and they are the main source of inoculum for long distance dispersal (Banville *et al.*, 1996; Jeger *et al.*, 1996; Tsror, 2010).

The life cycle of this fungus comprises two phases: an asexual and a sexual. The asexual phase is the most commonly observed in the fields. Infection starts with hyphae growing on the plant surface from mycelia or sclerotia. Then the fungus forms appressoria to penetrate plant cells. Initial infection is followed by the release of enzymes that degrade cell walls, kill the cells, and promote the spread of hyphae in the dead cells (Banville *et al.*, 1996; Tsror, 2010). The sexual phase is characterized by formation of hymenia on the stem base. Environmental factors, such as air and soil temperature, relative humidity, wind velocity and concentration of O_2 and CO_2 can determine the occurrence of *T. cucumeris.* The Basidiospore dispersal occurs almost exclusively during the night, but little is known about the relative importance of basidiospores as primary and secondary inoculum in the epidemiology of these diseases *(*Naito, 2006*).*

Previous studies have employed various methods to examine the variation among isolates of *R. solani* AG-3. Results of those studies have shown that populations of this pathogen are genetically variable (Ceresini *et al.*, 2002a; Ceresini *et al.*, 2002b; Campion *et al.*, 2003; Justesen *et al.*, 2003), with differences in virulence among isolates (Carling and Leiner, 1990; Bains and Bisht, 1995; Lehtonen *et al.*, 2008) and differences in their sensitivity to fungicides (Campion *et al.*, 2003; Lehtonen *et al.*, 2008). Those characteristics are important when proposing control measures of the diseases, because variable populations are more susceptible to overcome the control measures imposed in order to diminish their populations and to reduce the effect of the diseases in the fields.

The amount and distribution of genetic variation within and among populations is called genetic structure and is determined by the evolutionary history of populations, then its study gives insights into the evolutionary processes that shaped a population structure (McDonald and Linde, 2002) and can be used to predict the evolutionary potential of the pathogens and to formulate control strategies of the diseases.

In Colombia potato is grown at the high lands in the Andean zone (2500-3200 masl). The departments that have the highest production are Cundinamarca, Boyacá, Nariño and Antioquia; they contribute with 70% of the national potato production. Seed production is mainly domestic, with local production and informal exchange among farmers; only a few of them use certified tuber-seed, and this has contributed to the spread of this pathogen and actually it is present in all the production areas of potato. This pathogen is recognized only by few farmers in Colombia, basically because there is no information about its importance, distribution and biology. This research was proposed with the aim to go in deep in the knowledge of this pathogen and the disease in potato crops in Colombia. The main goals were: (i) to identify the Anastomosis Groups associated with symptoms in the main producing potato areas in Colombia (ii) to characterize the genetic population structure of this pathogen and (iii) to study the response of this fungus to stress factors. Each main objective is organized as a chapter of this document. The information generated can be used for basic and applied purposes, for example to know the evolutionary and adaptive history of this fungus as well as to propose management strategies of the disease.

To obtain the isolates, commercial potato fields were visited on seven of the main potato producing states in Colombia. Plants were reviewed for aboveground symptoms and for the presence of cankers at the base of the stems. The farmers were inquired about their knowledge of the disease and symptoms in their fields. Symptomatic plants were transported to the laboratory to do the isolation of the fungus. The Anastomosis Groups (AG) were identified by PCR using specific primers for AG-3, sequencing the ribosomal DNA using universal primers for fungi and by somatic compatibility.

The population structure was studied by characterizing and evaluating microsatellite markers which were obtained from the genome of *R. solani* AG-3. Polymorphisms at the SSR markers were analyzed by capillary electrophoresis followed by data analysis using several software, in order to study the frequencies of the alleles in the populations and how was this related to the evolutionary forces. SSR are effective markers that have been used in many species for studies of population genetics. SSR markers have many advantages over other markers such as ease analysis, high polymorphism rate, high reliability and codominance. Actually the availability of genomes sequenced makes their development more easy and achievable (Schlötterer, 2000).

The analysis of multilocus data allows determining the genotypic variability into the populations, and inferring the relative importance of each evolutionary force in shaping the genetic structure of the populations. The calculation of genetic distances between populations gives an estimate of isolation or relationship among populations. Mutation and genetic drift at selectively neutral loci lead to differentiation in the allele frequencies between populations and gene flow give cohesiveness to them (McDonald and Linde, 2002; Grünwald *et al.*, 2006). Multivariate analysis is a convenient way to represent the overall organization of genetic data, making statistic inferences about the spatial distribution of genetic diversity and to detect groups associated to ecological parameters.

The multilocus genotype of an individual helps to compute its probability of belonging to a given subpopulation. This is useful to determine the proportion of individuals that are immigrants and to identify the geographical origin of the individuals (de Meeus *et al.*, 2007). Additionally, different approaches based on Bayesian statistics and Markov Chain

Monte Carlo simulations have been developed, to find hide population structure due to unknown factors, that contributes to shape the genetic structure of the populations (Huelsenbeck *et al.*, 2011; Pritchard *et al.*, 2000)

By analyzing genetic and genotypic data, is possible to find evidences of random mating, inbreeding or asexuality into the populations. This allows inferring the predominant reproductive mode into the populations. Testing for linkage between pairs of loci, Hardy Weinberg Equilibrium and calculating the inbreeding coefficient (F_{IS} value) is possible to determine deviation of panmixia. Reproduction and mating systems have effect on the way that gene and genotypic diversity is distributed within and among individuals into the population. Recombining populations of pathogens can put together new combinations, particularly those related to virulence or resistance to fungicides generate more successful genotypes in agro-ecosystems (Milgroom, 1996; McDonald and Linde, 2002; Grünwald *et al.*, 2006; de Meeus *et al.*, 2007).

The study of the adaptation of agricultural pathogens includes their ability to emerge in agro-ecosystems. The emergence occur through several mechanisms, including host-tracking, host jumps, hybridization and horizontal gene transfer (Stukenbrock and McDonald, 2008). The evolution of virulence and fungicide resistance genes in response to control strategies of pathogen populations have interest in evolutionary studies (McDonald and Linde, 2002; Stukenbrock and McDonald, 2008). The base for evolution of organisms is their variability. In pathogens characters with effect in fitness are a good option to evaluate adaptability. Fitness can be assessed by measuring growth and meiotic or mitotic sporulation (Pringle and Taylor, 2002). The ability to improve fitness via adaptive evolution may be affected by environmental change, that decreases individual and population fitness and may also impact genetic variation and the evolution of polygenic traits (Hoffmann and Merila, 1999).

The evaluation of isolates of *R. solani* from different geographical origin to different stress factors is the first step in the knowledge of the ability of the Colombian populations to be adapted to them. Previous research has shown that isolates of this pathogen react differentially when they are challenged with fungicides *in vitro* (Campion *et al.*, 2003; Lehtonen *et al.*, 2008; Willi *et al.*, 2011), or temperatures out of its optimal (Willi *et al.*, 2011).

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Chapter 1. Characterization of Anastomosis Groups associated to stem canker in potato crops in Colombia

1.1. The state of the art

1.1.1. Rhizoctonia solani Kühn

Rhizoctonia solani Kühn [anamorph, teleomorph *Thanatephorus cucumeris* (Frank) Donk] is the most studied species within genus *Rhizoctonia*. The main characteristics of this specie were defined as follows: (i) Some shade brown hyphal pigmentation (ii) Branching near to the distal septum of cell in young vegetative hyphae, (iii) Constriction of hyphae and formation of septa at short distance from the point of origin of hyphal branches (Carling and Leiner, 1990) (iv) The presence of dolipore septa and (v) multinucleate cells in young vegetative hyphae. Characters never existing include presence of clamp connections, conidia, rhizomorphs and sclerotia differentiated on ring and medulla (Sneh *et al*., 1998). Morphology of teleomorphs also has been used as criteria for classification. The teleomorph of Rhizoctonia belongs to the subdivision Basidiomycotina; class Hymenomycetes and almost all Rhizoctonias belong to the Subclass Holobasidiomycetidae.

In nature *R. solani* mainly exists as vegetative hyphae and sclerotia. Sclerotia are composed of compact masses of cells generating tight hyphal clump that protects and preserves the fungus over non-optimal times (Sneh *et al*., 1998). The fungus is dispersed via mycelia or sclerotia present on tuber-seeds, in contaminated plant debris or in soil (Keijer, 1996).

The host range of *R. solani* is wide, it causes diseases on important crop plants including species in the Solanaceae, Fabaceae, Asteraceae, Poaceae and Brassicaceae as well as ornamental plants and forest trees (Ogoshi, 1996). Disease symptoms include leaf blights, leaf spots, damping-off, rot on roots, shoots and fruits, canker lesions on sprouts and stolons and sclerotial diseases (Ogoshi, 1996).

1.1.2. Grouping of *R. solani* isolates

Classification of *Rhizoctonia* spp. has evolved mainly from studies of isolates obtained from diseased plants. Affinities for hyphal fusion (anastomosis) was the first methodology used to group isolates in *Rhizoctonia* spp. Matsumoto (1921) proposed four categories to group strains based on hyphal anastomosis: perfect, imperfect, contact and no reaction. The cell death was included as criteria to evaluate relation between individuals. Categories currently used involve the previous criteria and are defined as C3 (Perfect reaction, this shows a very

close relationship indicating the same AG), C2 (more distant reaction, related isolates which are in the same AG but belong to different vegetative compatibility population (VCG), C1 represents a more distant relationship as can be found in AGs with subgroups and C0 (No reaction, different AG) (Carling, 1996).

Studies using restriction fragment length polymorphism (RFLP) of nuclear DNA (Andersen, 1996), RFLPs (Jeon *et al*., 2010) and sequencing of ribosomal DNA (rDNA) (Kuninaga *et al.*, 1997; González *et al.*, 2006; Sharon *et al.*, 2006; Sharon *et al.*, 2008)**,** and β-tubulin (González *et al*., 2006), analysis of soluble proteins (Reynolds *et al*., 1983), pectic zymograms (Balali *et al*., 2007), lectins (Kellens and Peumans, 1991; Hamshou *et al.*, 2007), profiles of whole-cell fatty acid composition (Stevens Johnk and Jones, 2001; Priyatmojo *et al.*, 2002a; Priyatmojo *et al.*, 2002b) and serology (Adams and Butler, 1979) have been used to classify groups in the *Rhizoctonia* complex and they support the anastomosis grouping. These approaches have clarified the taxonomy, genetic relationships and population structure of this complex pathogen. Currently, sequence analysis of ribosomal DNA (rDNA) is used as a simple and reliable methodology for the accurate designation of *Rhizoctonia* species and their subgroups (Kuninaga *et al.*, 1997; Sharon *et al.*, 2006; Sharon *et al.*, 2008).

13 AGs have been described, they are designed as AG-1 through AG-13 (Carling *et al.*, 2002; Truter and Wehner, 2004; Sharon *et al.*, 2008). The first AGs described (AG-1, -2, -3 and -4) produce the most destructive *Rhizoctonia* diseases around the world, and those described later are considered less destructive pathogens and have more restricted geographical distribution (Carling *et al*., 2002). Binucleated *Rhizoctonia* (BNR) species are sometimes considered as an additional AG since they are able to anastomose with some of the existing AGs (Kuninaga *et al*., 1979; Sneh *et al*., 1998). BNR species have been reported to be pathogenic (Rinehart *et al*., 2007), weakly pathogenic on different plant species (Martin, 1988; Olaya and Abawi, 1994) or no pathogenic.

Individual isolates belonging to the same AG still can differ in host range, virulence, and in morphological, genetic and physiological features. Intra-specific groups (ISG) within AGs and vegetative compatible populations (VCP) comprising highly similar isolates of *R. solani* have been identified by biochemical, serological, molecular and biological methods (Ogoshi, 1987; Kuninaga *et al.*, 1997; MacNish *et al.*, 1997). Isolates belonging to same VCP can be considered as clones, representing identical fungal strains, whereas ISGs within specific AGs are considered as subspecies. Subgroups have been identified in AGs -1, -2, -3, -4, -6, -8 and -9 (Sharon *et al*., 2008). The members each subgroup show hosts preferences, this can be the result of particular ability to produce virulence factors (Stodart *et al*., 2007).

Teleomorphs are originated under specific environmental conditions for each AG. Hymenium is composed by short hyphal cell that branch frequently, producing dense interwoven mats on which basidia are formed. The sterigmata arise from the basidia ranging in number from one to seven. The shape of basidiospores and basidia, the number and size of basidia,

basidiospores and sterigmata vary among species, being important characters for classification of *Rhizoctonia* spp (Sneh *et al.*, 1998; Naito, 2006).

1.1.3. Stem canker and black scurf on potato (*Solanum tuberosum* L.)

Black scurf and stem canker on potato caused by *Rhizoctonia solani* are economically important diseases, causing both quantitative and qualitative damage in potato crops throughout the world (Banville *et al.*, 1996; Jeger *et al.*, 1996b; Banville and Carling, 2001; Tsror, 2010). Quantitative losses occur due to infection of the stems, stolons and roots, that affects size and number of tubers (Carling *et al*., 1989). Qualitative losses occur mainly through the production of misshapen tubers and the development of sclerotia on the tuber surface downgrading their quality (James and McKenzie, 1972; Hide *et al.*, 1973; Frank and Leach, 1980; Anderson and Stretton, 1982; Carling *et al.*, 1989; Tsror and Peretz-Alon, 2005).

The severity of the symptoms of *Rhizoctonia* disease in potato depends on inoculum potential on tubers and in soil along with local climatic conditions. AG-3 is most virulent in cool growing conditions (Carling and Leiner, 1990; Bains and Bisht, 1995; Campion *et al.*, 2003; Justesen *et al.*, 2003). Economic impact of black scurf depend on the market of destine of the tubers. The highest impact occur on the tuber-seed market and depends on the norms of each country for the internal trade as well for the importation from external countries (Banville *et al.*, 1996). The effect of *Rhizoctonia* disease on the number, size distribution and tuber quality leads to yield losses ranging from 10% to 30% on marketable size tubers (Carling *et al*., 1989).

For a long time *R. solani* AG-3 was documented as host - specific and the main *R. solani* group infecting potato (Carling and Leiner, 1986; Carling and Leiner, 1990; Bains and Bisht, 1995; Balali *et al.*, 1995; Virgen-Calleros *et al.*, 2000; Banville and Carling, 2001; Ceresini *et al.*, 2002; Campion *et al.*, 2003; Justesen *et al.*, 2003; Truter and Wehner, 2004; Woodhall *et al.*, 2007; Lehtonen *et al.*, 2008a; Cedeño *et al.*, 2001). However, isolates of this AG have been reported to cause target spot of tobacco; tomato and eggplant. Analysis based on DNA sequence has lead to the subdivision of the AG-3 in subgroups potato (PT) (Johnk *et al*., 1993; Kuninaga *et al*., 2000), Tobacco (TB) (Kuninaga *et al*., 2000) and tomato (TM) (Kuninaga *et al*., 2000; Kuninaga *et al*., 2007).

Isolates belonging to AG-2-1 (Carling and Leiner, 1986; Cedeño *et al.*, 2001), AG-4 (Anguiz and Martin, 1989; Kuninaga *et al.*, 1997; Virgen-Calleros *et al.*, 2000; Truter and Wehner, 2004) and AG-5 (Bains and Bisht, 1995; Balali *et al.*, 1995; Virgen-Calleros *et al.*, 2000) are found frequently associated to stem canker on potato. Also AG-7 in Mexico (Carling *et al*., 1998) is reported to be pathogenic to potato. AG-1 has been isolated from potato roots (Bandy *et al.*, 1988) and AG-8 has been reported infecting and pruning potato roots, leading

to damping-off and reducing foliage, although no classical signs of *Rhizoctonia* disease were apparent (Carling and Leiner, 1990; Truter and Wehner, 2004). AG-9 has been found associated with potato and potato-growing soils, but it seems to be limited to the infection of young seedlings (Carling and Leiner, 1986). Artificial inoculation of potato with AG-13 led to formation of small stem canker lesions on shoots and roots (Carling *et al*., 2002). This indicates that in experimental situations some AGs are able to initiate limited necrosis although normally they do not infect potato. Binucleated *Rhizoctonia* (BNR) isolates have been isolated from potato plants, however they were mildly virulent or avirulent (Carling and Leiner, 1986) and have been proposed as an alternative to the disease control.

1.1.4. Disease cycle

1.1.4.1. Infection of stems and stolons

The infection process by *R. solani* on stems and stolons of potato plants starts when mycelia or hyphae from a germinating sclerotia grow attracted by chemical exudates from the plants (amino acids, sugars, organic acids and phenols (Keijer, 1996). Penetration may be mechanical or enzymatic (Weinhold and Sinclair, 1996). Mechanical penetration occurs when the fungus find a weak spot on the plant surface where it can break down the protecting layer. When inside the host, the fungus starts to grow inter- and intracellularly degrading the tissue, the result are necrotic lesions on epidermal tissue of shoots, roots and stolons or damping-off of the young seedlings (Demirci and Döken, 1998). In potato plants the disease potential varies over time, as the tissues and organs mature show less susceptibility. However a plant can escape to sprout pinch or stem canker and yet be severely affected by stolon infection (Banville *et al.*, 1996).

1.1.4.2. Infestation of tubers

Infestation of tubers with sclerotia starts when the mother plant begins to senesce. Sclerotia does not penetrate more than five or six cells deep in the periderm and does not result in physical damage of the tuber (Banville *et al.*, 1996). Formation of sclerotia on tubers is higher during the time that follows vine kill or natural plant senescence, this means that sclerotial formation is inhibited during active plant growth and the inhibition disappears gradually with the death of the mother plant and the tuber maturation (Dijst *et al.*, 1986; Dijst, 1988). Sclerotia may occur on progeny tubers from healthy, uninfected plants as well on infected plants. The absence of sclerotia does not mean that the pathogen is not present. *R. solani* is transmitted by contaminated seed tubers, providing the most efficient mechanism for its long-distance dispersal. Once established in soil, the mycelium and sclerotia of the pathogen are an additional source of primary inoculum.

1.1.4.3. Symptoms

Symptoms caused by *R. solani* AG-3 on potato are observed on below- and above-ground organs in different stages throughout the crop season (Carling and Leiner, 1986). Before emergence, the fungus attacks underground sprouts. Lesions formed near the growing tip may kill them resulting in the inhibition or the delay of plant emergence causing poor and uneven stands with weakened plants (Baker, 1970; Banville, 1989; Banville and Carling, 2001). Infection of growing plants lead to the formation of cankers on stems and roots, typically they are brown, dry and sunken lesions (Banville *et al.*, 1996; Jeger *et al.*, 1996a; Agrios, 2005). Cankers formed in the stem base may girdle the stem, interfering with the normal upwards water and downwards carbohydrate movements thus reducing plant vigor, weakening plants and lowering stands or stem numbers, or both; as a consequence, tuber yield is reduced (Jeger *et al.*, 1996a; Agrios, 2005). Aboveground symptoms include chlorosis and purpling of leaves and stunting (Hartill, 1989). In severe infection, small, green aerial tubers may be formed on the stems above the soil. The stems become resistant to infection after emergence, it is possible that new compensatory sprouts emerge successfully with no significant damage from previously affected stems (Lehtonen *et al*., 2008b).

As consequence of stolon infection the number, size and quality of tubers is reduced, due to malformation and cracking (Hide and Horrocks, 1994; Agrios, 2005; Fox, 2006). Black scurf, which is the most conspicuous sign of *Rhizoctonia* disease on potato, is characterized by the formation of black, irregular sclerotia of various sizes on the tuber surface; they are formed at the end of the crop cycle, particularly after vine death. Those structures are essential for the surviving and dispersal of *R. solani* (Banville, 1989; Jeger *et al.*, 1996b; Agrios, 2005; Tsror, 2010). An alteration in the size and number of infected tubers can occur, and in severe infections, tubers can be malformed and cracked (Hide *et al.*, 1973; Frank and Leach, 1980; Banville, 1989; Carling *et al.*, 1989; Jeger *et al.*, 1996a). Another symptom of the *Rhizoctonia* disease complex is a superficial white collar on the stem base; this symptom is caused by the teleomorph *T. cucumeris* (Banville and Carling, 2001). Despite its frequency in the field, the role of the sexual form of the fungus in the disease epidemiology is unclear (Jeger *et al*., 1996b).

1.1.4.4. Epidemiology

R. solani spreads to new growing areas on tuber-seed infested by sclerotia and contaminated plant debris in soil (Keijer, 1996; Tsror and Peretz-Alon, 2005). Tuber-borne inoculum is the main source of primary infection leading to stem canker symptoms on the underground plant parts. Soil-borne inoculum of *R. solani* include mycelia and sclerotia already inhabiting soil where the potato is planted (Balali *et al.*, 1995). Soil-borne infection emerges later in the season since the fungus needs some time to grow into proximity with its potato host (Carling and Leiner, 1986).

Alternative hosts can support the long survival of *R. solani* AG-3 in potato producing areas. *R. solani* AG-3 has been isolated from the underground organs of crop and weed plants (Jager *et al*., 1982; Windels and Nabben, 1989). In pathogenicity trials buckwheat, carrot, cauliflower, lucerne, oats, radish, clover, tobacco, tomato, wheat, bean, lettuce, maize, onion, sweet clover and sunflower were susceptible to AG-3 isolates (Carling and Leiner, 1986).The alternative hosts that can be either symptomatic or latently infected have to be considered in the integrated management of the disease.

In Colombia, potato is grown in the Andean highlands (2500 - 3200 masl). The departments with the largest production are Cundinamarca, Boyacá, Nariño and Antioquia, and they contribute with 70% of the national potato production (Espinal *et al.,* 2006). Seed production is mainly domestic. Although some potato growers use certified tuber-seed, it is impossible to guarantee tubers free of *R. solani* and the pathogen is easily dispersed between regions on infested seed tubers. In Colombia the knowledge about the relative importance of the distinct AGs on the etiology of *Rhizoctonia* diseases on potatoes and about the disease itself is still scarce. Farmers are not aware of underground symptoms on stems and in consequence there are no estimations about the importance of this pathogen in quality and tuber yield. The main goal of this research was to generate knowledge about the etiology of *Rhizoctonia* diseases on potatoes based on a large-scale population sampling in Colombia. The specific objectives were (i) to identify and characterize the relative importance of the distinct *R. solani* AGs associated with potato stem canker and black scurf diseases in Colombia using a PCR-based method and classical somatic compatibility reactions; and (ii) to determine the aggressiveness of each AG on potato and other plant species.

1.2. Materials and methods

1.2.1. Population sampling and establishment of a fungal isolate collection

Stem canker-diseased and early-sprouting potato plants were sampled from the main producing areas in Colombia as follows: six distinct fields in Cundinamarca, four fields in Boyacá, two fields in Nariño, Antioquia and Santander and one field in Cauca and Norte de Santander (Figure 1-1). In each field six to eight rows were sampled and between 20 to 30 symptomatic plants were collected. Rows were separated six meters between them. The infected plants were brought to the Laboratorio de Biotecnología Antonio Angarita Zerda (LBAAZ) of the Facultad de Agronomía at the Universidad Nacional de Colombia. Plants were washed with tap water, and infected stem segments and sclerotia from tubers were plated on selective medium for *R. solani* (Ko and Hora, 1979) modified (Castro *et al*., 1988; Ceresini *et al*., 1996) and stored at room temperature in the dark. After 24 to 48 h, pure cultures of *R. solani* were established by transferring hyphal tips from the growing colonies on the selective medium onto potato dextrose agar medium (PDA, Oxoid).

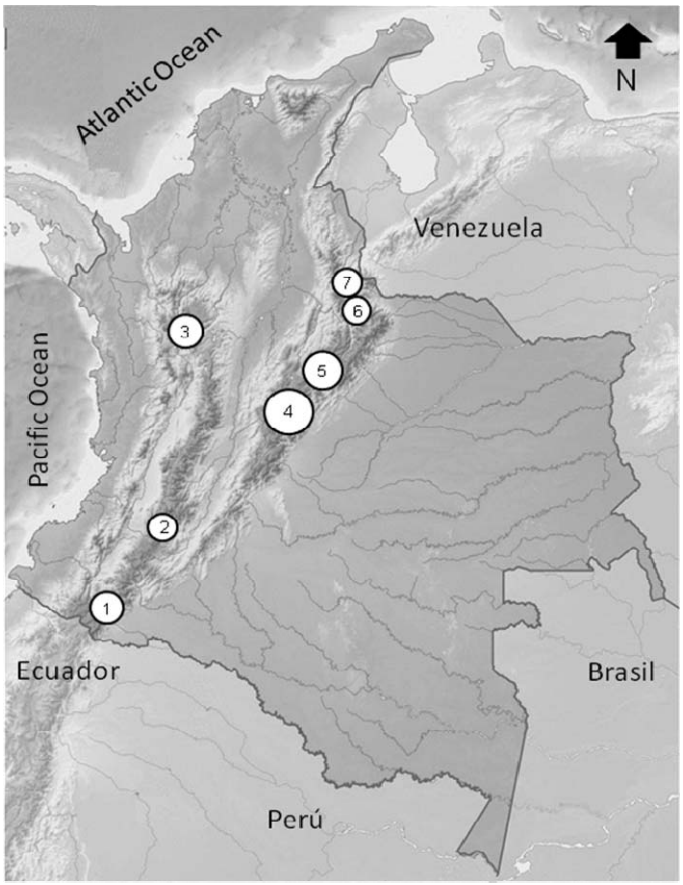


Figure 1-1. Geographical populations of *Rhizoctonia solani* AG-3 sampled from infested potato fields from Colombia. The following departments were sampled: 1. Nariño (N= 45), 2. Cauca (N=9), 3. Antioquia (N=63), 4. Cundinamarca (N=171), 5. Boyacá (N=110), 6. Santander (N=21) and 7. Norte de Santander (N=14). N: number of isolates obtained.

1.2.2. Anastomosis group identification

Mycelium for genomic DNA extraction from isolates collected was obtained from 5-day-old cultures. Each isolate was grown on plates containing PDA covered by a sterile cellophane sheet. After incubation at room temperature, mycelium from each isolate was harvested by scraping the mycelia from the cellophane membrane, followed by freezing and lyophilization. DNA was extracted with the QIAGEN DNeasy Plant Mini-Kit.

The anastomosis group was determined by selective amplification of the ribosomal DNA (rDNA) region of the fungus using specific primers for *R. solani* AG-3 (Lees *et al*., 2002). Polymerase chain reactions (PCR) were carried out in 10-µl volumes containing 10-50 ng of genomic DNA, 10 mm of KCl, 10 mm of $(NH_4)_2SO_4$, 20mM of Tris-HCl, 2mM of $MgSO_4$, 0.01% of Triton X-100, pH 8.8 (NEB), 0.2 µM of each primer (Microsynth), 0.1 mM of each dNTP and 0.06 U of *Taq* polymerase (NEB). PCR conditions comprised initial denaturation of

2 min at 96°C, followed by 35 cycles of denaturation for 30 s at 96°C, annealing for 45 s at 57°C and elongation for 45 s at 72°C, with a final extension step of 5 min at 72°C. PCR products were visualized with UV on agarose gels.

In order to determine the AG from isolates to which the AG-3 specific PCR assay resulted in negative amplifications, the ITS-rDNA region from these isolates and also from few positive controls were amplified using universal primers ITS5 (5'~GGAAGTAAAAGTCGTAACAAGG~3') and ITS4 (5'~TCCTCCGCTTATTGAT ATGC~3') (White *et al*., 1990). Sequencing reactions were prepared using BigDye TM v3.0 cycle Sequencing Kit (Applied Biosystems). Sequencing reactions were run on an ABI Prism 3100 genetic analyzer. Sequences were visualized and edited using the program Sequencher 4.0.5 (Gene Codes Corp).

All the sequences were analyzed with BLAST (Altschul *et al*., 1997) against the NCBI sequence database (National Center for Biotechnology Information, GenBank: http://www.ncbi.nlm.nih.gov) to detect similar sequences of known Anastomosis Groups. Sequence data of the individuals tested were imported and assembled using BioEdit v. 5.0.9 (Hall, 1999). Three sequences of *R. solani* AG-3 from the NCBI database (EF370434, AB19015 and AB19021) were used as references. Nucleotide identity among sequences was determined for each AG identified.

1.2.3. Microscopic and macroscopic hyphal interactions

Microscopic somatic compatibility reactions were determined on an essay using 60 isolates, collected in our study and its AG previously identified by ITS-rDNA sequencing. Pairings were made on slides containing a thin layer of water agar (WA) (Oxoid). Mycelial plugs (0.5 cm in diameter) from actively growing cultures (48 hours in PDA) were paired with mycelial plugs of tester isolates separated 1.5 cm each other. The slides were incubated at room temperature for 24 hours and the and the interaction was evaluated under light microscope, checking the type of interaction for 15 points of contact in each pairing, using safranin O staining (Bandoni, 1979).

The hyphal anastomosis categories evaluated were (i) C0, when paired hyphae grow without recognition; this indicates that the paired isolates belong to different AGs. (ii) C1 (contact reaction), there is only contact between hyphae. Apparent cell wall fusion but not membrane fusion, this reaction indicates that the paired isolates are distantly related, although may belong to the same AG. (iii) C2 (killing reaction), fusion of cell walls and cytoplasmatic membrane occurs, but exchanged cytoplasm does not remain viable and death at the fused cells occur. This indicates that the paired isolates belong to the same AG but are genetically distinct, and (iv) C3 (perfect fusion) in this case fusion of cell walls and membranes occurs. This reaction type indicates that the paired isolates are clones, or genetically identical (Cubeta and Vilgalys, 1997; McCabe *et al.*, 1999). A pair of separate isolates was considered

to being to the same AG if more than 50% of fusion contacts were C1, C2 and C3 reactions (Sneh *et al*., 1998)

An additional set of 21 AG-3 PT isolates was tested for macroscopic hyphal interactions to determine the somatic compatibility among isolates collected from different locations. To determine the macroscopic vegetative reaction between pairs of isolates, plates containing PDA were inoculated with disks of PDA colonized by each isolate and removed from the edge of an actively growing colony and located three cm apart each other. As control each isolate tested was duplicated in the plate, and then each plate contained four mycelia plugs from two different isolates. The 21 isolates were tested in all the possible combinations. Plates were incubated at 15 ^{o}C and were evaluated after 21 days. The macroscopic somatic reactions were defined as merge and tuft. In the category “merge”, the two cultures come together with little or no evidence of demarcation and in the category “tuft” there is an area of differentiation between the meeting of isolates, in this area a band of hyphae raises above the level of mycelium on the agar surface, in this reaction the color of the tuft mycelium is different from the color of the parent isolates (MacNish *et al*., 1997).

1.2.4. Pathogenicity tests

Three isolates of each anastomosis group were randomly chosen for the pathogenicity test. Prior to inoculation each isolate was grown for 48 hours on PDA. The following plants were tested as hosts: (i) seedlings of carrot (*Daucus carota* L.*)*, bean (*Phaseolus vulgaris* L.), corn (*Zea mays* L.), tomato (*Solanum lycopersicum* L.), lulo (*Solanum quitoense* Lam.), pea (*Pisum sativum* L.), raygrass (*Poa pratensis* L.), Wild radish (*Raphanus raphanistrum* L.) and mustard (*Brassica* sp. L.) (ii) Grass plantlets (*Pennisetum clandestinum* Hochst. ex Chiov.) rooting on peat. Potato sprouts (*Solanum tuberosum* L. cv. capiro) rooting on peat were used as susceptible host plant. Seedlings were transferred to pots containing autoclaved quartz sand five days before inoculation. Each plant was inoculated with a 5 mm PDA plug obtained from the margins of the fungal colonies (Carling and Leiner, 1986).

Plants were transferred to a growth chamber and incubated at 20°C with12 hour-photoperiod, watered with distilled water every two days and fertilized with nutrient solution (Murashige and Skoog, 1962) once a week. Fifteen days after inoculation the symptoms on stems and roots were evaluated. The following categories of symptoms were considered: (i) on stems, symptoms were classified as small superficial lesions (SS = mild symptoms with lesions size $<$ 5 mm), large superficial lesions (LSL = lesions > 5mm), cankers (C). (ii) On roots, besides determining the levels of disease incidence on roots, lesions categories were characterized similarly as on stems. Symptomatic and non-symptomatic plant tissue was transferred to selective media to check for the presence of the fungus.

1.3. Results

Symptoms observed in the field were the previously described in the literature. In the stem base there were cankers of different size depending on the age of the stem, being larger in young plants. Diseased plants are randomly distributed in the fields and aerial symptoms were not strong, plants showed less height, but purpling of the leaves was not commonly observed. It was possible to find in a row one diseased plant followed by five or more asymptomatic ones. Commonly plants with all the stems affected and the formation of roots above the point of infection were found (Figure 1-2). The sexual state was observed on plants older than two months.

Figure 1-2. Symptoms of stem canker on potato plants. Left: formation of roots above the canker. Right: Plant with diseased and healthy stems.

The isolates were identified as *Rhizoctonia* based on morphological characteristics. In young hyphae between eight to twelve nuclei were observed. The young mycelium was white, older isolates were brown. Microscopically the ramification of mycelia was in right angle, with a constriction in the base of hyphae (Figure 1-3).

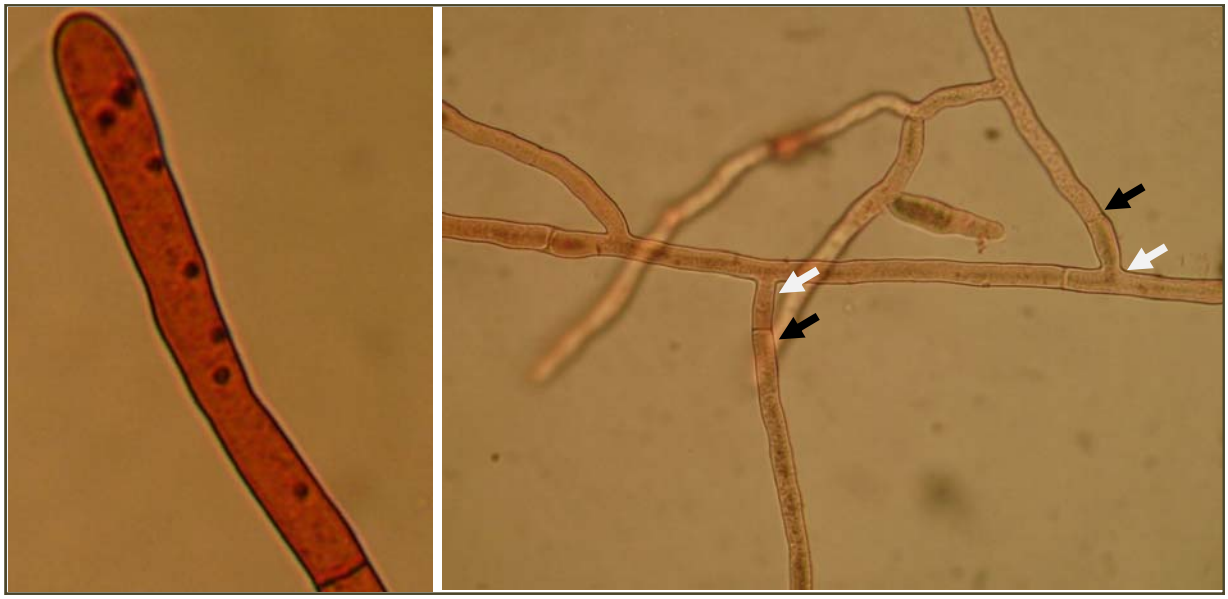


Figure 1-3. Microscopic characteristics of *Rhizoctonia solani* isolates collected in this research. Left: multinucleated cells (1000X, safranine O stain). Right: brown mycelia, ramification of mycelia in right angle, constriction in the hyphal base (white arrows) and septum formation near to the ramification (Black arrows) (400X, lactophenol blue stain).

1.3.1. Anastomosis group identification by PCR and sequencing of the ribosomal DNA

A total of 433 isolates of *R. solani* collected from symptomatic potato plants in Cundinamarca, Nariño, Antioquia, Boyacá, Santander and Norte de Santander states were obtained (Table 1-1). 387 isolates were positive for the AG-3 with the specific primers. Those samples amplified a 500 bp fragment according to the reported size. The results of positive isolates were verified by sequencing of the ITS-rDNA. Four additional isolates that were negative with specific primers were classified as AG-3 based on their ITS-rDNA sequence. The sequence of the ITS-rDNA of those four isolates showed a mutation point on nucleotide number nine into the forward primer, this can explain their failure to amplify. The ITS1-ITS2 regions of the rDNA from *R. solani* AG-3 sequences from this study showed high sequence similarity (98.3–100%) among them and with three AG-3 sequences from NCBI. The sequence analysis allows the discrimination between isolates AG-2-1 from AG-3.

Thus, by the criteria of amplifying with specific primers plus sequencing of the rDNA, 88.45 % of the collection was identified as AG-3. Few other isolates were identified as AG-2-1 (2.54 %), and binucleate *Rhizoctonia* (AG-A, AG-E and AG-I) (6.24 %). Around 3 % of the isolates could not be assigned to any anastomosis group, due to the poor sequence quality (Table 1-1); probably the last group belong to isolates with large nucleotide differences in the primer region.

Table 1-1. Number of isolates belonging to each anastomosis group of *Rhizoctonia solani* associated to symptoms of stem canker and black scurf on potatoes in Colombia.

Department	Municipality	Field	AG-2-1	AG-3	AG-A	AG-E	AG-I	NI	TOTAL
Cundinamarca	Subachoque	1	1	29	1	3	0	0	34
		2	0	20	0	0	1	0	21
	Cogua	1	2	24	3	1	3	0	33
		2	1	36	1	0	0	0	38
	Sibate	1	1	22	0	0	1	0	24
		2	3	15	1	0	1	1	21
Nariño	Pasto	1	0	18	0	2	1	1	22
		2	0	22	0	0	0	0	22
Antioquia	La Union	1	0	20	3	0	0	0	23
		2	0	35	1	0	0	2	38
Boyaca	Soraca	1	2	24	0	0	0	0	26
		2	1	32	2	0	0	0	35
	Ventaquemada	1	0	20	2	0	0	3	25
		2	0	22	0	0	0	2	24
Santander	Carcasi	1	0	11	0	0	0	1	12
		2	0	10	0	0	0	2	12
Norte de Santander	Chitaga	1	0	14	0	0	0	0	14
Cauca	Silvia	1	0	9	0	0	0	0	9
	Number		11	383	14	6	7	12	433
	%		2,54	88,45	3,23	1,39	1,62	2,77	

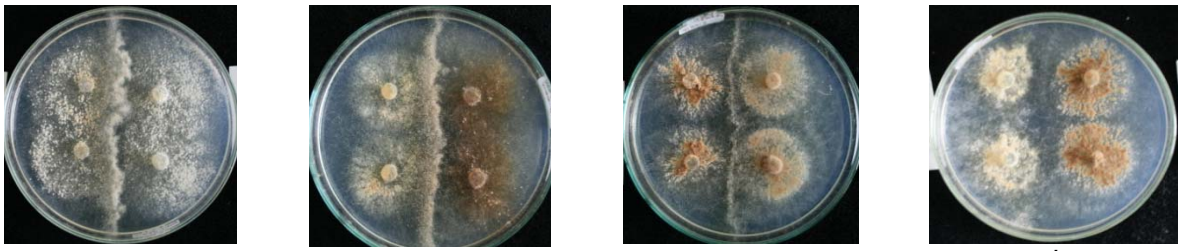
NI= Non identified

From the total of 433 isolates analyzed, 374 were isolated from cankers on the stems and 59 from sclerotia from the tubers seed that originated the plants collected. 55 of the isolates from sclerotia were identified as AG-3, two as AG-2-1, one as AG-A and one as AG-E. From the 374 isolates collected on stems, 328 were identified as AG-3, nine as AG2-1, 13 as AG-A, five as AG-E and 7 as AG-I.

1.3.2. Hyphal interactions

Microscopic somatic interactions between the AG-3 PT tester and the sample of 60 isolates of *R. solani* AG-3 resulted in positive anastomosis reactions. The frequency of reactions C1, C2 and C3 was higher than 50%, confirming the classification of isolates based on their ITS-rDNA sequences.

Somatically incompatible isolates were no detected in the microscopic test. However in the macroscopic somatic test the formation of tufts was common, this is evidence of somatic incompatibility among AG-3 isolates. The macroscopic interactions did not reproduce the categories of microscopic interactions observed. In fact, there was a continuing of somatic compatibility to incompatibility not detected in the microscopic test (Figure 1-4)



a b c d

21D4p Vs 21A7p 18B4p Vs. 15A1p 21A31p Vs. 13A6p2 12D2p Vs. 9C2p2

Figure 1-4. Macroscopic anastomosis reaction between *Rhizoctonia solani* AG-3 isolates. (a) Strong "tuft" reaction between isolates collected in the same field; (b) Strong "tuft" reaction between isolates collected in different field; (c) Mild "tuft" reaction between isolates collected in different field; and (d) "merge" reaction between isolates collected from different field. Numbers underneath each figure are codes for isolate pairs.

1.3.3. Pathogenicity tests

Symptoms observed in the different hosts correspond to the typical symptoms of canker reported previously, although severity varied among AGs (Table 1-2). The isolation from asymptomatic tissue was successful, showing that the absence of infection was not due to no viability on inoculum.

Table 1-2. Symptoms on different plant species caused by *Rhizoctonia solani* AG-2-1 and AG-3 recovered from potato fields in Colombia.

	AG-2-1				AG-3			
	Stem lesion	**Size (Cm)**	**Root lesion**	**Diseased roots (%)**	**Stem lesion**	**Size Cm**	**Root lesion**	**Diseased roots (%)**
Pisum sativum	SSL	< 0.01	SSL	34.5	NI	0	SSL/C	18.6
Phaseolus vulgaris	NI	0	SSL	45.3	NI	0	SSL	31.2
Solanum quitoense	SSL	0.40	NI	0	NI/C	0.70	NI	0
Zea mays	NI	0	SSL	40	SSL	0.40	SSL	60.0
Raphanus raphanistrum	C	0.83	NI	0	NI	0	NI	0
Brassica spp.	C	0.61	NI	0	NI	0	NI	0
Solanum tuberosum cv. capiro	C	1.03	NI	0	C	1.05	NI	0
Penissetum clandestinum	NI	0	NI	0	NI	0	NI	0
Poa pratense	NI	0	NI	0	NI	0	NI	0
Solanum lycopersicum	NI	0	C	0	NI/C	0.62	NI	0
Daucus carota	C	< 0.01	NI/C	7.4	NI	0	NI/C	49.9

Stem and root lesions were categorized as follows: Non-infected (NI); SSL (Superficial Small Lesion) and C (Canker). Size of the lesions is the average of five plants.

Isolates of *R. solani* AG-3 significantly affected stems of solanaceous hosts such as potato, tomato and lulo causing large cankers (> 5mm). No symptoms were observed on roots of

these plants. AG-3 isolates caused abundant small lesions on roots of peas, beans, corn and carrots, but they did not cause stems lesions. The other plants (*Brassica* spp*., R. raphanistrum, P. clandestinum* and *P. pratense*) inoculated with AG-3 did not present symptoms. Besides potatoes, isolates of *R. solani* AG-2-1 affected the Brassicaceae hosts (yellow mustard and purple mustard), causing stem cankers from 0.4 to 10mm, but not on roots. However, these isolates produced small spots on a large proportion of roots from pea, bean, corn and carrot. The two grasses (*P. clandestinum* and *P. pratense*) evaluated did not show any symptoms when inoculated with AG-2-1 nor AG-3, showing that they are not suitable host for those AGs.

Symptoms caused by binucleate *Rhizoctonia* isolates (AG-A, AG-E and AG-I) were characterized by the presence of many small superficial lesions on roots. Soft symptoms are characteristic of some binucleate isolates of *R. solani,* and they have been used as biocontrol organisms

1.4. Discussion

This is the first large scale population study carried out in Colombia aiming at determining the distribution of AGs associated with *Rhizoctonia* diseases on potatoes and assessing the influence of the various AGs in symptom expression on different hosts.

Previously was reported that the main AG associated to stem canker is AG-3, and the Colombian population was not an exception. The most proportion of isolates belonged to this AG. Those results were confirmed by PCR with specific primers, sequencing of the rDNA and somatic compatibility.

The most efficient and accurate methodology to identify AGs was PCR and sequencing. The somatic compatibility tests confirmed the results about the AG, however the differentiation between perfect fusion and death reactions were not possible. Pairings amongst AG-3 isolates resulted in C0, C1 and C3 reactions and when AG-3 was paired with AG-2-1, C0 and C1 reactions were observed.

In this work the AG differentiation by somatic compatibility was not a reliable methodology. However the macroscopic somatic compatibility test allows to infer death reactions between pairs tested. Previously was shown that C2 and C3 reactions are highly correlated with the macroscopic vegetative reactions “tuft” and “merge” respectively (MacNish *et al*., 1997). For instance macroscopic vegetative reactions can be used to predict microscopic anastomosis reactions and vice-versa. Dead reaction was the common reaction into the isolates tested, and was evident due to the barrage formation (Figure 1-3), a macroscopic indicator of vegetative incompatibility between isolates belonging to the same AG (MacNish *et al*., 1997). In Basidiomycetous fungi the somatic incompatibility is commonly restricted to secondary (multicariotic) mycelia, in *R. solani* has been associated to differences between the products

of the genes involved (Burnett, 2003) and has been proposed as a mechanism to prevent the horizontal transmission of malign elements like myco-viruses in groups like aspergilli (Pál *et al*., 2007).

Variation in the interaction among isolates was observed; in some pairings the “barrage” was strong with a huge amount of aerial mycelia (Figure 1-3 a, b) whereas in other cases the line was soft but well defined. 91.31% of the pairs formed barrage indicating genetic diversity among the isolates tested, at least in the genes related with somatic compatibility. 8.69% of the pairings showed “merge” reaction; those isolates came from different fields, except for one isolate from Nariño1 that showed “merge” reaction with five isolates belonging to different geographical origin.

AG-3 is the major and most aggressive group of *R. solani* on potato in Colombia. The symptoms were the typical canker reported on stems. Roots did not show symptoms; probably the environment was not suitable for root infection, considering that there are reports of symptoms on this organ. Our results about the prevalence of AG-3 on potato are in accord with previous worldwide reports (Carling and Leiner, 1986; Anguiz and Martin, 1989; Bains and Bisht, 1995; Balali *et al.*, 1995; Virgen-Calleros *et al.*, 2000; Campion *et al.*, 2003; Truter and Wehner, 2004; Woodhall *et al.*, 2007; Cedeño *et al.*, 2001). The AG-2-1 was also detected on stem cankers, but in lower frequency, and it caused only mild symptoms on potato.

Our results of the pathogenicity tests on different plants species showed that AG-3 is able to infect different hosts but there were differences among plants in severity and affected organ. Potato, tomato and lulo exhibited stem cankers; pea, bean, corn and carrot showed infection on the roots. Those results point at the limited efficiency of plant rotation as a disease management practice, because plants that usually are found in the fields may harbor AG-3, and although the amount of disease is low, those plants may contribute to maintain and increase the inoculum of *R. solani* AG-3 in soil.

Although host specific all AGs have proved to cause some level of damage in hosts different to their original host. This is relevant considering that AG-3 live on alternate hosts. Although the severity of the lesions is low, the pathogen can on alternate hosts increasing the potential of inoculum in absence of its primary host (potato). Crop rotation is one of the main strategies of control of stem canker and black scurf, and then it is necessary to select carefully the plant species for this practice to avoid the increase of inoculum by alternate hosts. The absence of infection on grasses shows that they are a proper option for rotation.

AG-2-1 was exclusively found in samples from Boyacá and Cundinamarca. Its occurrence on potato in these regions may be attributed to the abundance of two Brassicaceae hosts, yellow (*R. raphanistrum*) and purple mustard (*Brassica* spp.), that are common weeds in potato fields (Arrieta, 2000). In fact, this AG has been reported as a Brassicaceae pathogen (Carling and Leiner, 1986).

The presence of individuals of different AGs on lesions does not necessarily indicate a pathogenic relationship between potato and those strains (Carling and Leiner, 1986). The ability of AG-2-1 isolates to cause cankers on potato stems under artificial inoculation, indicates that potato is a suitable host for this AG and suggests that its presence in the field can enhance symptom expression of AG-3, the primary AG associated with potato.

The AGs -4, -5, -7 and -8 previously have been reported associated to stem canker on potato in different countries (Anguiz and Martin, 1989; Bains and Bisht, 1995; Balali *et al.*, 1995; Campion *et al.*, 2003; Truter and Wehner, 2004; Woodhall *et al.*, 2007; Cedeño *et al.*, 2001), however in this study they were not identified. The variability on the range of AGs present in potato fields in different countries could be related to the distribution of their hosts (weeds and wild plants) and the species used for crop rotation.

Binucleated isolates (AG-A, AG-E and AG-I) were present in stem canker lesions in potato. Those Rhizoctonias have diverse ecological preferences: some are non-pathogenic and rather used for biological control of pathogenic *Rhizoctonia* because they cause a mild infection on the plants and may act as antagonists of pathogenic *Rhizoctonia* improving the defense responses of plants (Carling and Leiner, 1986; Poromarto *et al.*, 1998). Other binucleated *Rhizoctonia* can cause serious diseases, like "*damping off*" on different species of plants (Martin, 1988; Escande and Echandi, 1991; Demirci *et al.*, 2002). In this study, pathogenicity tests with binucleated isolates indicated only mild symptoms restricted to stem surface, and plants did not exhibit deep cankers. These results suggest that they could be potentially exploited as bio-control, although an in-depth study of their effects on potatoes is necessary.

1.5. Conclusions

PCR and sequence analysis were useful techniques to discriminate among AGs associated to stem canker and black scurf in Colombia.

Only two anastomosis groups (AG-2-1 and AG-3) were associated with potato stem canker and black scurf diseases in Colombia. The most common was AG-3.

The AGs identified not only infected potatoes but also other plant species such as tomato, lulo, bean, carrot and pea. Those plants sometimes are used as rotation crops which can help to the increase and survival of fungal inoculum.

The grasses were not infected with any of the AGs, this suggest that crop rotation with grasses is the best alternative to diminish the amount of inoculum in potato fields.

Sequence analysis showed high uniformity in nucleotide sequence among AG-3 isolates, corroborating that those genomic regions are not suitable for diversity analysis in the *Rhizoctonia* complex.

Macroscopic somatic compatibility tests showed high variability among the isolates tested, this suggest that the Colombian population of this pathogen is more diverse that previously was though.

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Chapter 2. Genetic diversity and population structure of *Rhizoctonia solani* AG-3 in Colombia

2.1. The state of the art

Plant pathogenic fungi are a large and heterogeneous group of organisms with the ability to cause disease in natural and agricultural ecosystems. Their life history and the ways in which they interact with their hosts are diverse ranging from species that establish biotrophic associations with hosts to species that can survive for long periods of time on dead host tissue or saprophytically in soil (Burdon and Silk, 1997). Their effects on plants are diverse, ranging from mild symptoms to catastrophes in which large areas of plant crops are destroyed (Strange and Scott, 2005).

Plant pathogens have great impact on the food production around the world; they are very difficult to manage, as consequence of their variability on time and space. Crop plants instead have a narrow genetic base and continuously are challenged by pathogens (Strange and Scott, 2005). One of the main goals of plant pathology is to formulate strategies for disease control. Methods are mainly based on genetic resistance in the host, and application of chemical fungicides. Usually the control practices loss effectiveness quickly, due to the ability of the pathogens to evolve and to be adapted to external factors (McDonald and McDermott, 1993; Martin and English, 1997; McDonald, 1997; McDonald and Linde, 2002).

Studies on plant pathogen populations have increased our knowledge regarding their evolutionary potential in the agro-ecosystems and have allowed the characterization of the most important processes governing pathogen evolution (McDonald and Linde, 2002). Along with epidemiology, population genetics help to describe the dynamic of the diseases on time and space; looking for the geographical origin of the pathogens, mechanism of dissemination, and the scale of distribution of genetic variation (from lesion into the plants, to plants, hosts, fields and countries) (McDonald, 2004).This knowledge is important to establish control strategies of pathogens according to the amount and distribution of the genetic diversity of the pathogen populations.

2.1.1. Sources of genetic variability in fungi

2.1.1.1. Mutation

Mutation is the ultimate source of genetic variation in plant pathogenic fungi. Mutation is any heritable change in the genetic material. This includes changes in single nucleotides as well

as the rearrangement of chromosomes such as inversions or translocations (Hartl and Clark, 2007). Changes in the DNA sequence create new alleles (McDonald and Linde, 2002). The main consequences of mutation on plant pathogen populations are: (i) the evolution of avirulence genes that break major resistance genes on the plant, and (ii) the generation of fungicide or antibiotic resistant strains (McDonald and Linde, 2002; McDonald, 2004). In plant pathogens mutations from avirulence to virulence are not common; and by themselves would not cause the breaking of resistance, but if a mutation is coupled to efficient directional selection, virulent strains can increase their frequency causing the loss of effectiveness of resistance genes (McDonald and Linde, 2002).

2.1.1.2. Genetic drift

Genetic drift occurs when a small and randomly subset of individuals from a population survive to catastrophic events reducing population size and when a small, random subset individuals colonizes new host populations (founder event). Allele frequencies between original and the surviving or founding populations will be different, over short periods of time genetic drift lead to unpredictable changes on the allele frequencies in plant pathogen populations (McDonald and Linde, 2002; McDonald, 2004). In plant pathogens founder events occur when a pathogen is introduced into a new area, this usually happens by the movement of contaminated propagation material as result of the breaking of quarantines. Control of diseases into the fields generates bottlenecks due to the reduction on population size of the pathogen; after control the populations arise from few isolates reducing the genetic background of the population (McDonald and Linde, 2002).

The probability of generation of new alleles is proportional to the size of the population, in consequence large populations will have more mutants than small ones, thus large populations likely contain a reservoir of virulence or fungicide resistant genes (McDonald and McDermott, 1993; Burnett, 2003). The consequences of genetic drift are the decrease of diversity into the populations and the increase on the differentiation among populations, both of them due to loss of variation in the random sampling of genotypes (McDonald and McDermott, 1993).

2.1.1.3. Gene Flow

Commonly referred as migration, gene flow is the movement individuals (or their gametes) among sub-populations. The main consequences of gene flow are: (i) Introduction of novel genetic material, (ii) homogenization among populations, (iii) foundation of new populations (founder effect) (McDonald and McDermott, 1993; McDonald and Linde, 2002; Burnett, 2003; de Meeus *et al.*, 2007; Hartl and Clark, 2007). Gene flow is a simple evolutionary force; however it has a great relevance in the genetic diversity of the populations. If the process of migration leads to the foundation of new pathogen populations the immigrants can be established causing large epidemics. If the pathogen already exists the recognition of gene

flow events usually is restricted to instances that lead to obvious shifts in the genetic aggressiveness of the local populations (Burdon and Silk, 1997). The average of gene flow over generations will determine the degree of differentiation between populations (McDermott and McDonald, 1993; McDonald and Linde, 2002; de Meeus *et al.*, 2007; Hartl and Clark, 2007).

2.1.1.4. Reproduction and matting system

Organisms can transmit genes to the next generation by clonal reproduction or via mating and recombination. For clonal organisms their genomes would be an exact copy of the parental, as consequence all parts of the genome of the offspring's will have the same evolutionary history. In contrast, the progeny that result of the mating and meiotic recombination of genetically different parental will have different evolutionary histories (Taylor *et al.*, 1999). Recombination in plant pathogenic fungi occurs either through sexual reproduction or somatic hybridization (Burdon and Silk, 1997). Somatic hybridization may increases genotypic diversity in a pathogen population, but their importance varies both within and among species (Burdon and Silk, 1997).

Reproduction and mating systems can determine how the gene diversity is distributed within and among individuals in a population (McDonald and Linde, 2002). Mating systems determines the crossability between individuals. Pathogen species can range from strict inbreeding to obligate outcrossing with intermediate species with mixed reproduction. Outcrossing organisms put together new combinations of genes, increasing genotype diversity and the potential for rapid adaptation in a changing environment (McDonald, 2004; de Meeus *et al.*, 2007). Asexual and inbreeding pathogens have the potential to keep together well co-adapted combination of alleles, leading to low genotypic diversity (Taylor *et al.*, 1999; McDonald and Linde, 2002; McDonald, 2004).

Pathogenic fungi with mixed reproduction systems take advantage of both conditions. Meiotic recombination puts together new combinations of alleles that are tested in the local environment, then both asexual reproduction and selection operate to keep together combinations of alleles beneficial to the organism in the local environment; in this way a large population of well adapted individuals is created. As consequence populations with mixed reproduction are predicted to be more successful in nature (McDonald and Linde, 2002; McDonald, 2004). The contribution of sexual and asexual reproduction determines how fast new combinations of genes are generated. If those combinations involve virulence or fungicide resistance genes, the pathogen will be successful in breaking resistance and overcoming the effect of fungicides (McDonald and McDermott, 1993; McDonald and Linde, 2002).

2.1.1.5. Selection

Selection is a directional process that leads to changes in allele frequencies or genotypes (McDonald and Linde, 2002; de Meeus *et al.*, 2007). There are two kind of selection: 1) Directional selection; is the selection for or against a specific gene or gene combination which decrease the genetic variation in populations, and 2) Disruptive or balancing selection, that is the selection for or against several genes or gene combination, this increases genetic variation in populations (McDonald and Linde, 2002; McDonald, 2004; de Meeus *et al.*, 2007).

Selection is imposed to each pathogen deme by a range of biotic and abiotic factors. Host-related factors (major resistance genes; variation in presence, quantity, and relative balance of phenolic compounds; variations in morphology, etc.) frequently are seen as important, but their effect on population diversity is unpredictable. In a uniform environment, selection may favor particular genotypes (thus reducing its diversity). The survival of mutant genotypes requires variation in fitness among individuals. The evolutionary potential of a population is proportional to the amount of genetic diversity in genes that have an effect on fitness (McDonald, 1997; McDonald and Linde, 2002; de Meeus *et al.*, 2007).

2.1.2. Dynamics of diversity

The interplay of selection, genetic drift, migration and mutation has a major effect on the genetic structure and diversity of all pathogen populations (McDonald and Linde, 2002). The relative roles of these factors may change between different pathogen-host associations, between stages in the epidemiological cycle, and between associations in agricultural and natural ecosystems (Burdon and Silk, 1997).

The evolutionary forces are continuously intermeshing to generate the overall variation encountered within a species in a continuous and dynamic process. In the agroecosystems pathogens may be considered as colonizing species subject to frequent local extinction and recolonization events (McDermott and McDonald, 1993). Mutation will generate new alleles, if the pathogen is random mating those alleles will be mixed generating novel combinations. Selection chooses the best combination of alleles in a particular environment. Finally under clonal reproduction the new allele combination will be multiplied and the migration moves the genotypes to new areas. If the environment is favorable they can be established in the new geographical areas, in the new fields farmers cause bottlenecks by using different measures to reduce their effect on the crops (McDonald and Linde, 2002).

Viewing pathogens in a metapopulation context reinforces the importance of the ecological scenary to explain the generation and maintenance of diversity in pathogen populations. The interplay of fungal life history and host population size induces asynchrony in pathogen behavior among demes and affects the probability of local drift, extinction, and recolonization in time and space. Drift and migration are opposing forces acting to reduce and increase

within-population genotype diversity respectively and simultaneously increasing and decreasing the variability between populations. Their combined effect leads to a dynamic, ever-changing patchwork of distinct, individual pathogen demes, which may be strengthened by differences in the local selective biotic and abiotic environment (Burdon and Silk, 1997).

2.1.3. Population genetics on plant pathogenic fungi

Population genetics study the processes that lead to genetic changes in populations over time and space (McDonald, 1997). The main goal of population genetics is to understand the evolutionary processes shaping and maintaining genetic variation within and among populations over time and space. Changes in genotype or allele frequencies in populations are considered evolutionary changes; they are fundamental for genetic analysis (Milgroom and Peever, 2003; McDonald, 2004). Pathogen populations constantly have to be adapted to changes in their environment. In agricultural ecosystems, environmental changes include resistant varieties, applications of fungicides and fertilizers, irrigation, and crop rotation (McDonald, 1997). Control strategies, if they are expected to be effective must target a population of pathogen instead of an individual. Thus, plant pathologists should focus more effort on the genetics of populations to understand how populations will evolve in response to different control strategies.

2.1.4. Genetic structure of populations

Genetic structure is defined as the amount and distribution of genetic variation within and among populations, this attribute is determined by the evolutionary history and the potential of change of the organisms in a population (McDonald, 1997; McDonald and Linde, 2002). There are two types of diversity contributing to genetic structure: gene and genotype diversity. Gene diversity is the number and frequencies of alleles at individual loci in a population, it increases as the number of alleles increases and the relative frequencies of those alleles become more similar (McDonald and Linde, 2002). Genotype diversity refers to the number and frequencies of multilocus genotypes, or genetically distinct individuals in a population (Anderson and Kohn, 1995; Taylor *et al.*, 1999; McDonald and Linde, 2002; Halkett *et al.*, 2005).

The genetic structure of pathogen populations offers insights in their evolutionary potential. Understanding the genetic structure of pathogen populations is useful to infer the life histories and the evolutionary processes that shape the populations in agroecosystems (McDonald and Linde, 2002). The knowledge on diversity, its geographical distribution, and their relation to environmental issues could be useful to optimize the breeding programs and also to predict fungicide resistance (McDonald and Linde, 2002). Knowledge on the distribution of genetic diversity within and among populations can be used to identify migration patterns and to reveal cryptic recombination (Ceresini *et al.*, 2002b). The degree of similarity between geographically separated populations provides evidence of gene flow. The gene flow has

important effect on the effectiveness of control strategies, due to the movement of novel virulence genes or genotypes well adapted to the new cropping areas (McDonald and Linde, 2002).

A model proposed to determine the evolutionary risk of plant pathogens depends on their biological, ecological and genetic characteristics (Table 2-1). The model considers that pathogens with mixed reproduction have the highest risk of evolution, the sexual cycle, generate new combinations of alleles (genotypes). The recombinant genotypes can migrate to different environments where they are tested. The most fit combinations of alleles are held together and multiplied through asexual reproduction and selected clones may increase their frequency. The clone(s) with highest fitness can become distributed over a wide area through genotype flow (McDonald and Linde, 2002).

Table 2-1. Factors conditioning the evolutionary risk of plant pathogens

Highest risk of evolution	Lowest risk of evolution
(1) High mutation rate. Transposable elements active.	(1) Low mutation rate. No transposons.
(2) Large effective population sizes. Large overseasoning population. Extinction of local populations rare. No genetic drift, no loss of alleles.	(2) Small effective population sizes. No over-seasoning propagules. Extinction of local populations common. Significant genetic drift, loss of alleles.
(3) High gene/genotype flow. Sexual propagules dispersed by air over long distances.	(3) Low gene/genotype flow. Asexual propagules soil-borne. Quarantines effective.
(4) Mixed reproduction system. Sexual outcrossing and asexual propagules produced.	(4) Asexual reproduction system. Only asexual propagules produced.
(5) Efficient directional selection R-gene deployed in genetically uniform monoculture and continuously over large areas.	(5) Disruptive selection. R-genes deployed in mixtures/multilines. R-genes deployed as rotations in time or space.

Source: McDonald and Linde, 2002

2.1.5. Molecular markers and the study of population genetics

Population genetics deals mainly with genetic processes. To understand the influence of selection, inbreeding, genetic drift, gene flow and mutation in population genetics, is necessary to describe and quantify the amount of genetic variation in a population and the pattern of genetic variation among populations (Nei, 1978; Hedrick, 2005). The most common approach to study population genetics is through molecular markers. Actually there is a wide range of molecular markers available. The choice of the genetic marker depends mainly on the question to answer, on the experience and competence of the researchers and on laboratory facilities (McCartney *et al.*, 2003). In population genetics the molecular markers must help to test ecological and epidemiological hypothesis and to distinguish biological processes in the populations of pathogens, not only to quantify its diversity.

Genetic markers represent genetic differences between individual organisms or species. All genetic markers occupy genomic positions within chromosomes (like genes) (Collard *et al.*, 2005). In fungi the markers used include (i) morphological (phenotypic traits or characters), (ii) physiological (mating types, somatic incompatibility and fungicide resistance), (iii) pathogenic, (iv) cytological and (v) molecular (biochemical and DNA based) (Burnett, 2003). The most widely used are DNA markers, predominantly due to their abundance in genomes (Schlötterer, 2004). The preferred markers in population genetics are characterized by its neutrality, codominance and polymorphism (Collard *et al.*, 2005; Hartl and Clark, 2007).

Microsatellites are widespread markers. They are tandemly repeated sequences of 1–6 nucleotides found at high frequency in the nuclear genomes of most taxa. They are also known as simple sequence repeats (SSR), variable number tandem repeats (VNTR) and short tandem repeats (STR). A microsatellite locus typically varies in length between 5 and 40 repeats. Dinucleotide, trinucleotide and tetranucleotide repeats are the most common motifs choice in molecular genetic studies (Schlötterer, 2000, 2004)

Typically microsatellites display high mutation rate (among 10^2 and 10^6 mutations per locus per generation), generating the high levels of allelic diversity (Schlötterer, 2000; Karaoglu *et al.*, 2004). The mutation rate of SSR markers varies depending on the locus, the length of the repeated motif, the specie and sometimes the allele (Benali *et al.*, 2011). Microsatellites gain and lose repeat units by DNA-replication slippage and by proofreading errors during DNA replication. Both mechanisms primarily change the number of repeats and thus the length of the repeat string (Schlötterer, 2000; Ellegren, 2004; Karaoglu *et al.*, 2004).

2.1.6. The soil-borne plant pathogenic fungus *Rhizoctonia solani* Kühn

Rhizoctonia solani Kühn (teleomorph *Thanatephorus cucumeris* (Frank) Donk) is a soil-borne Basidiomycete fungus. The anastomosis group three sub-group PT (AG-3PT) is the causal organism of the Rhizoctonia disease complex in potato (Wilson *et al.*, 2008) which results in two different symptoms: stem canker and black scurf on the tubers (Tsror, 2010).

On the last years the studies on genetic diversity of *R. solani* have increased. The most studied are the populations belonging to the AG-1, as pathogen of rice, soybean and maize. Analysis with codominant RFLP loci in a population of AG1-IA collected in Texas (United States) found evidence for clonal reproduction by finding the same genotype many times, in the same and in separated fields, however more than a half of the loci were in Hardy-Weinberg Equilibrium and only a few of all possible pairs of loci were in linkage disequilibrium, which indicates that recombination is also occurring (Rosewich *et al.*, 1999). The same situation has been found on the subsequent studies on this AG on Brazil (Ciampi *et al.*, 2008); China and India (Bernardes de Asis *et al.*, 2009), using SSR markers, the results suggest a reproductive mode varying from strictly recombining to a mixed reproductive

system in which there are recombination events followed by clonal expansion during the growing season (Bernardes de Asis *et al.*, 2009).

R. solani AG-3 has been extensively studied (Ogoshi, 1987), however many aspects of its biology and epidemiology still remain unresolved. *R. solani* AG-3 has been considered asexual, producing mycelia and sclerotia as the main structures for dispersion and surviving (Cubeta and Vilgalys, 1997). In the fields, the sexual stage (*T. cucumeris*) is observed, however the role of basidiospores on the cycle life of this pathogen and in the epidemiological cycle of the disease is unknown. Ceresini *et al.,* (2002b) analyzing data from PCR-RFLP molecular markers found evidence of both colonality and recombination defining the population structure of *R. solani* AG-3 in North Caroline (USA).

Studies using molecular markers (Ceresini *et al.*, 2002a; Ceresini *et al.*, 2002b; Justesen *et al.*, 2003; Balali *et al.*, 2007), biochemical markers (Balali *et al.*, 2007), and phenotypic traits (Campion *et al.*, 2003; Lehtonen *et al.*, 2008) have shown high diversity levels in *R. solani* AG-3 populations around the world. The study on five North American populations showed that the gene flow is the main force shaping the geographic structure of this pathogen, the lack of population differentiation is consequence of the tuber-borne genotypes introduced each cycle to the fields (Ceresini *et al.,* 2000).

Despite of the importance of black scurf and stem canker in Colombia, the pathogen biology still remains poorly understood and the most important questions about the genetic structure of *R. solani* AG-3 populations remain unanswered. The main objective of this research was to evaluate the genetic diversity of *R. solani* AG-3 using SSR markers. The analysis of the data will allow us to answer the following questions: (1) Are the Colombian populations of *R. solani* AG-3 genetically diverse? This is a confirmatory question considering de variability found on populations around the world in previous studies, (2) Are those populations genetically subdivided or gene flow is shaping their genetic structure? In Colombia there is a continuous trade of tuber seed and the ruling for low inoculum content (maximum 10% of sclerotia on tuber seed) was established only 2003; this situation surely has contributed to the no differentiation of the populations of *R. solani* AG-3 in Colombia, however the hypothesis is that populations close to Venezuela and to Ecuador are genetically different from those in the Colombian Central Andes. And (3) Is the population structure consistent with a random mating hypothesis? How frequently recombination occurs? The hypothesis is that although recombination can occur this process is not enough strong and frequent in the Colombian population of *R. solani* AG-3

2.2. Materials and Methods

2.2.1 Sampling, fungal isolation and DNA extraction from *Rhizoctonia solani* AG-3

Seven of the main producing potato states in Colombia were sampled: Nariño and Cauca in the south of the country, Antioquia in the west, Cundinamarca and Boyacá in the middle, Santander and Norte de Santander in the north of the country (Table 2-2). Those States are responsible for approximately 85% of the potato production in Colombia (Espinal *et al.*, 2006).

Paired sympatric populations were sampled, six in Cundinamarca, four in Boyaca, two in Antioquia, two in Santander and two in Nariño; and one population in Cauca and one in Norte de Santander (Table 2-2). Samples from plants between two and three months of age exhibiting symptoms of stem canker were collected from naturally infected potato plants *Solanum tuberosum* Gr. andigena and Gr. *Phureja* in commercial farms. In each field 20-30 symptomatic plants were collected distributed along the field, normally in each field seven rows and five plants per row were sampled, with ten meters between rows, sometimes the tuber seed was collected and the isolation of the fungus from sclerotia was made when they were present.

The fungus was isolated transferring fragments of infected stems and sclerotia into plates containing selective media (Ko and Hora, 1971) and incubated at room temperature (20°C) in the dark. Pure cultures of *R. solani* were established by transferring hyphal tips from de colonies growing in the selective medium for 24 to 48 h to plates with potato dextrose agar (PDA). Sclerotia from 25-day-old cultures from each isolate growing on PDA plates with a sterile cellophane sheet were transferred to 1.8-ml cryotubes (Nunc CryoLine System, Denmark) containing anhydrous silica gel for long-term storage at 4°C. Mycelia for genomic DNA extraction was obtained from 5-day-old cultures on PDA containing a sterile cellophane membrane. After incubation at room temperature, mycelium from each isolate was harvested by scraping the culture from the cellophane membrane, the mycelia was frozen and lyophilized. DNA was extracted with the DNeasy Plant Mini-Kit (Qiagen). The AG-3 isolates were determined by PCR with specific primers for the ITS-5.8S region of the ribosomal DNA (Lees *et al.*, 2002).

Table 2-2. Geographic origin of the populations of *Rhizoctonia solani* AG-3 used in this study

State	Municipality	Population name	Geographical coordinates		Altitude (masl)	Potato cultivar	Sampling year
Cundinamarca	Cogua	Cogua1	5º 04´ 32" N	73º 58´33" W	2730	*S. tuberosum* Gr. phureja	2006
		Cogua2	5º 04´ 22" N	73º 58´45" W	2722	*S. tuberosum* Gr. phureja	2006
	Subachoque	Subach1	4º 57´ 26" N	74º 11´59" W	2931	*S. tuberosum* R-12	2006
		Subach2	4º 57´ 11" N	74º 12´ 56" W	3095	*S. tuberosum* R-13	2006
	Sibate	Sibate1	4º 25´ 26" N	74º 15´08" W	3250	*S. tuberosum* ICA-Morita	2006
		Sibate2	4º 25´16" N	74º 14´56" W	3296	*S. tuberosum* Parda Pastusa	2006
Nariño	Pasto	Pasto1	01,14486 N	77,34147W	3095	*S. tuberosum* Parda Pastusa	2007
		Pasto2	01.13798 N	77,31053N	3084	*S. tuberosum* Parda Pastusa	2007
Antioquia	La Union	LaUnion1	5º 58´ 22" N	75º 22´ 59" W	2505	*S. tuberosum* Capiro	2008
		LaUnion2	6º 00´ 26" N	75º 21´ 40" W	2504	*S. tuberosum* Capiro	2008
Boyacá	Ventaquemada	Ventaq1	5º 23´34,3" N	73º 27´49,1" W	2918	*S. tuberosum* Parda Pastusa	2007
		Ventaq2	5º 25´00,2" N	73º 27´25,3" W	2907	*S. tuberosum* ICA Unica	2009
	Soraca	Soraca1	5º 30´ 09" N	73º 20´22,09" W	2809	*S. tuberosum* Gr. phureja	2007
		Soraca2	5º 30´ 45" N	73º 19´33" W	2849	*S. tuberosum* Capiro	2009
Santander	Carcasi	Carcasi1	6º 39´418" N	72º 34´610" W	2950	*S. tuberosum* Parda Pastusa	2008
		Carcasi2	6º 4´470" N	72º 33´181" W	3296	*S. tuberosum* Parda Pastusa	2008
Norte de Santander	Chitaga	Chitaga	7º 03´062" N	72º40´638"W	2989	*S. tuberosum* Parda Pastusa	2008
Cauca	Silvia	Silvia	2º 31´30N	76º19,09W	3238	*S. tuberosum* Gr. phureja	2007

[a] masl = meters above sea level

2.2.2. Microsatellite genotyping

Tandem repeats were brought by the leader of the sequencing project of *R. solani* AG-3*. Twenty-two trinucleotide, four tetranucleotide and seven pentanucleotide microsatellite motifs were selected for developing the markers. Primers were designed from flanking regions using the software Primer 3 (Rozen and Skaletsky, 2000). Polymorphism was initially assessed on agarose gels using 16 isolates randomly chosen from two populations of *R. solani* AG-3 (Subachoque1 and Cogua1). Then they were evaluated by capillary electrophoresis and the best conditions were established for the subsequent analysis.

Each individual from each of the 18 populations of *R. solani* AG-3 was genotyped using a specific set of polymorphic Simple Sequence Repeat (SSR) loci using fluorescent-labeled forward primers (Ferrucho *et al.*, 2009). Isolates from the states of Cundinamarca, Boyacá, Antioquia and Nariño were processed in the plant pathology department of the Institute of Integrative Biology, ETH Zurich. Polymerase chain reaction (PCR) amplifications were performed in 96 well plates in a total volume of 15 µl containing 10–50 ng of total DNA, 1,5 µl of 10X reaction buffer (10 mM KCl, 10 mM $(NH4)_2SO_4$, 20 mM Tris-HCl, 2 mM $MgSO_4$, 0.01% Triton X-100, pH 8.8, NEB), 0.1–0.2 µm of each labeled and non-labeled primer (NEB and Microsynth), 0.1 mM of each dNTP, and 0.06 µL of *Taq* DNA polymerase (5 U/µL NEB). Isolates collected on the states of Boyacá, Santander, Norte de Santander and Cauca were processed in the Institute of Genetics of the National University of Colombia; following the conditions described previously but using Invitrogen supplies.

PCR conditions for all loci were: initial denaturation at 96 °C for 4 min, 35 cycles of denaturation for 30 s at 96 °C, annealing for 30 s at specific temperature for each loci (Ferrucho *et al.*, 2009) and elongation for 45 s at 72 °C, and a final extension cycle of 30 min. Multiplex PCR reactions were conducted for 9 loci in three groups: (Group A) TC_AG3_1, TC_AG3_7 and TC_AG3_14; (Group B) TC_AG3_6, TC_AG3_9 and TC_AG3_18; (Group C) TC_AG3_8, TC_AG3_10 and TC_AG3_19. Individual PCR amplifications were conducted for loci TC_AG3_0 and TC_AG3_16. For fragment analyses, 1–2 µL of fluorescent-labeled PCR product were mixed in three distinct sets: set 1 (Group A + Group B); set 2 (Group C + TC_AG3_16), and set 3 (TC_AG3 _0). Each set was electrophoresed using an ABI PRISM 3100 (Applied Biosystems) automated sequencer along with a fluorescently labeled size standard (GeneScan-500 LIZ; Applied Biosystems). In each set, the PCR products were mixed with sterile distilled water to a final volume of 30 µL. The isolate RH002 (isolated from sclerotia on tuber) was included as control in every run of 94 samples. The genotyping of one of the two populations used for describing the microsatellites was repeated. The statistical binning of the alleles was conducted using the software GeneMapper version 4.0 (ABI).

2.2.3. Data analysis

2.2.3.1. Microsatellite information content

For the data analysis was assumed that *R. solani* AG-3 is a functional diploid (Ceresini *et al.*, 2002b; Justesen *et al.*, 2003), and the data support this supposition. Except for a few cases one or two alleles for each SSR loci were found. The isolates with more than two peaks in the chromatogram were eliminated of the analysis. The initial analysis for the 11 microsatellite markers included the range of repeats for each locus, the total and average number of alleles per locus and per sampled field, the identification of private alleles (i.e., those present in only one population); all them derived from the information of allele frequencies and computed by the program CONVERT version 1.31 (Glaubitz, 2004).

2.2.3.2. Genotype diversity

Each isolate was assigned to a multilocus microsatellite genotype (MLMGs) using GENODIVE (Meirmans and Van Tienderen, 2004), isolates with the same MLMG were treated as clones. Indices of clonal diversity were calculated as follows: (i) number of MLMGs per population, (ii) site specific MLMGs, (iii) clonal fraction or the proportion of isolates originated from asexual reproduction, calculated as 1- (number of different multilocus genotypes) / (total number of isolates) (Zhan *et al.*, 2003), (iv) Stoddart and Taylor's genotypic diversity calculated as $G_0 = 1/\Sigma pi2$, where *pi* is the frequency of the *i*th genotype (Stoddart and Taylor, 1988), and (v) its evenness, an indicator for how the genotypes are distributed within the population (Grünwald *et al.*, 2003). These measures were calculated using the program GENODIVE. If pairs of populations differed in their genotype diversity indices was tested using a bootstrapping approach (resampling with replacement), where the individuals were resampled from the populations and the diversity indices were compared after every replicate (Manly, 1990) using 1,000 permutations with subsampling matching the size to the smallest population (Grünwald *et al.*, 2003). Finally the clone correction was performed to select only one individual of each MLMG to execute the subsequent tests.

2.2.3.3. Gene diversity and population differentiation

Allelic richness and the expected heterozygosity were calculated as indexes of gene diversity using the program FSTAT 2.9.3.2 (Goudet, 1995). Nei's unbiased gene diversity or expected heterozygosity was estimated as $n/(n-1) \times (1 - \Sigma_i p_i^2)$, where p is the observed frequency of the *i*th allele and n is the sample size (Nei, 1978). Allelic richness was estimated as the mean number of alleles per locus (El Mousadik and Petit, 1996) for a standardized sample size of five individuals, using rarefaction (Hurlbert, 1971) as described by (Petit *et al.*, 1998). Finally differences in allelic richness and gene diversity among pairs of populations were tested using a bootstrapping approach based on 1,000 permutations for calculating *p*- values.

2.2.3.4. Population differentiation

The distribution of gene diversity based on hierarchical analysis of molecular variance (AMOVA) was evaluated. The fixation indices (*F* statistics) were calculated to quantify differentiation between pairs of populations and to assess the degree of population subdivision, based on the sum of squared size differences for microsatellite loci (R_{ST}) (Slatkin, 1995). The null distribution of pairwise *F* statistics values under the hypothesis of no differentiation between two populations was obtained by permutating haplotypes between populations. Genetic differentiation between populations was considered significant when $P \leq 0.05$. The AMOVA was conducted to partition the variance components due to among group (states) effect, among-populations-within-group effect, and within-population effect. Significance of the fixation indexes was tested using 10.100 permutations by a nonparametric approach (Excoffier *et al.*, 1992) using the program ARLEQUIN 3.5.1.2 (Excoffier and Lischer, 2010).

2.2.3.5. Reproductive mode: Gametic and Hardy-Weinberg Equilibrium tests

To assess the contribution of sexual recombination to the genetic structure of the Colombian populations of *R. solani* AG-3, the tests of Hardy-Weinberg Equilibrium (HWE) and multilocus association were performed to determine the associations within and among loci, respectively. Tests for HWE were executed for each locus within each population with the program ARLEQUIN 3.5.1.2. This test is analogous to Fisher's exact test on a two by two contingency table but extended to a contingency table of arbitrary size (Guo and Thompson, 1992; Raymond and Rousset, 1995). *P* values were obtained using a Markov Chain Monte Carlo (MCMC) approach, generating an exact probability distribution not biased by rare alleles or low sample size (Raymond and Rousset, 1995).

The hypothesis of independence among locus of different MLMGs was tested using the Fisher's exact test (Garnier-Gere and Dillmann, 1992) based on an MCMC algorithm (with 10.000 batches and 10.000 iterations per batch) (Raymond and Rousset, 1995) and implemented in GENEPOP version 3.4. To avoid the error type I the significance levels were adjusted following the Bonferroni correction method for multiple test (Rice, 1989). The multilocus index of association (*IA*) (Maynard Smith *et al.*, 1993) and $\bar{r}_D$ (an alternative measure of *I*A) were tested for its significance with 10.000 randomizations (Agapow and Burt, 2001). Those tests are implemented in MULTILOCUS version 1.3. The hypothesis of complete panmixia was tested comparing the observed data set to data sets in which sexual recombination is imposed on the data by randomly shuffling the alleles among individuals, independently for each locus. An *IA* significantly different from zero means disequilibrium, $\bar{r}_D$ ranges from zero when there is no disequilibrium to one when there is disequilibrium among tested loci.

The inbreeding coefficient (F_{IS}) across loci was calculated to test for a significant deficit or excess of heterozygote (when compared with HWE expectations) (Weir and Cockerman, 1984), this test was based on 10,000 permutations using the program ARLEQUIN 3.5.1.2. When significant, this test can explain deviation from HWE and GE as inbreeding in the population.

2.2.3.6. Test for Admixture and hidden population structure

A possible cause of the departure from HWE and gametic disequilibrium between pairs of loci into the populations is the Wahlund effect. A Bayesian statistical procedure implemented in STRUCTURE version 2.3.2 was used (Falush *et al.*, 2003; Hubisz *et al.*, 2009; Pritchard *et al.*, 2000). The test determines whether there are individuals immigrants in a sample with respect to their reference geographical population. This program calculates the membership coefficients (*Q*) of every MLMG sampled and assign individuals (probabilistically) to populations based on their genotypes. Additionally, identifies admixed individuals whose genetic composition is drawn from more than one population (Hubisz *et al.*, 2009; Pritchard *et al.*, 2000). An initial run was executed considering all the geographical populations, and a simulation of the best K was made (Evanno *et al.*, 2005). Then three runs of MCMC simulations were performed with a burn-in period of 2.000.000 generations followed by 1.000.000 iterations each run. Parameters were set to the admixture model with the prior number of clusters corresponding to the number of fields sampled (K=18) and using prior information of the populations as the initial condition for assign MLMGs. The prior R_{ST} values used were those previously calculated with ARLEQUIN 3.5.1.2. In a second analysis the same priors were used except that individuals were not assigned to the sampled populations, and k varied from two to 18.

2.2.3.7. Population bottleneck and demographic parameters

The test for recent founder effects or historical bottlenecks was made with the program BOTTLENECK V 1.2 (Piry *et al.*, 1999). The number of alleles at one locus is reduced faster than the gene diversity when the size of the population is reduced, this because rare alleles are more readily affected by drift than more frequent ones. When population size is restored the average number of alleles increases faster than the gene diversity until reaching mutation-drift equilibrium. The test evaluates the heterozygosity excess or deficiency, under the assumption of mutation-drift equilibrium for each sample and for each locus, using an approach based on coalescence (Piry *et al.*, 1999). The program was run using the strict stepwise mutation model (SMM); the deviations from mutation-drift equilibrium across all loci in each population were assessed for statistical significance using the sign test and Wilcoxon's rank test, which is suggested when using less than 20 polymorphic loci (Cornuet and Luikart, 1996).

2.3. Results

2.3.1. Microsatellite information, genotype and gene diversity

From 32 microsatellite loci evaluated, 11 were used to characterize the genetic diversity in 18 populations of *R. solani* AG-3 in Colombia, South America. All loci were polymorphic, except for the loci TC_AG3_8 and TC_AG3_10 that were monomorphic in some populations. 99 alleles were revealed by the analysis of the microsatellite loci. The number of alleles at each locus varied from two (TC_AG3_0) to 14 (TC_AG3_16), with an average of 9 per loci (Annex 2-A). Allele frequencies were not evenly distributed for the loci in all the populations. As a trend each locus had two or three alleles with frequencies higher than 20% and they corresponded to sizes differing in one repetition. In locus TC_AG3_8 alleles with frequencies higher than 85 % were observed. Frequencies of the most common alleles varied among populations but no consistent patterns among loci were observed. A total of 18 private alleles were found in whole population. Nine populations had private alleles; La Union2, Cogua1, Sibate1, Cogua2, Soraca2, pasto1, pasto2, LaUnion1 and Chitaga.

295 MLMGs were found into the sample of 355 isolates analyzed (clonal fraction = 15%) (Table 2-3). The MLMGs were evenly distributed into the populations (evenness = 0.89). Low clonal fractions were observed for almost all populations. Subachoque2 and Ventaquemada2 showed the higher values of clonal fraction (0. 33 and 0.28 respectively) and the lowest clonal fraction was found in Carcasi2 and Silvia with no repeated clones. Clones of a particular MLMG were commonly found within the same field. 58 MLMGs were shared among populations and there was one MLMG repeated 11 times into the whole population. Subachoque1 shared the highest number of MLMGs (8 shared MLMGs from 23 isolates). The populations Sibate1 and Pasto1 did not share MLMGs with any population. The mean genotypic diversity, estimated using Stoddart and Taylor´s index, was 14.5, and scaled to the sample size this value was 84.9%.

We checked for differences among genotypes from tuber seed and stems in the same plant. Pairs of isolates collected on different stems in the same plant were commonly different (56% of the tested pairs) and several isolates from stem canker and sclerotia on tuber seed (25 pairs) had different genotypes (80%) (data not shown).

2.3.2. Gene diversity and population differentiation

All 295 MLMGs of *R. solani* AG-3 were heterozygous at almost all loci, but locus TC_AG3_8 was monomorphic for the populations Subachoque2, Sibate1, Pasto1 and Silvia and locus TC_AG3_10 was monomorphic for the population Silvia. The expected heterozygosity (HE = Nei's unbiased gene diversity) ranged from 0.45 to 0.66 across the populations (Table 2-3). The overall allelic richness across populations was 3.3.

Table 2-3. Overall measures of clonal diversity of *Rhizoctonia solani* AG-3 infecting potatoes in Colombia

State	Population	Sample size (N) [a]	Number of genotypes	Site specific genotypes [b]	Clonal fraction	(Go) [c]	Eveness [d, e]	H_E [d, f, g]	Allelic richness [d, g, h]	Private alleles [i]
Cundinamarca	Subachoque1	28	23	15 (8)	0,18	85,22	0,85	0,6197	3,28	0
	Subachoque2	18	12	6 (6)	0,33	84,38	0,84	0,6170	3,25	0
	Cogua1	23	21	20 (1)	0,09	93,30	0,93	0,6373	3,57	3
	Cogua2	33	26	21 (5)	0,21	82,13	0,82	0,6200	3,33	2
	Sibate1	21	17	17 (0)	0,19	89,45	0,90	0,5715	3,18	3
	Sibate2	12	11	10 (1)	0,08	93,51	0,94	0,6600	3,88	0
Nariño	Pasto1	18	17	17 (0)	0,06	95,29	0,95	0,5723	3,16	1
	Pasto2	22	19	16 (3)	0,14	84,91	0,85	0,5574	3,17	1
Antioquia	LaUnion1	17	14	10 (4)	0,18	82,57	0,83	0,6379	3,39	1
	LaUnion2	35	28	22 (6)	0,20	85,79	0,86	0,6210	3,39	4
Boyaca	Soraca1	22	18	16 (2)	0,18	89,63	0,90	0,5742	3,24	0
	Soraca2	23	17	13 (4)	0,26	75,89	0,76	0,6343	3,52	2
	Ventaq1	20	17	16 (1)	0,15	90,50	0,91	0,5785	3,29	0
	Ventaq2	21	15	10 (5)	0,29	89,09	0,89	0,5824	3,07	0
Santander	Carcasi1	10	9	7 (2)	0,10	92,59	0,93	0,6535	3,83	0
	Carcasi2	11	11	8 (3)	0,00	100,00	1,00	0,6082	3,26	0
Norte de Santander	Chitaga	14	13	7 (6)	0,07	94,23	0,94	0,5604	3,04	1
Cauca	Silvia	7	7	6 (1)	0,00	100,00	1,00	0,4545	2,74	0
	Overall	355	295	237 (58)	0,15	89,36	0,89	0,5978	3,31	18

a N, sample size of each population
b Number of genotypes shared with other(s) populations are showed in brackets
c G_o Stoddart and Taylor's genotypic diversity scaled to sample size (Stoddart, 1983; Stoddart and Taylor, 1988), values scaled by the maximum number of expected genotypes
d Mean followed by the same letter are not significantly distinct (p = 0.05) based on pairwise bootstrap test for differences in clonal diversity indices between populations calculated with GenoDive (Meirmans and Van Tienderen, 2004); 1000 permutations with subsampling to match the size of the smallest population
e; an evenness value = 1.0 indicates that all genotypes have equal frequencies
f Nei's unbiased gene diversity (Nei, 1978), also known as expected heterozygosity, averaged over all loci, corrected for sample size
g To test whether pairwise samples differed for Nei's unbiased gene diversity and allelic richness, we used FSTAT v.2.9.3.2 (Goudet, 1995), based on 1,000 permutations
h Calculated according to El Mousadik and Petit (El Mousadik and Petit, 1996)
i Alleles occurring in only one population, calculated with Convert 1.3

AMOVA estimates showed that variation among states represented only 1.57 % of the total variance. The variation among fields into states represents 0.82% of the total and the variation within fields accounts for the 93.5% of the total variability (Table 2-4). Pairwise analysis for population differentiation showed that the evaluated populations are genetically similar with low genetic distance among them. The overall R_{ST} value was 0.026 (p-value < 0.01). Of 153 of pairs of populations evaluated 43 were significant for differentiation when the R_{ST} index was used (p-value <0.05). 3 pairs showed low differentiation (R_{ST} <0.05), 36 pairs have moderate differentiation (R_{ST} between 0.05 and 0.15) and four pairs with high differentiation (R_{ST} from 0.15 to 0.25) (Annex 2-B).

Table 2-4. Hierarchical distribution of gene diversity among populations of *Rhizoctonia solani* AG-3 collected from potato symptomatic plants in Colombia

		Distance method: sum of squared size differences (R_{ST})				
Source of variation	df	Sum of squares	Variance components	Percentage of variation	Fixation indices	p-value
Among groups	10	1.301.656	0.96822	1.57	FCT: 0.0157	0.0743
Among populations within groups	7	553.725	0.50378	0.82	FSC: 0.0083	0.2111
Among individuals within populations	277	17.381.131	253.659	4.11	FIS : 0.0421	0.0977
Within individuals	295	17.014.000	5.767.458	93.50	FIT : 0.0649	0.0195
Total	589	36.250.512	6.168.317			

Analysis of molecular variance performed using the program ARLEQUIN version 3.5.1.2 (Excoffier and Lischer, 2010). Distance method based on the sum of squared size differences (R_{ST}) between two haplotypes, for microsatellite data. 10100 permutations were used. The groups correspond to the number of counties.

The highest level of subdivision was observed when comparing populations from different states. The groups of populations located at the south of the country (Nariño and Cauca states) are differentiated from those located in the Cundinamarca, Boyacá, Santander and Norte de Santander states. The samples from different fields in each municipality were pooled in one single population and the previous results for population differentiation were corroborated (Table 2-5). Geographically close populations presented R_{ST} values close to zero or their values were not significantly different from zero.

2.3.3. Reproductive mode: Gametic and Hardy-Weinberg Equilibrium tests

HWE and gametic disequilibrium between pairs of loci were tested with the clone-corrected data set. The exact test for HWE showed that average 78.4% of loci were in HWE in the populations (Table 2-6). The highest number of loci in HWE were present in populations Ventaquemada2 (11/11), Subachoque2 (10/10) and Silvia (9/9), and the lowest number of loci in HWE were present in populations Cogua1, Cogua2, Sibate2 and Soraca2 with four loci out of equilibrium each one.

Table 2-5. Measures of differentiation among populations of *Rhizoctonia solani* AG-3 in Colombia

Contrasts between counties			R_{ST}	***p*-value**
Sibate	*versus*	Subachoque	0.0375	0.016
Sibate	*versus*	Ventaquemada	0.0438	0.001
Sibate	*versus*	Antioquia	0.0286	0.021
Pasto	*versus*	Subachoque	0.0712	0.001
Pasto	*versus*	Cogua	0.0390	0.000
Pasto	*versus*	Ventaquemada	0.0718	0.000
Pasto	*versus*	Soraca	0.0496	0.003
Pasto	*versus*	Antioquia	0.0330	0.010
Pasto	*versus*	Carcasi	0.0901	0.000
Pasto	*versus*	Chitaga	0.0436	0.044
Silvia	*versus*	Subachoque	0.1068	0.014
Silvia	*versus*	Ventaquemada	0.1216	0.001
Silvia	*versus*	Antioquia	0.0770	0.015
Silvia	*versus*	Chitaga	0.1377	0.000
Contrast between states				
Cundinamarca	*versus*	Narino	0,024	0,033

Only pairs of populations with significant differences are presented. Distances were computed as the sum of squared size differences between two haplotypes (Slatkin, 1995) based on 10100 permutations.

Loci TC_AG3_9 was out of HWE in nine populations and TC_AG3_6 in seven of the 18 populations, and in most of the cases it was due to heterocigote excess. Heterocigote deficit was found in populations Sibate2 (four loci); Pasto1 and Soraca2 with three loci with heterocigote deficit each one; Cogua2, Pasto2, Ventaquemada1, LaUnion2 and Chitaga with two loci, the last populations presented one or no loci with heterocigote deficit. Locus TCAG3_10 present heterocigote deficit in eight populations.

The test for gametic disequilibrium followed by Bonferroni correction for multiple tests was significant only for a few pairs of loci in some populations. Populations with more than 10% of locus pairs in disequilibrium were: Subachoque1 (16.36%), Cogua2 (25.45%) and LaUnion2 (14.55%). Estimates of IA and $\bar{r}_D$ were significantly different from zero (p-value <0.001) for almost all populations, however their values were low (Table 2-6).

Population Pasto1 presented departure from HWE proportions in 3 of 10 loci, this population showed a significant value of F_{IS} (0.2487 p-value=0.0186), the same situation was present in the population Soraca2; this has 7 of 11 loci out of HWE, F_{IS} of 0.2764 (p-value=0.008) this is consistent with inbreeding. The overall FIS value was 0.081 (p-value= 0.0019), when populations Pasto1 and Soraca2 were removed from the analysis the overall F_{IS} value was not significant different form zero.

2.3.4. Admixture and hidden population structure

The analysis was performed assuming both recent ancestry and permanent (current) gene flow (admixture with correlated allele frequencies), supported in the results of AMOVA and R_{ST}. The simulation of the best K using the information generated by STRUCTURE supports for the existence of two genetically distinct populations from the 18 geographic locations (Figure 2-1 and Figure 2-2).

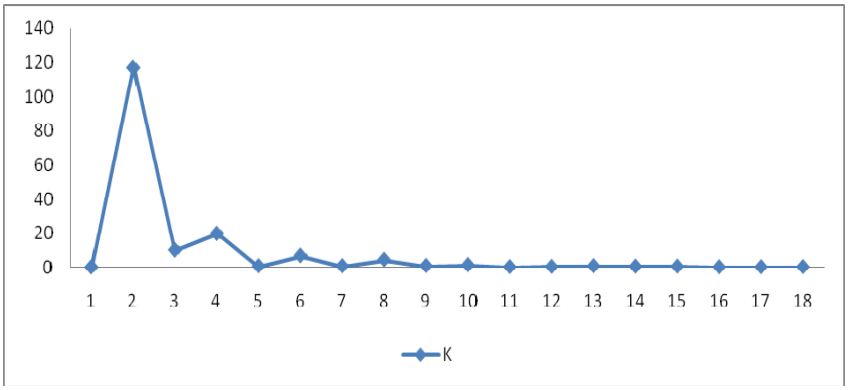

Figure 2-1. Number of simulated populations (Evanno *et al.*, 2005)

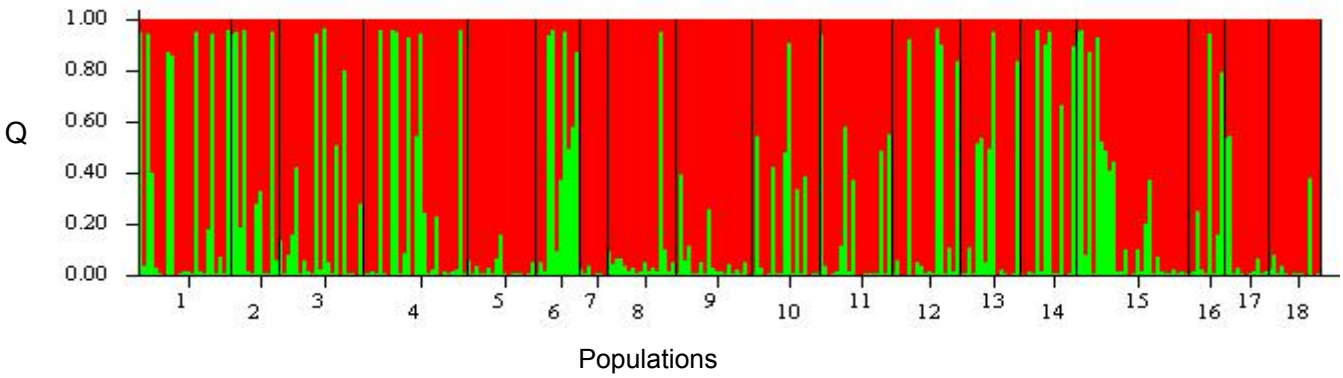

Figure 2-2. STRUCTURE inferred membership coefficient for MLMGs *Rhizoctonia solani* AG-3 in Colombia. Each vertical bar represents one MLMG. Each color represents the most likely ancestry of the cluster from which the genotype or partial genotype was derived. Individuals with multiple colors are admixed genotypes. The bar length indicates its membership coefficient (*Q*) to the distinctly colored populations. Populations 1=Subachoque1, 2=Subachoque2, 3=Cogua1, 4=Cogua2, 5=Sibate1, 6=Sibate2, 7=Silvia, 8=Pasto1, 9=Pasto2, 10=Ventaquemada1, 11= Soraca1, 12= Soraca2, 13= Ventaquemada2, 14=LaUnion1, 15=LaUnion2, 16=Carcasi1 17=Carcasi2 and 18=Chitaga

Admixture was detected in the overall sample. 20.67% (N=61) of the evaluated genotypes have a mixed background of the two genetic populations. The amount of admixture varied considerably among populations and all the populations present admixed MLMGs.

Table 2-6. Tests for random association of alleles within each locus and between pairs of loci populations of *Rhizoctonia solani* AG-3 in Colombia

State	Location	Clone corrected N	Number of loci under HWE [a]	F_{IS} [b]	p- value [b]	I_A [c]	rbarD [c]	p- value [d]	Locus pairs at significant disequilibrium [e]	%
Cundinamarca	Subachoque1	23	9/11	-0,0439	0,6510	1,3979	0.1439	< 0,0010	9/55	16,36
	Subachoque2	12	10/10*	0,1385	0,2141	1,2592	0,1430	< 0,0010	2/45	4,44
	Cogua1	21	7/11	0,1755	0,0655	0,2851	0,0293	0,3100	1/55	1,82
	Cogua2	26	7/11	0,0162	0,4321	1,4109	0,1429	< 0,0010	14/55	25,45
	Sibate1	17	7/10*	0,0477	0,3098	0,9931	0,1125	< 0,0010	3/55	5,45
	Sibate2	11	7/11	0,0128	0,4604	0,5048	0,0510	0,4190	0/55	0,00
Narino	Pasto1	17	6/10*	0,2487	0,0186	0,7759	0,0885	< 0,0010	0/45	0,00
	Pasto2	19	9/11	0,0908	0,2033	0,6503	0,0670	0,0020	0/55	0,00
Antioquia	LaUnion1	14	9/11	-0,0294	0,5503	1,1763	0,1225	< 0,0010	3/55	5,45
	LaUnion2	28	8/11	-0,0254	0,5806	0,8859	0,0916	< 0,0010	8/55	14,55
Boyaca	Soraca1	18	8/11	0,2764	0,0088	0,3726	0,0387	0,2650	0/51	0,00
	Soraca2	17	7/11	0,1142	0,1975	0,5130	0,0519	0,0530	0/55	0,00
	Ventaquemada1	17	8/11	-0,0348	0,6011	1,0026	0,1032	0,0010	0/55	0,00
	Ventaquemada2	15	11/11	-0,1050	0,7448	1,2943	0,1338	< 0,0010	0/55	0,00
Santander	Carcasi1	9	10/11	0,0276	0,4594	0,6409	0,0654	0,4270	0/48	16,36
	Carcasi2	11	11/11	-0,2143	0,8407	0,6972	0,0724	0,2300	0/55	0,00
Norte de Santander	Chitaga	13	9/11	-0,1881	0,8407	0,9325	0,0982	< 0,0010	0/55	0,00
Cauca	Silvia	7	9/9**	-0,0731	0,6314	0,7317	0,0930	0,0230	0/36	0,00

a HWE (Hardy Weinberg) test performed according to an exact test analogous to Fisher exact test, using a Marcov chain with forecasted length of 100,000 (Guo and Thompson, 1992); * one and ** two monomorphic loci

b Population specific FIS indices and p-values calculated based on 10.100 permutations using ARLEQUIN 3.5.1.2 (Excoffier and Lischer, 2010)

c IA is an index of multilocus gametic disequilibrium (for the random association of alleles among distinct loci pairs) (Maynard Smith *et al.*, 1993)

d Testing for complete panmixia based on 10.000 randomizations (H0); for diploid data, the two alleles at a locus are shuffled together (associations between alleles at a locus are maintained in the randomized data sets). This test is purely for associations between loci (Agapow and Burt, 2001)

e Number of pairs of loci with significant disequilibrium according to Fisher exact test (probability test) using both a Markov chain with 10.000 batches and 1.000 iteration/batch, implemented by Genepop (Raymond and Rousset, 1995), after Bonferroni correction for multiple comparisons (Rice, 1989)

2.3.5. Population bottleneck and demographic parameters

The test for bottlenecks or founder events was no significant in any of the tested populations (Table 2-7). Populations Pasto2 and Ventaquemada1 presented high number of loci with significant heterocigote deficit; this is consistent with population expansion.

Table 2-7.Test for the presence of a historic population bottleneck

Population	HD/HE	Exp. HE.	Sign Test	One tail for deficit
Subachoque1	4/7	6.22	0.43	0.81
Subachoque2	2/8	5.73	0.12	0.92
Cogua1	7/4	6.44	0.11	0.05
Cogua2	4/7	6.32	0.46	0.41
Sibate1	4/6	5.76	0.57	0,78
Sibate2	6/5	6.36	0.29	0,23
Pasto1	6/4	5.73	0.21	0,18
Pasto2	8/3	6.37	0.04	0,03
La Union2	5/6	6.40	0.52	0,71
La Union2	7/4	6.13	0.16	0,38
Silvia	6/3	5.18	0.13	0,15
Ventaquemada1	9/2	6.39	0.003	0,04
Ventaquemada2	4/7	6.27	0.45	0,68
Soraca1	8/3	6.28	0.03	0,06
Soraca2	6/5	6.34	0.30	0,16
Carcasi1	6/5	6.30	0.30	0,41
Carcasi2	5/6	6.44	0.50	0,26
Chitaga	6/5	6.57	0.25	0,23

HD and HE deficit and excess heterozygosity at 11 unlinked loci. HE is generated by simulations under the assumption of mutation drift equilibrium. Data from BOTTLENECK V 1.2

2.4. Discussion

The study of population structure and dynamics of *R. solani* AG-3 requires differentiation of the relative influence of life-history traits and historical processes in shaping present-day populations. This is the first study describing deeply the population structure of *R. solani* AG-3 in Colombia. A wide sampling was realized, including all the main producing areas and some counties with low production. We hypothesized that the main evolutionary force shaping the population structure of this pathogen is the gene flow, considering that *R. solani* AG-3 is dispersed long distance through human-mediated movement of contaminated tuber-seed (Simons and Gilligan, 1997) .

2.4.1. Genetic and genotypic diversity

The Colombian population is genetic and genotypically diverse. Our results supports the high variability detected in pathogenicity and morphology (Campion *et al.*, 2003; Lehtonen *et al.*, 2008), and genetic diversity (Kuninaga *et al.*, 1997; Ceresini *et al.*, 2002a; Ceresini *et al.*, 2002b; Justesen *et al.*, 2003; Balali *et al.*, 2007; Woodhall *et al.*, 2007).This is an important insight in the population studies of this pathogen because genetically diverse populations can react easily and quickly to stress factors as those imposed to control their populations in the fields (McDonald and Linde, 2002).

2.4.2. Population differentiation

Several approaches have been used to estimate the amount of genetic differentiation into the populations (Hedrick, 2005). Wright developed an approach to partition the genetic variation in a subdivided population using three statistics in terms of correlation among alleles. Nei (1977) showed that those coefficients can be expressed in terms of allele and genotype frequencies. Thus F_{ST} is a coefficient for evaluating population differentiation, it ranges from 0 to 1, with cero indicating no differentiation among the populations evaluated, and 1 showing that populations are totally different (Hedrick, 2005). Another way for testing population differentiation is AMOVA, which estimates population differentiation making a hierarchical analysis of variance. AMOVA make partition of the total variance in their components due to intra-individual differences, inter-individual differences, or inter-population level differences, it depends on the level tested (Excoffier *et al.*, 1992).

The low pairwise population subdivision of *R. solani* AG-3 in Colombia indicates that migration prevents the differentiation of the geographical populations; this is due to the movement of infected tuber-seed among counties. Populations of Pasto and Cauca had a moderate differentiation with the last populations; this is probably due to a low interchange of tuber-seed among them, and a higher inoculum movement from Ecuador. Those results support observations about the efficient dispersion of this pathogen in contaminated tuber seed among geographical distant populations. Long-distance dispersal of sclerotia and mycelia is suggested by the presence of the same multilocus genotypes in different counties. According to this 58 MLMGs were shared among populations and eight MLMGs were found more than once in geographically distant populations. MLMGs one, three and 13 were found in geographically separated populations, however the common situation was to find at least one MLMG shared between fields in the same municipality or state. This show a higher interchange of tuber seeds among fields into the same state and more restricted among states.

In Colombia there are 30 registered varieties, however five are mainly cultivated (Diacol capiro, Parda pastusa, Pastusa suprema, Sabanera and Criolla) (Espinal *et al.*, 2006) , this brings to the fungus a uniform agro-ecosystem and could be a cause for the

differentiation among populations. However, low pairwise population subdivision of *R. solani* AG-3 was found among isolates collected on different varieties, indicating that there is a strong force that prevents the differentiation. The movement of infected tuber seed among counties is the most plausible cause of the low differentiation. The highest level of subdivision was observed among groups of populations located at the south of the country (Nariño and Cauca states) and those located in the Cundinamarca, Boyacá, Santander and Norte de Santander states. Those populations are geographically distant and probably have a low interchange of tuber-seed with the other populations. Close populations were not differentiated. Lack of population structure has been described previously in populations of *R. solani* AG-3 sampled from stem potato plants in USA and Denmark (Ceresini *et al.*, 2002b; Justesen *et al.*, 2003).

On previous studies was proposed the genetic differentiation among isolates found in soil from those from plants on rice (Banniza *et al.*, 1999) and potato (Justesen *et al.*, 2003), however our results show that there are no specific MLMGs associated to black scurf on tubers or to stem canker. In some cases the MLMG on tuber seed was the same on stem canker showing that tuber-borne inoculum could start the infection. The opposite situation also was found, the MLMG on sclerotia on tuber seeds was different from MLMG on stem canker, which is evidence that the lesion was initiated by soil-borne inoculum. Previously was proposed that the infection of potato plants by *R. solani* AG-3 may be initiated by soil-borne as well as tuber-borne inoculum and their relative importance depends upon inoculum density and the environmental factors (Frank and Leach, 1980). However tubers are exposed to the soil inoculum more time than stems, as consequence it is more likely that isolates sampled from tubers mainly represent the population present in the soil (Justesen *et al.*, 2003).

AMOVA and F_{ST} results were confirmed by the analysis with STRUCTURE, low differentiation among populations, additionally those results showed that there are migrant individuals in populations and that many individuals have a genetic background from different populations. This is explained because the pathogen moves frequently through tuber seed contaminated and supports the occurrence of sexual recombination generating admixed genotypes as previously was shown (Ceresini *et al.*, 2002b).

2.4.3. Reproductive mode

The sexual stage of *R. solani* AG-3 is known, however for a long time was proposed that this pathogen was prevalent asexual in the fields. The hypothesis of clonality for this pathogen in Colombia was tested. The high genotypic diversity along with the low clonal fraction, the high proportion of loci on HWE, gametic equilibrium for most of the pairs of microsatellite loci and F_{IS} values not significant different from zero, confirm previous findings for random mating in populations of *R. solani* AG-3 (Ceresini *et al.*, 2002b).

Moreover we found unique MLMGs that might be the products of sexual reproduction that creates unique individuals by recombination during meiosis (Halkett *et al.*, 2005).

Clonal reproduction also was detected. Some loci deviated from HWE and a significant deficit of heterozygotes was observed in almost all populations, indicating nonrandom mating. The departure from HWE of some loci may be due to inbreeding. Additionally almost all the populations were characterized by some loci on gametic disequilibrium and significant values of IA and $\bar{r}_D$. Non-random associations among alleles at different loci can be generated by asexual reproduction, nonrandom mating, linkage, selection, population structure (isolated populations, low gene flow) or genetic drift (founder events, small populations) (Milgroom, 1996; Taylor *et al.*, 1999; Halkett *et al.*, 2005).

Populations of *R. solani* AG-1 from different countries present a reproductive mode varying from strictly recombining to a mixed reproductive system in which there are recombination events followed by clonal expansion during the growing season (Rosewich *et al.*, 1999; Ciampi *et al.*, 2008; Bernardes-de-Assis *et al.*, 2009).

The evidence for clonal reproduction and recombination suggest a structure named “epidemic structure”, this was described on bacteria (Maynard Smith *et al.*, 1993). As in bacteria, fungi can exhibit population epidemic structure in which sexual recombination occurs; suddenly a successful clone may increase in proportion, this clone predominates for a time, and finally can disappear as result of recombination. This situation is detected when there is a single or a few multilocus genotypes showing wide distribution, especially in epidemic areas. The frequently recovering of the same genotype could be taken as evidence of clonality, but when the clones are eliminated and the analysis is re-run, the populations behave as recombining, it explains bias in the tests for recombination towards clonality, and populations really have a random mating behavior (Taylor *et al.*, 1999).

Clonality produces a clear pattern into the populations: for a series of polymorphic loci, the same multilocus genotype is recovered over long distances or periods of time and loci are in GD. In the other side if reproduction is sexual and mating is random, multi locus genotypes are not repeatedly recovered, and there is no association between alleles (Anderson and Kohn, 1995; Milgroom, 1996).

Formally the mating system of *T. cucumeris* AG-3 is not known. Is suggested that it is homotallic (Cubeta and Vilgalys, 1997), however there is no enough evidence for this supposition. Our results suggest it is a heterothallic fungus. Homothallic fungi present a kind of inbreeding which means that mating occurs among close relatives, however our and previous results show the opposite situation: high variability in pathogenicity, morphology (Campion *et al.*, 2003; Lehtonen *et al.*, 2008) and genotypic diversity (Kuninaga *et al.*, 1997; Ceresini *et al.*, 2002a; Ceresini *et al.*, 2002b; Justesen *et al.*, 2003;

Balali *et al.*, 2007; Woodhall *et al.*, 2007), which is not consistent with inbreeding or a selfing organism.

2.4.4. Demographic parameters

The Colombian populations did not present evidence of bottlenecks or founder events. Genetic drift acts more quickly in small populations reducing genetic variation, bottlenecks can reduce drastically the genetic variation of the populations, even if the bottleneck does not last for very many generations. In plant pathogen populations the control of the diseases are the most important force causing reduction in their size. The management of *R. solani* AG-3 in Colombia is recent, and it uses Thifluzamide in the soil and applications of *Trichoderma* sp., however the results show that this activity has not affected substantially the population size of this pathogen in the localities evaluated. Populations Pasto2 and Ventaquemada1 presented gene diversity deficiency across loci, this show that those populations have experienced recent population expansion, which is the result of a high increasing in the population size.

Modern plant pathogen populations are shaped by the evolutionary forces that have acted on their ancestral populations (McDonald and Linde, 2002). Genetic polymorphism may be indicative of evolutionary adaptation, which plays a key role for survival of a population in the changing environment. The high genetic and genotypic diversity, the gene flow and the evidence of a mixed reproductive mode within Colombian populations of *R. solani* AG-3, are characteristics for pathogens with high evolutionary potential (McDonald and Linde, 2002). Pathogens with high evolutionary potential require special attention when generating control measures such as fungicide applications and resistance genes. Additionally the gene flow, through contaminated tuber-seed must be minimized in order to avoid dissemination of virulence genes and the generation of advantageous genotypes.

2.5. Conclusions

The Colombian population of *Rhizoctonia solani* AG-3 is not geographically structured. These results support observations about dispersal of this pathogen in contaminated tuber seed which is the most efficient mechanism of dispersion of this fungus between geographical distant populations.

The analysis with structure showed two genetic pools associated to the geographical populations of *R. solani* AG-3 en Colombia

Frequent human mediated dispersal of asexual propagules (mycelia and sclerotia) among populations would lead to the widespread distribution of clones among populations. Long-

distance dispersal of sclerotia and mycelia is suggested by the presence of the same multilocus genotypes in different counties.

Although clones are important for dispersion, it does not seem that the predominant mode of reproduction is the asexual, at least at the spatial scales sampled.

Recombination although sporadic has significant effect into the populations. This is an important insight in the population studies of pathogens; meiotic recombination contributes to the genetic and genotypic diversity generating individuals with new allele combinations that can be advantageous.

This research brings evidence for no preference among isolates infecting stems and causing black scurf, however it is necessary to propose a deep study to evaluate the inoculum source of initial infections on stems and tubers.

Association of MLMGs by cultivar was not found, tests on susceptibility of cultivars to *R. solani* AG-3 show that susceptibility in the host depends on the isolate used and that cultivars do no select for genotypes of the fungus.

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Annex 2-A. Information content of eleven microsatellite loci used for multilocus genotyping of individuals of *Rhizoctonia solani* AG-3 from Colombian potato crops

Microsatellite locus	TC_AG3_0	TC_AG3_1	TC_AG3_6	TC_AG3_7	TC_AG3_8	TC_AG3_9	TC_AG3_10	TC_AG3_14	TC_AG3_16	TC_AG3_18	TC_AG3_19	Total number of alleles
Motif	3-nucleotide	3-nucleotide	3-nucleotide	3-nucleotide	3-nucleotide	3-nucleotide	3-nucleotide	3-nucleotide - Compound	3-nucleotide	3-nucleotide	3-nucleotide	
Allele size range (bp)	147-156	141-201	192-246	207-234	214-244	220-259	244-250	284-320	315-363	318-321	320-359	
Repeat numbers	1-4	1-21	3-21	1-10	1-11	1-14	7-9	2-14	12-28	4-17	2-15	
Subach1 (N=28)	2	6	4	6	2	6	2	3	5	5	6	47
Subach2 (N=18)	2	5	5	4	1	5	2	3	5	4	5	41
Cogua1 (23)	2	8	7	5	3	6	4	4	7	6	7	59
Cogua2 (N=33)	2	6	4	7	3	5	2	4	5	5	8	51
Sibate1 (N=21)	2	7	6	4	1	6	2	4	4	3	5	44
Sibate2 (N=12)	2	7	8	4	3	6	2	6	6	4	5	53
Pasto1 (N=18)	2	9	7	3	1	5	2	4	8	4	5	50
Pasto2 (N=22)	2	8	6	4	4	6	2	4	6	5	6	53
LaUnion1 (N=17)	2	7	4	6	2	5	3	3	6	4	5	47
LaUnion2(N=35)	2	8	6	5	2	7	2	4	8	4	7	55
Soraca1 (N=22)	2	5	7	6	2	7	2	4	5	4	6	50
Soraca2 (N=23)	2	5	6	6	3	7	2	5	6	5	6	53
Ventaq1 (N=20)	2	5	8	5	3	5	2	6	6	4	6	52
Ventaq2 (N=21)	2	4	4	5	2	4	2	3	5	4	6	41
Carcasi1 (N=10)	2	5	7	4	2	6	3	5	5	4	6	49
Carcasi2 (N=11)	2	5	4	4	2	6	3	4	5	4	6	45
Chitaga (N=14)	2	6	4	3	3	5	3	5	3	3	6	43
Silvia (N=7)	2	5	4	3	1	4	1	4	3	2	4	33
Total number of alleles	2	11	12	9	7	13	4	11	14	7	9	99

N: Sample size for each population. Data without cloning correction

Annex 2-B. Measures of differentiation among populations of *Rhizoctonia solani* AG-3 infecting potato in based on R_{ST} values*

	Subach1	Subach2	Cogua1	Cogua2	Sibate1	Sibate2	Silvia	Pasto1	Pasto2	Vent1	Soraca1	Soraca2	Vent2	LaUnion1	LaUnion2	Carc1	Carc2	Chitaga
Subach1	0.000	0.229	0.284	0.487	0.193	0.512	0.214	0.043	0.054	0.042	0.243	0.931	0.398	0.093	0.592	0.152	0.162	0.199
Subach2	0.011	0.000	0.659	0.417	0.013	0.182	0.024	0.004	0.070	0.557	0.305	0.511	0.696	0.445	0.724	0.284	0.476	0.832
Cogua1	0.007	-0.010	0.000	0.701	0.003	0.555	0.009	0.005	0.014	0.679	0.532	0.579	0.993	0.803	0.396	0.396	0.325	0.803
Cogua2	0.002	-0.001	-0.015	0.000	0.018	0.463	0.044	0.011	0.015	0.282	0.611	0.564	0.651	0.260	0.407	0.578	0.197	0.405
Sibate1	0.017	0.060	0.067	0.078	0.000	0.093	0.172	0.466	0.131	0.000	0.005	0.246	0.016	0.004	0.050	0.001	0.012	0.020
Sibate2	0.001	0.019	-0.009	0.000	0.042	0.000	0.012	0.021	0.004	0.141	0.893	0.912	0.911	0.146	0.179	0.435	0.214	0.106
Silvia	0.027	0.093	0.111	0.104	0.029	0.138	0.000	0.121	0.148	0.001	0.008	0.223	0.009	0.004	0.083	0.007	0.004	0.000
Pasto1	0.041	0.081	0.073	0.100	0.001	0.088	0.053	0.000	0.136	0.004	0.006	0.095	0.018	0.019	0.023	0.002	0.026	0.036
Pasto2	0.034	0.030	0.054	0.085	0.024	0.103	0.036	0.029	0.000	0.009	0.009	0.200	0.028	0.029	0.220	0.002	0.148	0.061
Ventaq1	0.040	-0.009	-0.013	0.009	0.110	0.028	**0.170**	0.114	0.076	0.000	0.133	0.206	0.602	0.881	0.152	0.371	0.417	0.810
Soraca1	0.013	0.004	-0.006	-0.011	0.071	-0.029	0.099	0.083	0.061	0.020	0.000	0.865	0.718	0.223	0.268	0.348	0.195	0.111
Soraca2	-0.019	-0.003	-0.005	-0.004	0.016	-0.029	0.033	0.040	0.020	0.018	-0.017	0.000	0.993	0.300	0.811	0.511	0.775	0.434
Ventaq2	0.006	-0.011	-0.027	-0.014	0.069	-0.034	0.136	0.075	0.058	-0.011	-0.014	-0.033	0.000	0.759	0.331	0.408	0.547	0.354
LaUnion1	0.033	-0.004	-0.018	0.014	0.093	0.032	**0.163**	0.083	0.060	-0.027	0.012	0.010	-0.019	0.000	0.187	0.248	0.627	0.783
LaUnion2	-0.004	-0.010	-0.001	-0.001	0.033	0.018	0.045	0.046	0.010	0.016	0.006	-0.013	0.004	0.014	0.000	0.078	0.227	0.412
Carcasi1	0.035	0.009	-0.001	-0.013	0.137	-0.003	**0.181**	**0.165**	0.138	-0.001	0.004	-0.003	0.004	0.013	0.042	0.000	0.182	0.108
Carcasi2	0.026	-0.007	0.001	0.023	0.087	0.019	0.124	0.076	0.024	-0.009	0.013	-0.016	-0.010	-0.020	0.011	0.020	0.000	0.283
Chitaga	0.019	-0.020	-0.019	-0.002	0.062	0.033	0.138	0.060	0.039	-0.024	0.022	0.002	0.004	-0.024	-0.003	0.032	0.001	0.000

* Distances were computed as the sum of squared size differences between two haplotypes using ARLEQUIN 3.5.1.2 (Excoffier and Lischer, 2010). The R_{ST} values are located below the diagonal and above diagonal its significance. Significant values at $p< 0.05$ are shown in red. Test based on 1,100 permutations.

Chapter 3. Diversity and adaptability of *Rhizoctonia solani* AG-3

3.1 The state of the art

Fungi are one of the most diverse groups of organisms in nature. Approximately 70.000 species have been described, but is estimated to exist about 1.5 million of fungal species (Kiffer and Morelet, 1997). Most of them obtain nutrients living in close association with other organisms. Many fungi are pathogenic and have strong impact on human health or lead to severe economic losses due to crop and animal diseases (Strange and Scott, 2005). The emergence of fungal diseases of crop plants during the past century, probably is due to human activities that have modified the ecosystems at global scale (e.g., climate warming, widespread deforestation, habitat fragmentation, urbanization, changes in agricultural practices and global trade) (Kareiva *et al.*, 2007). The intensification and globalization of agriculture as well as the increases of international trade and travel have broken many natural barriers to pathogen dispersal causing the redistribution of many organisms (Kolar and Lodge, 2001).

Plant pathogens are very successful in agro-ecosystems causing serious diseases. They produce great deal of inoculum, their latent period is very short; have very efficient and fast dispersion means (water, wind, vectors, propagation material) and produce different kind of substances for attacking and manipulate the plant for their own benefit (Strange and Scott, 2005). Farmers are constantly challenging pathogens using practices to reduce plant diseases into the agro-ecosystems, causing strong directional selection on their populations.

3.1.1. Genetic diversity and the evolutionary forces

Molecular analyses have revealed high genetic diversity in the populations of plant pathogens. Prevalence of diversity in populations depends on the fitness of the individuals, that is determined by the combined ability of an organism to survive and reproduce (Leach *et al.*, 2001; Kassen and Rainey, 2004), it is measured as the ability of one genotype to leave offspring relative to others. The mechanisms underlying changes in fitness are the interactions among genotypes and between genotypes and the environment. For plant pathogens several traits, such as reproductive rate, growth, infection efficiency and amount of disease (aggressiveness), have been used to measure fitness (Leonard and Czochor, 1980).

Population genetics is focused mainly on genetic processes, such as mutation, genetic drift, gene flow, mating system and natural selection; those are called evolutionary forces and are responsible for the extent of genetic variation in every population of living organisms (McDonald and McDermott, 1993; McDonald, 2004). Although all of them are determinants for the genetic structure of the populations of organisms in this chapter we are interested mainly in selection and gene flow, as factors controlling the amount and distribution of variability into the populations.

Selection is a directional process that leads to changes (increasing or decreasing) in allele or genotypes frequencies in response to a specific environmental factor (McDonald and Linde, 2002; de Meeus *et al.*, 2007). Selection is a frequency-dependent process, the fitness of particular genotypes depend on its frequency into the population (de Meeus *et al.*, 2007). Changes in genotype or gene frequencies are evolutionary changes that occur on micro-evolutionary scales of time.

The short generation times and large population sizes of microbial populations are ideal conditions for natural selection. A pathogen can evolve over time to be adapted to the host in many ways. Mutation provides the natural variability upon which selection can act. An organism with an inherently high mutation rate would be more likely to give rise to mutations that avoid recognition without loss of fitness (Leach *et al.*, 2001).

3.1.2. Variation in time and space

Actually ecologists and population geneticists recognize that the arrangement of the environment (landscape) is connected to the maintenance of diversity. They have started to relate spatial variability with disease risk and its intensity (Ostfeld *et al.*, 2005). The variability in the amount and distribution of diseases in time space is caused by the diversity and the distribution of well adapted genotypes of the pathogen. Each environment has its own constraints to the pathogens, this mean that the environment selects for the organisms in a region.

Heterogeneous environments are composed by many niches that may vary in either space or time (Kassen, 2002; Kassen and Rainey, 2004). As consequence different types may be favored in each niche, so diversity is maintained according to the niche exclusion principle. Natural selection eliminates the fittest types under any given set of growth conditions, leading to a loss of diversity (Kassen and Rainey, 2004). Evolutionary theory predicts that in a spatially heterogeneous environment, selection allows the emergence of ecological specialists or different types being adapted to different niches (Kassen and Rainey, 2004).

Migration of plant pathogens forces them to survive and be adapted to novel environments. Local adaptation is consequence of adaptation to particular host genotypes

and selection pressures (Sicard *et al.*, 2007). Local adaptation or the enhanced performance on sympatric host populations can result on rapidly evolving parasites adapted to the most common host genotypes and to the environment (Belotte *et al.*, 2003; Sicard *et al.*, 2007).

Many problems in evolutionary biology are focused on phenotypic variation. Phenotypic variation allows the differentiation among species and among individuals into species. Traits that are often measured and given in quantitative continuous values are referred as quantitative traits (Hartl and Clark, 2007). Quantitative traits include size, fitness components and rate of growth, they are usually attributed to polygenic effects and are the result of the interaction among the genes and the environment (Hedrick, 2005).

3.1.3. Differentiation of soil-borne fungi in time and space

Soil is a diverse and complex environment. Soil composition has strong effect on ecological and evolutionary processes into the populations, in scales ranging from the soil pores until landscape (Ettema and Wardle, 2002). The composition microbial communities in the soil changes along environmental gradients, such as temperature, soil properties and geographical locations, if selection in these different environments is often strong can lead to ecological specialization (Kassen and Rainey, 2004).

Local environment modify the activity of the organisms (i.e. nutrients uptake, predation risk, competence and indirectly the growth, reproduction, movement and surviving). Some environmental factors (i.e. temperature, water potential) can be the same at macro and micro-scales, others (nutrient contents, moist and pH) are not uniform and do not reflect conditions affecting individuals or microbial communities (Franklin and Mills, 2007; Ritz, 2007), then more detailed studies are necessary to identify microenvironments regulating populations of microorganisms in the soil.

In soil-borne diseases the factors allowing the contact of host and pathogen are two: (i) inoculum density and (ii) growth rate, which are critical in the disease dynamic (Otten *et al.*, 2003; Otten *et al.*, 2005; Cunniffe and Gilligan, 2009). Relationship among rate of radial growth of colonies of *R. solani* and the biomass accumulation depend on the physical, biological and chemical properties of the soil, which are determinant for the dynamic of epidemics; they modify the colonization efficiency as well as the growth of fungi changing their ability for the invasion. Transmission of *R. solani* is determined by its ability for growing on or through soil. Exploration of mycelia from the inoculum source to the host occurs in a heterogeneous environment through a soil pore networks, cracks, aggregate surfaces and water films, those factors increase the tortuosity and reduce the connectivity of the soil pores (Gilligan and Bailey, 1997; Otten *et al.*, 1999; Gill *et al.*, 2000; Otten *et al.*, 2001).

The distribution of nutrient into the soil has strong effect upon the spatial organization of fungal mycelia. Most resources are heterogeneously distributed in the soil, and the invasion and persistence of parasites and saprophytes depends on its ability to colonize the substrate, that is determined by the endogenous availability of nutrients and the ability of translocation into the colony, the growth and the distance to susceptible hosts (Ritz, 2007).

For a long time researchers were interested in the evolutionary processes leading to the adaptation by selection affecting the frequencies of races or fungicide resistant phenotypes of pathogens (Milgroom and Peever, 2003). Actually biologists are aware on adaptation of different phenotypic and physiological traits particularly those that represent an advantage for the parasites populations. This forces us to think in selection and adaptation of plant pathogenic fungi as a highlight in research, especially under climate change perspective. In Colombia the potato production is distributed along the high lands in the Andean mountains, from 2400 to 3200 masl representing variable environments including climate, agricultural practices and soil properties that can influence to the populations to react differentially to stress factors. For that reason the evaluation of the responses of isolates of *R. solani* AG-3 to two factors causing stress (temperature and one fungicide) and the study of differences in aggressiveness among isolates collected on different geographical regions was proposed in this part of the research.

3.2 Materials and methods

3.2.1. Fungal populations for study

Individuals from 10 geographical populations of *R. solani* AG-3 were chosen from the collection of the Potato Pathology Group of the Agronomy Faculty at National University in Bogotá. The isolates tested included individuals of the main producing areas of potato in Colombia. The number of isolates tested in the different experiments was variable (Table 3-1). For the pathogenicity test on stems half of the isolates tested for black scurf, temperatures and fungicide sensitivity were used. The isolates chosen were previously characterized with SSR markers.

3.2.2. Preliminary tests

Preliminary tests were made with 9 isolates randomly selected to choose the proper levels of the factors subject of evaluation. First was checked if there were differences in the growth of the isolates using variable volumes of PDA (Potato Dextrose Agar) in the Petri plates (15, 20 and 25 ml) and the minimum number of repetitions required for the tests in PDA *in vitro*. In a second test, six doses of the fungicide thifluzamide (0, 0.1 1.0, 10, 100 and 1000 ppm) were evaluated to select one where the isolates show variability in their

growth. The third test checked for the differential response to the temperatures, growing the isolates at five temperatures (4, 10, 20, 30 and 40 ^{o}C). The isolates were kept in different spaces for each temperature (at 4 ^{o}C in a cold room, at 10 and 20 ^{o}C in a fitotron Labline Biotronette without light, at 30 ^{o}C in an incubator WTBbinder, and at 40 ^{o}C in an incubator (#lab line instrumentals). For all the tests *in vitro* the evaluation of the colony size was recorded at 24, 48 and 72 hours after inoculation of the Petri dishes in order to define the accurate time to make the evaluation.

Table 3-1.Number of isolates used for each analysis

Municipality	Test	
	Black scurf Temperature response Fungicide sensitivity (N)	Stem canker (N)
Subachoque	13	7
Cogua	14	7
Sibate	12	6
Silvia	7	6
Pasto	11	5
Ventaquemada	11	7
Soraca	15	6
LaUnion	10	6
Carcasi	10	6
Chitaga	8	4
Total	111	60

The isolates used for the pathogenicity tests were included in the test of infestation of sclerotia on tubers, temperature response and fungicide sensitivity

3.2.3. Response to temperatures and sensitivity to the fungicide thifluzamide

Fungal isolates from long-term storage were plated on PDA dishes and grown for five days at room temperature. 24 hours before the experiment each isolate was grown and the plates for the assay were prepared as follows: (i) Plates with PDA were prepared for the temperature assays and (ii) plates with 20 ml of PDA amended with 14.2 ppm of the fungicide. A plug of mycelia of 5 mm of diameter was took from the growing area with a cork borer and was transferred to the center of the plates. Plates for each treatment were kept at each temperature evaluated (for 15 and 25 ^{o}C in a fitotron labline Biotronette without light). The temperature used for the fungicide assay was 25 ^{o}C. Each isolate was replicated across three plates. Each treatment was conducted by one person during the course of a single day. After 48 hours the size of colonies from each plate were delimited with a permanent marker and all the isolates were kept in the previous conditions of

temperature. For each plate the time to the formation of sclerotia was recorded. The plates were scanned (hp Scanjet 3570c), and the colony sizes after 48 hours of growing were determined with the image analysis software ASSESS (Lamari, 2002). The data obtained with ASSES were transformed from pixels to cm^2.

An additional trial testing the *in vitro* response of 22 isolates to the fungicide thifluzamide (at 3 and 14.2 ppm) along the time was prepared. The isolates were grown in presence of the fungicide for 96 hours and the growth of the colony was determined each 24 hours. After 96 hours a plug of the micelial growth was transferred to a new plate with PDA.

3.2.4. Pathogenicity tests

Tubers of the cultivar Diacol capiro were used for the pathogenicity tests. Each tuber was disinfested superficially; first the sclerotia were removed manually and then the tubers were immersed in 1% of sodium hypochlorite for one minute and washed twice with distilled water. Tubers were stored in paper bags, in the dark to promote the development of sprouts.

3.2.4.1. Pathogenicity on stems

After a month of storage in the paper bags, uniform sprouts (1-1.5 cm) were took and transferred to pots with sterile quartz sand. The sand was autoclaved twice at 15 PSI for 30 minutes in two consecutive days. Pots were kept in a growing room for two weeks and then plants were inoculated. For the inoculation, fungal isolates were taken from long-term storage, plated on PDA and grown for five days at room temperature. 24 hour before the inoculation each isolate was transferred to a new plate with PDA. One plug of mycelia of 5 mm of diameter was used to inoculate each growing plant in the stem base. Each isolate was inoculated on five plants. Plants were kept on a growing room, at 20 ^oC, with 12 hours of photoperiod, and were watered whenever the substrate was dry and fertilized twice a week using nutrient solution (Murashige and Skoog, 1962). Two months after the inoculation, plants were harvested, then washed and the disease was evaluated as average of the lesion size (mm) on stems, roots and stolons.

3.2.4.2. Infestation on tubers

After a month of sprouting, uniform tubers were took and transferred to bags with soil. The soil used was collected in a not growing potato field and was evaluated for *R. solani* AG-3 by PCR using specific primers (Lees *et al.*, 2002). Two weeks after sow plants were inoculated with two plugs of mycelia of 5 mm of diameter close to the stem base. Each isolate was inoculated on five plants. Plants were kept in an open area, fertilized with 15-15-15 twice (30 grams per plant, the first fertilization at sow and the second at hilling) and

foliar aspersion with micronutrients three times in the cycle. The control of pests and diseases was effectuated whenever was necessary. After six months tubers were collected, washed and the black scurf evaluated using a severity scale (James, 1971). Additionally incidence of the disease on tubers in each plant was recorded.

3.2.5. Data analysis

Data from preliminary tests were analyzed with excel. The optimal dose of the fungicide was estimated using the procedure NLIM (SAS V9.0). Temperature response was estimated as the proportion of the growth relative to the optimal (25 °C), and the fungicide sensitivity as the proportion of the colony size in the presence of fungicide relative to the colony size in the absence of fungicide. Descriptive analysis of the evaluated variables was executed using excel. Analysis of variance was carried out to test for the effects of temperature and fungicide on radial growth of the fungi and on the pathogenicity of the isolates on potato plants (SAS V9.0). A cluster analysis was executed using the software SAS V9.0., in order to identify groups of individuals associated to geographical regions with similar behavior in the evaluated variables.

3.3. Results

3.3.1. Preliminary tests

The preliminary tests showed that *R. solani* AG-3 has a wide capability to react to different factors of stress imposed to the individuals. The higher growth rate was found at temperatures among 20 and 30 ^{o}C, and was close zero at 4 ^{o}C and at 40 ^{o}C for almost all the isolates tested (Annex 3-A). In average the formation of sclerotia started at 6 days for all the temperatures tested. The formation of sclerotia was inhibited at 4 and 40 ^{o}C (Annex 3-B).

The fungicide thifluzamide diminished the growth of the pathogen in all the doses evaluated. At 100 ppm the growth was completely inhibited (Annex 3-C). For the response analysis a dose of 14.2 ppm was evaluated, although this is a high dose this allows to detect variation in the response of the isolates and to detect differences among the individuals tested (data not shown).

The colony sizes were independent of the volume of media in the Petri dish, then for the temperature assays a fixed volume of PDA was not used.

3.3.2. Temperature response

The growth of the isolates was higher at 25 °C for all the isolates tested in all the municipalities (Figure 3-1). For both temperatures the growth was higher in the isolates from Chitaga, and the lower in isolates from Silvia. The difference among municipalities was not significant at both temperatures. In each municipality there were isolates that grew well at 15 °C and not at 25 °C and others that had better response at 25 °C.

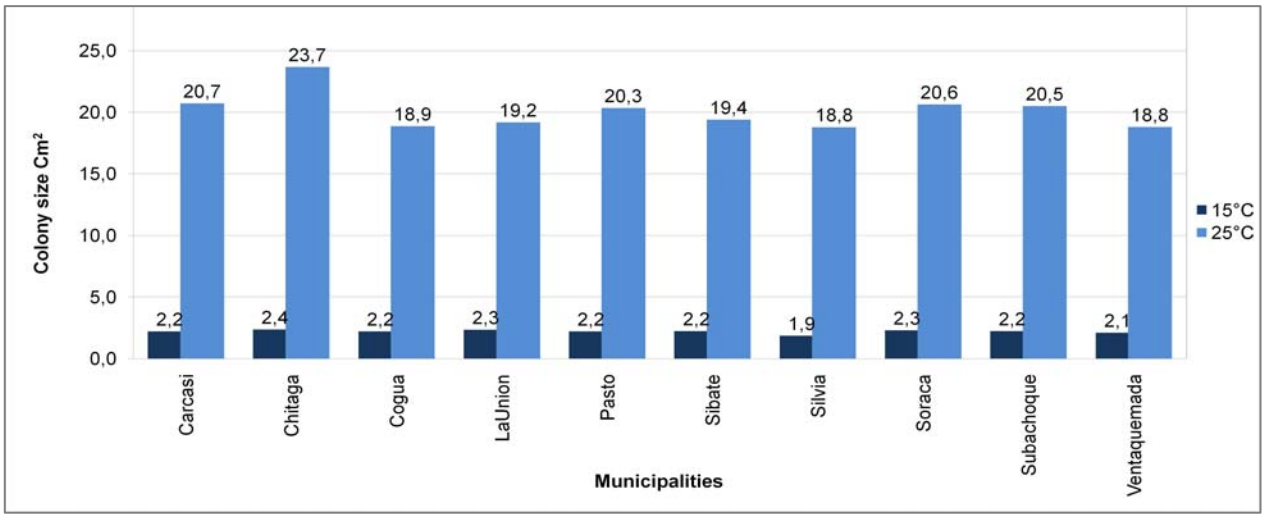

Figure 3-1. Average of colony size (Cm^2) 48 hours after inoculation of petri dishes, in isolates of *R. solani* AG-3 collected on different municipalities and grown at 15 and 25 °C

The test for differences in the average growth of isolates was significant for isolates of Pasto and Silvia at 25 °C, at 15 °C there were no differences among isolates into the municipalities. The response of the isolates to low temperature was high in the populations of Pasto, La Union and Ventaquemada (Figure 3-2).

3.3.3. Sensitivity to Thifluzamide

All the isolates were affected by the fungicide. The isolates less affected were those from the municipalities Cogua and Subachoque (Figure 3-3). In Cogua there was an isolate that was poorly affected; this increased the average of the size of the colony. The differences were located mainly among isolates in each municipality not among municipalities, particularly in isolates from Ventaquemada, Subachoque, Soraca and Silvia.

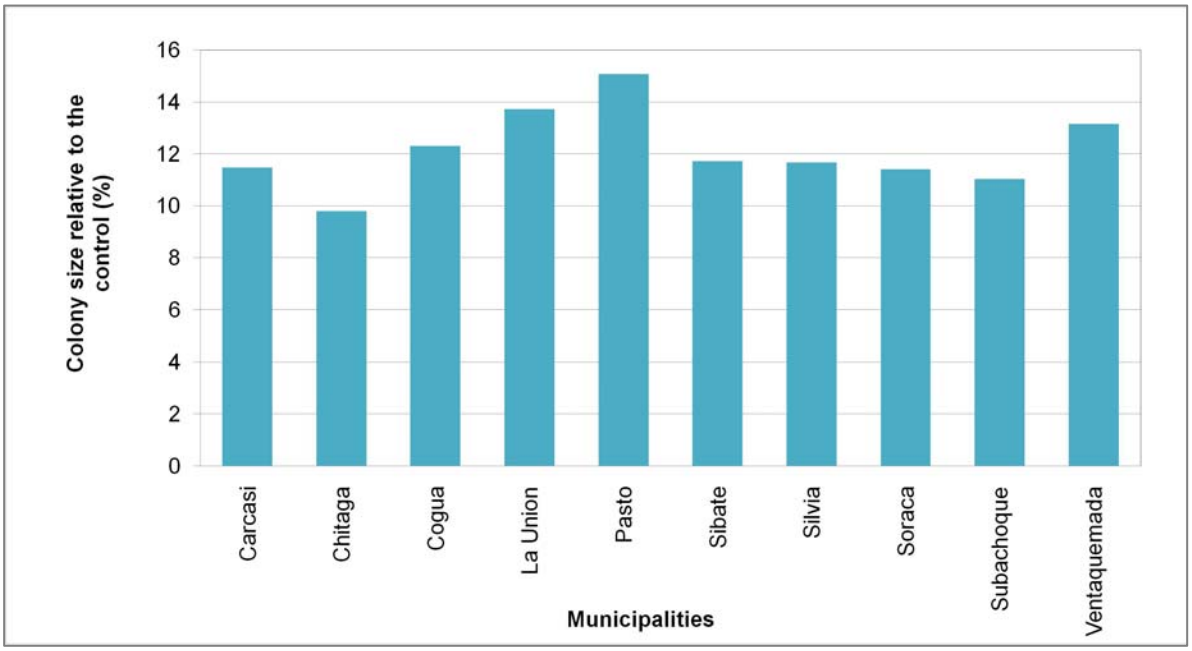

Figure 3-2. Proportion of growth of the isolates relative to the optimal temperature under *in vitro* conditions

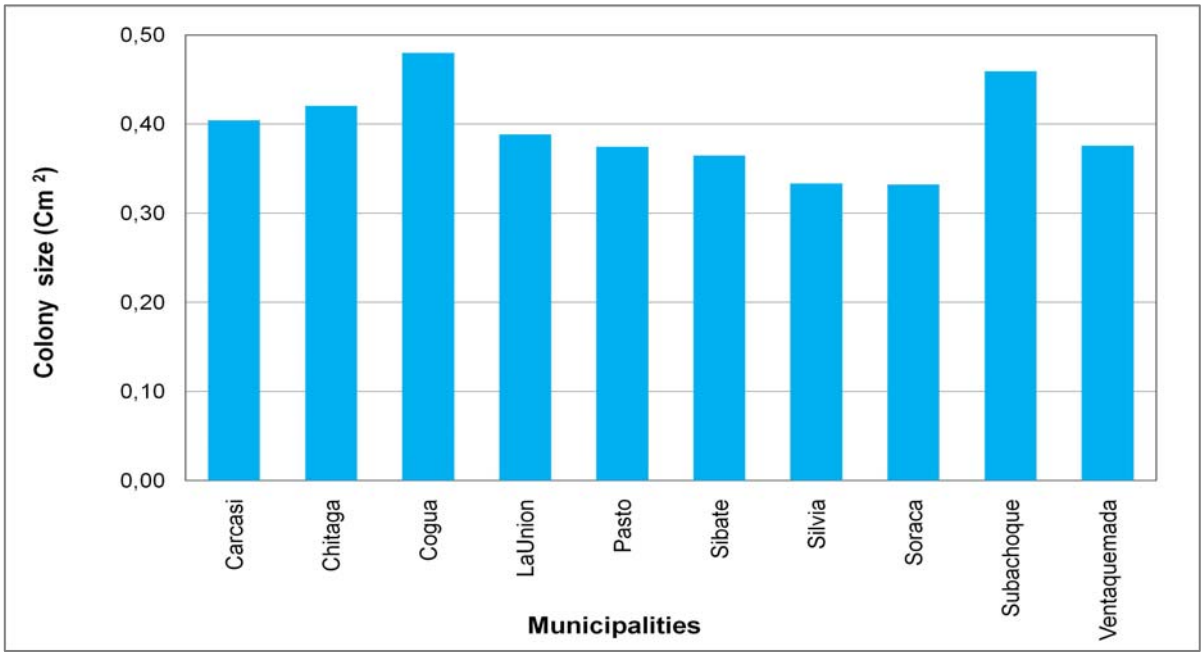

Figure 3-3. Average of colony size (Cm^2) in isolates collected on different municipalities and grown in presence of thifluzamide (14.2 ppm). Area of isolates in Petri dishes without fungicide was around 20 Cm^2

All the isolates were sensitive to thifluzamide, at 14 ppm. All the isolates showed a small colony in presence of the fungicide at 48 hours, and the proportion of growth relative to the control was less than 5 % (Figure 3-4), however the isolates continued its growth and were less inhibited in time.

The test *in vitro* with 22 isolates growing in presence of the fungicide and evaluated for 96 hours, showed that the fungicide inhibits the growth in the first 48 hours, after that the fungus diminish its sensitivity. At 3 ppm the response is quicker than at 14.2 ppm, in the first case at 24 hours the fungus started slowly to grow and in presence of 14.2 ppm of the fungicide at 48 hours.

3.3.4. Pathogenicity tests

Only a few isolates produced cankers on the stems of potato sprouts planted on quartz sand. The infection of different organs was independent, so amount and size of lesions on stolons were not related to amount or size of lesions on roots (Figure 3-5, Annex 3-D and Annex 3-I). There were statistically significant differences in the size of lesions caused by isolates collected on different municipalities (Annex 3-E). There were significant differences in the number and in size of lesions in stolons and roots among isolates into municipalities.

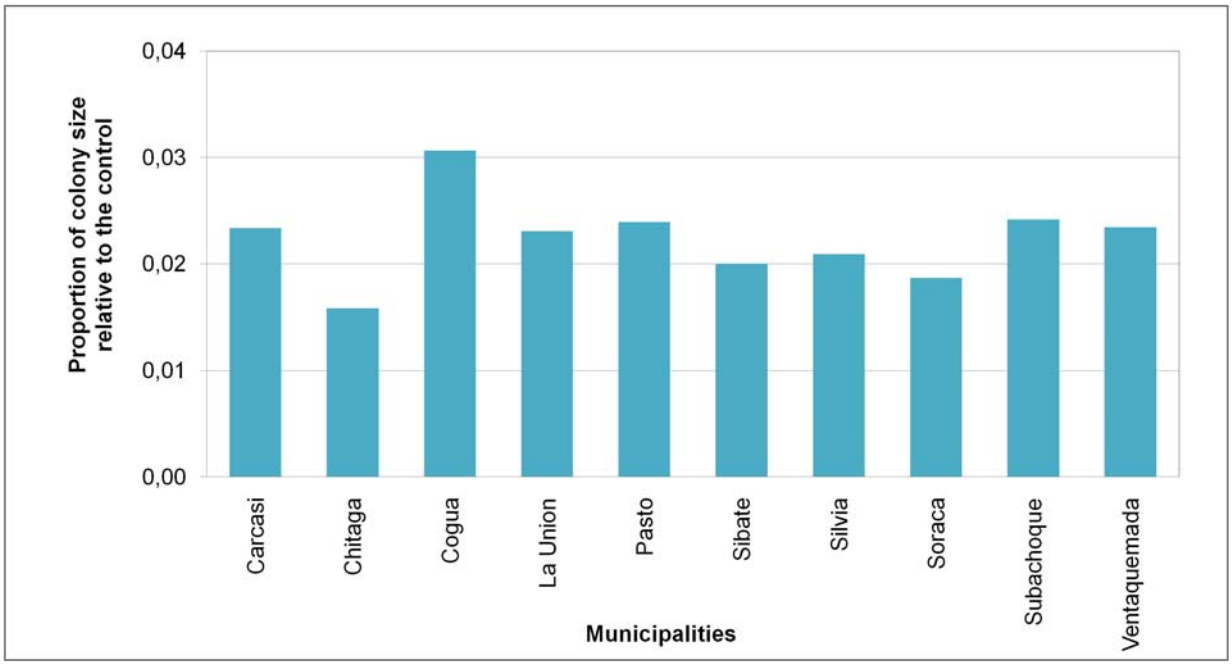

Figure 3-4. Proportion of growth of the isolates in presence of 14.2 ppm of fungicide relative to the growth without the fungicide

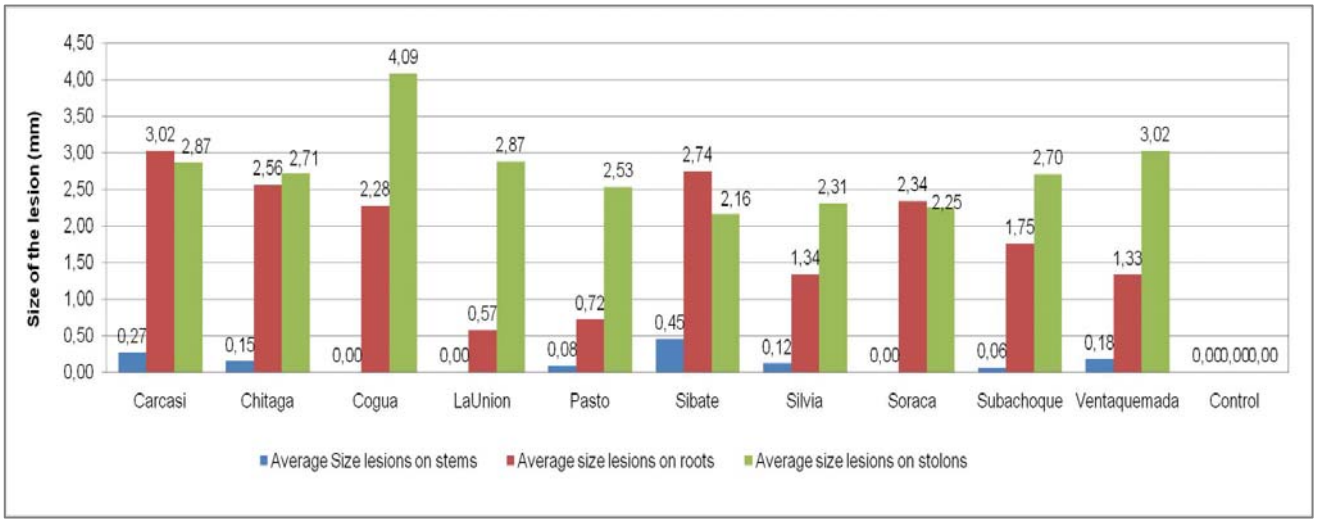

Figure 3-5. Size lesion average on potato plants (cv Capiro) caused by isolates of *R. solani* AG-3 collected on different municipalities in Colombia.

The sclerotia infestation on tubers was mild. The highest value was present in plants inoculated with isolates collected on Silvia with 1.75 % of the surface of tubers covered by sclerotia (Figure 3-6). The test for differences among municipalities was significant (Annex 3-E), however the values were small falling in one category in the severity scale.

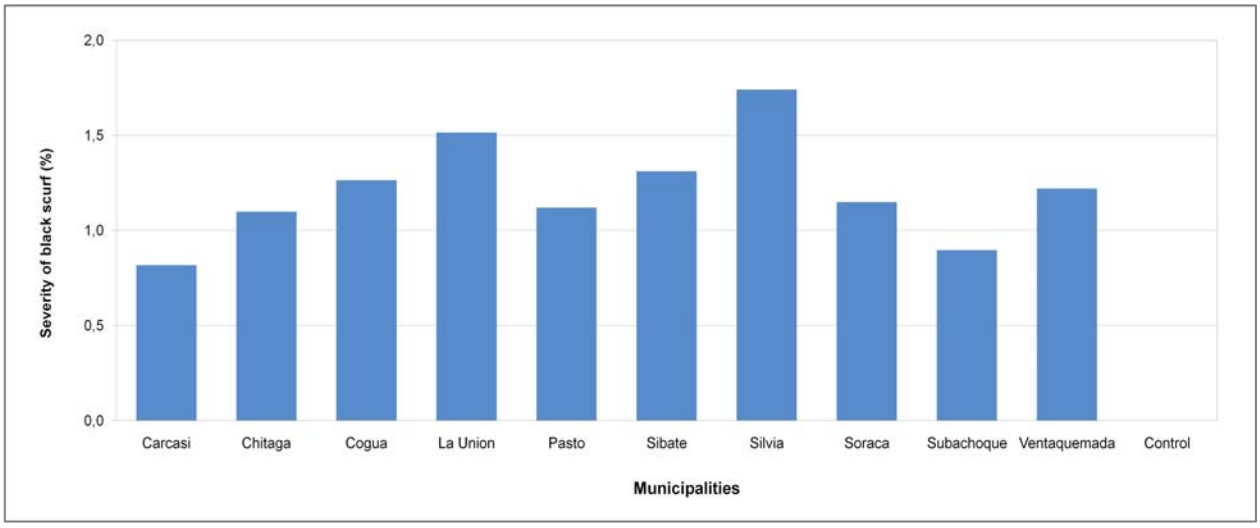

Figure 3-6. Percent of sclerotia on the tuber surface of the cv Capiro caused by isolates of *R. solani* AG-3 collected on different municipalities in Colombia.

There were differences in the incidence of black scurf on tubers among isolates collected on different fields (P value <0.05), and among localities. The isolates from Cogua, Sibate and Ventaquemada showed the highest levels of incidence of sclerotia on tubers, and the lowest was found in plants inoculated with isolates from Subachoque (Figure 3-7).

The pathogenicity of the isolates was not dependent on their origin (stems or sclerotia from tubers). Isolates obtained from sclerotia did not produced higher levels of black scurf. In each municipality there were a few isolates that produce high levels of sclerotia. The same result were obtained with isolates collected from stem cankers, they did not produce higher levels of cankers (Annex 3-F).

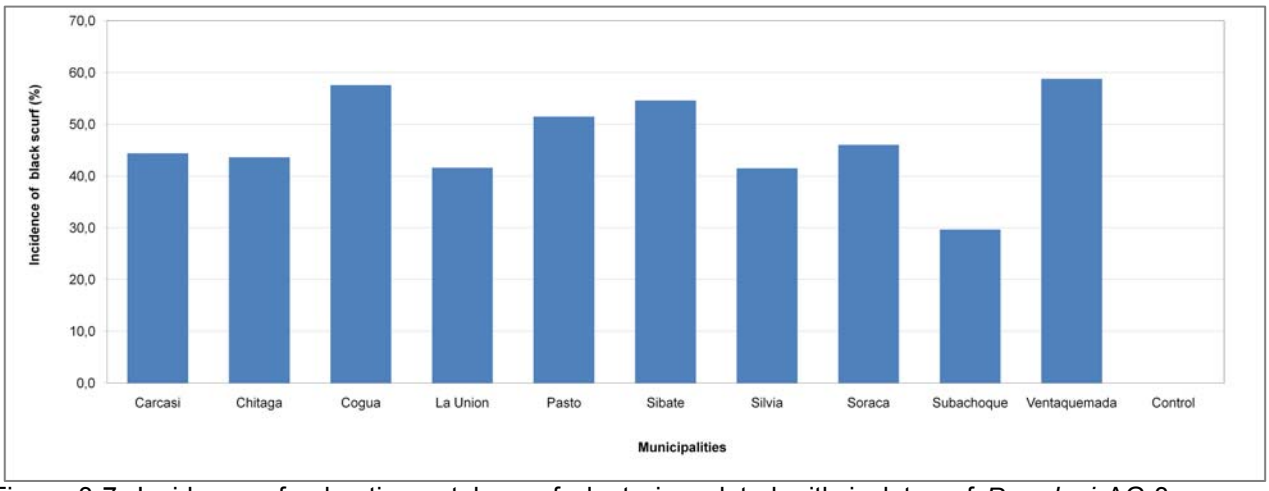

Figure 3-7. Incidence of sclerotia on tubers of plants inoculated with isolates of *R. solani* AG-3 collected on different municipalities in Colombia

The cluster analysis showed what was found with the descriptive analysis. There was no pattern of response associated with geographical location of the isolates but in each municipality there are isolates with differential responses to the stress factors evaluated.

3.4. Discussion

This was an exploratory research to study the variation on phenotypic and physiological traits, in isolates of *R. solani* AG-3 collected from potato infected plants in Colombia. We were interested in determine if the pathogen is adaptable to stress conditions, like fungicides and temperatures. Mycelial growth was selected as variable because is linked to fitness in this pathogen. Additionally differences in virulence and physiological responses were tested in the same isolates in order to identify individuals associated to geographical location or to physiological responses.

The life cycle of *R. solani* AG-3 has an asexual and a sexual phase. During the asexual phase, the fungus produces mycelia and sclerotia as survival structures. Infection starts with hyphae growing on surface of the plant, later appresoria are formed previous to the penetration of the plant cells. Initial infection is followed by the release of enzymes that degrade cell walls, kill the cells, and promote the spread of hyphae in dead cells (Lehtonen

et al., 2008b). Mycelial growth is therefore an important fitness component for this species because the fungus grows and damages host plants with asexual hyphae

The isolates showed high variation in the response to the two temperatures and to the fungicide evaluated. The smaller average area of the colony was found with Thifluzamide at 14.2 ppm (0.53 cm^2), then the growth at 15 ^oC (2.23 cm^2). At 25 ^oC the largest size of the colonies was observed (20.16 cm^2).

These results are in agreement with previous reports about the ability of *R. solani* to overcome the stress caused by fungicides and temperatures (24 and 27 ^oC) (Willi *et al.*, 2011). The isolates tested in this research showed high variation in the response to the two temperatures and to the fungicide evaluated, however the average among populations was similar. This is evidence that although there is variation in the response among isolates into the fields, the variation among them does not change. This shows the ability of this pathogen to be adapted under different climate regimens as well as to different crop management in different geographical regions.

The optimal temperature for the growth *in vitro* of *R. solani* AG-3 was among 20 and 30 ^oC. In a previous research was found that the optimal temperature for mycelial growth, production and germination of sclerotia is around 23 ^oC (Ritchie *et al.*, 2009). The soil temperature in the regions of potato production in Colombia is around 17 ^oC. The favorable response of the isolates to temperature above 15 ^oC, is an important concern respect to the response of the populations of this fungus in the climate warming context, and is a demonstration that the fungus can be adapted rapidly to global climate change (Willi *et al.*, 2011). On field conditions less incidence and severity is reported as the soil temperature increases, however in the studies did not considered the change in different soil properties that can generate an unfavorable environment to the pathogen (Simons and Gilligan, 1997).

The inhibition of the growth of *R. solani* AG-3 below 10 ^oC, suggest that the pathogen have less colonization ability in cold environments and as consequence less disease intensity is expected. However the pathogen survives and can be disseminated mainly as mycelia

The quick response against the fungicide was remarkable. In Colombia this is the single fungicide registered to the management of the disease, however it is not widely used, for instance the response of the isolates is not explained by the intensive use of the fungicide. In fact there was a wide response among isolates. This fungicide inhibits the succinate dehydrogenase enzyme within the tricarboxylic acid cycle (DowAgrosciences) and although is supposed to have a long residual control of *Rhizoctonia* diseases, from these results is clear that the fungus recovers its ability to grow actively after 48 hours of being exposed to the fungicide *in vitro* showing actual ability of the fungus to be adapted to this fungicide or fungistatic effect

It has been postulated that there are differences in virulence among isolates AG-3 collected from soil, tubers and potato stems (Carling and Leiner, 1990). However in this research there was no association, the size of the lesions on the plants was independent on the origin of the isolate (stem canker or sclerotia on tuber seed). This shows that the virulence of isolates of *R. solani* AG-3 does not depend on the origin of the isolate (stems, tubers or soil) but on the isolate in self (Carling and Leiner, 1990).

Under controlled environments the growth and expansion of the colonies of *R. solani* is controlled by the physical, chemical and biological properties of the substrate (Ritz, 2007). In this experiment we have more infection in stolons and roots than in stems as usually is expected. There are two hypothesis associated to this response, the first associated to the plant, the sprouts used were planted superficially, in this condition, the sprouts were green all the time. Previously was postulated that the resistance of the stems to the pathogen is associated to the presence of chlorophyll in the autotrophic phase (Carling and Leiner, 1990) and to the physical resistance of the tissue. The second hypothesis is related to the substrate, quartz sand allow higher expansion of the fungus (Otten *et al.*, 2001) and because is an inorganic substrate that does not bring any nutrient pressed to the fungus to move inside and parasite young roots and stolons.

The evolutionary forces (mutation, migration, genetic drift, selection and reproduction system) have influence in the amount of variation in the populations of organisms; but he selective factors causing differentiation among populations are natural selection and random genetic drift. Comparative studies of quantitative genetic and neutral marker differentiation assess for the relative roles of natural selection and random genetic drift in the divergence among populations (Leionen *et al.*, 2008). Most of the phenotypic evolution likely is driven by natural selection; however in plant pathogen fungi the genetic drift is common as bottlenecks caused fungicides and founder events. Small Ne increases the rate of genetic drift providing more scope for non adaptive differentiation, (England *et al.*, 2003). The populations of *R. solani* AG-3 evaluated in this study have a high effect of migration among populations, the permanent gene and genotype flow hide the effects of natural selection and genetic drift in the populations, this can be the main reason why association among isolates to geographical origin was not found and the wide variability in the response to the factors of stress evaluated.

Genetic and phenotypic diversity in the population of organisms is shaped by natural selection and adaptive evolution. The process of adaptive evolution depends on how much heritable genetic variation exists for the traits that are exposed to selection. Much of the variation observed in nature is neutral with respect to fitness, being determined by stochastic processes choosing among ecologically equivalent types (Kocher, 2004). From an ecological perspective, diversity is supported mainly by divergent natural selection, which is plausible when there are free niches or the organism is highly competitive and displaces to its competitors. The variety of niches available depends largely on the

physical structure of the environment. Complex, heterogeneous environments provide more niches and therefore maintain higher diversity than simpler environments.

In soil, spatial variation is high with a huge richness of niches determined by the biotic and abiotic environment explaining the high biological diversity in this kind of environments. The soil-borne pathogen *R. solani* is a complex specie, it affects a wide range of hosts including dicots and monocots, and has been adapted to different organs into the hosts (underground and above ground). In the sub-specific groups (AGs and subgroups) the variability also is high showing the evolutionary potential of this specie, which is adapted to a wide range of ecological conditions, being able to occupy a wide range of niches in different agriculture ecosystems.

3.5. Conclusion

Populations with high genetic variation have shown to have better potential to adapt to changing environments. Populations of *Rhizoctonia solani* AG-3 in Colombia are highly diverse, in genetic and phenotypic traits. Isolates in each population showed a different response to the temperatures, fungicides and presented differences in aggressiveness on potato stems and tubers, this show that those populations have high evolutionary potential with consequences for the management of the diseases caused by the pathogen.

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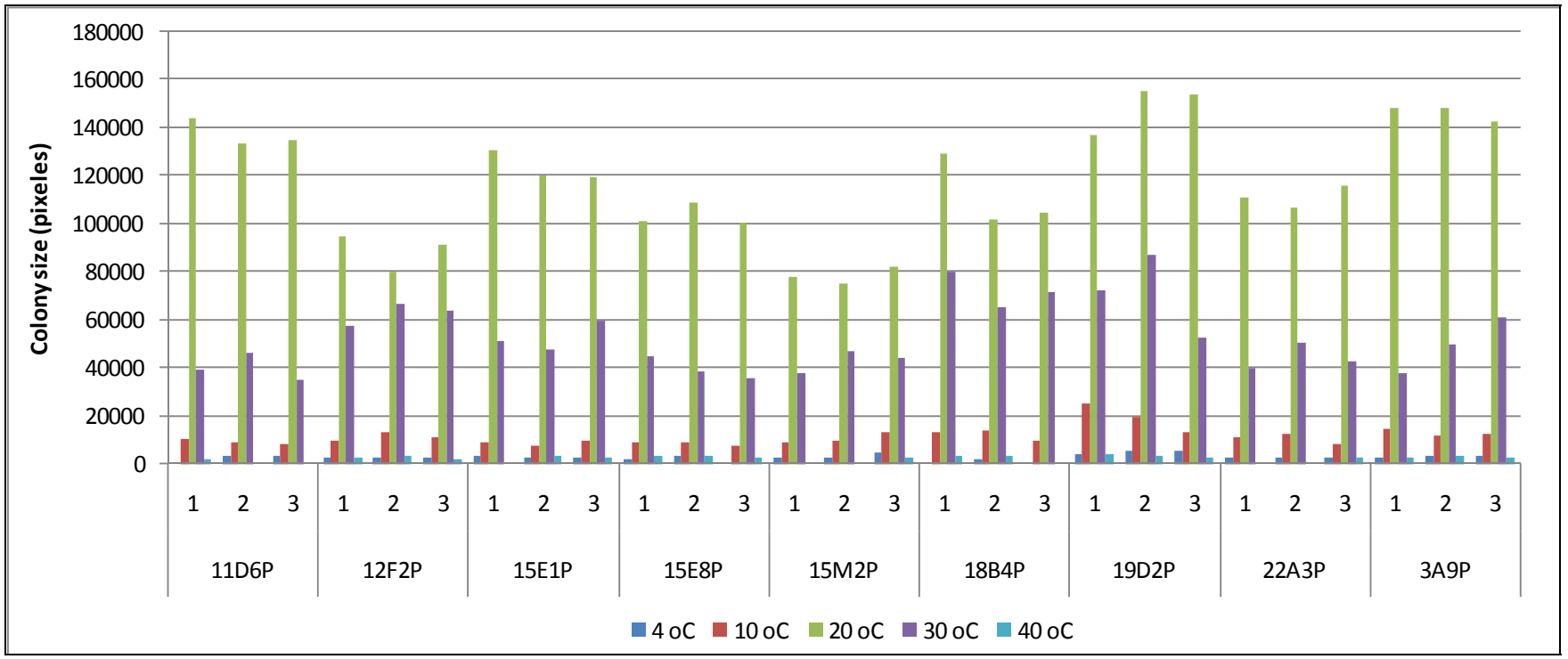
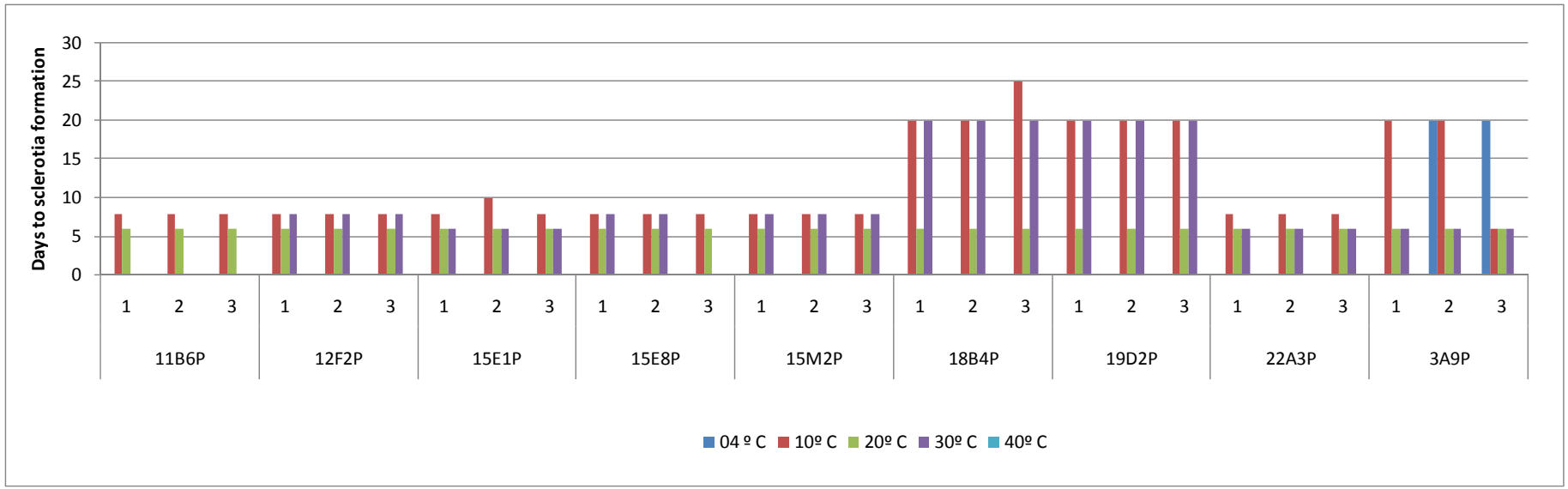
Annex 3-A. Growth *in vitro* of isolates of *Rhizoctonia solani* AG-3 in response to four temperatures after 48 hours of incubation



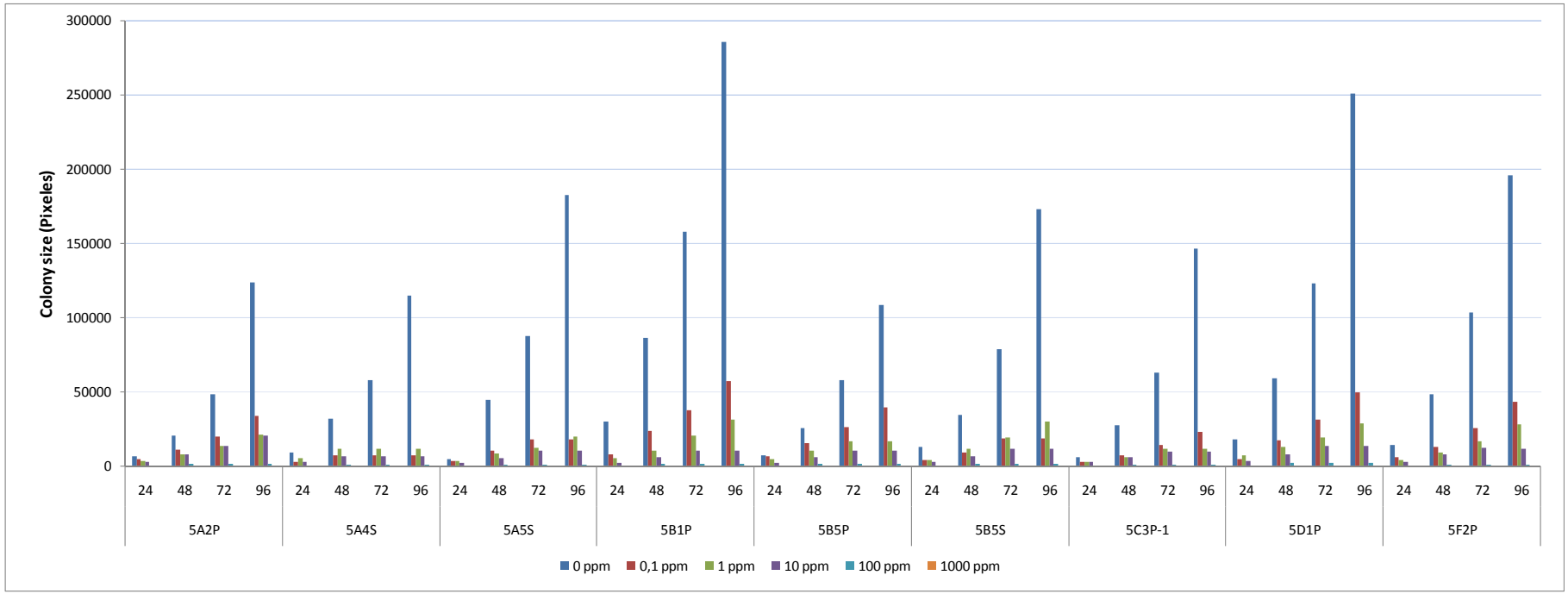
1, 2 and 3 are the number of repetitions for each isolate.

Annex 3-B. Time to sclerotia formation in isolates of *Rhizoctonia solani* AG-3 growing in four temperatures



1, 2 and 3 are the number of repetition for each isolate.

Annex 3-C. Growth of isolates of *Rhizoctonia solani* AG-3 *in vitro* in presence of different doses of the fungicide Thifluzamide



Evaluations of colony size were done at 24, 48, 72 and 96 hours after inoculation on Petri dishes.

Annex 3-D. Size of lesions on stems, stolons and roots generated by the artificial inoculation with isolates of *R. solani* AG-3 collected on different municipalities in Colombia

Municipality	Isolate	Average Size of lesions on stems	Average size of lesions on roots	Average size of lesions on stolons
Carcasi	19A2SKH	2,20	2,80	1,84
	19B1P	0,00	6,26	3,24
	19C2S	0,00	1,02	2,83
	19D2P	0,00	0,00	2,34
	19D4P	0,00	3,86	2,12
	19D5S	0,00	3,22	4,72
	19E1SKH	0,00	3,55	2,79
Average Carcasi		0,31	2,96	2,84
Chitaga	22A3P	0,00	3,46	4,31
	22B25P	0,00	0,00	1,69
	22C3P	0,00	7,94	2,89
	22C4P	0,80	2,49	2,65
	22H3P	0,00	1,91	2,09
	22I2P	0,00	2,29	2,51
Average Chitaga		0,13	3,01	2,69
Cogua	4A11P	0,00	0,48	5,98
	4A12P	0,00	1,42	6,22
	4A14P	0,00	2,90	4,45
	4A3P	0,00	0,74	2,41
	4A7P	0,00	0,49	2,50
	4AP	0,00	4,78	3,57
	4B6P	0,00	6,48	2,54
Average Cogua		0,00	2,47	3,95
LaUnion	16A1P	0,00	0,00	3,66
	16B10P-2	0,00	1,91	4,31
	16C10P	0,00	1,18	1,19
	16C2P	0,00	0,00	1,77
	16D7P	0,00	0,00	2,50
	16E7S	0,00	0,00	3,43
Average La Union		0,00	0,52	2,81
Pasto	10A3P-1	0,44	0,91	0,74
	10B8P	0,00	0,00	3,03
	10C1P-2	0,00	0,61	3,41
	10C3P	0,00	1,85	2,94
	10E3P-2	0,00	0,57	2,58
	10F5P	0,00	0,55	2,80
Average Pasto		0,07	0,75	2,58
Sibate	6A2S	0,00	5,70	2,23
	6A5P	0,76	2,37	2,49
	6C4S	0,87	1,51	2,18
	6D4P	0,00	3,64	1,12
	6D6P	0,00	2,89	1,91
	6F1P	0,00	0,31	3,65
	6F2P	1,51	2,56	1,50
Average Sibate		0,45	2,71	2,15
Silvia	7A4P	0,26	0,69	1,91
	7B1P	0,45	1,18	2,16
	7B2P	0,00	3,04	2,50
	7B5P	0,00	0,00	2,31
	7C2P	0,00	3,02	1,38
	7P	0,00	0,12	3,60
Average Silvia		0,12	1,34	2,31
Soraca	12A4P	0,00	1,69	3,85
	12A6P	0,00	5,44	1,08

	12B2P	0,00	1,51	2,33
	12BTOYA	0,00	1,41	1,56
	12C4P	0,00	2,94	0,60
	12C5P-2	0,00	2,33	2,36
	12D1P	0,00	1,07	3,97
Average Soraca		0,00	2,34	2,25
Subachoque	1A11P	0,40	1,23	2,34
	1B2P	0,00	1,67	2,79
	1D2P	0,00	0,00	1,98
	1E1P-2	0,00	2,19	4,15
	1E3P	0,00	1,66	3,92
	1E4P	0,00	2,03	1,42
	1E9S	0,00	3,54	2,89
Average Subachoque		0,06	1,76	2,78
Ventaquemada	11B2P	0,00	1,31	2,27
	11B5P-1	1,23	2,43	1,05
	11B6P	0,00	0,00	4,17
	11C4P	0,00	1,97	2,88
	11D1P	0,00	0,00	4,60
	11D3P	0,00	1,29	2,23
	11F4P	0,00	2,56	4,46
Average Ventaquemada		0,18	1,37	3,10
Control	*	0,00	0,00	0,00
Average Control		0,00	0,00	0,00
General Average		0,11	1,57	2,27

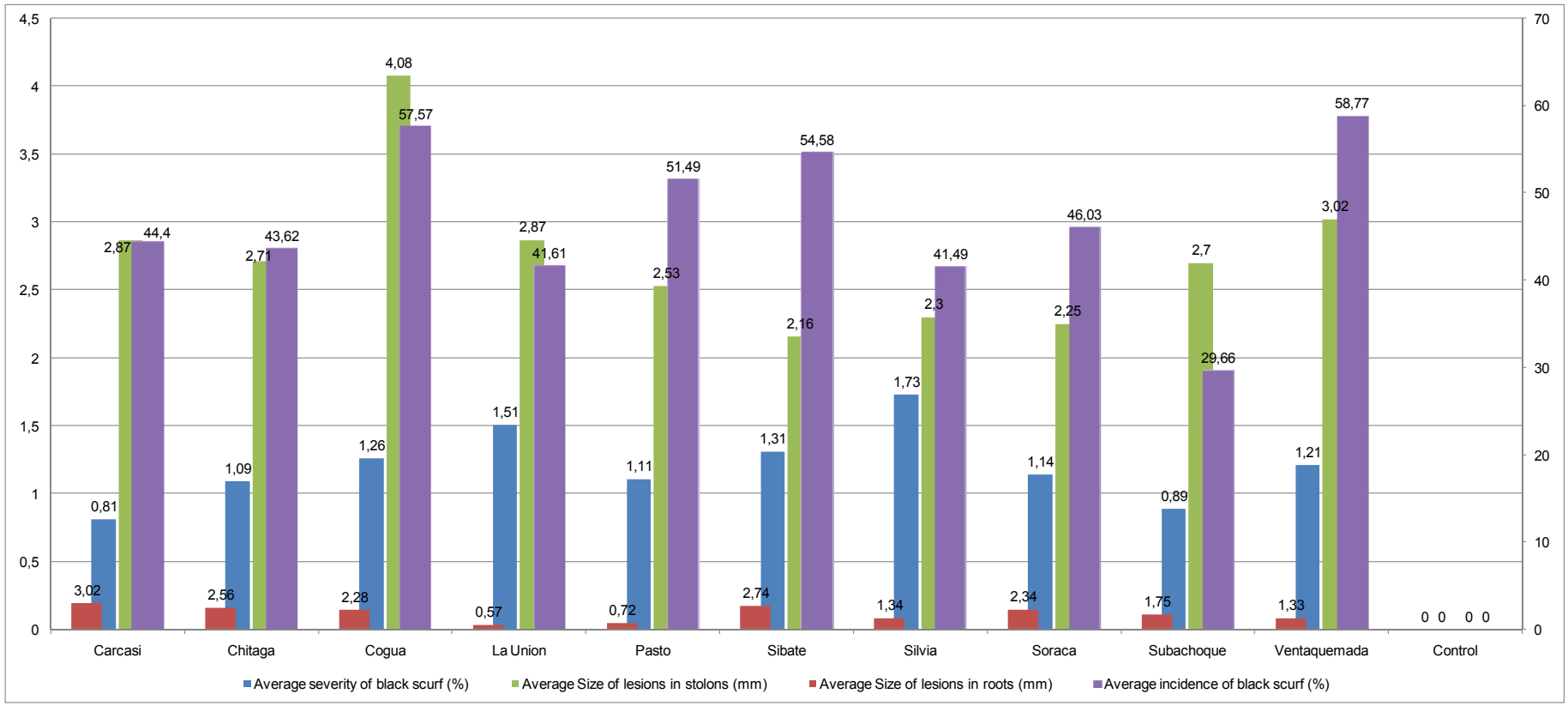
*Plants inoculated with a plug of PDA

Annex 3-E: Test for statistic differences among the variables evaluated in pathogenicity tests with isolates of *Rhizoctonia solai* AG-3.

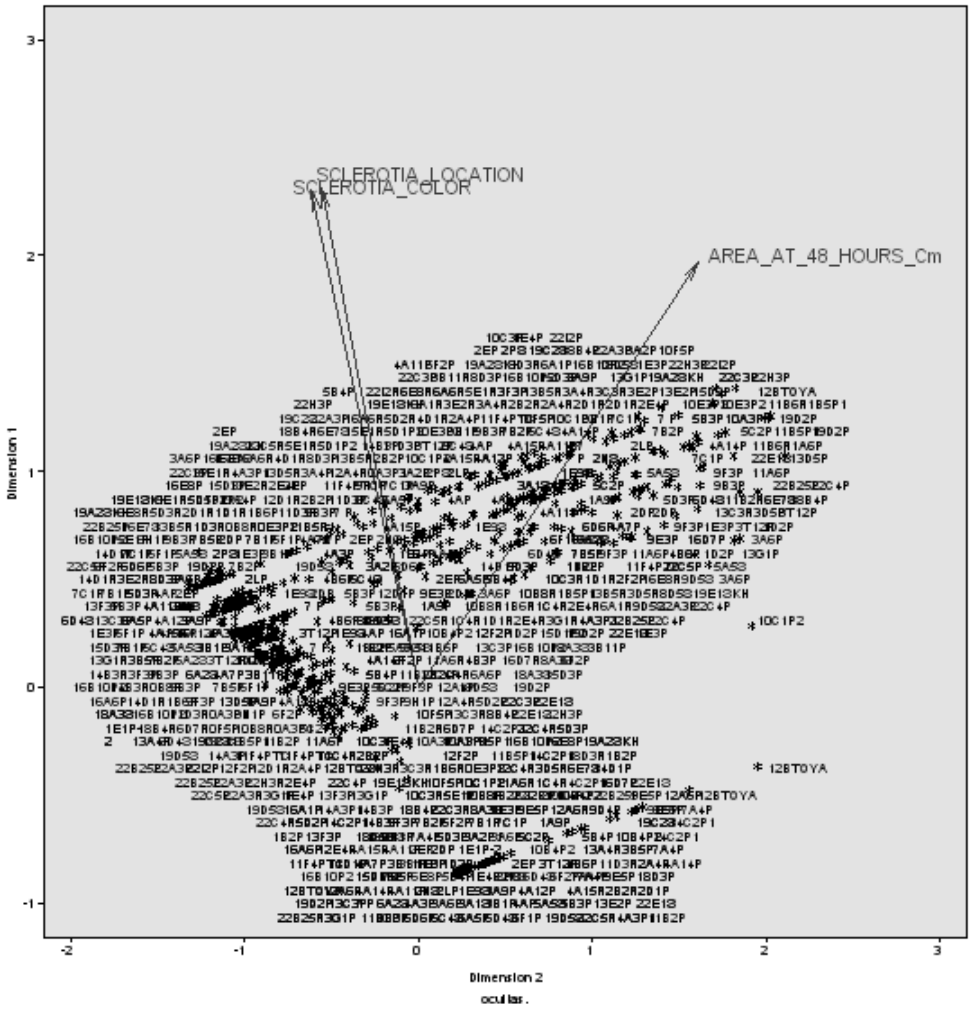
	Size of lesions in stolons		Size of lesions in roots		Incidence of black scurf		Severity of black scurf	
Municipality	Group*	Average	Group	Average	Group	Average	Group	Average
Subachoque	B	2,70	ABC	1,75	C	29,66	B	0,89
Cogua	A	4,08	AB	2,28	BA	57,57	BA	1,26
Sibate	B	2,16	A	2,74	BA	54,58	BA	1,31
Silvia	B	2,30	ABCD	1,34	BC	41,49	A	1,73
Pasto	B	2,53	BCD	0,72	BA	51,49	BA	1,11
Ventaquemada	AB	3,02	ABCD	1,33	A	58,77	BA	1,21
Soraca	B	2,25	AB	2,34	ABC	46,03	BA	1,14
La Union	B	2,87	CD	0,57	BC	41,61	BA	1,51
Carcasi	B	2,87	A	3,02	ABC	44,40	B	0,81
Chitaga	B	2,71	A	2,56	ABC	43,62	BA	1,09
Control	C	0	D	0	D	0	C	0

* Values with the same letter show no differences among average values. Test of Duncan of pairwise comparisons among means ($\alpha = 0,05$).

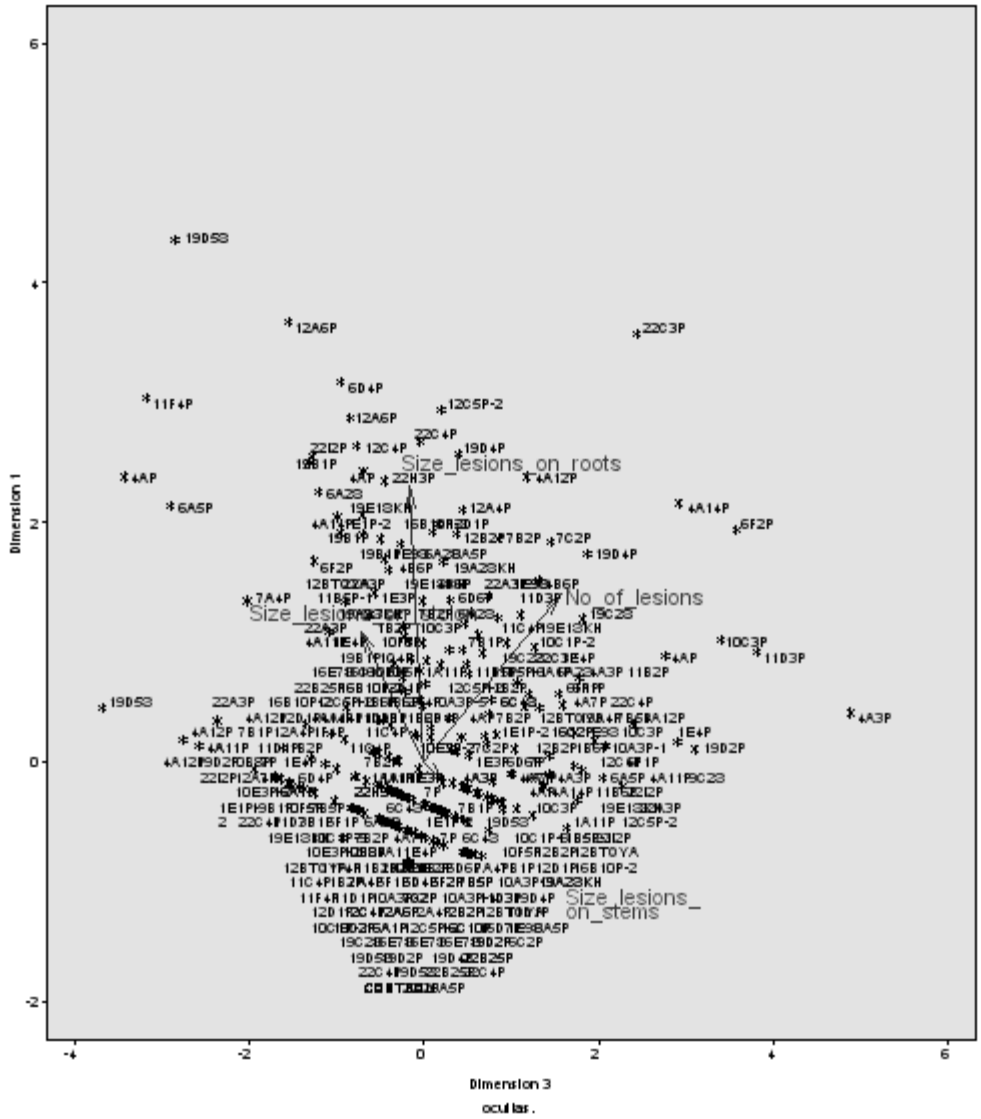
Annex 3-F. Symptoms induced by *Rhizoctonia solani* AG-3 in plants of the cv. Diacol capiro



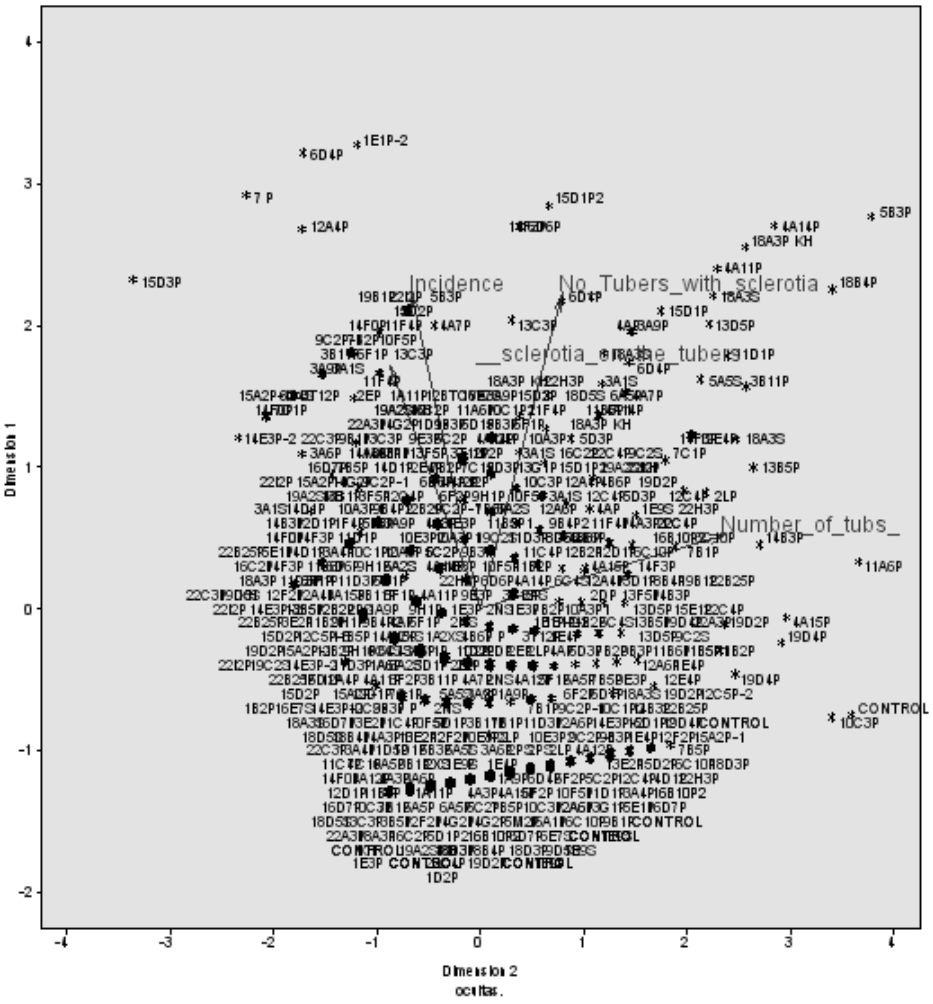
Annex 3-G. Principal component analysis for the test in vitro



Annex 3-H. Principal component analysis for the scores of the disease on stems, roots and stolons



Annex 3-I. Principal component analysis for the scores of the disease on tubers



Chapter 4. Final Considerations

R. solani AG-3 is a soil-borne fungus widely distributed everywhere potato is cultivated. This fungus causes the diseases stem canker and black scurf on tubers (Hide *et al.*, 1985). Damage on sprouts diminishes the number of areal stems and in consequence the foliar area in the plants. Cankers on stems alter the uptake and translocation of water and minerals to the leaves and the movement of photoassimilates to the tubers. The losses caused by this pathogen are no well-defined, however it has been demonstrated that affects the size distribution of tubers (Simons and Gilligan, 1997b). In Colombia the disease is not considered important, in fact when we visited potato fields for sampling, most farmers were not aware about the symptoms on stems. With the exception of farmers that trade with specialized markets, black scurf has no impact in the price of tubers for the internal market only for seed-tuber and the production for industry.

In this research we generated important knowledge about the biology of the *R. solani* AG-3 populations in Colombia. With this work we demonstrate the importance of basic studies on the biology of this pathogen to understand the development of the disease and some epidemiological aspects. In the next sections I will present the summary of the research calling the attention to relevant aspects that should be considered for the control of the disease.

The ample sampling allowed to cover the main and representative localities along the country and gave us the possibility of drawing powerful conclusions about the populations of *R. solani* AG-3 in Colombia, as the first step to propose and reconsider the management measures of the diseases in Colombia.

4.1. Anastomosis groups

Plant pathogenic isolates of *R. solani* are divided in intraspecific groups based on differences in pathogenicity, cultural appearance, morphology, physiology, ecology and DNA sequence (Anderson and Stretton, 1982; Ogoshi, 1987; Andersen, 1996; Sharon *et al.*, 2008). Those intraspecific groups are called Anastomosis Groups (AG). In different geographical regions where potato is produced, research on AGs has shown that AG-3 is the most prevalent and the group that causes the most serious damage on plants and the higher infestation on tubers. Additionally several AGs have been associated to symptoms and have been isolated from soils where potato is grown (Table 4-1). In this research two AGs were associated with symptoms on plants and tubers, AG-3 and AG-2-1. There was no relationship between the AG and the organ of the plant from where it was isolated.

Table 4-1. Anastomosis Groups associated to Rhizoctonia diseases in potato crops around the world

Country	AGs	Source	Reference
Australia	AG-3, AG-4, AG-5 y AG-8	Stems, roots, tubers and soil	(Balali *et al.*, 1995)
Canada	AG-3, AG-4 y AG-5	Stems, stolons, roots	(Bains and Bisht, 1995)
Chile	AG-3 and non identified isolates	Tubers	Castro, 2005
Finland	AG2-1, AG-3 and AG-5	Stems, stolons, tubers	(Lehtonen *et al.*, 2008a)
France	AG2-1, AG-3 y AG-5	Stems, hymenia and tubers	(Campion *et al.*, 2003)
Great Britain	AG2-1, AG-3 y AG-5	Stems, stolons, roots, tubers	(Woodhall *et al.*, 2007)
India	AG-3 and AG-4	Stems, roots and soil	(Suresh and Mall, 1982)
Mexico	AG-3 and AG-4	Stems, stolons, tubers	(Virgen-Calleros *et al.*, 2000)
Pakistan	AG-3, AG-4, AG-5	Tubers	(Rauf *et al.*, 2007)
Peru	AG-3, AG-4 and non identified isolates	Stems and tubers	(Anguiz and Martin, 1989)
South Africa	AG-3, AG-5, AG-7 y AG-8	Stems, stolons, roots and soil	(Truter and Wehner, 2004)
USA	AG-3, AG-5 and non identified isolates	Stems	(Bandy *et al.*, 1988)
USA and Alaska	AG2-1, AG-3 and non identified isolates	Stems	(Carling and Leiner, 1986)
Venezuela	AG2-1 y AG-3	Stems, stolons, roots, tubers	(Cedeño *et al.*, 2001)

The AG-2-1 was restricted to localities where weeds of Brassicaceae family are common. Therefore, the reduction of those weeds on potato fields may contribute to the reduction of inoculum and the consequent diminution the symptoms of stem canker and black scurf caused for this AG on stems of potato plants.

In Colombia potato is grown as the main crop in most geographical areas. The most common rotation is with grasses, which have not been reported as hosts of AG-3 and in the pathogenicity tests done in this study, they were not affected by AG-3 or AG-2-1. In a previous work the use of ryegrass as crop rotation diminished the severity of stem canker on potato (Talbot, 2003). This makes to the grasses as the best crop rotation for control of the two AGs prevalent in Colombia, especially considering that AG-3 was able to infect several other species in controlled conditions.

4.2. Genotypic and physiologic variability of *R. solani* AG-3

R. solani AG-3 has been reported variable in all the places where their populations have been evaluated. The variability is manifest not only at the genetic level but in its physiologic and phenotypic characteristics.

Individual variation has great interest from an evolutionary perspective, given that selection and evolution of traits arise from variable individuals present into the populations (Bennett, 1997). Individuals of the Colombian populations of *R. solani* AG-3 are highly variable, from a genetic perspective this pathogen has high evolutionary risk as has been proposed for pathogens with mixed reproduction and high and efficient gene flow (McDonald and Linde, 2002). Additionally results of this study show that this pathogen has the ability to grow well at temperatures above and under the optimal, in fact the

temperature optimal *in vitro* is higher than expected considering that the temperatures on the regions were the samples were taken are mainly from 15 to 22 ^{o}C.

The evolutionary potential of populations of organisms depends on the interaction of the evolutionary forces. In the Colombian populations of *R. solani* AG-3, the main evolutionary force acting was found to be gene flow, an unexpected condition for a soil-borne fungus, condition that is explained for the movement of tuber seed among localities. In Colombia the regulation for seed production of *R. solani* on tubers allow until 10% of tuber surface with sclerotia, additionally farmers exchange seed informally and only few of them use certified seed-tubers, providing the means for homogenization of whole population; which was confirmed by the presence of two genetic populations from the 18 geographical populations sampled.

The second force that acts in the structure of the Colombian populations of *R. solani* AG-3 is its mode of reproduction. The data revealed cycles of sexual reproduction followed by asexual multiplication. This condition gives an advantage to those populations. Meiotic recombination puts together new combinations of alleles, and the asexual multiplication increases the frequency of those new combinations of alleles in the populations. Pathogen populations with mixed reproduction show particular genetic structure, called epidemic structure. The sexual recombination gives raise an excess of recombinant genotypes, the most fit then dominates in the asexual cycles. Many fungi that have being assumed mitosporic, exhibit characteristics of recombination in their population genetic structure, and that truly asexual lineages if they exist, do not appear to persist for evolutionarily significant lengths of time (Taylor *et al.*, 1999).

Evidence for epidemic structure has been found in populations of *R. solani* AG1-IA suggesting a reproductive mode varying from strictly recombining to a mixed system in which there are recombination events followed by clonal expansion during the growing season. The evidences for those conclusions where the same genotype recovered many times in the same and in separated fields and in the opposite several loci in HWE and a few of all possible pairs of loci in linkage disequilibrium (Rosewich *et al.*, 1999; Ciampi *et al.*, 2008; Bernardes-de-Assis *et al.*, 2009)

There was no evidence for recent founder events or bottlenecks in the populations and the absence of structure does not allow inferring selection associated to geographical regions. The SSR as neutral markers, are not able to identify patterns of genetic variation associated to selection.

The variability in the response to temperatures, the sensitivity to the fungicide and the aggressiveness on potato plants are important clues in the evolutionary history of this pathogen and additional studies are necessary to understand if those characteristics are adaptive, evolutionary or phenotypic plasticity of the individuals into the populations of *R. solani* AG-3.

4.3 Variability of *R. solani* AG-3 and the control of the diseases black scurf and stem canker

The control strategies for the diseases caused by *R. solani* AG-3 are varied but have low individual effect. The genetic, pathogenic and physiologic complexity of this pathogen shows that an integrate approach is necessary. The knowledge here generated gives the possibility of proposing the proper approach for the management of this pathogen from a population view and considering its genetic and physiological variability.

Microbial communities are evolving entities that interact. Only one individual rarely ever cause serious epidemics. Disease symptoms typically manifest once the pathogen populations reach certain thresholds. The severity of the disease is also influenced by evolution within the pathogen population, that occurs within an ecological context defined by the host and the climatic conditions (Kassen and Rainey, 2004). Then, it is important to have always in mind that in the fields, the populations of pathogens interact with plants, where pathogens are variable in time and space and plants have a narrow genetic base consequence of human selection process.

In the potato crops in Colombia, *R. solani* AG-3 maintain high genetic, physiological and pathogenic variability and is able to be adapted to the fungicide thifluzamide and to react positively to the increase of temperature. Those characteristics improve its adaptability. The growth of the fungus is reduced in response to stress, however it is not inhibited completely. In those conditions the development of the disease will be slower, allowing to the plants improve their defenses against the attack of the fungus. The differences among isolates from one locality, allows to infer that within fields the disease can be expressed in differential ways, with isolates more inhibited, the plants do not get ill, and in the places where are located the isolates best adapted, the incidence and severity will be higher.

The possibility of diminishing *R. solani* AG-3 in soils is remote. The production of resistance structures allows it to survive in soils for long periods of time. With rotation the population of the fungus diminishes but never will be close to zero. Additionally, the use of seed with some degree of sclerotia and mycelia always adds inoculums to the soil. Therefore the control on the movement of tubers with visible structures must be considered as a main tool of management of the disease along with crop rotation.

4.3.1 Legal control

The use of pathogen free seed-tubers is the most important tactic, because in this way the inoculum potential is reduced. Previous studies have shown that tuber-borne as well as soil-borne inoculum is important in the severity of the disease and the symptoms are more severe when both of them are present in the field (Frank and Leach, 1980; Carling *et al.*, 1989; Scholte, 1989; Tsror and Peretz-Alon, 2005). The epidemics are strong when

the inoculum on tubers is higher than 10% of tuber surface covered by sclerotia (Simons and Gilligan, 1997).

With low levels of inoculum on tubers, the degree of the disease caused probably is not significant, however there are three main consequences of the arrival of new genotypes; (i) continuous introduction of new genotypes into the populations, that can be successful in the new environment (ii) Recombination between foreigner and native genotypes produce more novel genotypes with new combination of alleles and (iii) the continuous addition of resistance structures (sclerotia) to soil increases the potential of inoculum on the long run.

Additionally the variation in the response to stress factors of the isolates evaluated show that this pathogen has a high evolutionary potential, therefore their populations react positively to differences in environments.

4.3.2. Cultural practices

Cultural practices together with host resistance generate an inadequate environment to the pathogen. Practices that favor rapid emergence reduce the risk of root and stem cankers (Jeger *et al.*, 1996). This occur when the colonization of the pathogen is diminished and the host advantaged. Invasion of *R. solani* occurs in a physical, chemical and biological heterogeneous environment through a web of pores, cracks and aggregates (Otten *et al.*, 1999; Otten and Gilligan, 2006). In that environment the pathogen interacts with soil particles, water films, organisms and with the host to cause the diseases. Modification of the components of soil can diminish the effect of the soil diseases caused by *R. solani*. The main characteristic of the soil having an important effect on the development of epidemics is soil water, high contents affect the pathogen (Otten *et al.*, 1999; Ritz, 2007), for what pre-irrigation of dry soils before planting reduces the rate of growth of the fungus (Banville *et al.*, 1996). Additionally selection of soils with low organic matter, low porosity and moderate density can contribute to diminish the colonization of the substrate and delay the contact of the fungus with the host.

Successive cropping of potato in the same field will increase the inoculum potential in the soil, and long-term rotations diminish the viability of that inoculum. The continuous cropping of potato may enhance the incidence and severity of stem canker due to the increase in soil-borne inoculum density (Honeycutt *et al.*, 1996)**.** Rotations of three years or longer are suggested to reduce damage caused by *R. solani* (Banville *et al.*, 1996). However farmers must be cautious about the plant species used for rotation, avoiding those that can host AG-3.

Some studies have shown that diseases can increase in potato fields with certain crop rotations. *Rhizoctonia* disease incidence and severity is reduced in most rotations,

compared with the continuous cropping of potato. Canola, barley or sweet corn prior to potato had the lowest levels of *Rhizoctonia* disease and the best tuber quality, in the opposite soybean, green bean, broccoli and clover increased the diseases in potato (Larkin and Honeycutt, 2006; Tsror, 2010).

Our results showed that, not only potato but carrot, tomato, lulo, pea, bean, and corn were infected by isolates of *R. solani* AG-3, therefore those species are not recommended for rotation. The best options seem to be grasses (*P. clandestinum* and *P. pratense*) that were not affected when were inoculated with AG-3 isolates.

4.3.3. Chemical control

Fungicide application is the most common alternative for disease management (Banville *et al.*, 1996) but this option is not sustainable because of the impact on non target soil microorganisms and the potential risk of resistance to fungicides.

In Colombia it is necessary to think on alternative options, the only registered fungicide for stem canker and black scurf management, showed short time effect in the inhibition of the fungus (24 hours at 3 ppm and 48 hours at 14 ppm), although the manufacturer cite that this molecule is stable to hydrolysis and that the half-life in soil ranges from 95 to 155 days. The mode of action of thifluzamide and other carboxamides are thought to have a medium to high level of potential resistance (FRAC, 2011). Resistance to thifluzamide has been documented for specific fungi, but is not a wide spread issue. Our results would indicate that even though this fungicide is very stable in soil, the low inhibition of micelial growth in time makes its use as the sole management strategy, very unreliable

4.3.4. Resistant varieties

Resistant cultivars are the desirable option to enhance the control of *R. solani* disease in the field. Different studies have reported potato germplasm with different responses to the pathogen (Naz *et al.*, 2008; Olanya *et al.*, 2009) though, the responses are weak and no resistant cultivars have been identified or developed (Jeger *et al*., 1996). In a recent study, systemic induction of resistance in sprouts upon infection with a virulent isolate of *R. solani* was demonstrated and the secondary infection of the sprouts was inhibited (Lehtonen *et al.*, 2008b).

The high variability of *R. solani* and the effect of gene flow and sexual reproduction situate this fungus in the category of high evolutionary risk, which need an approach for host resistance based on more than one resistance gene. Until the *R. solani – S. tuberosum* interaction is completely elucidated, breeding for resistance does not seem to be an option for the management of the black scurf and stem cankers on potato.

4.3.5. Biological control

Different organisms have been used to suppress *R. solani*, including bacteria (actinomycetes, *Bacillus* and fluorescent *Pseudomonas*) and fungi (*Trichoderma*, *Verticillium*, binucleated Rhizoctonias and hypovirulent isolates of *R. solani*), all of them reduce the disease or inhibit the pathogen in controlled assays, however in natural conditions usually fails to reduce disease severity or to stimulate plant growth (Tsror, 2010). Combination of organisms have been reported to give better results in the control of stem canker severity by 40-49% in greenhouse trials (Brewer and Larkin, 2005).

Microbial communities are evolving entities. Groups of individuals in a population are interacting genotypes and whose driving mechanisms are the interactions among them. These interactions may range from antagonistic to beneficial, and the populations can themselves evolve, sometimes fortuitous and unexpected (Kassen and Rainey, 2004). Epidemics of soil-borne pathogens depend on the ability of the pathogen to contact the host. The organisms used for biological control block the sites for infection acting against the pathogen infection(Bailey and Gilligan, 1997).

In laboratory usually one strain of the pathogen is challenged with one strain of the biocontrol agent, in this one by one interaction is easy to depict conclusions about the beneficial effect of the biocontrol against the disease. However in the fields there are many different individuals in the populations of the pathogen that respond differentially, under that situation only one strain of the biocontrol agent is fighting against a complex population of the pathogen.

When biological control of diseases is used in field conditions it is necessary to consider the complex environmental and the genetic diversity of the pathogen populations as well as the diversity of the interacting organisms, all those factors contribute to the failure or success of biological control in the field.

The management of *R. solani* AG-3 in Colombia requires an integrated approach. The main objective must be to decrease its evolutionary potential, which can be achieved reducing the population size into the fields and limiting the movement of inoculum among localities.

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