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Diseases of coffee with particular reference to those affecting stems and roots in Colombia

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By

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DECLARATION OF AUTORSHIP

I, the undersigned, hereby declare that the thesis submitted herewith for the degree of *Philosophiae Doctor*, to the University of Pretoria, contains my own independent work and has hitherto not been submitted for any degree at any other University.

Bertha Lucía Castro Caicedo

May 2014

Pretoria, South Africa

DEDICATION

To my parents, Claudio and Martha Fanny,
to whom I owe what I am.

To my brothers and sister, Claudio, Gilberto, Maria Elena,
Javier, Jaime, Jairo, Alvaro, Germán, William
and Giovanny, as to be examples of overcoming,
oneness, love and honesty.

To my children, Adriana and Juan José,
who are a vital part of my existence

To all of them that have always supported me in all my struggles unconditionally through their love and patience. And of course to Father Creator of the Universe for the opportunity and conscience of being a co-creator in this wonderful world.

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PREFACE

Coffee is the most important agricultural export product for Colombia and plays a crucial role for social stability in rural areas. There are more that 563 000 families producing coffee on ~915 793 hectares of land and most Colombian coffee growers have plots no larger than 2 ha (5 acres) on average. Based on this resource, Colombia has been a recognized leader in the cultivation of high quality coffee, relying exclusively on *Coffea arabica*, one of the two most cultivated species of coffee in the world.

Coffee production globally, including in Colombia, is constrained by a number of fungal pathogens. Of the diseases affecting coffee, the coffee leaf rust (CLR) caused by *Hemileia vastatrix* is the most important. Despite the development of resistant varieties, and the fact that CLR seldom kills the plants, this disease still causes economic losses in areas planted with susceptible varieties. Other diseases, however, are responsible for decreasing production by causing plant death. Of these, the root rot known as "llagas radicales" and Ceratocystis trunk canker, known as "llaga macana" are of considerable importance in Colombia. Both diseases are caused by soil-borne fungi, in the genera *Rosellinia* and *Ceratocystis* (*Ce*) respectively. The exact identity of the *Rosellinia* species in Colombia, however, needs clarification and this is included in the objectives of this thesis. Llaga macana, in Colombia has been ascribed to two species, *Ce. colombiana* and *Ce. papillata.*

The aim of the research reported in this thesis was to provide a clear identification of *Rosellinia* species that are affecting coffee and other hosts in Colombian coffee growing areas. Special enphasis was placed on the genetic resistance against Ceratocystis canker in coffee genotypes. The results of studies to address these aims are presented in six chapters.

The first chapter provides an overall review of the literature on the genus *Coffea,* its classification and origin, conditions for cultivation, selection and breeding, diseases caused by *Rosellinia, Ceratocystis*, and economic importance. In Chapter two, studies to characterize the cause of "llagas radicales" using molecular studies (DNA sequence data and pathogenicity tests) on *Rosellinia* isolates from coffee and other

plants in Colombian coffee growing areas are presented. The third chapter reports on the results of resistance evaluations against *Ce. colombiana* and *Ce papillata* in selected accessions of *C. canephora, C. liberica*, *C. arabica* and Timor Hybrid (*C. canephora* x *C. arabica*). Chapters four and five deal with the assessment of resistance to *Ce. colombiana* and *H. vastatrix* in two different populations of advanced genotypes of interspecific coffee hybrids derived from *C. canephora* and *C. arabica* backcrosed to *C. arabica*. In the final chapter, I consider opportunities to select advanced genotypes of *C. arabica,* resistant to both *Ceratocystis* species and CLR.

It is my hope that the results of the research undertaken for this thesis will contribute to the continued and improved sustainable production of coffee, not only in Colombia, but globally. This is especially regarding the development of coffee genotypes with resistance to both CLR and *Ceratocystis* species.

Chapter 1

Literature Review: Coffee production and diseases caused by species of Rosellinia and *Ceratocystis*

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ABSTRACT

Coffee trees belong to the botanical genus *Coffea* (family Rubiaceae). Arabica coffee (*Coffea arabica*) originated in Ethiopia and was first cultivated in Yemen in the 15th century. Arabica coffee was introduced to the Asian and American continents during the 17th and 18th century respectively. Today, this species accounts for 70% of the world coffee production, with *C.canephora* (robusta) constituting ~30% of the production. Coffee has contributed significantly to the economic and cultural development of the countries where it is grown. In 2013, the exporting countries produced approximately 8.5 million metric tonnes (MMT) of green coffee beans, with Brazil, Vietnam, Indonesia and Colombia as the main producers. Significant research programs have focused on providing high-yielding *C. arabica* cultivars with resistance to pests and diseases, drought, low temperatures and acidic soils. In Colombia, coffee is grown on 915 793 hectares, sustained by approximately 563 000 Colombian coffee growing families. Colombia exported 0.7 MMT of coffee in 2013, worth approximately USD 3 500 000 000. Coffee production has been significantly threatened by leaf rust caused by the fungus *Hemileia vastatrix,* which is present in all coffee growing countries globally, and results in substantial economic losses. In Colombia two groups of soil borne pathogens, *Rosellinia* species that cause root rot diseases, and *Ceratocystis* species which causes canker (“llaga macana”) disease, also reduce productivity. This review presents a summary of knowledge regarding coffee species, their economic importance, breeding of coffee to improve production and details of the latter two diseases, primarily in Colombia.

1. INTRODUCTION

Coffee is one of the most valuable primary agricultural products in world trade and its cultivation, processing, trade, transportation and marketing provide employment for approximately (~) 125 million people worldwide (ICO 2013). Coffee is grown in more than 70 tropical countries and its production is predominantly a "small-holder" enterprise with orchards that cover more than 11 000 000 hectares (ha) globally. An estimated 25 000 000 farmers in Africa, Latin America and Asia depend on coffee production for their livelihoods (Waller *et al.* 2007). Commercial production is based on two species, *Coffea arabica* L. ("Arabica") and *C. canephora* P. ("Robusta"). In Colombia, *C. arabica* is exclusively cultivated and is the most important agricultural product for the country. This provides an income for approximately 563 000 families and currently, there are close to 915 793 ha under coffee cultivation (FEDERACAFE 2013).

Coffee production globally is threatened by several biotic and abiotic factors. Of these, fungal diseases continue to have a significant negative impact on coffee production, annually. Arguably the best known fungal disease is the coffee leaf rust (CLR) or orange coffee rust, caused by the *Hemileia vastatrix* Berkeley & Broome. It has been considered as one of the most catastrophic foliage diseases in all major coffee-producing countries (Kushalappa 1989; Van der Vossen 2009; ICO 2013). Coffee berry disease (CBD), caused by the fungus *Colletotrichum kahawae* Waller & Bridge, is also of considerable importance, but is prevalent only in Africa (Van der Vossen 2009). Diseases caused by soil borne pathogens include wilt disease ("tracheomycosis") caused by *Gibberella xylarioides* Heim & Saccas, Fusarium bark disease caused by *G. stilboides* Gordon: Booth (Rutherford 2006), stem canker caused by *Ceratocystis* species (Castaño 1951; Fernández 1964; Marin *et al.* 2003b; Van Wyk *et al*. 2010) and root rots caused by *Armillaria* and *Rosellinia* species (Fernández and López 1964; Waller *et al.* 2007).

This Chapter reviews knowledge regarding the origin, evolution, plantation practices, genetic resources and economic impact of coffee globally, but especially in Colombia. Attention is given only to commercial, cultivated coffee species and hybrids. Special attention is given to two groups of soil-borne fungal pathogens, *Rosellinia* species and species in the *Ceratocystis fimbriata* Ell. & Halst *sensu lato* (*s.l.*) complex. The focus on

the latter two diseases is linked to the fact that they are the major topic of the thesis that follows this review.

2. THE GENUS *COFFEA*

2.1. Classification and origin

The Rubiaceae includes some 500 genera and over 6 000 species in the tropics. The sub-genus *Coffea* includes 103 species, with 59 found in Madagascar, 41 in tropical Africa and three in the Mascarenes (Mauritius and Reunion). The most important species in the sub-genus *Coffea* include *C. arabica* (Arabica coffee), *C. canephora* (robusta coffee) and *C. liberica* Hiern (Liberian coffee) (Charrier and Berthaud 1985; Herrera *et al.* 2011). These species occur naturally in three biogeographic areas of the African Continent (Figure 1), known as the Malgache region (Madagascar), East-Central and West Africa or Guineo-Congolese (Anthony *et al.* 1999; Lashermes *et al.* 2009). *Coffea arabica* is indigenous to the highland forests of Ethiopia and northerm Kenya, *C. canephora* is native to the forests of West Africa and *C. liberica* is from the humid tropical forests of Western and Central Africa (Lashermes *et al.* 2009).

2.2. Cultivation, selection and breeding

Coffea arabica was introduced as a cultivated crop into India, Sri Lanka (then Ceylon) and Java (Indonesia) in the 17th century. The British spread coffee as a crop into their African territories, such as Malawi (then Nyasaland) in 1878 and then to Uganda in 1900. The introduction to southern Kenya and Cameroon took place in 1963 and 1984 (Anthony *et al*. 1987). Coffee introduction to the American continent dates back to the early 18th century in Martinique and Brazil (Waller *et al.* 2007). Later in the 18th century, coffee cultivation also spread in the Caribbean Basin (Cuba, Puerto Rico, Santo Domingo, etc.), Mexico and Colombia (Anthony *et al*. 1999).

Coffea arabica is an allotetraploid (2n=4x=44), is autogamous and mostly inbreeding, with only 10-12% outcrossing. It can be crossed with most diploid species. The average arabica plant is a large bush with dark-green oval leaves (Figure 2a). Historically, the bulk of the world's commercial coffee production has been derived from "typica" and *C. arabica* var. *bourbon,* (Waller *et al.* 2007).

Coffea canephora originated in West and Central Africa, today Tanzania and Uganda (Wringley 1988). This is a diploid (2n=2x=22) plant which is self-sterile and relies on cross-pollination, which results in much more variability than in *C. arabica.* It is a robust shrub or small tree growing up to 10 meters in height. It is larger than *C. arabica* and produces greater yields (Figure 2b). Growing wild in African equatorial forests, *C. canephora* has been widely distributed globally, being more adaptable than *C. arabica* (Waller *et al*. 2007; Lashermes *et al.* 2009). Some accessions of *C. canephora* are resistant to CLR and to nematodes (Bertrand *et al.* 2000; 2001; Fazuoli *et al.* 2002). *Coffea canephora* is less demanding than *C. arabica* and it is cheaper to grow. It is used in blends, mainly for instant coffee (Waller *et al.* 2007).

Coffea liberica is a native tree of the humid tropical forests of Western and Central Africa (Bridson 1985, cited by Herrera *et al.* 2011). It is a diploid (2n=2x=22) plant that is a self-sterile, lowland species, growing in warm equatorial forests (Lashermes *et al*. 2009). It grows as a large strong tree, up to 18 meters in height, and it has large leathery leaves (Figure 2c). There are two botanical groups of *C. liberica*, *C. liberica* var. *liberica* and *C. liberica* var. *dewevrei* (excelsa) (Berthaud and Guillaumet 1978). Although these two varieties produce a low cup quality, small areas are cultivated in Malaysia and West Africa (Waller *et al.* 2007). Some accessions of *C. liberica* are registered as CLR resistant (Prakash *et al.* 2004).

Natural hybrids between *C. arabica* and diploid species have produced valuable material used in breeding programs, mainly for selecting resistance to CLR (Bettencourt and Carvalho 1968; Eskes *et al.* 1990). One of the most important natural hybrids came from the Timor Hybrid (HDT), a natural *C. arabica/C. canephora* hybrid (Figure 2d). Some HDT introductions produced by the Coffee Rust Research Center (CIFC) in Portugal (eg. 832/1, 832/2, 1343, 2570) have been crossed with CLR-susceptible commercial varieties of *C. arabica* grown in different countries of the world (Varzea and Marques 2005).

The introgression of genes from the diploid species (*C. canephora/C. liberica)* into commercial tetraploid varieties (*C. arabica*) has been successfully used by many coffee breeders (Charrier and Eskes 2004; Prakash *et al.* 2006). This has improved the agronomic performance, and resistance to pests and pathogens. Commercially cultivated coffee species include several varieties of *C. arabica*, selected based on drink quality,

resistance to pests and diseases and drought tolerance. Selections are also based on performance under different conditions of climate, soil and methods of cultivation (Charrier and Berthaud 1985; Bertrand *et al*. 1999; Van der Vossen 2009). In Colombia, CLR resistant varieties have been developed through crossing *C. arabica* var. Caturra and the HDT/1343. These commercial varieties are "Colombia" (Castillo and Moreno 1988), "Tabi" (Moreno 2002) and "Castillo®" (Alvarado *et al.* 2005).

2.3. Conditions for plantation growing

Coffea arabica grows between 25°N and 24°S and 1 000 and 2 000 m above sea level with an optimum temperature range from 15-25°C (Smith 1989; Van der Vossen 2009). *Coffea arabica* is grown under shade or full sunlight. *Coffea canephora* and *C. liberica* grow best under warmer conditions (24-30°C), typical of the lowland tropics, and are less tolerant to cold temperatures (< 10°C is damaging). Coffee needs an annual rainfall of 1 100 to 3 000 mm (Waller *et al.* 2007). Well-drained, open-textured volcanic soils with pHs < 6 and adequate mineral content are best for coffee production (Clarke and Macrae 1988; Waller *et al.* 2007).

Coffee is propagateD with seeds and vegetative techniques. In the first case, the freshly picked coffee seeds (typically named beans) can either be planted immediately or dried for later use. Seeds are pre-germinated by spreading them on sand beds and removed as soon as radicals emerge, typically after 60-75 days (Mestre 1971). Coffee seedlings are planted in nursery beds in polybags, and in coffee fields when they have 9 to 12 pairs of leaves (Mestre 1971). Vegetative propagation is possible with shoots, leaves and roots, as well as by grafting techniques (Indian Coffee 2010; Hidalgo and Rojas 2007). Vegetative propagation is common in robusta and coffee hybrids (Indian Coffee 2010). In some countries, such as Brazil, Costa Rica, Guatemala, Honduras, India, Kenya and Salvador, commercial-scale disease-resistant hybrid plants have been produced also by micro-propagation (Clarke and Macrae 1988; Etienne *et al*. 1999; Waller *et al*. 2007).

Plants of *C. arabica* produce berries between 1 and 3 years after planting, while *C. canephora* takes longer to begin fruiting. The period from flowering to berry maturity is between 6 to 9 months for *C. arabica*, and 10 to 12 months for Robusta (Wringley 1988). Coffee harvesting is commonly done by handpicking or in a few places using mechanical

methods (Norris 2001). The pulp is removed from the berries using disk or drum pulpers (Mitchel 1988). The remaining part of the mesocarp (the mucilage) is removed either by fermentation, or mechanically. After washing, coffee beans are dried to obtain a moisture content of 10 to 12%. In mass production systems, mechanical dryers are used but in small farms solar drying is most common (Ramírez *et al.* 2002). Drying yields the "green coffee" beans that represent the commonly traded product, which is later roasted. All the structures that envelop the bean must be removed mechanically, using a variety of devices such as screens and knives. Finally, beans (green coffee) are graded by size to improve density and color separation, and to meet specific client requirements before they are sent to the roasters (Vincent 1987). All post-arvesting processes impact coffee quality (Marin *et al*. 2003a).

After 6 to 10 years of production, plants of *C. arabica* must be pruned. The objectives of this practice are, to keep the trees in a manageable shape, to remove dead and old unproductive stems and branches, to encourage the growth of new stems and branches and to regulate the crop in years of heavy bearing (Clarke and Macrae 1988). There are different methods of pruning according to the variety. Dwarf varieties, such as 'Caturra'and 'Colombia' are usually stumped after 6 or 7 years of production. This form of "renovation" is known in Colombia as "zoqueo" (Mestre and Salazar 1995).

2.4. Global economic importance

Coffee is consumed worldwide and represents one of the most valuable primary agricultural products in world trade. For many years, coffee has been second in value only to oil, as a source of foreign exchange for developing countries (Waller *et al*. 2007). The total annual production in 2013 was estimated to be 8.7 MMT with about a 18% increase in the last twenty years. World consumption is estimated to be 142 000 000 bags in 2013 (ICO 2013). Approximately 100 million people are employed in the coffee industry including in the growing, processing and marketing of the crop. The crop is grown by some 25-30 million farmers (Waller *et al.* 2007). Brazil has been the largest coffee producer in the world for many decades, with Vietnam currently the second largest producer followed by Indonesia and Colombia (ICO 2013).

2.5. The coffee industry in Colombia

"Colombian coffee" is a 100% washed arabica coffee ('Caturra', 'Typica', 'Colombia', 'Tabi' and 'Castillo'®). It is grown between 1° and 11°15 North latitude and 72° to 78° West latitude, and up to 2 000 meters above sea level (FEDERACAFE 2013). Coffee in Colombia is grown in the hilly west part on the country (Figure 3), in an area covering ~915 793 ha. There are approximately 563 000 producers in 588 municipalities occurring in 20 departments. In 2013, coffee production in Colombia amounted to 10 900 000 bags (60kg) of which 8 561 549 bags were exported (ICO 2013).

3. COFFEE DISEASES

Coffee production globally is negatively impacted by a number of diseases affecting different parts of the plant (Table 1). Most of these are caused by fungi, a few by bacteria, nematodes, viruses and mycoplasmas. The three most severe diseases are caused by fungi. All of these originated from Africa and only one has a worldwide distribution, namely, coffee leaf rust (CLR) caused by *Hemileia vastatrix* Berk &Br. CLR is the best known and arguable the most damaging coffee disease, resulting in crop losses of 20-40% when no control measures are used (Van der Vossen 2005). The second most severe disease is coffee berry disease (CBD), caused by *Colletotrichum kahawae* Waller and Bridge. This disease is a major cause of crop loss in arabica coffee in Africa, where it is still confined (Bridge *et al*. 2008). The third severe disease is coffee wilt disease (CWD), also known as Tracheomycosis, caused by the vascular fungus *Gibberella xylarioides* (Heim and Saccas). This disease has only been found in Africa and kills arabica and robusta coffee trees (Waller *et al*. 2007). Other than these diseases mentioned above, coffee is also affected by foliar pathogens that can cause economic losses in different regions, such as American leaf spot caused by the fungus *Mycena citricolor* (Berk &Kurt) Sacc, iron spot by *Cercospora coffeicola* Berk & Cooke, pink diease, attributed to *Phanerochaete salmonicolor* (Berk & Broome) Julich and dieback caused by *Phoma* spp. (Waller *et al*. 2007). Other disease problems include those caused by soil-borne pathogens such as *Rhizoctonia solani* Kün, *Ceratocystis fimbriata* s.l., *Rosellinia* spp. *Armillaria mellea* (Vahl) P. umm and some *Meloidogyne* spp. In addition, there are other minor problems caused by bacteria, such as *Xylella fastidiosa* Wells, Raju, Hung

Weisburg, Madelco-Paul&Brenner and *Pseudomonas syringae* van Hall. (Waller *et al*. 2007).

Among the major diseases that significantly reduce Colombian coffee production are CLR caused by the obligate pathogen *Hemileia vastatrix* (Rivillas *et al*. 2011; Cristancho *et al*. 2012), pink disease caused by *Phanerochaete salmonicolor* (Rivillas 2008) and iron spot caused by *Cercospora coffeicola* (Leguizamon 1997). Other important diseases caused by soil-borne-pathogens infecting stems and roots are Ceratocystis canker (Castro *et al*. 2003), caused by species in the *Ceratocystis fimbriata sensu lato* (s.l) complex, root rot caused by *Rosellinia* species (Fernández and López 1964; Castro and Serna 2009).

3.1. Coffee root rot caused by *Rosellinia* species

Rosellinia species have been recorded from many countries of the world. Most of the species are saprophytes and some are endophytes which occasionally become pathogenic. Only a few species occur as pathogens causing root rot in temperate and tropical zones on a wide variety of host plants (Ten Hoopen and Krauss 2006).

3.1.1. Taxonomy

The Ascomycete genus *Rosellinia* was erected by De Notaris in 1844 using the name *Sphaeria* with *S. aquila* (Fr.:Fr.) De Not. as the type species. *Rosellinia necatrix* is mentioned for the first time affecting vineyards in Germany and France in 1877 (Behdad 1975, cited by Ten Hoopen and Krauss 2006). And Hartig (1883, Cited by Ten Hoopen and Krauss 2006) described the anamorph as *Demathophora necatrix* on this host. Saccardo established 10 sub-genera in 1882 (Sivanesan and Holyday 1972). The genus *Rosellinia* (Subdivision Ascomycotina) is classified in the class Euascomycetes, Subclass Pyrenomycetes, order Sphaeriales (syn. Xylariales) and family Xylariaceae (Hsieh *et al.* 2010; Pliego *et al*. 2012). The genus includes more than 100 species, among them economically important root rot pathogens such as *R. necatrix* Berl.:Pril., *R. pepo* Pat, *R. bunodes* (Berk & Broom) Sacc., *R. arcuata* Petch and *R. desmazieresii* (Ber. & Br.) Sacc. (Ten Hoopen and Krauss 2006).

Ro*sellinia bunodes* was described as *Sphaeria bunodes* by Berkeley and Broom in 1873, and as *R. bunodes* (Berk. et Br.) Sacc. in 1882 (Saccas 1956). Sivanesan and Holliday

(1972) suggested that the conidial state of *R. bunodes* is probably of the *Demathophora* type.

Rosellinia pepo was described in 1908 (Booth and Holliday 1972). *Graphium* is the conidial state (Saccas 1956; Bermudez and Carranza 1992). However, similar to *R. bunodes,* it appears to be of the *Dematophora* type (Booth and Holliday 1972; Oliveira *et al.* 2008).

Other species of *Rosellinia* that attack coffee as well as their taxonomic descriptions are mentioned by Saccas (1956), e.g. *R. coffeae* Sacc., *R. didolotii* Sacc., *R. echinocarpa* Sacc., *R. lobayensis* Sacc., *R. mastoidiformis* Sacc., and *R. megalospora* Sacc. Another species of some importance is *R. arcuata,* described in 1916, which affects coffee and tea (*Camellia sinensis*), in India, Sri Lanka and Africa (Kannan 1995).

Rosellinia species have mostly been characterized based only on morphology (Petrini and Petrini 2005), with only limited DNA sequence data available for species in the genus. Molecular tools have provided important means to elucidate genetic variation and phylogenetic relationships among global members of the Family Xylariaceae (Bahl *et al*. 2005; Peláez *et al*. 2008; López and Ruano 2008; Hsieh *et al*. 2010; Pliego *et al*. 2012). Most work has, however been focussed on *R. necatrix* (Pérez *et al.* 2002; Bahl *et al.* 2005; López and Ruano 2008; Takemoto *et al.* 2009a; 2009b; Armengol *et al.* 2010; Hsieh *et al.* 2010), and the classification of many other species still requires considerable study. Sequencing of the internal transcribed spacer regions (ITS), fragments of the β-tubulin (BT), adenosine triphosphatase (ATP) and translation elongation factor 1α (TEF) gene regions and random amplified polymorphic DNA (RAPD) amplifications have mostly been used for identification of *R. necatrix* (López *et al*. 2004; Takemoto *et al*. 2009a, 2009b). At the population level, inter-simple sequence repeat (ISSR) markers have been used to study *R. necatrix* diversity in *Cyperus esculentus* L. (Armengol *et al*. 2010). Through these methods, new species such as *R. capetribulencis*, *R. aquila* and *R. compacta* have been described (Bahl *et al*. 2005; Takemoto *et al.* 2009a, 2009b). However, there is still a lack of information on tropical *Rosellinia* spp. that cause damage to commercially propagated plants such as coffee. In Colombia, López *et al*. (2004), for example, studied the genetic variability in 12 isolates of *R. bunodes* and *R. pepo* obtained from potato, coffee and cocoa. He used DNA-RAPD and ITS markers and found two

main clades for these fungi. The potato isolates formed one clade and *R. bunodes* and *R. pepo* formed a monophyletic group exhibiting high variability.

3.1.2. Morphology

Most *Rosellinia* species exhibit three morphological states. These include a teleomorph with asci and ascospores to which the name *Rosellinia* has been applied, an anamorph state in which conidia are produced by a *Graphium*-like synnematal state and a vegetative state, including mycelium and rhizomorphs, which is sometimes referred to as the *Demathophora* state (Saccas 1956; Bermudez and Carranza 1992; Sarasola and Sarasola 1975).

Rosellinia bunodes and *R. pepo* have mycelium characterized by pear-shaped septal unions (Figure 4a). Synnematal conidiophores (Figure 4b) (*Graphium*-like state) have conidiophores that may be di- or trichotomously branched, sub-hyaline to light brown and variable in length (Figure 4c, d). According to Bermúdez and Carranza (1992), conidia (ameroconidia) of *R. bunodes* are ellipsoidal and hyaline to light brown in color, 6.0-7.0 µm x 3.0–3.5–4.0 µm, while those of *R. pepo* are 5.0-9.0 µm x 2.0-3.0 µm in size (Figure 4e).

The teleomorph state of *R. bunodes* and *R. pepo* includes stromata (Figure 5a) with dark perithecia that are leathery and hard (Saccas 1956). *Rosellinia bunodes* has stromata with the surface ornamented with polygonal brown scales (Figure 5b), while perithecia of *R. pepo* are not ornamented (Figure 5c). Perithecia of *R. bunodes* have ostioles that are papilate-hemispherical and they contain asci which are cylindrical to globose, with paraphyses (Figure 5d). Ascospores are dark-brown with filamentous ends (Figure 5e). *Rosellinia pepo* has cylindrical asci (Figure 5f) with dark-brown ascospores without filamentous ends (Figure 5g) (San Martin and Rogers 1995; Petrini and Petrini 2005).

3.1.3. Geographic distribution and host range

Rosellinia spp. are found in many parts of the world (Table 2). *Rosellinia necatrix* is the most common species and has been reported from about 170 species in 63 genera and 30 families of plants. A USDA report in 2011 (Annonymous 2011) presented 437 records

for *R. necatrix* affecting plants on all five continents in temperate, subtropical and tropical zones. There are 22 reports of *R. pepo* and 102 reports of *R. bunodes* in tropical areas of Central and South America. Other, less known species such as *R. arcuata* are known mainly from Africa and Asia, *R. dezmazieresii* is currently known only from Europe, *R. radiciperda*, a pathogen of apple, is known only from New Zealand and *R. minor* has been found only in North America and Europe (Ten Hoopen and Krauss 2006). In Colombia, Chardon and Toro (1930, cited by Fernández and López 1964), provided the first description of *R. bunodes* affecting root in coffee plants. Subsequently, Fernández and López (1964) tested the pathogenicity of *R. bunodes* (black rot) and *R. pepo* (stellate rot) on coffee plants in Colombia. After that, several species of hosts have been found affected by both species in Colombia. Among these hosts are, casava (*Manihot esculenta* Crantz), coffee, cocoa (*Theobroma cacao* L.), macadamia (*Macadamia integrifolia* F. Muell.), morera (*Morus indica* L), potato (*Solanum tuberosum L.*), and some forest trees, such as *Cedrela odorata* L., *Podocarpus oleifolius* D. Don. and *Tabebuia rosae* DC. among others (Fernández and López 1964; Guerrero 1990; Aranzazu and Botero 1998; Realpe *et al*. 2006; Castro and Serna 2009).

3.1.4. Ecology

Some members of the *Xylariaceae* have a widespread capacity to destroy lignocellulose substrates and to cause various types of diseases, largely based on extensive tissue degradation (Rogers 2000). Fernández and López (1964) noted that in coffee, the parasitic ability of these fungi is incidental and that it depends on the substrate. If there are insufficient nutrients, the fungi do not enter the pathogenic phase. Aranzazu (1996) and Merchan (1988), noted that *R. pepo* lives as a saphropyte in roots of cocoa and becomes pathogenic as the trees start to die.

Soil moisture and pH are important factors in disease development caused by *R. bunodes* and *R. pepo.* López and Fernández (1966), found that *R. bunodes* and *R. pepo* isolates had more mycelial growth in soil with 50-60 and 70% of moisture. However *R. bunodes* caused 100% of infection in coffee seedlings planted in soils with moisture of 50 to 110%, while *R. p*epo lost 30% of its pathogenicity in soils with excess moisture >70%. Root diseases caused by *Rosellinia* spp. are often associated with acidic soils, rich in organic matter (Ofong *et al.* 1991; Mendoza *et al.* 2003). *Rosellinia bunodes* and *R. pepo*

affected coffee seedlings in soils at a pH from 4.0 to 7.0 and the greatest mortality at pH 5.2 (López and Fernández 1966). Castro and Serna (2009), studied the *Rosellinia* disease in coffee-cassava production systems in the Quindio Province (Central Colombia), at 1 250 to 1 500 msl and average temperatures from 22 to 23°C. These authors found that 58% of the affected coffee plants were infected by *R. pepo* and 42% corresponded to *R. bunodes*. Only 5% of the affected plants were associated with cassava debris and 95% with roots of forest or fruit trees.

3.1.5. Disease symptoms and spread

Rosellinia root rots occur in patches that extend in circular or non-definite patterns. The pathogens infect neighbouring plants, spreading through direct root contacts (Ten Hoopen and Krauss 2006; Castro and Serna 2009). In some tropical species, identification has been based on a combination of disease symptoms and the presence of the *Dematophora* anamorph state (Fernández and López 1964; Merchan 1988; Aranzazu 1996; Realpe *et al.* 2006). In coffee, mycelium of *R. bunodes* spreads superficially on the bark of the roots and hyphae penetrate the cortical cells, invading the internal tissues and forming masses of mycelium (Ibarra *et al.* 1999). These appear as macroscopic, small black specks or streaks under the bark, embedded between root tissues (Figure 6a). In contrast, *R. pepo* produces white mycelium in a star/fan shaped mat (Figure 6b) (Fernández and López 1964; Realpe *et al*. 2006).

In cocoa, coffee and macadamia, the first external symptoms caused by *R. bunodes* and/or *R. pepo* occur when the fungi reach the base of the plant (Fernández and López 1964; Realpe *et al.* 2006). These symptoms include yellowing, wilting and drying of the leaves and branches, and finally tree death (Figure 7). Disease development in adult coffee plants can last from months to years and depends on the age of the plants. For example, in coffee nurseries, seedlings died 15 days after inoculation with *R. bunodes,* while 5 month-old-plants took 45 days to die after inoculation (Ibarra *et al.* 1999).

3.1.6. Economic, social and environmental impact.

Coffee in Colombia plays a crucial role for social stability in rural areas, where 95% of farms correspond to smallholders with <2 ha (5 acres) on average (FEDERACAFE 2013). Therefore even a small reduction in coffee yields or a modest increase in

production costs caused by a disease or pest can have a huge impact on producers. Since their first report of *R. bunodes* and *R. pepo* on coffee plants in Colombia (Chardon and Toro 1930, cited by Fernández and López 1964), several authors have reported losses caused by, and the costs of managing these diseases (Fernández and López 1964; Castro and Esquivel 1991; Ibarra *et al.* 1999; Castro and Rivillas 2002; Castro and Serna 2007). For example, Castro and Serna (2007) estimated losses in the province of Quindio (Colombia) to be at least $1 054 266 (Colombian pesos) ha^{-1} in 2006 (ca USD 458). They also estimated the costs of control of patches of 100 coffee plants affected by the pathogen to be $282 900 Colombian pesos (ca USD$ 123).

Rosellinia species represent a serious threat not only for coffee cultivation in Colombia, but also for other forest or fruit trees associated with coffee stands (Castro and Serna 2007). Special concern has been noted due the case of erradication of old coffee plants suggested as a renovation program in Colombia, during the last years (FEDERACAFE 2007). Such is the case of more than 250 foci of *R. bunodes* affecting new coffee planted on farms in a few provinces recorded in 2013 (Cenicafe unpublished). Here, management activities include the isolation of plants/areas with trenches require uprooting trees, soil solarization, fungicide/fumigant applications, stumps burning on the spot (Nitta 2002, cited by Ten Hoopen and Krauss 2006; Aranzazu *et al*. 1999; Gutierrez *et al.* 2006; Ruano and López 2009).

3.1.7. Isolation, culturing and storage of cultures

Rosellinia species can be isolated from infected roots that are covered with strands of white mycelium or from under the bark, by transferring mycelium directly onto potato-dextrose-agar (PDA) or malt extract agar (MEA), amended with antibiotics and thiamin (100 ug /l). The pieces of root containing the fungus must be carefully washed using hypochlorite (1.0%) or soap, rinsed several times with sterile distilled water, and dried well before placement on media (Mendoza 2000; Ten Hoopen *et al.* 2004). Some authors have used trapping techniques (Eguchi *et al.* 2009). Ibarra *et al.* (1999) and Realpe *et al.* (2006) suggest the use of MEA acidified to a pH between 4.5 and 8.5 and incubation at temperatures between 22 to 27°C in the dark to isolate *R. bunodes* and *R. pepo*. Uetake *et al.* (2001), cultured *R. necatrix* on oatmeal agar (OA) at 25°C. Ten Hoopen *et al.*

(2004), recommend storage of *R. necatrix* and *R. pepo* in liquid nitrogen on different substrates.

3.1.8. Methods for inoculation

It is well known that *Rosellinia* spp. need an enriched food base in order to artificially infect plants (Fernández and López 1964). These authors also mention that fungus can lose pathogenicity after being sub-cultured more than twice. Different substrates have been used to multiply *Rosellinia* isolates in order to test their pathogenicity or for fungicide screening and resistance tests. For instance, autoclaved pieces of sweet corn cob and coffee branches were used to grow *R. bunodes* and *R. pepo* to inoculate roots of coffee plants (Fernández and López 1964). Sterilized grains of wheat (*Triticum aestivum*) or sorghum (*Sorghum bicolor*) have also been used to produce inoculum of *R. bunodes* for pathogeniciy tests on coffee and cocoa plants (Esquivel *et al.* 1992; Ibarra *et al.* 1999; García *et al*. 2005). In these cases, the inoculum was added to the soil or placed in contact with the roots. Coffee seedlings died 15-20 days after inoculation and nursery plants after 30 days (Ibarra *et al*. 1999). Similarly Realpe *et al*. (2006) used grains of sorghum to produce *R. pepo* for inoculations on macadamia plants.

3.1.9. Disease management

Management of diseases caused by *Rosellinia* species includes a combination of chemical, cultural, physical and biological approaches. Cultural control measures consist of isolation of diseased plants/areas, the removal and destruction of all plants within the affected area, and eradication of the pathogen by soil solarization. Application of fungicides such as benomyl, fluazinam, thiophanate-metyl and/or biocontrol agents such as mycoparasitic fungi or bacteria, complement the control measures (Freeman *et al.* 1990; Castro 1995; Aranzazu and Botero 1998; Sharma and Sharma 2002b; Ten Hoopen and Krauss 2006; Ruano and López 2009; García-Velasco *et al*. 2012). Some researchers mention control using mycorrhiza (Castro and Rivillas 2002) and others suggest the use of mycoviruses to induce hypovirulence in *R. necatrix* (Lee *et al.* 2003; Sasaki *et al.* 2005; Kanematsu *et al.* 2010).

Very little research has been conducted to find resistance to *Rosellinia.* Resistance to *R. necatrix* has been recorded in some hosts such as apple (*Malus domestica* Borkh), avocado (*Persea americana* Mill.), and olive (*Olea europea*) (Sztejnberg and Madar 1980; Lee *et al*. 2000, cited by Ten Hoopen and Krauss 2006; Barceló *et al*. 2007). In *Musa*, Belalcazar (1991), noticed less susceptibility to *R. pepo* in AAA cultivars than plantains. Wellman (1954), reported some level of resistance to *Rosellinia* in *C. canephora*; however, some accessions of this species, as well as *C. liberica* and Timor Hybrid were affected by *R. bunodes* and *R. pepo* (Castro 2012 umpublished).

3.2. Coffee stem canker caused by the *Ceratocystis fimbriata s. l.* complex

Zimmermann (1900, cited by Pontis 1951) provided the first report of canker caused by *Rostrella coffeae* Zimm. in coffee plants in Java. Later, the disease attributed to *Ceratocystis fimbriata* (Ell. & Halst.) Hunt. was subsequently found in other coffee-growing countries causing stunting of plants and economic losses (Schieber and Echandi 1961; Fernández 1964; Baker *et al*. 2003; Castro *et al*. 2003).

3.2.1. Taxonomy

The genus *Ceratocystis* (Phylum: Ascomycota, Class: Sordariomycetes, Order: Microascales, Familly: Ceratocystidaceae) was first reported causing rot of sweet potato (*Ipomoea batatas* L.) in 1890 in New Jersey, USA and the type species, *C. fimbriata* Halsted & Hunt sensu stricto (s.s), was described by Halsted & Fairchild in 1891. However, the taxonomy of the genus was steeped in controversy with *Ceratocystis* species being confused with a number of other morphologically similar genera, especially species in the Ophiostomatales such as the genus *Ophiostoma* (Upadhyay 1993). With the advent of DNA sequence technologies and phylogenetic analyses the family level classification of *Ceratocystis* species has been clarified (Hausner *et al*. 1992; 1993; Spatafora and Blackwell 1994; Witthuhn *et al*. 1999; Harrington *et al*. 2001; Paulin-Mahadi *et al*. 2002; Hausner 2005; Reblová 2006; De Beer *et al*. 2013; De Beer and Wingfield 2013; Wingfield *et al*. 2013). The genus is now classified in the Ceratocystidaceae and is recognized by phialidic anamorphs (Thielaviopsis-like).

At the genus and species level, *Ceratocystis* is still undergoing considerable study and changes. Based on more refined morphological descriptions, DNA tools, and ecological roles, three species complex are recognized, the *C. coerulescens* complex, *C. fimbriata* complex and the *C. moniliformis* complex. Within these phylogenetic groups, significant work remains to be conducted to clarify species boundaries as contain numerous cryptic species. For example, several species previously referred to as *C. fimbriata*, were recognized as distintic and novel using DNA sequence data species (Barnes *et al.* 2003; Marin *et al.* 2003b; Johnson *et al.* 2005; Van Wyk *et al.* 2006; 2010; 2011; 2013; Marin *et al.* 2005; 2006; Engelbrecht *et al*. 2007; Kamgan *et al.* 2008; Rodas *et al*. 2008; Heath *et al.* 2009; Wingfield *et al*. 2013).

The identity of *Ceratocystis* species causing disease of coffee plants has also been affected by taxonomic changes. The first association of a *Ceratocystis* species with coffee disease can be traced back to 1900 in Java, when Zimmerman described *Rostrella coffeae* Zimm. as the cause of "kanker" (Zimmerman 1900, cited by Pontis 1951). Later, Obregon (1936), cited by Pontis (1951) described symptoms of yellowing, wilting and death of coffee plants in some provinces of Colombia. The external symptoms included violet discoloured tissues at the tree base, leading to the disease being called "ink disease". Pontis (1951), described a similar disease of coffee in Venezuela and Colombia, caused by *Ceratostomella fimbriata* (Ell. & Halst.) Elliott. The disease, also atributed to *Ceratostomella,* was named “llaga macana” by Castaño (1951, 1953). Shieber and Echandi (1961) and Fernández (1964) mention *Ceratocystis fimbriata* (Ell. & Halst.) Hunt., as the causative agent of coffee canker. Recent studies of *Ceratocystis* isolates from Colombian coffee-growing areas (Barnes *et al.* 2001; Marin *et al.* 2003b; Johnson *et al*. 2005)indicated two phylogenetic lineages in the *C. fimbriata* s.l. complex, which were later recognized as distinct species, *C. colombiana* Van Wyk & Wingf. and *C. papillata* Van Wyk & Wingf. (Van Wyk *et al.* 2010).

3.2.2. Morphology

Reproductive structures of *Ceratocystis* species are morphologically similar. Teleomorphs produce ascomata (perithecia) with long necks that terminate in ostiolar hyphae (Figure 8a). The ascomata produce asci that are irregularly distributed, thin-walled and evanescent. Asci produce ascospores that are carried by the ostiolar hyphae

and accumulate in slimy masses at the apices of the ascomatal necks (Hunt 1956; Upadhyay 1981). Asci are consistently evanescent and are generally not seen. Species in the *C. fimbriata* complex have ascospores with hat-shaped sheaths (Figure 8b). The anamorphs of *Ceratocystis* species, traditionally treated as *Chalara* (Corda) Rabenh., are now accommodated in *Thielaviopsis* (Paulin-Mahady *et al.* 2002). It is characterized by two types of conidia produced on *Chalara*-type phialides (Nag Raj and Kendrick 1993). Pigmented chlamydospores with thick walls are also produced in chains at the tips of specialized hyphae (Figure 8c). Conidiophores give rise to both barrel-shaped conidia (Figures 8d and 8e) and cylindrical conidia (Figure 8f) (Paulin-Mahady *et al.* 2002).

Van Wyk *et al.* (2010) described clear morphological differences between *C. colombiana* and *C. papilllata* mainly in the ascomata. *Ceratocystis colombiana* thus has ascomata with a globose base, while *C. papillata* has globose ascomatal bases with a papillate apex.

3.2.3. Geographic distribution and host range

According to the CABI (Commonwealth Agricultural Bureaux International) crop protection compendium lists (www.cabi.org), more than 30 economically important plants are affected by *Ceratocystis* species, mainly species of woody plants on all five continents in temperate, subtropical and tropical zones. On coffee plants, *Ceratocystis* species have been reported from Brazil, (Upadhyay 1981), Colombia (Castaño 1951; Van Wyk *et al*. 2010), Costa Rica (Echandi and Fernández 1962), Cuba (Izquierdo 1988), Guatemala (Schieber and Echandi 1961), India (Bath *et al*. 2002), Indonesia (Waller *et al*. 2007) and Venezuela (Pontis 1951). In Colombia, *Ceratocystis spp.* have been found as saprophytes in soil of all coffee areas, as well as in plants of coffee and other hosts such cocoa (*Theobroma cacao* L.), mandarin (*Citrus reticulata* Blanco), Valencia orange (*Citrus sinensis* Osbeck), Mineola tangelo, lemon (*Citrus limon* Burmman), tambor (*Schizolobium parahyba* (Well.)S.F. Blake) and guanabana (*Annona muricata* L.) (Capera *et al*. 1995; Marin *et al.* 2003b; Van Wyk *et al.* 2010).

3.2.4. Ecology

Oliveira *et al.* (1995) suggested that *C. fimbriata* grows best at temperatures from 18 to 28°C and is able to produce ascospores within a week. They further suggested that the fungus probably survives adverse conditions as mycelium within the plant host, while perithecium and ascospore production is favored by wet weather. In Colombian coffee areas C*eratocystis* species have been found in almost all soils (Castaño 1953; Fernández 1964; Capera *et al*. 1995; Castro and Montoya 1997; Castro 1999; Marin *et al.* 2003b). In the coffee-growing regions of Colombia, the soils are volcanic, sandy, stony and clayey, with pHs between 5.0 to 6.0 and variable organic matter. Temperatures range from 18 to 25°C, rainfall varies from 1 500 to 3 500 mm yr^{-1} and there are 1 400 to 2 400 hours of sunshine (Gómez *et al*. 1991).

3.2.5. Disease, symptoms and spread

Ceratocystis species cause stem cankers on coffee, characterized by hardening of the phloem tissues (Figure 9). This is the origin of the disease's common name, “macana” meaning hard wood (Castaño 1951; Pontis 1951). Adult plants affected by *C. fimbriata s.l.* are randomly scattered in orchards. The first symptoms are chlorosis of the foliage, followed by wilting of leaves, die-back and death of the trees (Castaño 1951; 1953; Fernández 1964; Castro 1999). Vascular streaking and dark brown-coloured lesions sometimes tinged with purple/red are evident beneath the bark above and below the infection site. In advanced stages, the lesions girdle trunks and killi plants. When infection occurs on coffee trees that have been “renovated” by pruning ("zoqueo"), the new sprouts are affected and die (Figure 10).

Wounds, made by anthropogenic activities, insects and other agents are important infection courts for *Ceratocystis* species (Malloch and Blackwell 1993). In Colombian coffee areas, the principal wounds made by farmers's shoes, lead to basal cankers on stems (Castro 1999), whereas prunning ("zoqueo") and other cultivation practices are secondary importance. Water splash can diseminated pathogen-infested soil to wounds (Castaño 1953; Castro and Montoya 1997; Castro *et al*. 2003). Although insects are thought not to be involved (Castaño 1953), this factor deserves further study.

3.2.6. Economic, social and environmental impact.

The disease can cause losses from 20 to 40% mostly in slope zones and stem pruning ("zoqueo") (Castro 1999). In the Colombian Central area in 2002, Castro *et al*. (2003) reported losses of $ 495 000 (Colombian pesos) $ha^{-1}year^{-1}$ due the loss of 10 to 20% of adult coffee plants ha^{-1}.

Fungicides are used to prevent the disease during the renovation by "zoca" (Castro and Montoya 1997; Castro and Zuluaga 2012). They are sprayed with conventional equipment and cause environmental pollution and negative impacts on human health.

3.2.7. Isolation, culturing and storage of cultures

Pieces of de-barked young cofee stems can be buried in moist soil for 8-10 days as *Ceratocystis* baits (Castro 1994). Alternative, symptomatic material can be incubated in humid chambers (Fernández 1964), or placed between discs of freshly cut carrot, under conditions of high humidity, to stimulate perithecial production (Moller and De Vay 1968a). Resultant masses of ascospores can be placed onto the surface of media such as 1-2% malt extract agar (Seifert *et al.* 1993), oatmeal agar, or V8-juice agar amended with thiamine (100 ug /l) and antibiotics (100mg /l of tetracycline; 200 mg/l of choramphenicol and 200 mg /l of penicillin) (Marin *et al.* 2003b). Petri dishes are then incubated at room temperature (20-25°C) with or without light. Lyophilized sporulating cultures have been viable for 20-40 years, when stored in a cool, dark place, and other measures have available for storage at -20°C or in liquid nitrogen (Seifert *et al.* 1993).

3.2.8. Methods for inoculation

Different methods of inoculation have been used to evaluate pathogenicity, chemical control measures and genetic resistance in coffee. Seedlings or adult trees can be inoculated by placing *Ceratocystis* isolates in contact with freshly wounded roots, trunks or shoots. Wounds can be made by removing the bark with a cork borer or a knife. The inoculum can be a plug of medium bearing the fungus (Pontis 1951; Castaño 1953; Fernández 1964; Marin *et al*. 2003b; Van Wyk *et al*. 2010), or a suspension of ascospores

(Schreiber and Stipes 1966; Castro 1994; Marin *et al.* 2003b; Castro and Cortina 2009). For screening fungicides and biological control treatments, a suspension of ascospores has been placed directly onto fresh pruned coffee plants ("zocas") (Castro and Montoya 1994; Castro and Rivillas 2003; Castro and Zuluaga 2012). Castro (1994) suggested growing *C. fimbriata* on pieces of young coffee stem without bark in humid chambers to induce the formation of perithecia. These could then be used to prepare ascopore suspensions, which are placed in sterilized water, adding Triton X- 100 (0,01ml/l) and sonicated for 1 minute. After that, different concentrations of ascospores are prepared using a neubauer chamber for inoculations. After inoculation, the wounds should be wrapped with humid chamber and Parafilm® to maintain moisture and to reduce the chances of contamination (Castro and Cortina 2009; Mirzae *et al.* 2009). Between 8-10 days after inoculation, black, carbon-like structures develop followed by darkly- stained lesions (Fernández 1964; Castro and Cortina 2009).

Ceratocystis isolates vary from weakly pathogenic to very aggressive on coffee seedlings (Castaño 1953; Fernández 1964; Marin *et al*. 2003b). To preserve their pathogenicity, Fernández (1964) sugested maintaining the fungus on pieces of young coffee stem without bark.

3.2.9. Disease management

Cultural and sanitary measures are effective for disease control in some cases. Disinfecting machetes and pruning tools between can help to prevent infection after “zoqueo” (Castro and Montoya 1994). Gómez and Castro (2004) developed a “contact applicator” device to apply preventive fungicides onto coffee stumps. Recently Castro and Zuluaga (2012) identified synthetic anticorrosion paints that enhance fungicide persistence after "zoqueo". Castro and Rivillas (2003) also mention the application of *Trichoderma* spp. to prevent infection by *C. fimbriata* s.l. in coffee plants.

In *C. arabica,* genetic resistance in one line of var. Borbón has been reported (Fernández 1964). There are also reports of ‘immunity” in some lines of *C. canephora* and *C. liberica* (Echandi and Fernández 1962; Izquierdo 1988). Resistance to infection by *Ceratocystis* species is usually characterized by small areas of xylem discoloration and

formation of callus and lignified tissues that contains lesions (Fernández 1964; Castro and Cortina 2009).

4. CONCLUSIONS

Two coffee species, *C. arabica* (70%) and *C. canephora* (30%) are responsible for virtually all of the consumed beverage. Brazil has been the largest coffee producer in the world for many decades. Vietnam is currently the second largest producer, followed by Indonesia and Colombia. Major declines in coffee export earnings would have serious economic and political repercussions for these countries.

Among the diseases that negatively affect coffee production, coffee leaf rust caused by *H. vastatrix* is the most important. Other important diseases prevalent in Colombian coffee growing areas are caused by the soil borne fungi *Rosellinia* species and *Ce. fimbriata s.l.* complex. They have attracted attention in recent years, but relatively little is known about them in Colombia. Thus, the aim of the studies presented in this thesis are to increase the body of knowledge regarding these pathogens.

The studies presented in this thesis are generally of a practical nature and thus focus mostly on opportunities to reduce losses due to disease. Some attention is given to the identification of the pathogens, specifically in the case of *Rosellinia*. Overall, the best available options to reduce losses lie in the development and use disease-tolerant plants. In this regard, substantial attention has been given to *H. vastatrix*, but less work has been done on Ceratocystis canker and Rosellinia root rot. A core aim of studies presented in this thesis has been to rectify this situation and synthesize knowledge on the three diseases and the genetic opportunities to reduce losses that they cause.

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Figure 1. Distribution of *Coffea* species in three biogeographic zones: Guineo-Congolese (in blue), East Africa (in red) and Malgache Floristic region (in green) (Anthony *et al.* 1999).

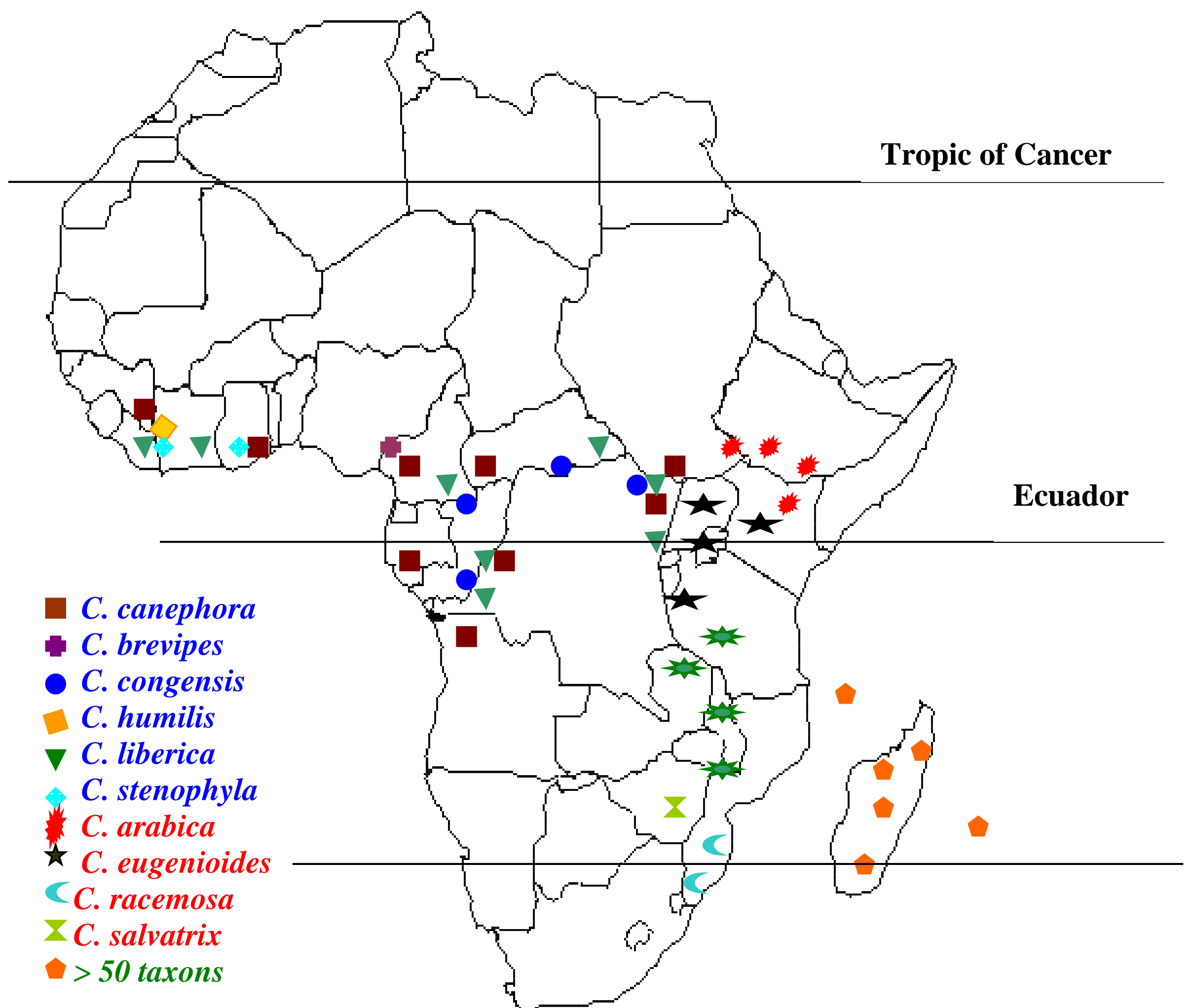

Tropic of Cancer
Ecuador
C. canephora
C. brevipes
C. congensis
C. humilis
C. liberica
C. stenophyla
C. arabica
C. eugenioides
C. racemosa
C. salvatrix
> 50 taxons

Figure 2. Adult plants of: (a) *Coffea arabica,* (b) *Coffea canephora,* (c) *Coffea liberica,* and (d) Timor Hybrid.

a

b

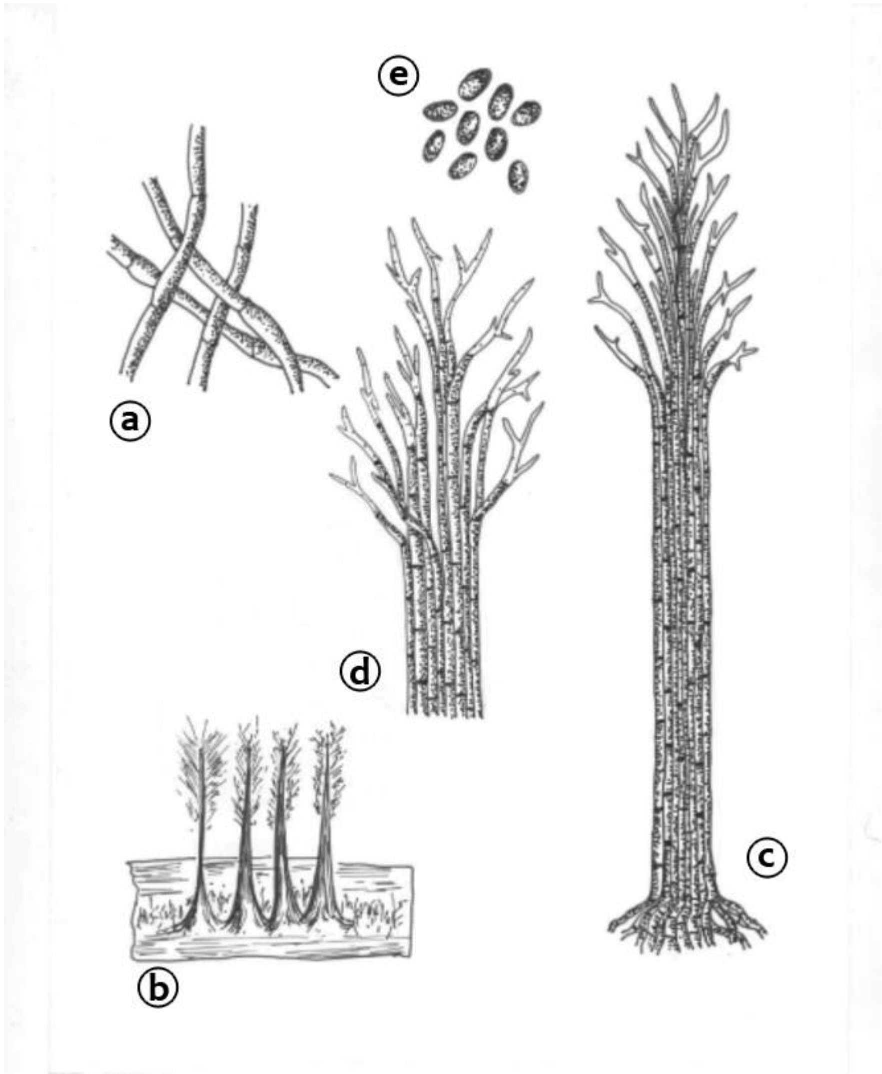
c

d

Figure 3. Coffee-growing areas (yellow) in Colombia (FEDERACAFE 2013).

COLOMBIA
Zona Cafetera
N
78°
72°
11°50'
1°
LA GUAJIRA
ATLANTICO
MAGDALENA
CESAR
N. DE SDER.
SUCRE
CORDOBA
BOLIVAR
ANTIOQUIA
SANTANDER
ARAUCA
CHOCO
BOYACA
CASANARE
CALDAS
CUNDINAMARCA
VICHADA
QUINDIO
TOLIMA
META
VALLE
CAUCA
GUAINIA
HUILA
GUAVIARE
NARIÑO
PUTUMAYO
CAQUETA
VAUPES
AMAZONAS
1 : 8000000

Figure 4. Morphological characteristics of the anamorphs of *Rosselinia* species: (a) mycelium with pear-shaped septal union, (b) synnemata of the *Graphium*-like state, (c) conidiophores of the synnematal state, and (e) hyaline ameroconidia (drawings by Juliana Restrepo from the reproduced by Saccas 1956).


e
a
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c

Figure 5. Morphology of teleomorphs of *Rosellinia* species: (a) stromata, (b) stroma – perithecium of *R. bunodes,* (c) stromata– perithecium of *R. pepo*, (d) asci and paraphyses of *R. bunodes,* (e) ascospores of *R. bunodes,* (f) asci and paraphyses of *R. pepo,* and (g) Ascospores of *R. pepo*. (drawings by Juliana Restrepo from the reproduced by Saccas 1956).

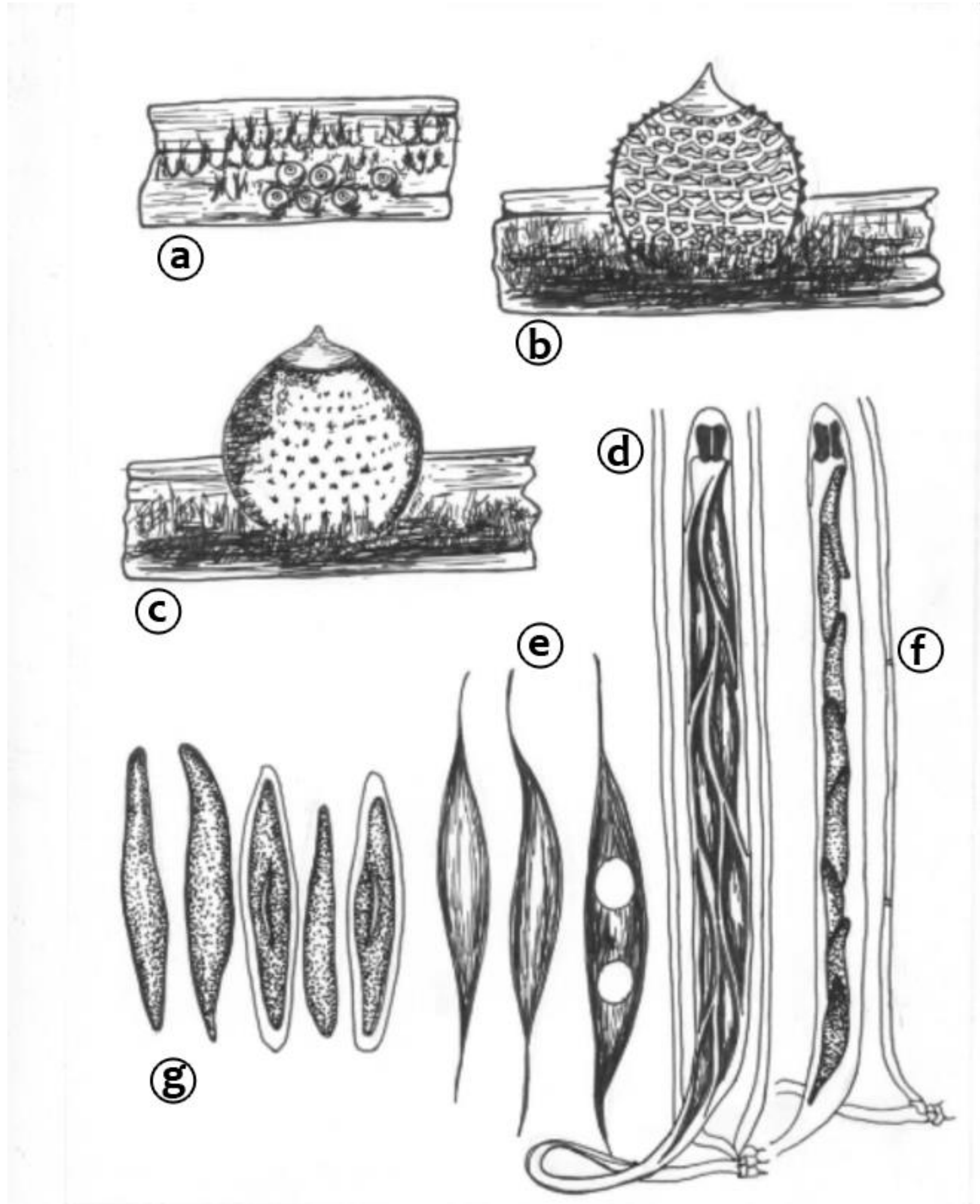
a
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Figure 6. Signs and symptoms of coffee disease caused by *Rosellinia* species: (a) *R. bunodes* root infection visible as black specks or streaks under the bark and embedded in root tissues, (b) white, fan shaped mycelium of *R. pepo* under the bark of a coffee root.

a

b

Figure 7. Wilting and death of 12-month-old plants of *Coffea arabica* var. Castillo®, caused by *Rosellinia bunodes*.

Figure 8. Morphological features of *Ceratocystis fimbriata* (*s.l.*): (a) perithecium with long neck terminating in ostiolar hyphae, (b) hat-shaped ascospores, (c) chlamydospores, (d) barrel-shaped conidia, (e) conidiophore, (f) cylindrical conidia, and (g) mycelium (drawings by Juliana Restrepo).

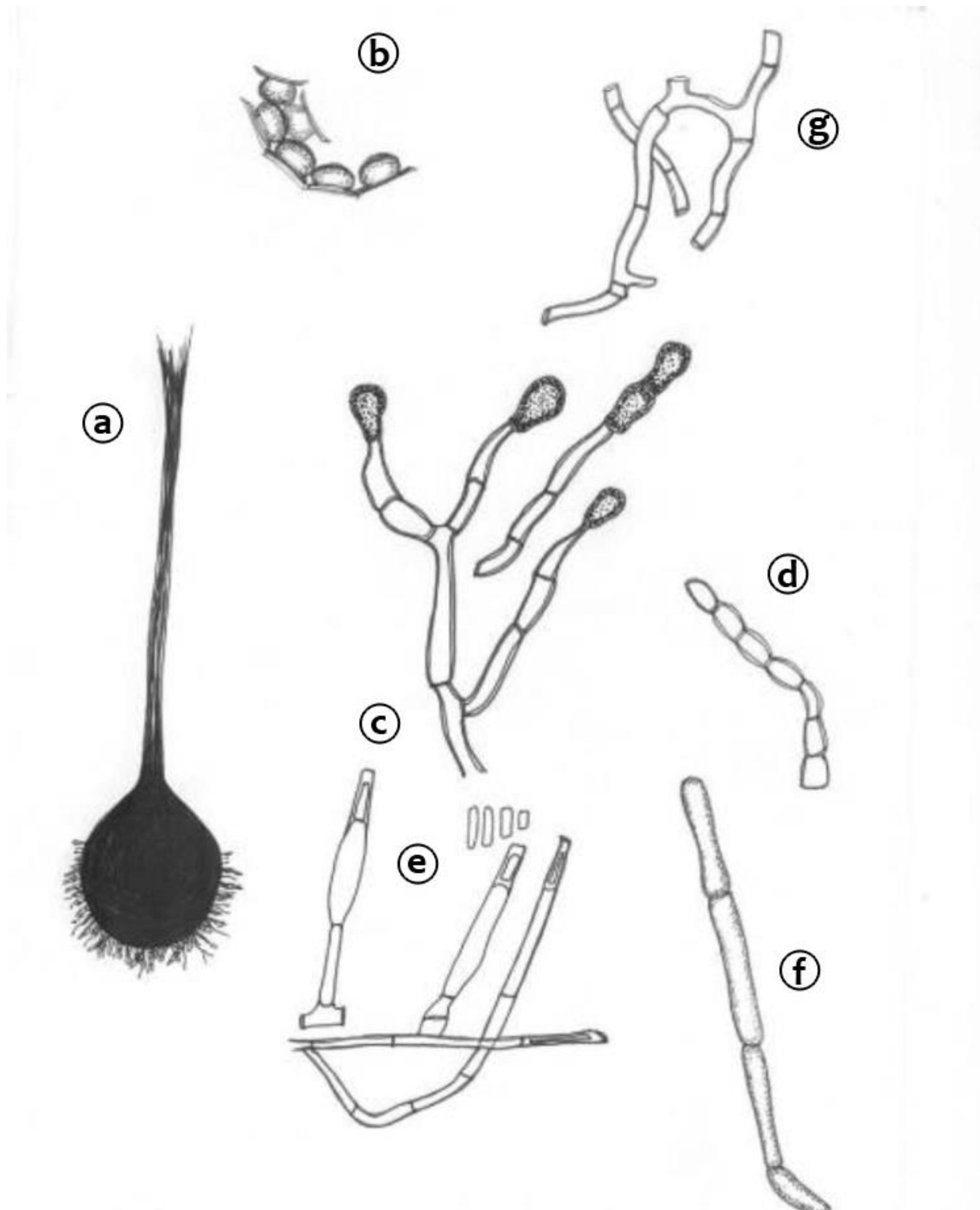
a
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Figure 9. Stem canker of coffee caused by *Ceratocystis fimbriata s.l.*

Figure 10. Dry branches and dead new sprout on coffee plant affected by "llaga macana ", caused by *Ceratocystis fimbriata s. l.*

Table 1. Diseases of cultivated *Coffea* species, their causal agents and geographical distribution.

Disease and pathogen(s)	Plant part(s) affected	Geographic distribution(s)	References
CAUSED BY FUNGI			
Anthracnose *Colletotrichum* spp.	Branches, leaves and floral buds	Colombia, Vietnam	Gil 2001
Américan Leaf spot *Mycena citricolor* (Berk. & Curt.) Sacc	Leaves and berries	Central and South América	Wang and Avelino 1999; Waller *et al*. 2007
Berry Blotch or Iron Spot *Cercospora coffeicola* Berk & Cooke *Mycosphaerella coffeicola* (Cooke) J.A. Stev. & Wellman.	Leaves and berries	All coffee countries	Waller *et al.* 2007
Black root rot *Rosellinia bunodes* (Berk et Br.) Sacc.	Root	Caribbean Island; Central and South America; Southeast Asia Ubangui-Chari (Central African Republic)	Saccas 1956; Fernández and López 1964; Sivanesan and Holiday 1972
Black rot *Ceratobasidium anceps (*Bres & Syd.*);* *Corticium koleroga (*Cooke) *Höhn; Koleroga noxia* Donk (Bres&Syd) H.S. Jacks and *Pellicularia koleroga* Cooke	Leaves, berries and young shoots	Central and South America, India	Waller *et al.* 2007
Brown root, Stump rot *Phellinus noxius* (Corner) G. Cunn. (Previously *Phomes noxius* Corner)	Roots	India	Kannan 1995
Coffee berry disease (CBD) *Colletotrichum kahawae* J.M. Waller & P.D. Bri	Flowers, leaves, unopened Inflorescences and ripe berries	Cameroon, Ethiopia, Kenya, Malawi, Tanzania, Uganda, Zaire Zimbabwe,	Agwanda *et al*. 1997; Bridge *et al.* 2008

Coffee leaf rust (CLR) *Hemileia vastatrix* Berk. & Broome	Leaves	All coffee producing countries	Kushalappa and Eskes 1989; Waller *et al*. 2007
Coffee powdery rust *Hemileia coffeicola* Maublanc & Roger	Leaves	Cameroon, Congo, Cote d' Ivore, Nigeria, Togo and Uganda	Gil 2003; Waller *et al.* 2007
Damping- Off *Rhizoctonia solani* Kühn	Seedlings Hypocotyls	All coffee producing countries	Gaitán and Leguizamón 1992
Dieback *Phoma costaricensis* Echandi *Phoma tarda* (R.B. Tewart) H. Verm.	Growing tissue, meristematic buds, branches and leaves	Central and South América, India.	Gómez and Bustamante 1977; Kannan 1995; Gil and Leguizamón 2000
Fusarium Bark Disease (FBD) *Fusarium lateritium* Nees var *longum* Wollenw	Vascular tissues	Kenya, Madagascar, Malawi, Tanzania	Waller *et al.* 2007
Greasy spot or oil spot *Colletotrichum gloeosporioides* (Penz) Pens & Sacc.	Leaves and berries	Colombia, Costa Rica, and Brazil	Leguizamón and Baeza 1973; Ferreira *et al.* 2010
Leaf-stem spot *Myrothecium roridum* Tode	Leaf Stem	Colombia, Costa Rica, Brazil, Guatemala, India	Urhan 1951; Schieber and Zentmayer 1968; Silveira *et al*. 2007; Bhat *et al*. 2011
Pink disease *Phanerochaete salmoniclor* (Berk & Br.) Julich	Leaves, berries, shoots and main stem.	Central and South América	Galvis 2003; Waller 2007
Root rot *Rosellinia arcuata* Petch.	Root	India; Sri Lanka, and Ubangui Chari (Central African Republic)	Saccas 1956; Kannan 1995; Sivanesan and Holliday 1972

Black root rot *Rosellinia bunodes* (Berk et Br.) Sacc.	Root	Caribbean Island; Central and South América; Ubangui-Chari; Southeast Asia	Saccas 1956; Fernández and López 1964; Sivanesan and Holiday 1972
Star root rot *Rosellinia pepo* Pat**.**	Root	Caribbean Island; Central and South América; Ubangui-Chari; Southeast Asia	Saccas 1956:Fernández and López 1964; Sivanesan and Holiday 1972
Root rot *Armillaria mellea* (Vahl) P. umm	Roots	Kenya and Uganda	Wallet *et al*. 2007
Sooty mold *Capnodium* and *Fumago*	Leaves and berries	Colombia, Guatemala	Hernández 1967; Cárdenas 1985
Stem canker stain *Ceratocystis fimbriata* Ellis & Halst. *sensu lato* *Ceratocystis colombiana* M. van Wyk & M.J. Wingf., *Ceratocystis papillata*, Van Wyk & M.J. Wingf.	Trunk	Caribbean, Central and South América, India Colombia	Pontis 1951 Bhat *et al*. 2002 Fernández 1964; Van Wyk *et al.* 2010
Vascular wilt *Fusarium solani* (Mart.) Saac. var *minus* Wollenw.	Seedlings	India	Govindarajan and Subramanian 1968
Wilt disease or Tracheomycosis *Gibberella xylarioides* R. Heim & Saccas. (*Fusarium xylarioides* Steyaert*)*	Vascular tissues	D.R. of Congo, Ethiopia, Ivory Coast, Puerto Rico, Tanzania, Uganda	Adugna *et al.* 2005 (Cited by Waller *et al*. 2007); Lepoint *et al.* 2005; Geiser *et al.* 2005; Waller *et al.* 2007
Wilt, Santavery root *Fusarium javanicum* Koord	Roots	India	Muthappa 1977
CAUSED BY BACTERIA			

Atrophy of branches			
Xylella fastidiosa Wells, Raju, Hung, Weisburg, Mandelco-Paul & Brenner.	Branches, leaves	Costa Rica, Brazil	Montero *et al.* 2007; Zambolim *et al.* 2005
Halo blight			
Pseudomonas syringae van Hall pv. *garcae* (Amaral, Texeira and Pinheiro) Young, Due & Wilkie	Leaves, branches, flowers and young fruits	Brazil, Kenya	Kairu 1997
Leaf blight			
Pseudomonas cichorii (Swingle) Stapp	Leaves	Brazil	Oliveira *et al*. 1991
CAUSED BY NEMATODES			
Corky-root			
Meloidogyne spp	Roots	Caribean, Central and South America	Bertrand *et al*. 2000
Root-lesion			
Pratylenchus coffeae (Zimmerman) Filipjev & Schuurmans Steckhoven.	Roots	Africa, Central, Caribbean and South América, and Southeast Asia,	Waller *et al.* 2007
CAUSED BY VIRUS			
Coffee ringspot (CoRSV)			
Coffeee ringspot virus	Leaves, berries and twigs	Colombia, Costa Rica, Brazil	Ocampo *et al*. 2002; Chagas *et al.* 2003; Kitajima and Chagas 2011
CAUSED BY PHYTOPLASMS			
Coffee crispiness			
Phytoplasm X- disease group**.**	Leaves and floral buds	Colombia	Galvis *et al.* 2007
CAUSED BY PHYTOMONAS			
Phloem necrosis			
Phytomonas	Phloem	Surinam, Guyana, Trinidad, Brazil	Dollet 1984
CAUSED BY ALGA			
Algaceous spot			
Cephaleuros virescens Kunze	Leaves, twigs and branches	Colombia, India and Indonesia	Wellman 1965; Cadena 1982

Table 2. Geographic and host range of *Rosellinia* species causing important diseases

Country/region	Hosts affected	*Rosellinia* sp.	References
ASIA			
India	*Malus domestica*	*Dematophora necatrix*	Sharma *et al.* 2005
	Coffea arabica	*R. arcuata*	Kannan 1995
	C. arabica, C. canephora	*R. bunodes*	Muthappa 1977
Indonesia	*Citrus spp. Coffea spp., Leucaena glauca*	*R. bunodes*	Waterston 1941
Japan	*Lupinus luteus, Malus prunifolia*	*R. compacta, R. necatrix*	Uetake *et al.* 2001; Takemoto *et al.* 2009b
	Vitis vinifera, Pyrus betulifolia, Malus spp.	*R. necatrix*	Sasaki *et al.*2005; Eguchi *et al.* 2009
Malasia	*Citrus* spp.	*R. bunodes*	Waterston 1941
Sri Lanka	*Citrus* spp., *Hevea brasiliensis*	*R. bunodes*	CABI 2008
Taiwan	*Camellia sinensis, Hevea brasiliensis, Serissa japonica*	*R. necatrix*	CABI 2008; Sun *et al.* 2008
ÁFRICA			
Kenya	*Coffea* spp., dicotiledonea wood, *Pinus patula*	*R. coffea , R. arcuata, R. necatrix, , R bunodes, R. pepo*	Saccas 1956,
Tanzania	*Pinus patula*	*R. necatrix*	CABI 2008
Other countries in French Equatorial África	*Coffea spp , Elaes guinensis, Hevea brasiliensis, Saccharum officinarum*	*Rosellinia* spp.	Saccas 1956
EUROPE			
England	*Buxus semipervirens*	*R. buxi*	Petrini 1993
	Picea orientalis	*R. minor*	
	Salix repens	*R. desmazieresii*	Ofong *et al.* 1991
France and Germany	*Eucalyptus sp., Ilex aquifolium, Salix sp. Salvia officinalis, Buxus sempervirens*	*R. aquila, R. buxi*	Petrini 1993, CABI 2008
	Vitis vinifera	*R. necatrix*	Tourveille 1986 (cited by Ten Hoopen and Krauss 2006)
Greece	*Buxus semipervirens*	*R. buxi*	Petrini 1993

	Ficus carica	*R. necatrix*	Vagelas *et al.* 2009
Portugal	*Ficus carica, Jasminum sambac, Malus spp., Populus fastigiata, Prunus avium,Prunus persica, Prunus spp., Vitis vinifera*		Teixeira *et al*. 1995
Spain	*Camellia japonica, Cyperus esculentus Olea europaea, Persea américana*	*R. necatrix*	Pérez *et al.* 2002; Ruano *et al*. 2007; López and Ruano 2008; Pliego *et al*. 2012; Armengol *et al*. 2010
Some other countries	*Corylus avellana, Morus alba, Picea abies, Polpulus pyramidalis, Prunus padus, Quercus sp., Sambucus sp.,, Salix sp., Quercus sp., Vitis vinifera*	*R. necatrix,*	Petrini 1993
	Alnus glutinosa, Fagus sylvatica, Lenzites betulina, Picea abies, Quercus robur, Salix repens.	*R. desmazieresii*	Petrini 1993
MIDDLE EAST			
Israel	*Coffea sp., Citrus aurantium, C.macrophilla, Eriobotrya japonica, Macadamia aurantifolia, Mangifera indica, Olea europaea, Persea Américana, Prunus armeniaca, P. persica, P. amigdalus, Punica granatum, Vitis vinifera.*	*Dematophora necatrix*	Sztejnberg and Madar 1980
NORTH AND CENTRAL AMÉRICA			
Costa Rica	*Alnus acuminata, Coffea arabica, Rosa sp., Theobroma cacao*	*R. bunodes, R. pepo*	Bermúdez and Carranza 1990 Wellman 1953; Bautista and Salazar 2000; García *et al.* 2005
Cuba	*Coffeasp. Citrus sp., Theobroma cacao*	*R. pepo, Dematophora necatrix, R. bunodes*	Arnold 1986; Herrera 1989
El Salvador	*Coffea spp*	*R. bunodes*	Alvarez 1953
Guadalupe	*C. arabica, C. excelsa, C.canephora*	*R. cofeae*	Saccas 1956

Guatemala	*C. arabica*	*Rosellinia sp.*	Hernández 1967
Jamaica	*C. arabica, Bixa orellana, Maranta arundinaceae, Theobroma cacao, Zingiber officinale*	*R. bunodes*	Waterston 1941
México	*Citrus spp., Coffea arabica, Hevea brasiliensis, Quercus spp.,*	*R. bunodes, R. aquila*	Watertson 1941; San Martín and Rogers 1995
	Rosa sp.	*R. necatrix*	García-Velasco *et al*. 2012
Puerto Rico	*Acalypha wilkesiana, Coffea arabica*, *Didymopanax morototoni, Eugenia* sp, *Hibiscus rosae, Inga fragifolia, Inga vera, Jazminum grandiflorum, Manihot utilissima,*	*R. bunodes, Rosellinia sp*	García 1945; CABI 2008
Rep. Dominicana	*Theobroma cacao*	*R. pepo*	Mendoza 2000
USA	*Acer pseudoplatanus, Eucalyptus sp., Ilex aquifolium, Salix sp. Salvia officinalis.*	*R. aquila*	Petrini 1993; CABI 2008
	Corylus avellana, Gleditschia triacanthos, Philadelphus coronarius, Polpulus pyramidalis, Pyrus malus, Quercus, Sambucus sp. Vitis vinifera	*R. necatrix*	CABI 2008
OCEANÍA			
Australia	*Acacia spp., Calamus spp., Cyclamen, Eucalyptus sp., Narcissus sp., Prunus dulcis, Ribes rose, Salix sp.*	*R. necatrix* *R. capetribulensis*	Fox 1999 Bahl *et al*. 2005
New Zealand	*Mallus domestica*	*R. radiciperda*	Petrini 2005
SOUTH AMERICA			
Argentina	*Alnus acuminata, Citrus limon*, *Humulus lupulus*, *Juglans regia*, *Ligustrum lucidum, Malus domestica, Melia azedarach*, *Olea europae*, *Persea americana*, *Pinus*	*R. necatrix*	Sir *et al*. 2012

	sp. Pirus comunis, Platanus acerifolia, *Populus, Prunus avium, P. dulcis, P. persica*, *Salix* sp., *Trifolium* sp., *Vitis vinifera*		
Brazil	*Coffea arabica, Thea sinesis, Theobroma cacao, Hevea brasiliensis,*	*Rosellinia pp., R. bunodes, R. necatrix, R. pepo* *R. pepo*	López 1992; Carvalho and Chalfoun 1998 López *et al*. 2006; Oliveira *et al*. 2008
	Syzygium aromaticum, Euterpe olaraceae, Myristica fragans		
Chile	*Citrus limonia, C. sinensis, Juglans regia, Malus pumila, Prunus avium, Pyrus communis, Vitis vinifera, Paeonia suffriticosa, Vitis vinifera*	*R. necatrix*	Mujica *et al*. 1980; Gilchrist *et al*. 2010
	Chusquea quila	*R. chusqueae*	Petrini 1993
Colombia	*Daucus carota, Morus indica,*	*Rosellinia sp*	Guerrero 1990; Aranzazu and Botero1998
	Solanum tuberosum	*Roselliniasp.*	López *et al.* 2004
	Coffeea arabica	*R. pepo, R. bunodes*	Fernández and López 1964; López *et. al* 2004, Castro and Serna 2009
	Theobroma cacao	*R.pepo*	Merchán 1988; Aranzazu 1996
	Macadamia integrifolia;	*R. pepo*	Realpe *et al*. 2006
	Citrus sp., Guajava spp., Inga sp. Musa sp. Manihot esculenta, Persea américana,	*R. pepo,* *R. bunodes*	Castro and Serna 2009
Ecuador	*Solanum tuberosum*	*Rosellinia* sp	Orellana 1978 (cited by Torres 2002)
	Chusquea quila	*R. chusqueae*	Petrini 1993
Paraguay	*Theobroma cacao*	*R. paraguayensis*	Waterston 1941

Chapter 2

Identification and genetic diversity of *Rosellinia* spp. associated with root rot of coffee in Colombia

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ABSTRACT.

The genus *Rosellinia* includes species that cause root rot on a wide range of herbaceous and woody hosts. In Colombia, these fungi cause serious diseases of potato, forest and fruit trees, as well as coffee plants. The aim of this study was to identify isolates of *Rosellinia* collected from coffee and other hosts using DNA sequence comparisons of the internal transcribed spacer (ITS) region. Pathogenicity tests were conducted on coffee seedlings to confirm the role of the collected species in coffee root disease. Twenty six isolates were obtained and these were grouped into two clades representing *R. bunodes* and *R. pepo*. Isolates from *Coffea arabica*, *Hevea brasiliensis, Macadamia integrifolia, Psidium guajava* and *Theobroma cacao* were identified as *R. pepo,* while *R. bunodes* was obtained only from coffee plants. Low levels of genetic variability were observed among isolates of the two species. Pathogenicity tests on coffee with *R. bunodes* resulted in 98% seedling death in an average of 10 days, while *R. pepo* killed 54% of inoculated seedlings in an average of 16 days confirming the compatibility of both species with this host. Pathogen characterization will be useful for further research in disease diagnosis, soil recovery and breeding for resistance.

1. INTRODUCTION

Based on symptoms and morphological characteristics, two species of *Rosellinia* are known in Colombian coffee growing areas, *Rosellinia bunodes* (Berk & Brome) Sacc., which causes a disease known as black root rot, and *R. pepo* Pat. caussing stellate root rot (Fernández and López 1964; Castro and Esquivel 1991). Other than in Colombia, these pathogens are known to affect coffee in Africa (Saccas 1956), Brazil (Ponte 1996), Costa Rica (Bautista and Salazar 2000), Cuba (Herrera 1989), El Salvador (Procafé 1996), Guatemala (Hernández 1967) and Puerto Rico (Garcia 1945), while *R. arcuata* Petch, and/or *R. bunodes* are mentioned infecting coffee in India (Sivanesan and Holliday 1972; Muthappa 1977; Kannan 1995).

Many *Rosellinia* spp. are saprophytes, some live endophytically and occasionally become pathogenic, and some species are well-known root pathogens on commercially grown plants such as potato (Guerrero 1990) and woody perennial trees in tropical and sub-tropical areas globally (Petrini and Petrini 2005; Ten Hoopen and Krauss 2006). Among the best known root pathogens are *R. necatrix* Berl.: Prill and *R. desmazieresii* (Berk. & Br.) Sacc. (= *R. quercina* Hart), mostly known from temperate climates causing diseases on pear, apple and grape in Japan (Eguchi *et al.* 2009; Takemoto *et al.* 2009*a*, 2009*b*), on avocado in Spain (López-Herrera 1998; Pliego *et al*. 2012) and in Argentina on peach, plum, apple, pear, grapevine and other hosts (Sarasola and Sarasola 1975). *Rosellinia bunodes, R. pepo* and *R. arcuata*, are known only from the tropics (Kannan 1995; Ten Hoopen and Krauss 2006). Various other *Rosellinia* spp. that infect coffee were mentioned by Saccas (1956), e.g. *R. coffeae* Sacc., *R. didolotii* Sacc, *R. echinocarpa* Sacc, *R. lobayensis* Sacc., *R. mastoidiformis* Sacc. and *R. megalospora* Sacc., but very little is known about these species.

Rosellinia bunodes and *R. pepo* occur in the soil as saprophytes (Aranzazu 1996). After infection of suitable living hosts, patches of dying plants extend in a circular pattern due to the pathogen´s spread through root contact or via mycelial aggregations (Fernández and López 1964; Merchán 1988; Aranzazu 1996; Bautista and Salazar 2000). Many shade and fruit trees grown in association with coffee (e.g. *Inga* sp., *Leucaena* sp., *Erytrina* sp., *Cordia alliodora* (Ruiz & Pav.) Oken, *Tabebuia rosea* DC, *Cedrela odorata* L., *Alnus acuminata* Kunth) are susceptible to infection by *Rosellinia* spp. and

are thought to provide initial sources of inoculum for coffee tree infection (Bermúdez and Carranza 1990; Aranzazu 1996; Castro and Serna 2009). In addition, debris of cassava (*Manihot esculenta* Crantz) left in the soil after co-cultivation with coffee has been mentioned as increasing the survival of the pathogen and thus damage due to subsequent *Rosellinia* infection in Colombian coffee growing areas (Castro and Serna 2009).

Infection of coffee plants by *Rosellinia* spp. results in chlorosis, wilt, die-back and death of plants. This may occur within a few weeks in the case of seedlings or young plants in the field or take up to three or four years after infection in the case of adult plants (Fernández and López 1964; Castro and Esquivel 1991; Ibarra *et al*. 1999). Important economic losses have been recorded by Castro and Serna (2009) in Colombian coffee growing areas. The diseases caused by *Rosellinia* spp. are also known to be difficult to control and numerous integrated measures have been investigated, with variable results (Merchán 1988; Ten Hoopen and Krauss 2006; Gutiérrez *et al*. 2006). Barceló *et al*. (2007), implemented a program aimed at selecting avocado rootstocks tolerant to white rot caused by *R. necatrix* in Spain. However, little research on resistance to tropical *Rosellinia* spp. has been published (Ten Hoopen and Krauss 2006).

The genus *Rosellinia* belongs to the family Xylariaceae (Class Euascomycetes, subclass Pyrenomycetes, order Sphaeriales, syn. Xylariales) and includes more than one hundred species (Pliego *et al*. 2012). Teleomorphic structures, such as ascospore morphology, are considered valuable taxonomic characters for the identification of *Rosellinia* spp. (Pérez-Jiménez *et al*. 2003; Petrini and Petrini 2005; Takemoto *et al*. 2009a, 2009b; Pliego *et al*. 2012). However, in tropical areas, stromata bearing fruiting bodies are rarely found in nature, making the identification of *Rosellinia* spp. reliant on characteristics of the anamorph (*Dematophora*) (Fernández and López 1964; Bermúdez and Carranza 1992; Ibarra *et al*. 1999; Realpe *et al*. 2006). A major diagnostic character at the generic level has been the presence of pear-shaped swellings at the septa of the hyphae (Saccas 1956; Sarasola and Sarasola 1975; Pérez-Jiménez 2006; Pliego *et al*. 2012). At the species level, *R. pepo* and *R. bunodes* have been distinguished based on the mycelial aggregates formed on the roots. *R. pepo* produces grayish cobweb-like strands, which become black and coalesce into a woolly mass. Beneath the bark, white, star-like fans can be observed macroscopically. *Rosellinia bunodes* shows black

branching strands firmly attached to the roots, forming black dots and lines embedded in the tissues (Waterston 1941; Fernández and López 1964; Ibarra *et al*. 1999; Realpe *et al*. 2006).

Rosellinia species have mostly been characterized based only on morphology (Petrini and Petrini 2005), with only limited DNA sequence data available for species in the genus. However, in the last decade, molecular tools have provided important means to elucidate genetic variation and phylogenetic relationships among global members of the Family Xylariaceae (Bahl *et al*. 2005; Peláez *et al*. 2008; Hsieh *et al*. 2010; Pliego *et al*. 2012). Sequencing of the internal transcribed spacer regions (ITS), fragments of the β-tubulin (BT), adenosine triphosphatase (ATP) and translation elongation factor 1α (TEF) gene regions and random amplified polymorphic DNA (RAPD) amplifications have mostly been used for identification of *R. necatrix* (López *et al*. 2008; Takemoto *et al*. 2009a, 2009b). ITS Scorpio primer pairs have been successfully developed for large-scale detection of *R. necatrix* by real-time Scorpion- polymerase chain reaction (PCR) in soils and in plant materials (Schena and Ippolito 2003; Ruano-Rosa *et al*. 2007), and recently Takemoto *et al*. (2011) developed a species-specific PCR diagnostic for *R. necatrix* and *R. compacta* Takemoto in Japan.

At the population level, inter-simple sequence repeat (ISSR) markers have been used to study *R. necatrix* diversity in *Cyperus esculentus* L. (Armengol *et al*. 2010). However, there is still a lack of information on tropical *Rosellinia* spp. that cause damage to commercially propagated plants such as coffee. López (2004), made a first attempt to study the genetic variability of *R. bunodes* and *R. pepo* from coffee, cocoa and potato in Colombia, using ITS and RAPD sequences and mentions high variability in these species.

The primary aim of this study was to identify the species of *Rosellinia* damaging coffee and other associated plants in the Central Colombian coffee growing area. A pathogenicity test through artificial inoculation was carried out to confirm compatibility of the species with coffee.

2. MATERIALS AND METHODS

2.1. Sample collection and fungal isolation

During 2008 and 2009, samples were collected from plants showing macroscopic signs of root rot caused by *Rosellinia* spp. Plant hosts sampled included coffee (*Coffea arabica* L.), macadamia (*Macadamia integrifolia* Maiden & Betche), rubber (*Hevea brasiliensis* Müll. Arg.), cocoa (*Theobroma cacao* L.) and guava (*Psidium guajava* L.). The areas sampled were located in the central coffee growing area of Colombia and included the Caldas, Risaralda and Quindío Provinces. Samples were selected and preliminary identifications were made based on in situ macroscopic observation of symptoms and signs as described by Fernández and López (1964) and Realpe *et al*. (2006).

Plants thought to have root rot were identified based on external symptoms, including wilting, yellowing or dead trees. For fungal isolations, small segments (4-5 cm) were removed from fresh roots of symptomatic plants and placed in 2% NaClO for 15 min, rinsed in sterile water and dried as described by López (2004) and Realpe *et al*. (2006). Small pieces of tissue, including fungal mycelium, were removed from the root sections and transferred to 2% Malt Extract Agar (MEA), (Oxoid), pH 5.7, containing thiamine (100µg/l) and antibiotic (100 mg/l rifampicin). Six to seven pieces were transferred to each Petri dish and the plates incubated at 24°C for three to four days in the dark. Resultant colonies were transferred to fresh medium to obtain pure isolates, which were distinguished by the pear-shaped swellings at the septa, characteristic of *Rosellinia* spp. (Saccas 1956; Realpe *et al*. 2006; Pérez- Jiménez 2006; Pliego *et al*. 2012). Isolates were stored in liquid nitrogen (-196°C) using the technique described by Ten Hoopen *et al*. (2004).

2.2. DNA extraction, amplification and sequencing

Twenty six isolates, identified as possible *Rosellinia* spp. based on morphology, and representing each of the hosts and areas sampled, were selected for characterization using DNA sequence comparisons. For each isolate, small pieces of mycelium from 15-day-old cultures were transferred to 100 ml Erlenmeyer flasks containing 100 ml Sabouraud medium (peptone-glucose-yeast extract). Flasks were incubated at 27°C, for

8 days in darkness, with continuous shaking at 150 rpm. The resultant mycelium was harvested by filtration through Whatman No.1 filter paper and DNA was extracted using the method of Lee and Taylor (1990). Resultant DNA was diluted 20-fold with distilled water and stored at -20°C until further use.

Amplification of the ITS1, 5.8S and ITS2 nuclear gene regions of the ribosomal RNA operon was performed for 26 isolates as described by Hillis *et al*. (1996) using the primers ITS1 (5´TCC GTA GGT GAA CCT GCG G3´) and ITS4 (5´TCC TCC GCT TAT TGA TAT GC 3´) (White *et al*. 1990). PCR reactions consisted of 1.25 U of Taq polymerase (Promega, Southhampton, UK), 2 mM MgCl2; 0.2 mM dNTPs; 1X PCR Buffer; 0.2 μM of each Primer (ITS1, ITS4) and 100 ng of template DNA. Reactions were conducted with an initial denaturation at 95°C for 5 min, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C. A final elongation step was at 72°C for 5 min. PCR products were separated on a 1.5 % agarose gel and stained with 1μl ethidium bromide. Amplified products were visualized under UV light and their molecular mass estimated by comparison with Lambda DNA/HindIII Marker.

PCR products were purified using PCR purification kit (QIAGEN). Sequencing reactions were conducted using BigDye terminator cycling conditions on an Applied Biosystems Automatic Sequencer 3730XL (Macrogen Inc, Seoul, Korea). Sequences were aligned with MAFFT 6 (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/) and a tree was generated using PAUP 4b10 (Swofford 2002). Analyses were done using the heuristic search option with 100 random addition sequence replications (Efron 1986). Sequences of known *Rosellinia* spp. were retrieved from Genbank (National Center for Biotechnology Information (NCBI) and incorporated into the analyses (Table 1). *Hypoxylon intermedium* (Achwein.) Y.M. Ju & J.D. Rogers (Sánchez *et al*. 2000) and *Amphisphaeria umbrina* (Fr.) de Not., both members of Xylariales, were used as outgroup taxa.

2.3. Pathogenicity tests

In order to preliminary evaluate the ability of the species of *Rosellinia* collected in the surveyed area to infect coffee, two pathogenicity tests (one in November and another in December 2009) were conducted under greenhouse conditions at Planalto-Cenicafé, in

Chinchiná, Colombia, using seedlings of *C. arabica* variety Caturra. Isolates encoded as RCQ 60 (CBS134099), obtained from coffee with black root rot in a farm of Quimbaya (Quindio), and RCACC 67 (CBS 134106) obtained from cocoa roots with star rot, in Palestina (Caldas), were grown on twice-autoclaved parboiled rice (Doña Pepa ®) placed in plastic bags. One mycelial disc (6 mm diameter) taken from cultures of each isolate growing on MEA plates was added to each bag, then incubated at 25°C in darkness for twenty five days, to allow the mycelium to completely colonize the rice.

Coffee seedlings (65-days-old) previously grown in sterilized sand, were planted in plastic pots (one plant/pot) containing 150 g of sterilized soil (sandy loam, pH = 4.9, organic matter = 10 %). Eight days after planting, the seedlings were inoculated with 0.18 g of rice/plant, placing the inoculum in contact with the roots. For the controls, uninoculated soil was used. The experimental unit was made up of ten seedlings placed individually in a row of plastic pots; the treatments corresponded to the isolates and five replicates were used per treatment as well as for the controls. Inoculated and control experimental units were placed in a fully randomized design in a greenhouse at an approximately 28°C day and 20°C night temperature regime.

In both trials, plants were checked daily for symptoms for 35 days post-inoculation. Symptomatic and dead plants were inspected for the presence of mycelium on their stems or roots to confirm infection by *Rosellinia* spp. Experimental data, including the number of dead plants and days to mortality, were statistically analyzed using ANOVA and Tukey´s mean test (p=0.05) (SAS Statistical Software 2010).

3. RESULTS

3.1. Sample collection and fungal isolations

Twenty coffee farms located at an altitude of between 1200 and 1500 (m) where patches with symptoms of root rot were present were sampled. In these plantations, areas including infected coffee plants ranged from three to 3000 trees. Cocoa plantations (two) and macadamia (three) had smaller numbers of infected trees ranging from three to 50 plants affected. Other hosts growing on coffee farms had fewer than 10 plants with symptoms per stand. Trees from which samples were collected showed typical

symptoms of root rot caused by *Rosellinia* spp. including foliage yellowing or wilting as well as dead plants. In the root collar area, the bark was cracked and small black lines and dots could be seen macroscopically, embedded in the wood (Figure 1a). These symptoms were most common on coffee and are similar to those recorded for *R. bunodes* (Fernández and López 1964; Ibarra *et al*. 1999; López 2004). Other plants, including cocoa, had white fan-shaped or stellate mycelial growth under the bark of roots (Figure 1b), which is typical for *R. pepo* (Waterston 1941; Sivanesan and Holliday 1972; Merchán 1988; Realpe *et al*. 2006).

Rosellinia spp. were successfully isolated from 90% of the root samples collected, resulting in a total of 26 putative isolates of the fungus (Table 2). Cultures were grouped based on the signs of the pathogen observed on the roots at the time of sampling. Colonies of morphological group A, representing 18 isolates obtained from roots with star rot, were initially white and then turned dark gray and olive green or dark brown. Colonies of group B isolates, consisting of eight cultures originated from black root rot, produced a fluffy mycelium that was also initially white and later turned gray, brown or black, or with some areas white and other black. However there was no consistency with regard to the color of the colonies in each group after ten days of cultivation. No other structures were observed in addition to the mycelium, either on infected roots or in culture after 25 days of cultivation. The most important morphological characteristic observed using light microscopy for these isolates was the pyriform-swellings at the junctions of the septa in the hyphae (Figure 2), as has been previously described for these fungi (Fernández and López 1964; Ibarra *et al*. 1999; López 2004; Realpe *et al*. 2006). The size range of the swellings was between 6.0 and 12.5 µm wide for both species, increasing as the mycelium aged. Strains of three (3) isolates of *R. bunodes* and eight (8) of *R. pepo* were deposited in the Centraalbureau voor Schimmelcultures (CBS, Netherlands). The deposit numbers are to be found in Table 2.

3.2. ITS sequence comparisons

PCR amplification of the ITS regions for 26 isolates putatively representing *Rosellinia* spp. yielded fragments of ~633 bp in length. All sequences generated for the phylogenetic analyses in this study were deposited in Genbank (Table 2). Parsimony analysis produced a data set with 363 constant characters, 101 parsimony-uninformative

and 257 parsimony informative characters. Four hundred and sixty four uninformative characters were excluded and thirty trees were obtained, from which one was chosen for presentation (Figure 3). This tree consisted of two major clades, the largest of these included 18 isolates from coffee and other hosts (colony type A), exhibiting little diversity, and strongly supported by a 100% bootstrap value. The reported sequence in this clade corresponds to *R. pepo* (AB 017659) from the CBS (Netherlands). The second clade, including 8 isolates from Colombia (colony type B), all obtained from coffee plants, was strongly supported by a 100% bootstrap value and was related to an *R. bunodes* sequence (AB 609598). The tree had a consistency index (CI) of 0.45, homoplasy index (HI) of 0.54, retention index (RI) of 0.76, and rescaled consistency index (RC) of 0.34 (TreeBase number TB2: S12799).

3.3. Pathogenicity tests

Isolates of both *R. bunodes* (RCQ 60) and *R. pepo* (RCACC 67) were pathogenic to *C. arabica* seedlings. In both trials, wilting symptoms caused by *R. bunodes* (RCQ 60) were seen as early as nine days post-inoculation on most of the seedlings. All seedlings were killed in the first trial and 98% in the second trial, within 10 to 11 days post-inoculation. Wet rot, as well as brown, sunken discoloration were noticed in the tissues at the bases of the seedlings, with fine white mycelium invading the roots infected by *R. bunodes.*

Wilt symptoms caused by *R. pepo* (RCACC 67) were evident 14 days post-inoculation on most of the seedlings in both trials. For the first trial, 62% of the seedlings were killed and 46% died in the second trial after 16 and 24 days post-inoculation respectively. Dry rot and brown tissue discoloration, but no sunken tissue, were observed at the bases of the seedlings, with gray mycelium invading the roots. Differences in mortality and days to death were statistically significant ($P < 0.0001$) in both tests. No mortality was found in any of the control plants for either of the tests.

4. DISCUSSION

This study provides the first detailed identification of *Rosellinia* species in Colombian coffee growing areas using DNA sequence data. Previous studies in the country relied on identification based only on morphology. We identified *R. bunodes* and *R. pepo*

affecting several hosts, including coffee plants in three provinces of Colombia. Symptoms, signs and molecular characterization of *R. bunodes* and *R. pepo* in this study are consistent with the etiology of the diseases known as black root rot (*R. bunodes*) and stellate root rot (*R. pepo*).

In this study, ITS sequence data confirmed that both *R. bunodes* and *R. pepo* are present in Colombia, confirming previous reports based on disease symptoms. Isolates obtained grouped into two distinct clades with 100% bootstrap support and separate from any other *Rosellinia* sp. for which sequence data are available in GenBank. Currently, there are more than 100 reported *Rosellinia* species known (Kirk *et al*. 2001), but molecular data for these species is minimal or non-existent for most. Most of the sequence data available for the genus in GenBank are for *R. necatrix*. Our data for *R. bunodes* broadens the single sequence report, previously available for this species.

The sequence data emerging from this study supports the reliability of observed differences in macroscopic characters on infected plant roots as diagnostic for discriminating between *R. pepo* and *R. bunodes*. Neither culture morphology nor microscopic features were sufficient to differentiate between species. Differences in mycelium color in culture were not consistent among isolates of the same species, or between species. Pyriform hyphal swellings at the junctions of septa were also observed for both species, as previously reported by Fernández and López (1964); López (2004) and Realpe *et al*. (2006). These swellings have also been reported for *R. necatrix* (Saccas 1956; Pérez-Jiménez 2006; Pliego *et al*. 2012). Our observations showed that these swellings develop in mature hyphae (more than 8- days-old) rather that in young mycelium. Eventually, synnemata and conidia were observed in some old cultures (more than 30 days), but these structures had dimensions very similar in *R. pepo* and *R. bunodes*, as reported by Saccas (1956) and Petrini and Petrini (2005). Thus, they did not add any taxonomic information useful for diagnostics. Fruiting bodies, such as stromata bearing perithecia were not observed, as frequently reported in other studies (Bermúdez and Carranza 1992; Ibarra *et al*. 1999). This might be related to the condition of the samples and the time required for these structures to appear after infection (Sarasola and Sarasola 1975; Texeira *et al*. 1995; Nakamura *et al*. 2000; Pliego *et al*. 2012). It could also mean that the pathogen does not require those parts of its life cycle under Colombian conditions where the temperature and humidity is high all year round. An

effort should be made, nonetheless, to locate such structures and thus complement the molecular taxonomic understanding of *Rosellinia* spp. in Colombia.

5. REFERENCES

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Table 1. Sequences of isolates retrieved from GenBank included in this study.

Taxon	Host and geographic origin	Culture Number	Gen Bank Accession Number	Reference
Amphisphaeria umbrina (Fr.) de Not.	Unknown	---	AF009805	---
Hypoxylon intermedium (Achwein.) Y.M. Ju & J.D. Rogers	Unknown	H4A	AJ390396	Sánchez *et al*. 2000
Rosellinia bambusae Henn.	*Dendrocalamus latiflorus* Munro (Taiwan)	ATCC 66430	AY908998	Peláez *et al*. 2008
Rosellinia bambusae Henn	*Calamus* sp. (Australia)	---	AY862573	Bahl *et al*. 2005
Rosellinia buxi Fabre	Unknown (England)	ATCC 32869	AY909000	Peláez *et al*. 2008
Rosellinia capetribulensis J. Bahl, Jeew K.D. Hyde	*Calamus* sp. (Australia)	---	AY862570	Bahl *et al*. 2005
Rosellinia corticium (Achwein.) Sacc.	Unknown	F-160.845	AY908999	Peláez *et al*. 2008
Rosellinia compacta Takemoto	Unknown (Japan)	---	AB430457	Takemoto *et al*. 2009b

Rosellinia compacta Takemoto	Unknown (Japan)	89112602	AB430456	Takemoto *et al*. (2009b)
Rosellinia mirabilis (Berk & Broome) Y.M. Ju & J.D. Rogers	*Calamus* sp. (Australia)	---	AY862572	Bahl *et al*. 2005
Rosellinia necatrix Berl. Ex Prill	*Ehretia microphylla* Lam (Taiwan)	R210	EF592569	Sun *et al*. 2008
Rosellinia necatrix Berl. Ex Prill	*Camellia sinensis* (L.) Kuntze (Taiwan)	R301	EF592564	Sun *et al*. 2008
Rosellinia necatrix Berl. Ex Prill	*Serissa japonica* (Thunb.) Thunb (Taiwan)	R203	EF592563	Sun *et al*. 2008
Rosellinia necatrix Berl. Ex Prill	Unknown (Japan)	W536	AB430450	Takemoto *et al*. 2009b
Rosellinia necatrix Berl. Ex Prill	*Acer morrisonense* Pax (Taiwan)	R101	EF592568	Sun *et al*. 2008
Rosellinia pepo Pat.	Unknown	CBS350.36	AB017659	---
Rosellinia quercina R. Hartig	Unknown	ATCC36702	AB017661	---

Rosellinia subiculata (Achwein.)Sacc.	Wood (Illinois, USA)	ATCC 58850	AY909002	Peláez *et al.* 2008
Rosellinia bunodes (Berk & Broome) Sac*c.*	*Hibiscus mutabilis* L. (Bahamas)	CBS. 347.36	AB609598.1	---

Table 2 Isolates of *Rosellinia* spp. from coffee and other hosts in Colombia used in this study and for which internal transcribed spacer (ITS) regions sequence data were generated.

Taxon	Culture Number	Host	Origin	Gen Bank Accession number
Rosellinia bunodes	RCQ 48.2 (CBS 134097)**	*Coffea arabica* L.	Circasia (Quindío)	JF263537
"	RCQ 68 (CBS 134098)**	"	"	JF263538
"	RCQ 67.2	"	"	JF263539
"	RCQ 67	"	"	JF263540
"	RCQ 66	"	"	JF263541
"	RCQ 65	"	"	JF263542
"	RCQ 60* (CBS 134099)**	"	Quimbaya (Quindío)	JF263543
"	RCQ 48	"	Circasia (Quindío)	JF263544
Rosellinia pepo	RCACC 65 (CBS 134100)**	*Theobroma cacao* L.	Palestina (Caldas)	JF263545
"	RMACC 45 (CBS 134101)**	*Macadamia integrifolia* Maiden &	Chinchiná (Caldas)	JF263546

		Betche		
“	RGUC 46 (CBS 134102)**	*Psidium guajava* L.	“	JF263547
“	RGUC 45	“	“	JF263548
“	RGUC 28	“	“	JF263549
“	RCR 14.2 (CBS 134103)**	*Coffea arabica* L.	Pereira (Risaralda)	JF263550
“	RCC 67 (CBS 134104)**	“	Chinchiná (Caldas)	JF263551
“	RCC 64.2	“	Palestina (Caldas)	JF263552
“	RCC 64	“	“	JF263553
“	RCC 60	“	Chinchiná (Caldas)	JF263554
“	RCAUR 18 (CBS 134105)**	*Hevea brasiliensis* Müll. Arg.	Pereira (Risaralda)	JF263555
“	RCAUR 17	″	“	JF263556
“	RCACC 67* (CBS 134106) **	*Theobroma cacao* L.	Palestina (Caldas)	JF263557
“	RCACC 66	“	“	JF263558
“	RCACC 36	“	“	JF263559
“	RMACQ 46 (CBS 134107)**	*Macadamia integrifolia* Maiden &	Buena Vista (Quindío)	JF263560

		Betche		
“	RCR 24	*Coffea arabica* L.	Pereira (Risaralda)	JF263561
“	RCR14	“	“	JF263562

* Isolates included in pathogenicity tests. ** CBS deposit number.

Codes: RCC: *Rosellinia*-Coffee-Caldas; RCQ: *Rosellinia*-Coffee-Quindío; RCR: *Rosellinia*-Coffee-Risaralda; RCACC: *Rosellinia*- Cocoa-Caldas; RMACC: *Rosellinia*-Macadamia-Caldas; RGUC: *Rosellinia*-Guava -Caldas: RCAUR: *Rosellinia*-Caucho (Hevea)-Risaralda; RMACQ: *Rosellinia*-Macadamia-Quindío.

Figure 1. Signs of *Rosellinia* infection observed on coffee trees. (a) Black streaks and spots caused by *R. bunodes*, (b) white mycelial stars under the bark caused by *R. pepo.*

Figure 2. Morphological characteristics of *Rosellinia bunodes* and *Rosellinia pepo* growing in culture. Typical pear-shaped swelling in the septa union of mycelia.

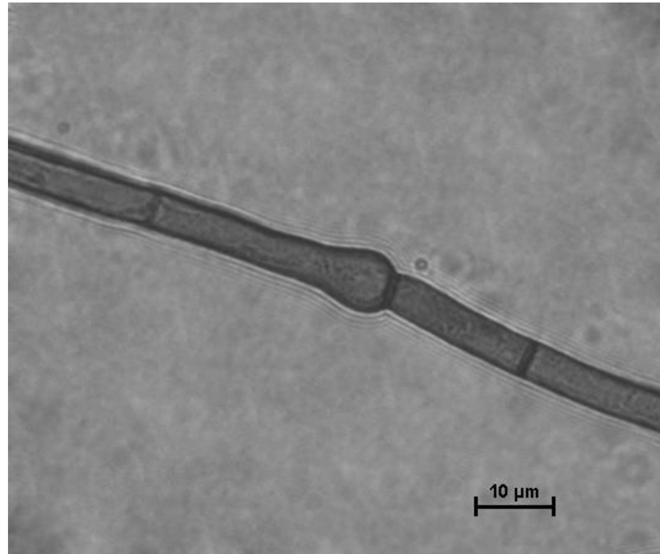
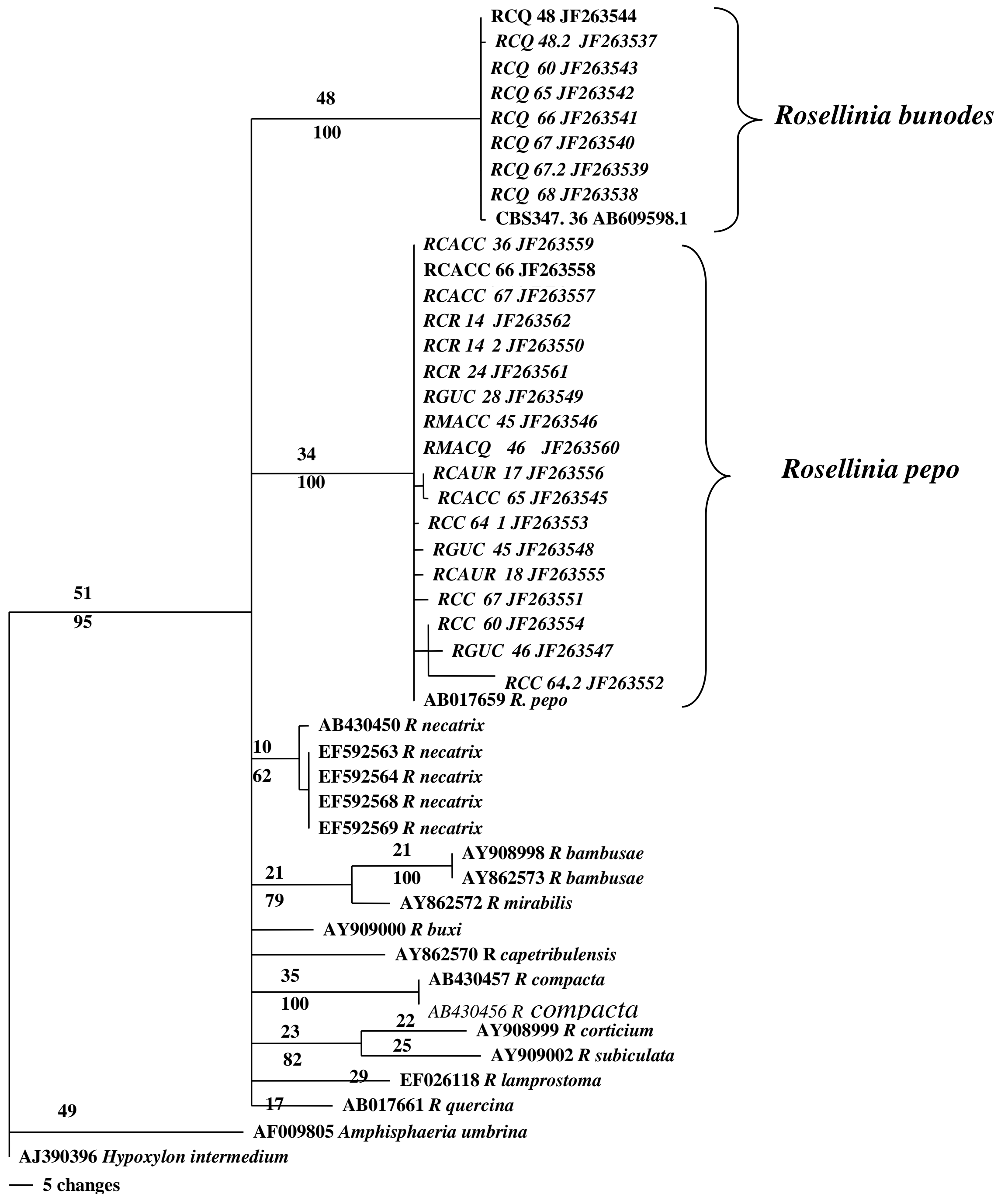

10 µm

Figure 3. The most parsimonious tree generated from DNA sequence data of the ITS regions for isolates of *Rosellinia* spp. Branch lengths are shown above and boostrap values below the branches. CI=0.4535; RI=0.7659; HI= 0.5465; RC=0.3474 Tree Length = 452.


RCQ 48 JF263544
RCQ 48.2 JF263537
RCQ 60 JF263543
RCQ 65 JF263542
RCQ 66 JF263541
RCQ 67 JF263540
RCQ 67.2 JF263539
RCQ 68 JF263538
CBS347. 36 AB609598.1
Rosellinia bunodes
48
100
RCACC 36 JF263559
RCACC 66 JF263558
RCACC 67 JF263557
RCR 14 JF263562
RCR 14 2 JF263550
RCR 24 JF263561
RGUC 28 JF263549
RMACC 45 JF263546
RMACQ 46 JF263560
RCAUR 17 JF263556
RCACC 65 JF263545
RCC 64 1 JF263553
RGUC 45 JF263548
RCAUR 18 JF263555
RCC 67 JF263551
RCC 60 JF263554
RGUC 46 JF263547
RCC 64.2 JF263552
AB017659 R. pepo
Rosellinia pepo
34
100
51
95
AB430450 R necatrix
EF592563 R necatrix
EF592564 R necatrix
EF592568 R necatrix
EF592569 R necatrix
10
62
AY908998 R bambusae
AY862573 R bambusae
AY862572 R mirabilis
21
100
21
79
AY909000 R buxi
AY862570 R capetribulensis
AB430457 R compacta
AB430456 R compacta
35
100
AY908999 R corticium
AY909002 R subiculata
22
25
23
82
EF026118 R lamprostoma
29
AB017661 R quercina
17
AF009805 Amphisphaeria umbrina
49
AJ390396 Hypoxylon intermedium
5 changes

Chapter 3

Assessment of resistance to stem canker caused by *Ceratocystis colombiana* and *Ceratocystis papillata* in Colombian coffee genotypes

ABSTRACT

Coffee stem canker disease, caused by *Ceratocystis colombiana* and *C. papillata,* results in the death of plants at all stages of development and significant losses to the Colombian coffee industry. Resistant genotypes are a most important tools for managing this disease. The aim of this study was to assess the responses of accessions of *Coffea canephora*, *C. liberica,* the Timor Hybrid (HDT), and succeptible *C. arabica* var. Caturra and var. Colombia to artificial inoculation with these pathogens. Ascospore suspensions of each species were applied to stem wounds, and plants were assessed for lesion development and mortality after 1 year. No plants of *C. canephora* and *C. liberica* died, whereas 12 and 10% of the HDT plants and all of the var. Colombia and Caturra plants were killed by *C. colombiana* and *C. papillata,* respectively. These results indicate that substantial resistance to Ceratocystis stem canker and wilt exists in *Coffea* which could be used to develop resistant cultivars.

1. INTRODUCTION

Coffee is cultivated in more than 70 tropical countries where production is predominantly a small-holder enterprise, with plantations covering more than 11 million ha worldwide (ICO 2013). Commercially cultivated coffee species include several varieties, mainly of *Coffea arabica* L. and *C. canephora* Pierre: A. Froehner; they, vary in drink quality, resistance to pests and diseases, drought tolerance, and performance in different climates, soils and cultivation systems (Van der Vossen 2009; Herrera *et al.* 2011).

Coffea arabica is a self-compatible tetraploid species (2n=4x=44 chromosomes) responsible for 70% of world coffee production. In contrast, *C. canephora* is a self-incompatible diploid species (2n=2x=22 chromosomes) representing most of the remaining 30% of world coffee production. The Timor Hybrid (HDT), is a self-fertile tetraploid (2n=44 chromosomes) hybrid between *C. arabica* and *C. canephora,* discovered in Timor (Bettencourt 1973). Some introductions of HDT have been made into coffee producing countries, including Colombia via the Coffee Rust Research Center (CIFC) in Portugal. These introductions have been used in breeding programs to produce valuable varieties, some of which resist Coffee Leaf Rust (CLR) caused by the fungus *Hemileia vastatrix* Berk. & Broome (Van der Vossen 2005).

Several breeding programs have developed durable resistance against CLR, using the dominant major resistance genes (Sh) identified in some *Coffea* species (Van der Vossen, 2005). Nine Sh genes have been identified, which confer resistance to more than 49 physiological races of the fungus. Genes S_H1, S_H2, and S_H4 are present in non-commercial varieties of *C. arabica* and S_H5 is present in commercial varieties of *C. arabica* such as Caturra, Typica and Borbón, S_H3 apparently comes from *C. liberica* W.Bull.: Hiern. (Prakash *et al.* 2005) and S_H6 to S_H9 have been introgressed from *C. canephora* into some introductions of the HDT. Among these HDT, e.g. CIFC-1343, CIFC- 832, CIFC-2570 (Bettencourt and Rodrigues 1988). In Colombia, HDT accession 1343 has been the source of major resistance genes transferred into the dwarf variety *C. arabica* var. Caturra (CLR susceptible), which resulted in the resistant varieties Colombia (Castillo and Moreno 1988) and Castillo® (Alvarado *et al.* 2005). HDT/1343 is also the parent of the Tabi variety, arising from cross between Typica and Borbon

(Moreno 2002). These varieties are currently planted in ~ 60% of Colombian coffee growing areas (FEDERACAFE, unpublished)

Despite the success of CLR breeding programs, there is evidence that some improved commercial varieties derived from the HDT have lost their resistance posible due the emergence of new races of the pathogen (Várzea and Marques 2005; Prakash *et al.* 2005; Alvarado 2005; Gichuru *et al*. 2012). Therefore, breeding programs have focused on efforts to broaden the genetic base of commercial coffee varieties, speciallly with genes from *C. canephora* or *C. liberica* (Varzea and Marques 2005; Herrera *et al*. 2002a and 2002b). *Coffea arabica* is characterized by its autogamous nature and low genetic diversity, whereas diploid coffee species such as *C. canephora,* are allogamous and genetically variable (Berthaud and Charrier 1988; Anthony *et al.* 2002). Resistance to other diseases, such as those caused by nematodes (Bertrand *et al*. 2001; Noir *et al.* 2003) and, possibly, coffee berry disease (CBD, caused by *Colletotrichum kahawae* Waller & Bridges (Gichuru *et al.* 2008)) are some of the desired traits that diploid species could provide in coffee breeding programs (Van der Vossen 2001).

Other than CLR, one of the most frequently encountered coffee diseases, is Ceratocystis stem canker (CSC), caused by species of *Ceratocystis (Ce)* well-known canker and wilt pathogens of trees and some agronomic crops (Kile 1993; Kile *et al*. 1996; Engelbrecht & Harrington 2005; Van Wyk *et al.* 2005; Al Adawi *et al*. 2006; Roux & Wingfield 2013; Wingfield *et al*. 2013). The taxonomy of these pathogens is confused and the name *Ce fimbriata s.l.* is used to refer to a complex of closely related species. Whitin the *Ce fimbriata s.l.* pecies complex, two species *Ce. colombiana* Van wyk. & Wingf. and *Ce. papillata* Van Wyk & Wilgf. have been identified in Colombia (Van Wyk *et al.* 2010).

Rostrella coffea (later *Ceratocystis fimbriata s.l.*) was first reported in coffee on coffee in Indonesia by Zimmerman (1890). Later, Obregon (1936) reported that the fungus caused “mal de tinta” (ink disease) on coffee plants in Colombia. Pontis (1951) described a similar disease in Venezuela and Colombia caused by *Ceratocystis fimbriata s.l.* This disease was named “llaga macana” by Castaño (1951; 1953) and by Fernández (1964) in Colombia. A similar disease has also been found on *C. arabica* in Costa Rica, Cuba, Guatemala, India and Surinam (Baker *et al*. 2003).

Llaga macana has resulted in 20% to 50% mortality of plants in some areas in Colombia, reducing productivity significantly (Castro *et al*. 2003). The management of this disease in coffee has been challenging because the pathogens are soil-borne and disease develops on stems that wounded during essential cultural practices (Castro *et al*. 2003). The most common control practice is the use of fungicides (Castro and Montoya 1994; Castro and Zuluaga 2012).

Genetic resistance is the most desirable and sustainable method of disease control in coffee cultivation. Resistance against Ceratocystis canker has been observed in a line of *C. arabica* var. Borbon in which small lesions and large amounts of callus develop after infection (Fernández 1964; Castro and Cortina 2009). Possible "immunity" has also been observed in *C. canephora* and *C. liberica,* probably linked to higher levels of chlorogenic or phenolic acids present in these species, as compared to the susceptible *C. arabica* (Echandi and Fernández 1961; Schieber and Echandi 1961; Izquierdo 1988).

The aim of this study was to evaluate resistance to Ceratocystis canker in accessions of *C. canephora*, *C. liberica* and the HDT (CIFC-1343) compared to the susceptible commercial varieties *C. arabica* var. Caturra and var. Colombia.

2. MATERIALS AND METHODS

2.1. Genotypes and experiments

Five accessions of *C. liberica,* six of *C. canephora* and one of the HDT, CIFC-1343 were screened for resistance to Ceratocystis canker. Plants of *C. arabica* var. Caturra and Var. Colombia were used as susceptible controls. The different accessions were selected from the germplasm collection of Cenicafé (Chinchiná, Colombia), and plants were propagated from seed, grown in plastic bags (22 x 35 cm) containing soil and 21 months old.

In April 2011, each accession was inoculated individually with *Ce. colombiana* isolate CMW5768 and *Ce. papillata* isolate CMW10844 (CMW culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa). The isolates were re-vitalized by inoculating 20 young coffee branches (4.0 cm long) from which the bark had been removed, and placing these inoculated stems in

moist chambers in Petri dishes for 6 days to obtain ascomata and ascospores. The method described by Castro and Cortina (2009) was used to prepare the inoculum and to inoculate the plants. Spore suspensions were prepared from ascospore masses transferred to sterile water supplemented with 0.06 ml/l of the surfactant Triton® X100 (Sigma Saint Louis Missouri, USA). The suspensions were sonicated at a frequency of 40 kHz for 30 seconds (Branson Ultrasonic cleaner/2510) to evenly disperse the ascospores.

The concentration of spores was determined with a hemacytometer and the inoculum dose was a 50 µl drop of ascospore suspension (7.0 x 10^{4}/ml^{-1}). Using a micropipette, the fungi were deposited in contact with the phloem, in an inverted 1.5 cm U-shaped wound made with sterile scalpel on the stems of plants, approximately 10.0 cm above soil level. Wounds were covered for 15 days with moistened cotton wool and Parafilm® (Pechiney Plastic Packaging, Chicago, IL) to reduce desiccation and the chance of contamination. Visual verification of pathogen colonization was made by observing the presence of black mycelial growth at the inoculation point as described by Fernández (1964).

Two experiments, one for each *Ceratocystis* species were established simultaneously. The experimental unit was 10 plants with three replicates per genotype (treatments), including controls that were inoculated with sterile distilled water. Plants were arranged in a fully randomized design (for each experiment) in an outdoor nursery at Cenicafé, Chinchiná, Colombia (5° N and 75°36' W) (historical annual means of 2560 mm rainfal, RH of 77.1%, and 1751 h of sunshine) (Cenicafé 2012).

2.2. Ceratocystis canker evaluation

All plants were monitored monthly for external symptom expression such as chlorosis, which was usually followed by death. After 4 months the development of lesions from the points of inoculation was determined sisually. After 1 year, a final assessment was made. Dead plants were counted and lesion sizes were measured (cm) on infected plants. The circumference of the stems (CS), width of necrotic lesions (WNL) and phloem lesion length (LL) in cm was evaluated for each plant. The circumference of the stem affected by the necrotic lesion (CSA) was expressed as girdling percentage

calculated as WNL/CS x 100. Data were analyzed with ANOVA and the F test ($P<0.01$) using the SAS Statistical Software 9.2. (SAS 2010). The presence or absence of callus completely covering the cankers was also observed for the inoculated plants.

3. RESULTS

3.1. Ceratocystis canker evaluation

After 3 to 4 months foliage on plants of *C. arabica* var. Caturra and var. Colombia, began to yellow, and after 6 months all these plants had died, irrespective of which *Ceratocystis* sp. was used for inoculation. Lesions spread both upward and downwards, girdling the stems (Figure 1a).

After 1 year, no mortality was observed in *C. liberica* plants and there was obvious callus development at the points of inoculation in all plants (Figure 1b). In a few *C. canephora* plants, lesions began to develop after 4 months. However, no mortality was observed at the time of the final evaluation. A few plants of *C. canephora* var. Ugandae CCC 712, *C. canephora* CCC 962 and *C. canephora* CCC 1015 had long and narrow lesions but also with obvious callus development (Figure 1c). In these accessions *Ce. Colombiana* caused lesions with an average CSA of 3.5% and LL of 0.85 cm, and *Ce papillata* caused lesions with an average CSA of 3.8% and LL of 0.85 cm.

A respective 12% and 10% of the HDT plants were killed by *Ce. colombiana* and *Ce. Papillata.* The remaining plants of this hybrid developed small lesions similar to those in *C. canephora* (Figure 1b). One year after inoculation, these resistant plants had on average 7.2% CSA and 0.5 cm LL caused by *Ce. colombiana* and 8.8 % CSA and 1.4 cm of LL caused by *C. papillata.* The values for CSA and LL in resistant plants of some accessions of *C. canephora* and the HDT/1343 were statistically different compared with the susceptible controls, Caturra and var. Colombia. But no differences were noticed between these resistant plants for either variable (Table 1). No disease developed in control plants.

4. DISCUSSION

In the present studies, defense reactions on plants of *C. canephora, C. liberica* and the HDT inoculated with *Ce. colombiana* and *Ce. papillata* were observed as callus formation and the absense of lesions. Similar reactions to *Ceratocystis* spp. Have been observed in other resistant host plants (Sandnes and Solheim 2002; Pilloti *et al*. 2009; Zauza *et al*. 2004; Sanches *et al*. 2008).

An important result of this study was the recognition of *Ceratocystis* canker resistance in the HDT, *C. liberica* and *C. canephora* . Notably, rust resistance genes also exist in C. *liberica* and *C. canephora* (Lashermes *et al*. 2000; Prakash *et al*. 2004; Van der Vossen 2005; Herrera *et al*. 2009). Therefore, it should be possible to introgress resistance to CLR and Ceratocystis canker from these taxa into improved *C. arabica* varieties. To that end advances have been in Colombia where (F_4) hybrids, with rust resistance have been developed with these species (Alvarado and Cortina 1997). In addition there are (F_4) interspecific hybrids between *C. canephora* and *C. arabica* var. Caturra backcrossed to Caturra, which combine resistance against CLR and Ceratocystis canker and are detailed in Chapter 4 (Castro *et al*. 2013).

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Figure 1. Different reactions against Ceratocystis canker in coffee genotypes, one year after inoculations with *Ceratocystis colombiana* and *Ce. papillata*. (a) A typical susceptible reaction, showing canker and lesion development on the stem of a coffee plant. (b) Typical resistant reactions and complete lack of lesion development as obtained for accessions of *Coffea liberica,* most *C. canephora* and some plants of the Timor Hybrid. (c) A typical resistance reaction in a few accessions of *C. canephora,* exhibiting long and narrow lesions with callus formation preventing stem girdling and plant death.

a
b
c

Table 1. Resistant and susceptible reactions in genotypes of *Coffea* spp. 1 year after inoculation with *Ceratocystis colombiana* and *Ce. papillata*. s.

Genotype	***Ceratocystis colombiana***		***Ceratocystis papillata***	
	CSA (%) (mean ± SD)	**LL (cm) (mean ± SD)**	**CSA (%) (mean ± SD)**	**LL (cm) (mean ± SD)**
C. liberica CCC 1025	0	0	0	0
C. liberica CCC 1029	0	0	0	0
C. liberica CCC1033	0	0	0	0
C. liberica CCC1035	0	0	0	0
C. liberica CCC 1022	0	0	0	0
C. canephora CCC 1006	0	0	0	0
C. canephora CCC 978	0	0	0	0
C. canephora CCC 980	0	0	0	0
***C**. canephora* (var. Ugandae CCC712**)**	4.1 ± 11.4 b	2.1 ± 5.4 b	0	0
C. canephora CCC 962	1.7 ± 6.4 b	0.3 ± 1.3b	2.0 ± 6.3 c	0.3 ± 1.2 b
C. canephora CCC 1015	4.8 ± 10.5 b	0.9 ± 2.1b	5.6 ± 11.0 bc	1.4 ± 2.6 b
Timor Hybrid/1343	7.2 ± 14.6 b	0.5 ± 0.9 b	8.8 ± 20.9 b	1.4 ± 2.7 b
C. arabica var. Colombia	100.0a	17.5 ± 4.8 a	100.0 a	13.6 ± 5.0 a
C. arabica var Caturra	100.0 a	16.2 ± 5.9 a	100.0a	12.3 ± 3.9 a

* Circumference of stem affected by necrotic lesion (CSA) and lesion length (LL) caused by the pathogens. Means followed by same letter do not differ significantly (Duncan´s test, $P<0.05$).

Chapter 4

New coffee (*Coffea arabica* L.) genotypes derived from *Coffea canephora* exhibiting high levels of resistance to leaf rust and Ceratocystis canker.

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ABSTRACT

The purpose of this study was to evaluate the resistance to coffee leaf rust (CLR), caused by *Hemileia vastatrix* and to Ceratocystis stem canker (CSC) in coffee genotypes derived from crosses of *Coffea arabica* var. Caturra with accessions of *C. canephora* backcrossed to Caturra. Twenty-three (F_3BC_1) progenies including *C. arabica* var. Caturra and var. Colombia as controls were established in a field experiment. CLR evaluations were made during five years of natural infection, using an incidence rating scale. For CSC, artificial stem inoculations were made with an isolate of *Ceratocystis colombiana* and the results were assessed after one year. The selection process also included agronomic aspects such as plant height, canopy diameter, number of branch pairs, yield and grain characteristics. Twenty progenies showed >70% of rust resistance. Twelve progenies exhibited >80% of CSC resistance, while no resistance was observed in the controls. Only three progenies performed well for all criteria, including resistance to both pathogens and agronomic characteristics.

1. INTRODUCTION

Two major diseases that are currently responsible for significant yield reduction in Colombian coffee production are coffee leaf rust (CLR), caused by the obligate pathogen *Hemileia vastatrix* Berk. & Broome and Ceratocystis stem canker (CSC), caused by the soil-borne fungi *Ceratocystis papillata* Van Wyk & Wingf. and *Ce. colombiana* Van Wyk & Wingf. (Van Wyk *et al*. 2010). Of these, CLR is the best known and arguably the most damaging coffee disease in the world, resulting in crop losses of 20-40% where no control measures are used (Van der Vossen 2005; Rivillas *et al*. 2011). Recently, reports from Central American countries suggest up to 80% defoliation caused by this disease (Cressey 2013).

At least 49 rust races have been identified in coffee, using a set of more than 40 coffee differentials, including the two commercial species cultivated globaly, *Coffea arabica* L. (tetraploid 2n= 4x= 44) and *C. canephora* Pierre: A. Froehner (diploid 2n= 2x =22) (Varzea and Marques 2005; Gichuru *et al*. 2012). The pathogen *H. vastatrix* has (with various periods of delay), followed the spread of *C. arabica* around the world. This includes the Americas, where the traditional Typica and Borbón varieties, and their derivatives, are susceptible to race II (genotype v5 v5), the most predominant CLR race in the world (Eskes *et al*. 1989; Van der Vossen 2005). In Colombia, where only *C.* a*rabica* has been cultivated, CLR race II was reported for the first time in 1983 affecting var. Caturra (Leguizamón *et al*. 1984). Subsequently, more than 10 different races have been identified (Castillo and Leguizamón 1992; Gil and Ocampo 1998; Alvarado 2005; Cristancho *et al*. 2007). After the severe epidemics reported in Colombia during 2008-2011, with losses above 30% (Cristancho *et al*. 2012), molecular studies showed that race II and its derivatives prevail in this country (Rozo *et al*. 2012).

Several breeding programs have developed durable resistance against CLR, using the dominant major resistance genes (Sh) identified in some *Coffea* species (Van der Vossen 2005). Nine dominant genes involved in resistance to *H. vastatrix* have been identified so far. Alone or associated, they confer resistance to more than 49 physiological races of the fungus. Genes S_H1, S_H2, S_H4 are present in non-commercial varieties of *C. arabica* and S_H5 present in commercial varieties of *C. arabica* such as Caturra, Typica and Bourbón, among others (Bettencourt and Rodriguez 1988). Gene

S_H3 apparently comes from *C. liberica* W.Bull.: Hiern. (Prakash *et al*. 2005) and genes S_H6 to S_H9 have been introgressed from *C. canephora* in some introductions of the Timor Hybrid (HDT), which is a self-fertile spontaneous tetraploid (2n=44*)* originating from *C. arabica* and *C. canephora* (Bettencourt and Rodrigues 1988). Among these HDT introductions are CIFC-1343, CIFC- 832, CIFC-2570, and their resistant genes come from some accessions of *C. canephora* (Bettencourt and Rodrigues 1988). Mahe *et al*. (2007) reported new sources of rust resistance in other natural interspecific hybrids from New Caledonia, and more recently, Brito *et al*. (2010) identified one additional resistance gene to race II of *H. vastatrix* in the HDT -UFV-427-15. Fernandes *et al*. (2012) suggested potential novel rust resistance genes from genomic studies.
Most coffee breeding programs have used the HDT as the main source of CLR resistance (Van der Vossen 2001). In Colombia, HDT accession 1343 has been the source of major resistance genes transferred into the dwarf variety *C. arabica* var. Caturra (CLR susceptible), which resulted in the resistant varieties Colombia (Castillo and Moreno 1988) and Castillo® (Alvarado *et al.* 2005). HDT/1343 is also the parent of the Tabi variety, arising from cross between Typica and Bourbón (Moreno 2002). These varieties are currently planted in ~ 60% of Colombian coffee growing areas (FEDERACAFE, 2013 unpublished).

Despite the success of CLR breeding programs, there is evidence that some improved commercial varieties derived from the HDT have lost their resistance due to the possible emergence of new virulent races of the pathogen (Várzea and Marques 2005; Prakash *et al.* 2005; Alvarado 2005; Gichuru *et al*. 2012). Variability in virulence of the pathogen could be due to natural mutation processes, but it could also arise from other mechanisms such as cryptic sex and hidden sexual reproduction of the pathogen (Carvalho *et al*. 2011). Therefore, breeding programs have focused on efforts to broaden the genetic base of commercial coffee varieties. This especially has been through the exploration of alternative resistant resources, mainly from diploid species such as *C. canephora* or *C. liberica* and the introgression of genes into *C. arabica* (Varzea and Marques 2005; Herrera *et al*. 2002a and 2002b). *Coffea arabica* is characterized by its autogamous nature and low genetic diversity, while diploid coffee species such as *C. canephora* are reported as allogamous with considerable variability (Berthaud and Charrier 1988; Anthony *et al.* 2002). This includes resistance to other diseases, such as

those caused by nematodes (Bertrand *et al.* 2001; Noir *et al.* 2003) and possibly to coffee berry disease (CBD) caused by *Colletotrichum kahawae* Waller & Bridges (Gichuru *et al.* 2008). Hence, the introgression of desired characters from diploid species into cultivars of *C. arabica* has been a priority in coffee breeding (Van der Vossen 2001).

Ceratocystis stem canker (CSC), known in most Latin-American coffee producing countries as “llaga macana” or “trunk canker” is an important disease of coffee in Colombia caused by the soil-borne pathogen *Ceratocystis fimbriata* Ell & Halst. *sensu lato* (s.l.). Chlorosis, dieback and wilt are the external symptoms in plants that have been affected in their vascular tissues, primarily in the stem, where the dark lesions extend upwards or downwards, girdling the trunk and causing tree death. Currently the disease is found in all the Colombian coffee-growing areas (Marin *et al*. 2003), and all commercial coffee varieties (*C. arabica*) planted in this country are susceptible to the disease (Castro *et al*. 2003). Mechanical injuries (fresh wounds) are the main sources of entry for the pathogen in coffee plants. These wounds arise from farmers stabilizing their boots on the trunks of trees in order to support themselves on the steep slopes where coffee is cultivated, and also from pruning (Castro *et al*. 2003).

Ceratocystis fimbriata s.l. complex is associated with vascular diseases in a large number of plants in many parts of the world. Webster and Butler (1967) recognized that *Ce. fimbriata* probably represented more than one entity. Baker *et al*. (2003) hypothesize that local populations of *Ce. fimbriata* in some Latin American plants have become specialized on different hosts. Studies developed during the last twelve years with isolates from both soil and plants in affected Colombian coffee areas have revealed two phylogenetic lineages (Barnes *et al.* 2001; Marin *et al*. 2003). Based on morphological and DNA sequence comparison of isolates from different hosts in Colombia (coffee, cocoa, citrus and native forest), Van Wyk *et al.* (2010) named the two lineages *Ce. colombiana* Van Wyk & Wingf. and *Ce. papillata* Van Wyk &Wingf. Both species are pathogenic, causing death of coffee plants artificially inoculated in their stems (Marin *et al*. 2003; Van Wyk *et al*. 2010).

Apparently all *C. arabica* commercial varieties are susceptible to CSC, however, an exceptional case of resistance has been reported in a line of *C. arabica* var. Bourbón.

Such resistance is characterized by the formation of lignified tissues surrounding infection sites and preventing the girdling of plant stems (Fernández 1964). In the "immune" *C. canephora* and *C. liberica,* necrotic lesions do not develop due to rapid wound closure (Echandi and Fernández 1961; Izquierdo 1988). Recently such "immunity" was also recorded in the Colombian National Center of Coffee Research (Cenicafé, Colombia) in some accessions of *C. canephora* and *C. liberica* in artificial inoculations with *Ce. colombiana* and *Ce. papillata* (unpublished).

Selection and breeding for disease resistant/tolerant coffee cultivars remains an ongoing challenge and priority in Colombia. Besides developing rust resistant in commercial coffee varieties, it has also been necessary to consider resistance to CSC. Using the resistant Bourbón line, Castro and Cortina (2009) developed Caturra-like genotypes with resistance to "llaga macana," but these were susceptible to rust. Hence, development of varieties resistant to both rust and CSC is necessary. One approach would be to develop promising genotypes from gene introgression into *C. arabica* by way of triploid hybrids, through crossing of *C. arabica* x *C. canephora* (Orozco 1976; Alvarado and Cortina 1997; Herrera *et al*. 2002a and 2002b). Following this strategy, researchers at Cenicafé crossed tetraploid *C. arabica* var. Caturra with accessions of diploid *C. canephora,* generating triploids backcrossed (BC) to Caturra. Based on rust resistant tetraploid F_2BC_1 genotypes, the aim of this study was to select F_3BC_1 progenies simultaneously resistant to both CLR and CSC. Furthermore, the process of selection included desirable agronomic characteristics and bean attributes that will be useful for future commercial varieties.

2. MATERIALS AND METHODS

2.1. Genotypes and field experiment

Twenty-three F_3 progenies produced by crossing *C. arabica* var. Caturra with three accessions of *C. canephora* and backcrossed with var. Caturra (F_3BC_1) were included in the study. The different parental accessions were selected from Cenicafé´s germplasm collection (Chinchiná, Colombia). These tetraploid interspecific hybrids, labeled as MEG 639, were generated by crossing the *C. arabica* var. Caturra as female parent (susceptible to both CLR and CSC) with accessions of *C. canephora* (BP.358-EA.93;

BP.358- EA.239 and BP.46-EA.131) known to be CLR resistant, and backcrossed to Caturra. Two controls, *C. arabica* var. Colombia (CLR resistant, but CSC susceptible) and var. Caturra (susceptible to both pathogens), were included.

Field plots were established at Cenicafé Central Experiment Station (04°58' NL, 75°39' W, 1381 m) in 1998. The site has an annual average precipitation of 2556 mm, sunshine of 1816 hours/year, HR of 78% and a mean temperature of 20.8°C (Cenicafé 2008). Trees were planted in a square lattice (5 x 5) and two replicates were used per treatment in the experimental design. The experimental unit was a line of 10 plants with distance of 1.0 x 1.0 m between plants and 2.5 m between blocks. Standard agronomic management was applied.

2.2. Coffee leaf rust evaluation

Incidence of *H. vastatrix* infection was evaluated from January 2000 to August 2005, during periods of heavy rust outbreaks as defined by Sierra *et al.* (1991). The rating scale of Eskes and Braghini (1981) was used for plant evaluation. This scale grades the whole plant as a unit of observation on a visual scale (0 to 9), where 0 = absence of sporulating lesions; 1= presence of one diseased branch; 2 to 8= gradual increase in number of diseased branches with sporulating lesions; and 9= maximum disease incidence. At each evaluation, the number of plants with rust was scored and grouped into three categories: uninfected plants (grade 0); plants with a low level, considered resistant (graded 1 to 4) and plants with high level of infection, as susceptible (graded 5 to 9). The maximum grade of each tree was recorded along with the frequency of plants of each grade, according to the criteria of Alvarado and Cortina (1997).

2.3. Ceratocystis canker evaluation

When coffee plants reached seven-years-old, they were inoculated with isolate CMW 34925 (ITS-rDNA sequence = GenBank accession KF300545 and CBS 135942) of *C. colombiana* (Van Wyk *et al.* 2010), and previously identified as highly virulent by Castro and Cortina (2009). The inoculum was prepared as previously described by Marin *et al*. (2003). Drops of 70 µl containing approximately 3.0 x 10^4 ascospores/ml, were inoculated into inverted U-shaped wounds, approximately 2.0 cm in diameter,

made on the stems at ~1.40 m above soil line. The spore suspension was inserted under the bark and sealed with Parafilm “M” ® (Pechiney Plastic Packaging, Chicago, IL).

Fifteen days after inoculation, the Parafilm was removed and pathogen colonization was verified. Red paint was applied to the trunks below the site of inoculation to further identify plants and to aid in later assessments. One year after inoculation, the size of the lesions under the stem bark was determined. Three measurements were made for each plant, including stem circumference (SC), width of necrotic lesion (WNL) and lesion length (LL). The width of stem affected (WSA) by the necrotic lesion was expressed as percentage of the stem circumference (SC) girdled (WNL/SC x 100).

2.4. Evaluation of agronomic characteristics

All plants were evaluated for plant height (cm), canopy diameter (cm) and number of branch pairs at 24 months of age. Yield data per plant were also recorded from 2001 to 2005 in kg of fresh berries per tree. The percentage of bean defects such as empty beans in ripe coffee fruits and defects of dry parchment beans such as “peaberry” ("caracol") and “triangle” were evaluated in two peaks of yield in 2001 and 2002. For empty beans, the floating method was used. The size of dry beans (Supreme type), determined as the percentage of husked beans (green coffee) retained by a 17/64 inches mesh. The assessment of these bean characteristics were made according to the methods of Castillo and Moreno (1988) and Moreno and Alvarado (2000), as well as the coffee quality standards (FEDERACAFE 1988).

2.5. Data analysis and selection of promising genotypes

For CLR, a frequency distribution per progeny was developed for each assessment and the maximum rating was considered with these results expressed as percentage of plants affected. Data were grouped based on a severity scale from 0 (uninfected plants), grade 1 to 4, and grade 5 to 9. Progeny with ≥ 70 % of plants graded 0-4 on the CLR scale were selected as resistant because at this level there is no effect on productivity (Eskes and Braghini 1981; Alvarado and Cortina 1997).

To analyze the CSC data, the mean values of the measurements for each coffee genotype were calculated and analysis of variance (ANOVA) was performed for the WSA and LL data at a significance level of P=0.05. Tukey's test (P=0.05) was used to compare the results for different progenies. All analyses were done using the SAS statistical program (SAS Statistical Software 2010). Resistant genotypes were selected as those where >80% of plants had WSA values lower than 50% and with lesions smaller than those of the susceptible controls, following the method previously used by Castro and Cortina (2009).

For agronomic characteristics, ANOVA was performed for plant height, canopy diameter and number of branch pairs as well as bean characteristics and annual yield over five years. The yield data were taken from harvests obtained over five years and the average (kg of fresh berries/tree/year) was transformed to kg of dry parchment coffee/plant/year, using the conversion factors of Montilla *et al*. (2008). Where statistical differences were found among the 23 progenies tested, Dunnett´s test (P= 0.05) was used to compare the results with those for var. Caturra. Average agronomic characteristics equal to or better than those of the controls were the criteria used to select promising plants for future use.

3. RESULTS

3.1. Coffee leaf rust evaluation

On the basis of 12 evaluations from 2000 to 2005, clear differences among progenies, as well as between the progenies and the two control varieties, were found (Figure 1). The var. Caturra was the most susceptible genotype tested, with all plants scoring between 5 and 9 on the rating scale. In contrast, progenies MEG 639-410, ME G639-475, MEG 639-705 and MEG 639-708 were the most resistant, with 100% of plants graded 0 to 4 on the disease assessment scale. Eighteen progenies had greater than 70% of the plants graded 1 to 4, and these were also considered resistant.

3.2. Ceratocystis canker evaluation

There was variability in the resistance reactions against CSC in the genotypes tested. Twelve progenies were statistically different to the susceptible control varieties in WSA, with <50% of the stem circumference affected by lesions (Figure 2). Eight of these progenies were also significantly different from the controls in LL (Figure 3). Progenies MEG 639-708, MEG 639-617, MEG 639-841, MEG 639-620, MEG 639-601, MEG 639-842, MEG 639-704, MEG 639-602, MEG 639-609, MEG 639-475, MEG 639-771 and MEG 639-705 were the most resistant to *C. colombiana* infection, compared with susceptible control varieties, which were killed by the pathogen. Progenies MEG 639-841, MEG 639-617 and MEG 639-708 exhibited the smallest lesions, suggesting a possible form of "immunity".

Among the progenies with resistance reactions, strong callus formation was observed surrounding the necrotic lesions (Figures 4a and 4b) with small amounts of discoloured tissue in the underlying wood (Figure 4c). In contrast, wood discoloration spread either upwards or downwards, and there was an absence of an obvious defense response in susceptible controls or susceptible progenies (Figure 4d).

3.3. Evaluation of agronomic characteristics

The average plant height ranged from 131 to 161 cm in the progenies, while var. Caturra and var Colombia were an average height of 133 cm and 144 cm, respectively. Statistical differences ($P < 0.0001$) were observed only for progenies MEG 639-410 and MEG 639-602, which were significantly taller than var. Caturra. No differences were noticed in the number of branches nor in canopy diameter for either the progenies or the controls. These phenotypic attributes are commercially accepted by Colombian coffee growers and also mentioned by Castillo and Moreno (1988) and Alvarado and Cortina (1997).

Variable bean characteristics were observed in the progenies (Table 1). Dunnett´s test showed that ten of these had a higher percentage of empty beans than var. Caturra (3.9%) and var. Colombia (5.9%), while the rest of the progenies had acceptable ranges of bean production as defined by Castillo and Moreno (1988). Similarly, the progenies

had higher peaberry values (average, 14.6%) than the two controls (average 8.2%). Dunnett´s test showed that 12 progenies had different values to those of var. Caturra (8.4%) and var. Colombia (9.6%). The frequency of triangle beans was low, with an average of 4%, which is within the acceptable range according to Castillo and Moreno (1988) and no differences were observed for this trait.

The average Supreme bean size (percent beans retained by a 17/64 inch screen) in the progenies was 61.0%, better than var. Caturra (41.0%), but lower than var. Colombia (66.0%). Dunnett´s test showed that 14 progenies and var. Colombia had larger beans than var. Caturra. Progenies MEG 639-771 (45.0 %) and MEG 639-818 (37.4%) had bean sizes that were much lower than expected (Table 1), according to the criteria established by Moreno and Alvarado (2000) for commercial varieties.

For average yield calculations (Table 1), differences (P< 0.0001) and Dunnet´s test showed that progenies MEG 639-602, MEG 639-705, MEG 639-722, MEG 639-771 and MEG 639-841 were more productive than var. Caturra. There were no progenies statistically less productive than the controls. Thus progenies had variable yield and bean attributes, similar to what has been observed by other studies (Alvarado and Cortina 1997). However, attributes such as bean size were close to the Colombian commercial varieties (Moreno and Alvarado 2000).

Based on their resistance to rust and Ceratocystis canker, as well as on their most important agronomic characteristics (yield and bean size), three progenies (MEG 639-601; MEG 639- 617 and MEG 639-704) were selected for future breeding development.

4. DISCUSSION

Since 1983, when CLR (Race II) was detected in Colombia (Leguizamón *et al*. 1984), there has been a gradual increase in disease severity, depending on weather conditions (Sierra *et al*. 1991; Rivillas *et al*. 2011; Rozo *et al*. 2012). During the course of the current project, the highest CLR incidence (>60%) was found on var. Caturra in 2003 and 2005. In contrast, very low levels (<30%) of rust were observed in 2004. This behavior is related to climatic conditions as well as plant vigor and size of the harvest, amongst other factors (Kushalappa 1989; Costa *et al.* 2006). Overall, results of this

study indicate that the selection of CLR resistant progeny made in earlier generations (F_2BC_1) assured an 87% level of resistance in the presently used progenies (F_3BC_1). The resistance found in var. Colombia indicates that, while the resistance genes in this variety come from the *C. canephora* through the HDT/1343 (Castillo and Moreno 1988), the rust resistant genes in the F_3 progenies studied are different from the ones present in the varieties Colombia, Tabi and Castillo®, whose resistance introgressed from the unique HDT/1343. This is evident in our study, because at least seven progenies had some plants totally resistant, while all progeny of var. Colombia were infected, although at a low level. This possibility was also raised by Mahe *et al.* (2007), who studied the genetic diversity and rust resistance in natural interspecific hybrids between *C. arabica* x *C. canephora* from New Caledonia (HNC) and found resistance to all rust races in some progeny. Based on molecular data, they suggested a high level of genetic diversity of *C. canephora* progenitors at the origin of HNCs. For Colombian coffee, this new source of resistance genes may have important consequences.

After the last severe rust outbreak of 2008-2011 (Cristancho *et al.* 2012), the susceptible var. Caturra has been replaced by commercial resistant multiline varieties derived from the HDT/1343. The increasing area planted with these HDT/1343 lines may intensify the selection pressure on the pathogen, favoring the emergence of compatible races and thus threatening resistance durability. Therefore, the incorporation of new genotypes with different rust resistant genes, such as those selected in this study, into current breeding programs should increase the stability and durability of CLR resistance.

The resistance reactions against CSC observed in the progenies selected are similar than those noticed as "immunity" by Echandi and Fernández (1961) in inoculations with *Ce. fimbriata s.l* on *C. canephora* and *C. liberica* in Guatemala, and by Izquierdo (1988) in Cuba on *C. canephora*. Thus, our results showed clear evidence of resistance to *Ceratocystis* infection, probably conferred by the parental *C. canephora* through the hybridization, and the resistance persisted after backcrossing to the susceptible var. Caturra.

The progenies selected in the present study may give rise to new resistant genes and may be valuable as efforts are made to reduce the damage caused by *Ceratocystis* spp. in Colombia.

This study has shown that it is possible to transfer desirable genes for resistance to the most important coffee pathogens to new genotypes. These genotypes will be valuable as new sources of resistance to these pathogens in the future.

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Figure 1. Maximum percentage of plants infected by *Hemileia vastatrix* per each progeny set in 12 assessments, based on Eskes and Braghini scale (0 to 9). Green bars represent uninfected plants (grade 0). Blue bars are for plants graded 1 to 4. And red bars are plants graded 5 to 9.

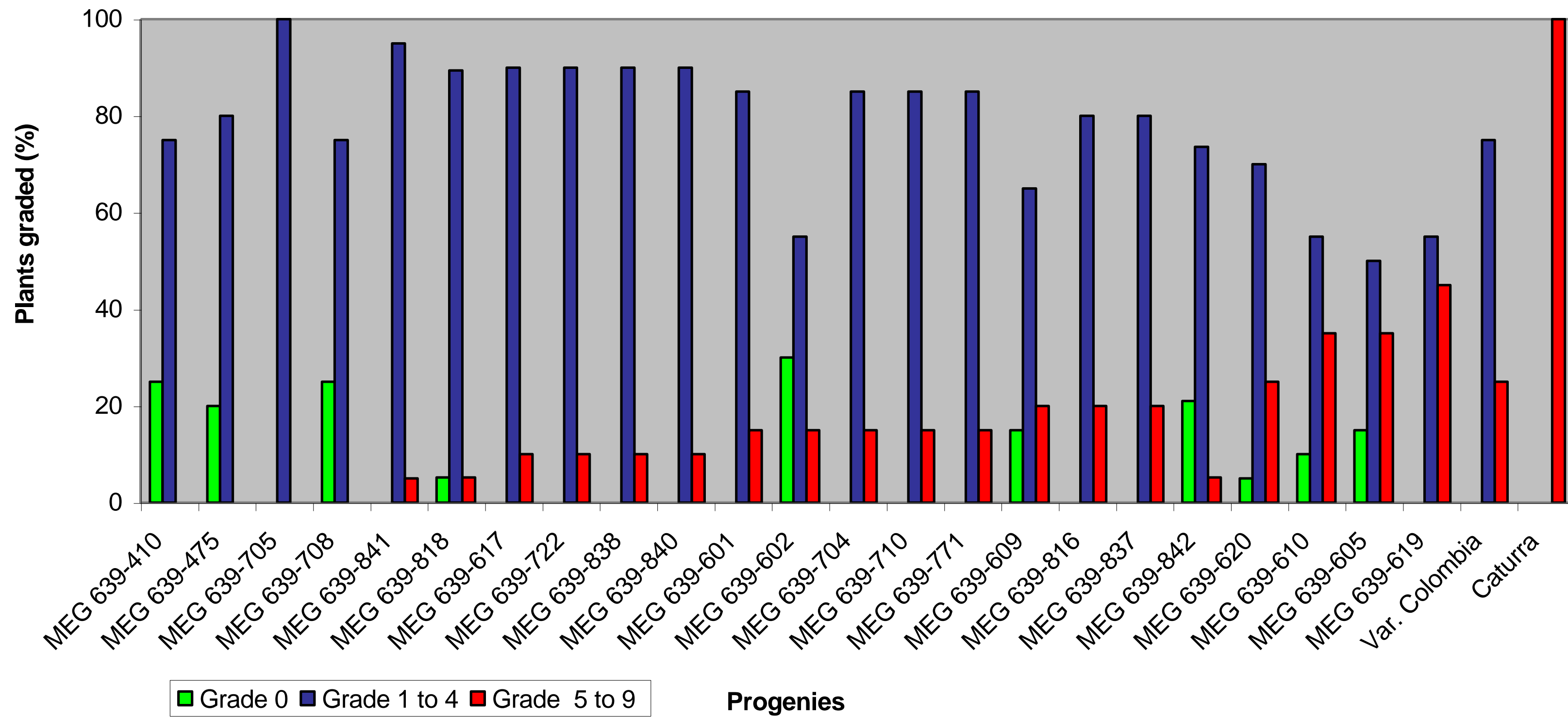

Plants graded (%)
100
80
60
40
20
0
MEG 639-410
MEG 639-475
MEG 639-705
MEG 639-708
MEG 639-841
MEG 639-818
MEG 639-617
MEG 639-722
MEG 639-838
MEG 639-840
MEG 639-601
MEG 639-602
MEG 639-704
MEG 639-710
MEG 639-771
MEG 639-609
MEG 639-816
MEG 639-837
MEG 639-842
MEG 639-620
MEG 639-610
MEG 639-605
MEG 639-619
Var. Colombia
Caturra
Grade 0
Grade 1 to 4
Grade 5 to 9
Progenies

Figure 2. Resistance reactions of coffee progenies to infection by *Ceratocystis colombiana*. Means (%) of width stem affected (WSA), one year after inoculation. Tukey´s tests (P=0.05) and bars represent the standard error (F= 0.05%).

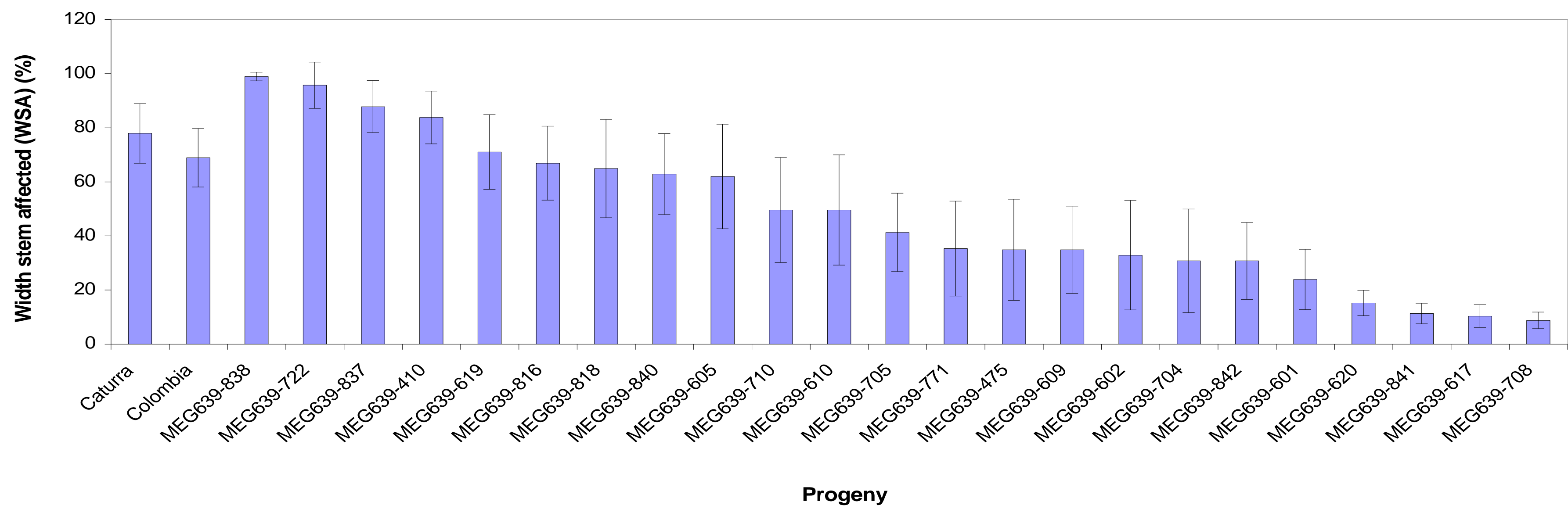
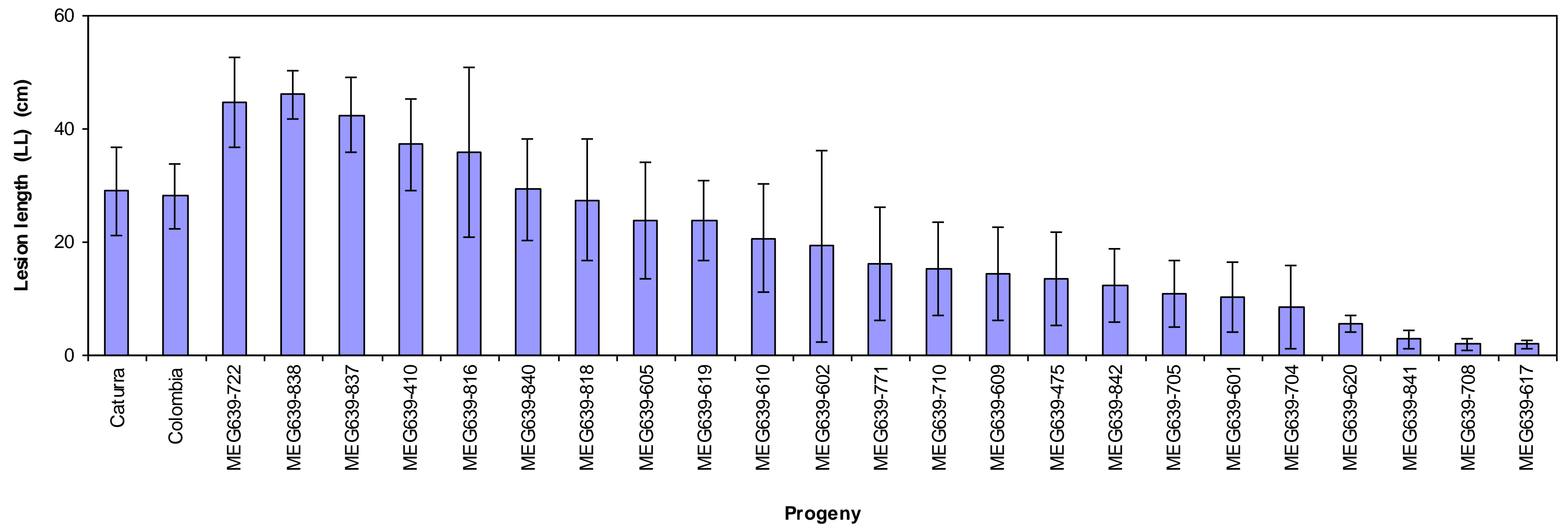

Width stem affected (WSA) (%)
0
20
40
60
80
100
120
Caturra
Colombia
MEG639-838
MEG639-722
MEG639-837
MEG639-410
MEG639-619
MEG639-816
MEG639-818
MEG639-840
MEG639-605
MEG639-710
MEG639-610
MEG639-705
MEG639-771
MEG639-475
MEG639-609
MEG639-602
MEG639-704
MEG639-842
MEG639-601
MEG639-620
MEG639-841
MEG639-617
MEG639-708
Progeny

Figure 3. Resistance reactions of coffee genotypes to infection by *Ceratocystis colombiana*. Means of lesion lengths (LL) in cm, assessed one year after inoculation. Tukey´test (P=0.05) and bars represent the standard error (F=0.05%).



60
40
20
0
Lesion length (LL) (cm)
Caturra
Colombia
MEG639-722
MEG639-838
MEG639-837
MEG639-410
MEG639-816
MEG639-840
MEG639-818
MEG639-605
MEG639-619
MEG639-610
MEG639-602
MEG639-771
MEG639-710
MEG639-609
MEG639-475
MEG639-842
MEG639-705
MEG639-601
MEG639-704
MEG639-620
MEG639-841
MEG639-708
MEG639-617
Progeny

Figure 4. Resistance reaction displayed by some progenies with strong callus formation (a, b) and reduced lesion area in the underlying wood (c), compared with wood discolouration (d) in controls and susceptible progenies F_3BC_1 derived from interspecific hybrids (*Coffea canephora x C. arabica*) one year after inoculation of *Ceratocystis colombiana.*

a

b

c

d

Table 1: Bean characteristics and yield (Kg of dry parchment coffee/plant/year) for each progeny.

Progeny nr.	Bean defects (%)			Bean size supreme[d] (%) (Mean ± SD	Yield (Kg) (Mean ± SD)
	Empty beans[a] (Mean ± SD)	Peaberry[b] (Mean ± SD)	Triangle[c] (Mean ± SD)		
MEG 639.410	5.1 ± 1.4	8.8 ± 2.0	5.8 ± 2.3	75.0 ± 3.8*	3.0 ± 0.9
MEG 639.475	7.8 ± 2.9	17.1 ± 4.1*	0.2 ± 0.2	53.5 ± 14.3	3.2 ± 1.2
MEG 639.601	6.6 ± 2.9	13.3 ± 4.0	3.2 ± 1.6	64.9 ± 4.3 *	3.2 ± 1.4
MEG 639.602	10.5 ± 4.1*	20.7 ± 8.4*	2.9 ± 2.1	54.6 ± 2.4	3.6 ± 1.2*
MEG 639.605	13.5 ± 5.8*	27.0 ± 13.3*	4.3 ± 2.3	58.7± 4.8*	3.0 ± 1.1
MEG 639.609	7.5 ± 3.0	12.6 ± 2.9	3.5 ± 1.3	50.5 ± 16.4	2.7 ± 1.1
MEG 639.610	6.4 ± 1.6	9.7 ± 2.4	6.7 ± 2.2	60.0 ± 8.8 *	3.1 ± 1.0
MEG 639.617	8.6 ± 3.2	10.7 ± 5.5	8.0 ± 1.8	70.3 ± 6.2*	3.1 ± 0.6
MEG 639.619	7.5 ± 3.8	11.0 ± 1.4	8.7 ± 2.4	55.6 ± 7.5	2.7 ± 0.9
MEG 639.620	11.1 ± 4.6*	18.7 ± 10.6*	1.9 ± 1.1	63.3 ±11.9*	3.1 ± 1.2
MEG 639.704	5.1 ± 1.7	12.4 ± 4.8	4.9 ± 2.6	66.6 ± 11.3*	3.1 ± 0.9
MEG 639.705	12.3 ± 7.7*	24.7 ± 9.2*	2.3 ± 0.8	64.5 ± 8.5*	4.0 ± 1.1*
MEG 639.708	7.2 ± 3.0	9.5 ± 1.9	5.9 ± 3.1	55.7 ± 9.2	3.1 ± 1.0
MEG 639.710	9.8 ± 4.0*	15.2 ± 7.9*	2.9 ± 1.3	61.8 ± 10.4*	2.6 ± 1.1
MEG 639.722	5.1 ± 1.3	8.1 ± 1.9	1.8 ± 0.6	70.6 ± 7.4*	4.2 ± 0.8*
MEG 639.771	9.9 ± 8.5*	15.7 ± 4.4*	7.0 ± 3.9	44.9 ± 19.2	3.5 ± 1.4*
MEG 639.816	9.5 ± 8.0*	18.2 ± 5.3*	0.9 ± 0.6	47.8 ± 16.9	3.2 ± 1.0
MEG 639.818	9.3 ± 4.5	20.8 ± 1.20*	0.5 ± 0.4	37.4 ± 19.6	3.3 ± 1.4
MEG 639.837	9.1 ± 3.3*	27.4 ± 11.4*	4.1 ± 2.3	62.2 ± 13.8*	3.1 ± 1.2
MEG 639.840	6.1 ± 2.8	14.1 ± 3.6	0.8 ± 0.6	74.9 ± 10.1*	3.1 ± 1.0
MEG 639.838	6.3 ± 3.6	16.3 ± 6.0*	4.1 ± 2.6	57.9 ± 13.6	2.9 ± 1.2
MEG 639.841	11.4 ± 10.2*	16.4 ± 3.5*	1.1 ± 0.7	70.3 ± 8.7*	3.5 ± 1.1*
MEG 639.842	4.7 ± 8.2*	12.3 ± 2.6	1.0 ± 0.6	73.8 ± 8.3*	3.1 ± 1.1
Var. Caturra	3.9 ± 1.0	8.4 ± 2.8	5.8 ± 1.2	41.0 ± 7.5	2.4 ± 0.8
Var. Colombia	5.9 ± 2.2	9.6 ± 2.6	2.3 ± 1.3	66.0 ±12.6*	3.2 ±1.0

[a] Empty beans: Average percent of 100 ripe coffee fruits floating in three samples of two harvest peaks

[b] Pea berry : Average percent in three samples of 400 dry parchment beans of two harvest peaks.

[c] Triangle: Average percent in three samples of 400 dry parchment beans of two harvest peaks

[d] Supreme: Average percent of three samples of 100 g of husked beans (green coffee) retained by a 17/64 inch screen.

*Statistical differences (0.05) according Dunnett's test are indicated for each character.

Chapter 5

Resistance to leaf rust and Ceratocystis canker in interspecific coffee hybrids

ABSTRACT

The aim of this study was to develop coffee genotypes with resistance to coffee leaf rust, caused by *Hemileia vastatrix,* and stem canker caused by *Ceratocystis* spp. Sixteen F_2 and F_3, hybrids between *Coffea arabica* cv. Caturra and *Coffea canephora*, backcrossed to Caturra were evaluated versus cv. Caturra and cv. Colombia susceptible controls. A field experiment was established in the Experimental Station of Naranjal, Chinchiná, Colombia. Coffee rust evaluations were based on natural infections for 5 years, using a 0 to 9 severity scale. For stem canker, resistance was assessed 1 year after artificial inoculation with *Ceratocystis colombiana.* Agronomic and bean characteristics were also evaluated. Eight progenies showed resistance to both diseases, only two of which had acceptable commercial performance.

1. INTRODUCTION

Coffee leaf rust (CLR) caused by the fungus *Hemileia vastatrix* Berkeley & Broome, is a significant constraint in the production of arabica coffee (*Coffea arabica* L.) and results in considerable costs due to the need for chemical control (Van der Vossen 2005; Rivillas *et al*. 2011). For more than 50 years, the natural interspecific hybrid between *C. arabica* and *C. canephora* Pierre: Froehner, known as Timor Hybrid (HDT), has been the most important source of CLR resistance (Rodrigues *et al*. 2000). In Colombia, *C. arabica* 'Colombia' (Castillo and Moreno 1988), 'Tabi' (Moreno 2002) and 'Castillo®' (Alvarado *et al*. 2008) have been developed based on HDT accession 1343. These varieties are cultivated in 35% of all Colombian coffee growing areas, whereas the remaining areas are planted to CLR-susceptible varieties such as Caturra and Typica (Rivillas *et al*. 2011).

In recent years, a loss of CLR resistance has been observed in some commercial varieties derived from the HDT (Alvarado 2005; Varzea and Marques 2005, Zambolim *et al*. 2005). The main cause of the break down appears to be the limited diversity of resistance genes that are present in these genotypes (Van der Vossen 2005; Varzea and Marques 2005). On the other hand, robusta coffee (*C. canephora*) and *C. liberica* Hiern exhibit a high level of durable resistance to CLR (Eskes 2005). Through gene introgression the later resistances have helped improve *C. arabica* (Lashermes *et al*. 2000; Herrera *et al*. 2002; Van der Vossen 2005; Prakash *et al*. 2005).

Ceratocystis stem canker (CSC), known in Latin American countries as llaga macana, or canker of the stem causes significant losses. In Colombia, the disease affects all commercial coffee varieties (Castro *et al.* 2003), and at least two species, *C. colombiana* Van Wyk & Wingf. and *C. papillata* Van Wyk & Wingf., have been identified as causal agents (Van Wyk *et al*. 2010). Limited research has been conducted to identify resistance to this pathogen. Fernández (1964) observed some resistance in a line of *C. arabica* var. Bourbon, and later Castro and Cortina (2009) used Bourbon crossed with Caturra to select eight progenies with resistance to CSC. These genotypes were, however, susceptible to CLR.

Resistance to CSC is characterized by limited xylem discoloration, as well as the formation of lignified tissues surrounding the lesions that prevent stem girdling (Fernández 1964; Castro and Cortina 2009). In contrast, in *Coffea canephora* and *C. liberica*, necrotic lesions do not develop (Schieber and Echandi 1961; Izquierdo 1988). The aim of this study was to select F_2BC_1 and F_3BC_1 progeny of interspecific hybrids between *C. canephora* crossed with *C. arabica* cv. Caturra in order to combine resistance to CLR and CSC. Furthermore, these progenies were screened for acceptable commercial value (agronomic performance and grain quality).

2. MATERIALS AND METHODS

2.1. Plant material and field experiment

One F_1 (F_1BC_1) progeny produced through crossing *C. arabica* cv. Caturra (Ca) with one accession of *C. liberica* (Lib) and backcrossed (BC) to Ca, encoded as MEG- 631-093; Four F_2 (F_2BC_1) and twelve F_3 (F_3BC_1) progenies produced through crossing Ca with accessions of *C. canephora* (Can) and BC to Ca were included in the study (Table 1**.** The parental accessions of Can and Lib were selected from among the live germplasm collection (Colombian Coffee Bank) in Cenicafé (Chinchiná, Colombia), to obtain triploid inter-specific hybrids. The progenies derived from Can had been selected from seed-generated plants with CLR resistance in previous generations. *Coffea arabica* cv. Ca (susceptible to CLR and to CSC), *C.arabica* cv. Colombia red berries and cv. Colombian yellow berries (CLR resistant, but susceptible to CSC) were included as controls. A field experiment was established in a plot at the Central Experimental Station, Naranjal, of Cenicafé (04°58' NL, 75°39' W., 1.381 m) during 1998. The site had an annual average precipitation of 2.556 mm, 1.816 hours/year of sunshine, RH of 78% and mean temperature of 20.8°C (Cenicafé 2008). Plants representing the progeny were planted in a lattice (5x4) and with two replicates per genotype. The experimental unit was a plot of ten plants with a distance of 1.60 x 1.0 m between plants and 2.5 m between blocks.

2.2. Coffee leaf rust evaluation

CLR severity was assessed from 2000 to 2005, during peak severities, as defined by Sierra *et al.* (1991) and with the scale of Eskes and Braghini (1981). Their pictorial assessment scale grades the whole plant, where 0 = absence of sporulating lesions; 1= presence of one diseased branch; 2 to 8= gradual increase in number of diseased branches with sporulating lesions; and 9= maximum disease. At each evaluation, the number of plants with rust was scored and grouped into three categories: uninfected plants (grade 0); plants with a low level (graded 1 to 4) and plants with high level of disease (graded 5 to 9). The maximum grade was noted along with the frequencies of plants for the mentioned grades, according Alvarado and Cortina (1997). Frequency distributions for each of the hybrid progeny were established for each assessment and the results were expressed as a percentage of plants. Data were grouped in the categories: 0; 1 to 4; more than 4. Progeny with more than 70 % of plants graded 0-4 were selected as resistant.

2.3. Ceratocystis canker evaluation

To evaluate resistance to Ceratocystis stem canker, 7 year-old plants were inoculated with isolate CMW34925 of *C. colombiana* (Van Wyk *et al.* 2010). The inoculum was prepared following the technique of Marin *et al*. (2003). Drops (70 µl) containing 3.0 x 10^4 ascospores/ml^{-1} were placed into inverted U-shaped wounds, approximately 2.0 cm in diameter, made on the stems of the plants, around 104.0 cm above soil level. The inoculum was deposited under the bark and covered with moist cotton wool and sealed with Parafilm®. After 1 year, disease development was measured as the size of lesions. Three measurements were made, in cm, for each plant: stem circumference (SC), width of necrotic lesion (WNL) and length of lesion (LL). The circumference of stem that was affected by the necrotic lesion (CSA) was expressed in % (WNL/SC x 100). Tukey's test (p=0.05) was used to satistically separate means CSA and LL data. Following criteria of Castro and Cortina (2009), resistant genotypes were those with more than 80% of plants with CSA less than 50% and with lesions samller than those on the susceptible controls.

2.4. Evaluation of agronomic and bean characteristics

All plants were evaluated for: plant height (cm), canopy diameter (cm) and number of branch pairs at 15 and 24 months of age. Yield (kg of fresh berry plant^{-1}) was also recorded from 2001 to 2005. Grain defects were recorded as: empty beans in ripe berries, peaberry and triangle beans. The size of dry beans (Supreme type %), defined as beans retained in a 17/64-inch mesh, was recorded during 2001 and 2002, as recommended by Castillo and Moreno (1988). ANOVA was calculated for all these variables as well for average yields for the cumulative yield (kg of fresh berry coffee plant^{-1}), and data were transformed to kg of dry parchment coffee plant^{-1}year^{-1} , as suggested by Montilla *et al.* (2008). Dunnett´s test was used in comparisons with Caturra data. All analyses were made using SAS (SAS Statistical Software 2010). Average agronomic characteristics equal to/or better than those of Colombia (Castillo and Moreno 1988) were used to select promising plants for future research.

3. RESULTS

3.1. Coffee leaf rust evaluation

Clear differences were observed among the progenies and the control varieties during the 5 years of rust evaluation (Figure 1). Low levels of CLR occurred in 2000 and 2001. However, in 2002, 30& of the 'Caturra' control plants reached assessment scales of <4, while all plants of twelve progenies and var. Colombia were graded 0 to 4 (data not shown). In 2003, CLR levels were even higher when 75% of the 'Caturra' plants reached assessment scales of < 4. In 2004, 95% of the 'Caturra' plants graded 5 to 7. The disease decreased in 2005, 75% of the 'Caturra' plants graded >4.

A maximum of 95% of the Caturra plants graded 5 to 9, in contrast to more than 70% of the plants of the twelve progenies being considered resistant graded 0 to 4. Among these, progenies MEG 639-562, MEG 639-836, MEG 639-884, MEG 639-565 (F_3RC_1), and MEG 623-40 (F_2 RC_1) were the most resistant with all plants of each grading <4. Both var. Colombia reached a maximum level of 85% of plants graded 0 to 4.

3.2. Ceratocystis canker evaluation

Significant variation was observed in CSC resistance in the progenies included in this study, as indicated by CSA ($F=8.5$, $p<0.0001$) and LL ($F= 11.1$, $p<0.0001$) (Figures 2A-2B). Progenies MEG 639-565, MEG 634-590, MEG 623-40, MEG 639-562, MEG 639-566, MEG 615-17, MEG 639-884, MEG 639-561 and MEG 623-04 were considered resistant and had CSA significantly lower than the controls; in contrast MEG 636-815 and MEG 636-946 were the most susceptible.

3.3. Evaluation of agronomic and bean characteristics

Statistical differences ($F=5.7$, $p<0.0001$) were observed in the heightof plants after 24 months. Plants of MEG 634-590 and MEG 639-836 were taller than var. Caturra, whereas those of MEG 639-562 and MEG 639-565 were smaller. Significant differences ($F=8.6$, $p<0.0001$) also were noticed in mean canopy diameters, as plants of MEG 634-590, MEG 639-836 and MEG 639-884 were larger and MEG 639-562 were smaller than Caturra. Branch pair numbers for MEG 639-561, MEG 639-562, MEG 639-565 and MEG 639-566 were lower than the controls ($F=6.3$, $p=<0.0001$) (Table 2).

Differences ($F=10.6$, $p<0.0001$) were observed in the proportions of empty beans among genotypes and Dunnett'test ($p=0.05$) indicated that five progenies had higher %s of empty beans than vars. Caturra (4.5%) and Colombia (7.9%) (Table 3). Less than 10% of ripe berries of the remaining progenies had this defect, which is acceptable (Alvarado and Cortina 1997). Similarly, differences ($F=33.25$, $p<0.0001$) occurred in peaberry proportions, and seven progenies had higher values (average of 19%) than commercially accepted genotypes (12%) and controls (11%). There were also differences in the frequency of triangular bean defects ($F=15.6$, $p<0.0001$), but at acceptable commercial levels (4%).

The grain size (% Supreme) in the progenies was on average 50% higher than in the controls (46%) (Table 3). Significant differences ($F=9.5$, $p<0.0001$) were observed for bean size, five progeny (MEG 636-834, MEG 636-946, MEG 639-561, MEG 639-566 and MEG 639- 884) had larger grains (Supreme type) than var. Caturra, while MEG 639-727 and MEG 623-04 had smaller beans. Among the five progenies that had larger

grain sizes (Supreme) than the controls only two MEG 639-561 and MEG 639-884 had resistance to both CLR and for CSC. Only MEG 636-816 was more productive than var. Caturra.

4. DISCUSSION

The progenies selected as CLR resistant had higher levels of resistance than those observed in India by Prakash *et al*. 2005 in similar hybrids. This is possibly due to the larger number of rust races and favourable climatic conditions prevailing in India than in Colombia. The present results suggest that different *C. canephora* accessions have different CLR resistance gene than HDT/1343 (SH_5 to SH_9) (Bettencourt *et al.* 1980), which was used to developed cultivars Colombia, Tabi and Castillo®. This assumption has been confirmed in the molecular study of Lashermes *et al*. (2000), which indicated high genetic diversity in *C. canephora.* These authors also argued that few resistant genes are found in HDT hybrids. Mahe *et al*. (2007) proposed that other interspecific hybrids, such as the diverse New-Caledonian natural interspecific hybrids between *C. arabica* and *C. canephora,* providing increased chances of finding durable CLR resistance genes.

The CLR resistant genotypes in this study may be most useful in areas where resistance in the derivatives of the HDT (called “Catimors”) has been eroded. Some examples of gradual loss of resistance are mentioned by Zambolim *et al*. (2005) in Brazil, such as ‘Icatu’ and ‘Catucai’ derived from the HDT (CIFC832/1, 832/2 and 2570). Similarly the variety ‘Cauvery’ in India (‘Catimor’ originated from one HDT plant designated as ‘UFV386-45’) was completely susceptible to CLR. In Colombia, some commercial plots planted to var. Costa Rica 95 (Caturra x HDT-832/1) have been affected by CLR during 2012 (unpublished data). Therefore, the results of this study imply that this additional source of resistance will increase the current set of genes present in the Colombian varieties.

On the other hand the reactions of resistance against CSC observed in the progenies selected resembles a possible "immunity" in the diploid *C. canephora* as it has been mentioned by Echandi and Fernández (1961) and Izquierdo (1988). In susceptible

progenies and controls, callus formation was not observed and the lesions spread longitudinally to girdle more than 50% of the stem circumference. Although no mortality was observed in the trial during the period of assessment, susceptible plants will be likely girdled completely and eventually die, as has been observed previously (Castro and Cortina 2009). Inoculation trials using the predominant species *Ce. colombiana* and *Ce. papillata* identified by Van Wyk *et al.* 2010 in Colombian coffee-growing areas would be useful in producing the next generation of progenies selected in order to confirm the resistance reactions.

Bean defects are frequently found in interspecific coffee hybrids as well in autopolyploids and these are related to sterility and chromosomal irregularities (Carvalho and Mónaco 1969, Herrera *et al*. 2002). These traits are important as they used to determine a factory processing coefficient, and this has been a serious obstacle with such hybrids in the past (Carvalho *et al*. 1983). Unfortunately some selected progenies with high levels of resistance to CLR and CSC were excluded due to problems with this trait. On the other hand, Supreme type is an attribute that has become important for Colombian coffee growers, because this is also related with the factory processing coefficient and thus greater commercial acceptance (Alvarado *et al*. 2008). The results showed variability in grain size among the progenies, predominating small grain. Similar divergences were noticed by Alvarado and Cortina (1997) in advanced generations (F_4RC_1) of these kind of hybrids. However in our study control varieties, Caturra and Colombia had also minor size (43.8% and 45.4% respectively) than the registered by Castillo and Moreno (1988), for Caturra (63%) and Colombia (83.0%).

On the trait production, most of the progenies had similar yields than the controls. It is possible linked to the backcross to var. Caturra. Althought Owuor and Van der Voseen (1981), suggested two backcrosses to recover the fertility, in this experiment only one backross was enough to achieve similar performance than Caturra.

Two progenies (MEG 639-561 and MEG 639-884) were identified as promising for future breeding programs; they combine the resistance to both pathogens with good agronomic performance. Some interspecific hybrids have displayed resistance to other pathogens such as Cercospora coffeicola (Patricio et al. 2010), to nematodes Meloidogyne exigua (Bertrand et al. 2001) or to coffee berry disease, Colletotrichum

kahawae J.M. Waller & Bridge (Omondi et al. 2001) as well as their combinations. However, no previous research has considered Ceratocystis canker. Future trials with genotypes selected on the basis of taste attributes will be made in order to complete the information to provide suitable commercial coffee varieties.

5. REFERENCES

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Table 1. Genealogy and codes of coffee inter-specific progenies studied, produced in the cross-breeding between *Coffea arabica* var. Caturra (Ca) and accessions of *C. canephora* (Can), backcrossed to Ca.

Hybrid	Cross	Progeny/generation	
		F_2RC_1	F_3RC_1
4158	[Ca x Can L.147-EA.263] x Ca*	MEG 634-590	
4224	Ca x (Can BP.4-EA.224)-EE.073	MEG 623-04	
4228	Ca x (Ca x Can 1-EA.21)-EE.132	MEG 623-40	
4340	[(Ca x Can) - ED.1 a 160] x Ca*	MEG 615-17	
4241	Ca x (Ca x Can BP.358- EA.239)-ED.93	EY.012	MEG 639-727
4283	Ca x (Hybrid 4158 - EI.69)	EZ.183	MEG 636-877
4284	Ca x (Hybrid 4158 - EI.69)	EZ.189	MEG 636-816
4284	Ca x (Hybrid 4158 - EI.69)	EZ.189	MEG 636-815
4285	Ca x (Hybrid 4158 - EI.69)	EZ.251	MEG 636-946
4285	Ca x (Hybrid 4158 - EI.69)	EZ.251	MEG 636-834
4341	[(Ca x Can) - EE.1 - 200] x Ca*	FB.379	MEG 639-566
4341	[(Ca x Can) - EE.1 - 200] x Ca*	FB.379	MEG 639-884
4341	[(Ca x Can) - EE.1 - 200] x Ca*	FB.379	MEG 639-565
4341	[(Ca x Can) - EE.1 - 200] x Ca*	FB.379	MEG 639-561
4341	[(Cat x Can) - EE.1 - 200] x Ca*	FB.379	MEG 639-562
4343	[(Cat x Can) - EI.1 - 167] x Ca*	FB.1180	MEG 639-836

(*) Free pollination.

Table 2. Average of plant agronomic traits for each progeny at 24 months - old.

Progeny nr.	Plant height (cm) (Mean ± SD)	Canopy diameter (cm) (Mean ± SD)	No. of branch pairs (Mean ± SD)
MEG 615-17	115.5 ± **14.9**	105.0 ± **32.5**	20.6 ± **5.1**
MEG 623-04	118.3 ± **10.4**	129.2 ± **18.4**	24.2 ± **2.9**
MEG 623-40	113.7 ± **10.2**	110.2 ± **26.6**	22.9 ± **3.1**
MEG 634-590	137.7 ± **12.8***	150.5 ± **19.0***	21.7 ± **3.3**
MEG 636-815	120.8 ± **11.0**	126.5 ± **17.4**	23.5 ± **2.3**
MEG 636-816	127.8 ± **13.6**	129.0 ± **19.0**	26.5 ± **3.2**
MEG 636-834	128.8 ± **12.4**	135.5 ± **16.2**	24.9 ± **2.7**
MEG 636-877	120.8 ± **13.5**	119.7 ± **19.0**	23.7 ± **2.4**
MEG 636-946	120.3 ± **35.0**	120.0 ± **34.6**	21.4 ± **4.3**
MEG 639-561	120.5 ± **17.0**	120.2 ± **24.9**	19.0 ± **3.1***
MEG 639-562	84.7 ± **18.3 ***	76.7 ± **28.7***	15.3 ± **7.5***
MEG 639-565	99.7 ± **22.1***	107.5 ± **22.5**	18.4 ± **5.4***
MEG 639-566	106.5 ± **29.0**	115.0 ± **21.7**	19.0 ± **5.6***
MEG 639-727	125.0 ± **16.5**	130.0 ± **34.3**	22.0 ± **2.9**
MEG 639-836	133.5 ± **10.8***	144.5 ± **17.6***	24.8 ± **2.5**
MEG 639-884	120.0 ± **27.1**	115.7 ± **20.8***	20.6 ± **5.0**
MEG 631-093	118.5 ± **15.05**	111.5 ± **23.5**	29.9 ± **3.3**
Var. Caturra	116.3 ± **18.9**	114.7 ± **27.8**	23.2 ± **4.2**
Var. Colombia red	118.8 ± **14.5**	127.2 ± **18.5**	23.4 ± **3.0**
Var. Colombia yellow	114.0 ± **14.0**	115.2 ± **25.4**	21.6 ± **2.7**

*Statistically significant differences from the vars. Caturra and Colombia controls, as determined by Dunnett 's HSD ($p<0.05$).

Table 3. Average of bean characteristics and yield (Kg of dry parchment coffee/plant-year).

Progeny nr.	Bean defects (%)			Grain size	Yield Average
	Empty beans[a] (Mean ± SD)	Peaberry[b] (Mean ± SD)	Triangle[c] (Mean ± SD)	Supreme type [d] (%) (Mean ± SD)	(kg) (Mean ± SD)
MEG 615-17	13.6 ± 4.5*	26.4 ± 7.5*	3.6 ± 2.2	46.7 ± 12.3	2.4 ± 1.9
MEG 623-04	17.1 ± 7.6*	23.7 ± 5.1*	0.6 ± 0.4*	23.7 ± 9.3	2.1 ± 0.9
MEG 623-40	26.4 ± 9.8*	45.1 ± 11.0*	0.3 ± 0.3*	44.5 ± 15.3	2.1 ± 0.6
MEG 634-590	19.0 ± 5.9*	36.5 ± 11.6*	0.3 ± 0.4*	35.9 ± 16.4	3.3 ± 1.3
MEG 636-815	5.4 ± 1.5	12.1 ± 2.7	2.8 ± 1.5*	52.3 ± 9.7	3.2 ± 0.6
MEG 636-816	6.4 ± 1.6	13.4 ± 1.2	1.7 ± 0.6*	47.7 ± 5.5	3.8 ± 0.9*
MEG 636-834	22.4 ±13.5*	10.9 ± 2.6	0.8 ± 0.5*	68.0 ± 4.6*	2.6 ± 0.7
MEG 636-877	8.0 ± 6.1	12.6 ± 3.4	1.8 ± 0.8*	48.8 ± 10.3	2.8 ± 0.7
MEG 636-946	7.3 ± 3.6	12.1 ± 4.4	1.4 ± 0.6*	58.5 ± 11.2*	2.7 ± 0.8
MEG 639-561	5.8 ± 1.9	12.3 ± 2.7	1.8 ± 1.1*	65.3 ± 4.3*	2.7 ± 0.7
MEG 639-562	3.5 ± 1.6	12.9 ± 4.5	7.5 ± 3.1*	41.6 ± 7.8	2.4 ± 0.7
MEG 639-565	7.9 ± 5.7	19.0 ± 5.9*	2.6 ± 1.4*	49.2 ± 8.2	2.8 ± 0.9
MEG 639-566	6.5 ± 2.5	16.1 ± 2.7*	6.1 ± 3.7*	66.3 ± 6.6*	2.4 ± 1.1
MEG 639-727	6.1 ± 2.5	7.9 ± 2.1	1.7 ± 0.5*	27.0 ± 12.2*	2.3 ± 0.7
MEG 639-836	7.6 ± 4.3	16.1 ± 4.9*	3.1 ± 1.4	51.8 ± 16.6	3.0 ± 0.8
MEG 639-884	6.7 ± 2.5	10.7 ± 3.1	3.0 ± 1.4*	67.2 ± 7.6*	2.5 ± 0.9
MEG 631-093	13.5 ± 5.1*	37.0 ± 8.3*	1.2 ± 1.2*	32.8 ± 6.8	3.1 ± 0.9
Var. Caturra	4.5 ± 1.9	10.3 ± 5.0	4.5 ± 1.7	43.8 ± 6.9	2.9 ± 0.8
Var Colombia red	7.2 ± 2.5	11.0 ± 2.8	0.6 ± 0.5*	45.4 ± 12.3	3.0 ± 0.9
Var. Colombia yellow	8.6 ± 6.9	11.5 ± 3.3	0.7 ± 0.3	48.7 ± 5.7	2.8 ± 0.8

[a] Empty beans: Average percent of 100 ripe coffee fruits floating in three samples of two harvest peaks

[b] Pea berry : Average percent in three samples of 400 dry parchment beans of two harvest peaks.

[c] Triangle: Average percent in three samples of 400 dry parchment beans of two harvest peaks

[d] Supreme: Average percent of three samples of 100 g of husked beans (green coffee) retained by a 17/64 inch screen.

*Statistical differences from the vars. Caturra and Colombia controls (0.05) according Dunnett's test HSD ($p<0.05$). are indicated for each character

Figure 1. Frequency distribution of rust (%) per progeny in twelve assessments (2000 to 2005) in The Central Experimental Station, Naranjal, of Cenicafé (Chinchiná, Colombia), based on Eskes and Braghini scale (0 to 9). Green bars represent uninfected plants (grade 0). Blue bars represent plants graded 1 to 4. And red bars are susceptible plants graded 5 to 9.

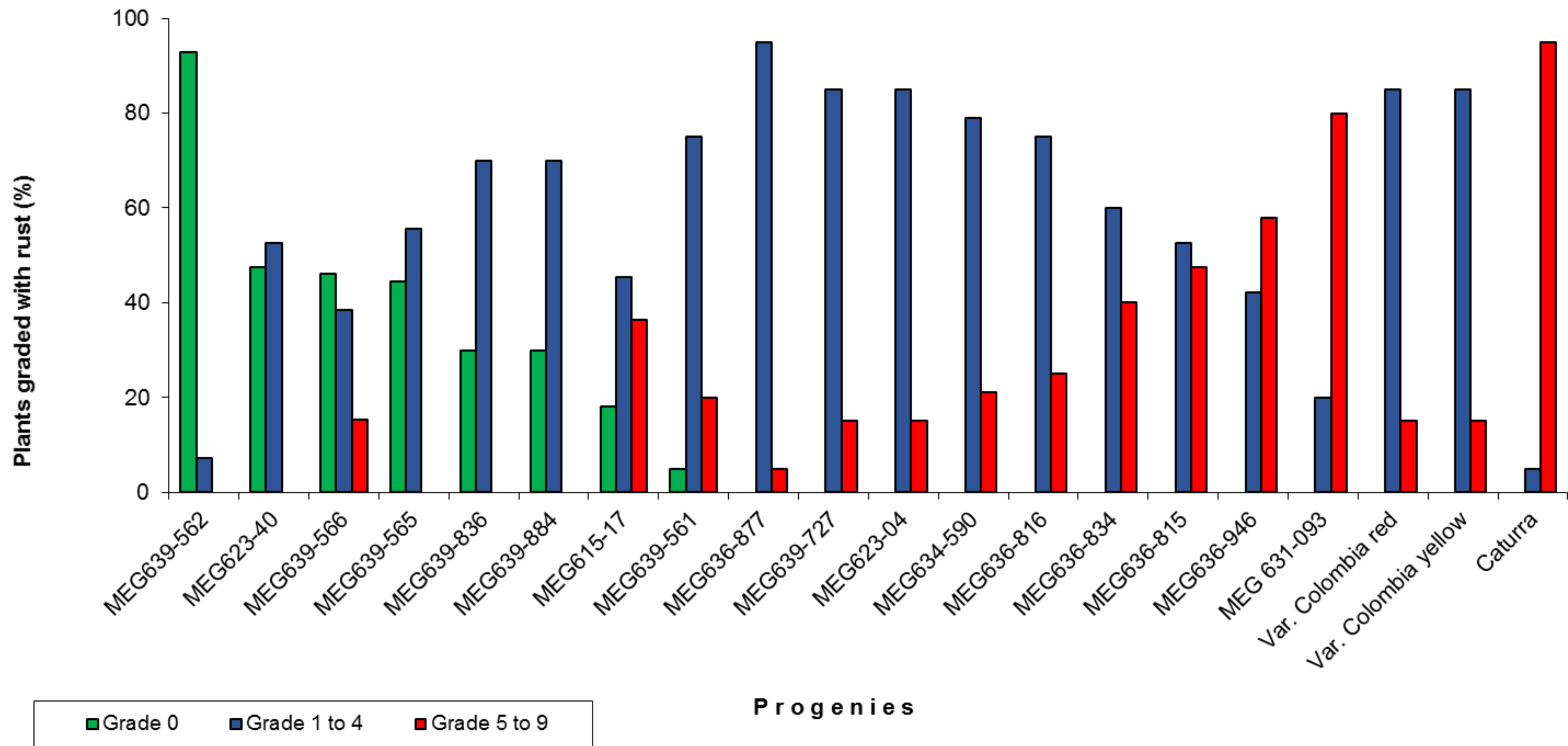
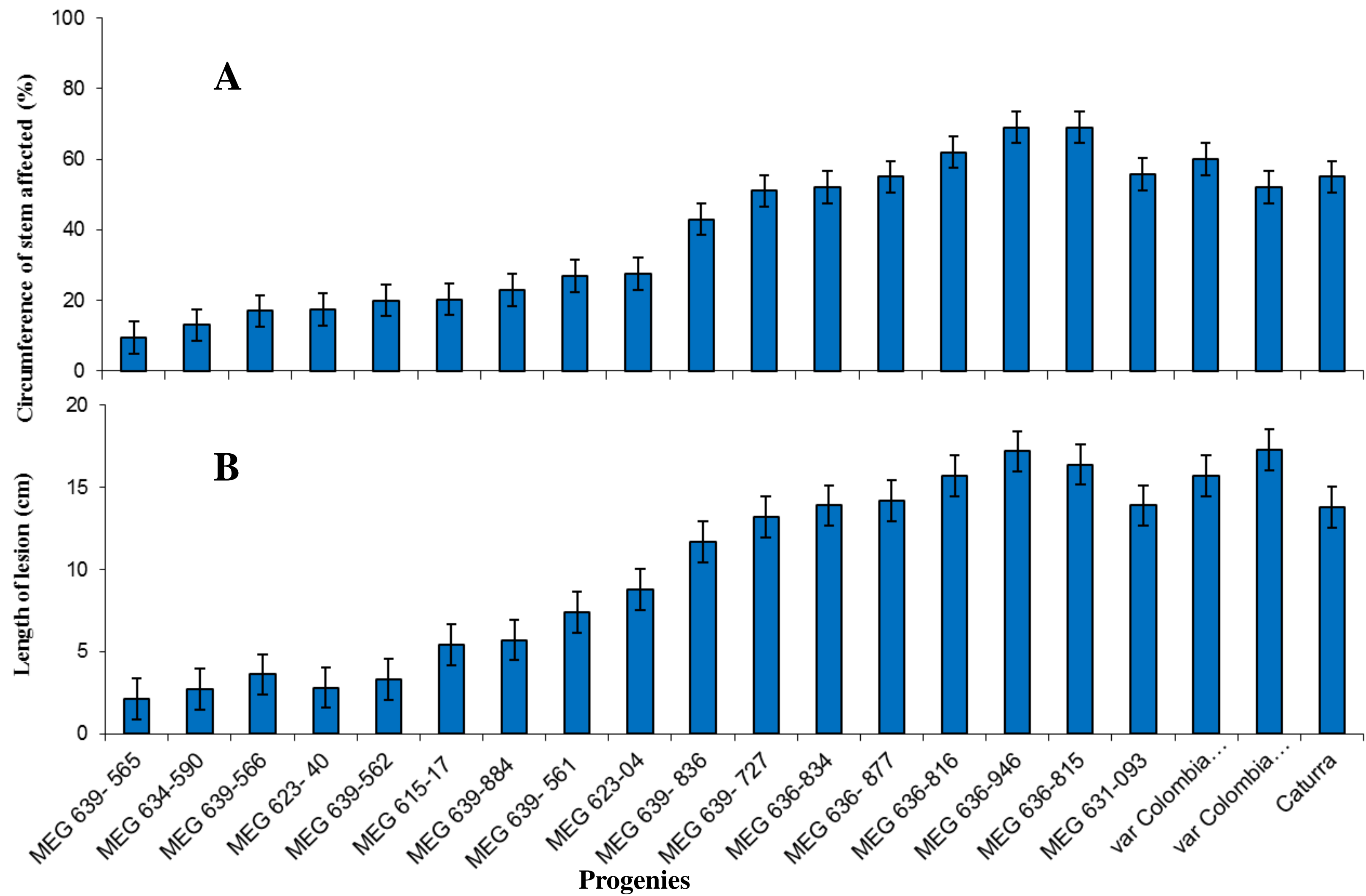

Plants graded with rust (%)
100
80
60
40
20
0
MEG639-562
MEG623-40
MEG639-566
MEG639-565
MEG639-836
MEG639-884
MEG615-17
MEG639-561
MEG636-877
MEG639-727
MEG623-04
MEG634-590
MEG636-816
MEG636-834
MEG636-815
MEG636-946
MEG 631-093
Var. Colombia red
Var. Colombia yellow
Caturra
Progenies
Grade 0
Grade 1 to 4
Grade 5 to 9

Figure 2. Evaluation of coffee progenies to infection by *Ceratocystis colombiana.* One year after inoculation. (A) Percentage of circumference of stem affected (CSA). (B) Length of lesion LL in cm. Tukey'test (P=0.05) and bars represent Standard Error (F=0.05%).


A
B
Circumference of stem affected (%)
Length of lesion (cm)
100
80
60
40
20
0
20
15
10
5
0
MEG 639- 565
MEG 634-590
MEG 639-566
MEG 623- 40
MEG 639-562
MEG 615-17
MEG 639-884
MEG 639- 561
MEG 623-04
MEG 639- 836
MEG 639- 727
MEG 636-834
MEG 636- 877
MEG 636-816
MEG 636-946
MEG 636-815
MEG 631-093
var Colombia…
var Colombia…
Caturra
Progenies

Chapter 6

Selection of advanced Coffea arabica genotypes combining resistance to rust (*Hemileia vastatrix*) and *Ceratocystis* species

ABSTRACT

Coffee Leaf Rust (CLR), caused by *Hemileia vastatrix*, and Ceratocystis Stem Canker (CSC), caused by the soil-borne fungi *Ceratocystis colombiana* and *Ce. papillata*, are two of the most important diseases of coffee in Colombia. High productivity *Coffea arabica* genotypes, resistant to rust and with good cup quality, have been developed and are in the process of being adopted by Colombian coffee growers. However, these varieties are susceptible to CSC resulting in the death of plants at any given age of development. The aim of this study was to select advanced progenies of *C. arabica* with combined resistance to both CLR and CSC. This was achieved through the selection of advanced progenies of *C. arabica* derived from conventional double genotype crosses including F_3 resistant progenies from a line of *C. arabica* var. Borbon (resistant to CSC but susceptible to CLR) crossed with *C. arabica* var Caturra (susceptible to both pathogens) and F_3 progenies crossed with the Timor Hybrid (HDT/1343), (resistant to CLR). Eight F_4 progenies were established in a field experiment and CLR susceptibility assessments were made using an incidence rating scale. *Ceratocystis colombiana* and *Ce. papillata* were inoculated into plant stems to obtain data on susceptibility to CSC. Agronomic and bean characteristics were also evaluated. All progenies had acceptable resistance to CLR, but only one of these had acceptable resistance to CSC, as well as agronomic performance and bean quality.

1. INTRODUCTION

Most of world's coffee production (70%) is based on cultivars of arabica coffee (*Coffea arabica* L.). The majority of these cultivars are, however, susceptible to coffee leaf rust (CLR) caused by the obligate fungus *Hemileia vastatrix* Berkeley & Broome (Basidiomycete, Puccinidiaceae). More than 49 physiological races of *H. vastatrix* have been identified globally (Varzea and Marques 2005; Gichuru *et al*. 2012). CLR race II (virulence gene v5) is the most aggressive and is the most prevalent race on all *C. arabica* cultivating countries, including Colombia (Leguizamón *et al*. 1984; Van der Vossen 2005; Rozo *et al*. 2012). On the other hand, nine dominant genes (S_H) involved in resistance to *H. vastatrix* have been characterized to date (Wagner and Bettencourt 1965; Bettencourt and Rodrigues 1988). Genes S_H1, S_H2 and S_H4 are present in non-commercial *C. arabica* varieties, while S_H5 (gene defeated by CLR race II) is present in commercial varieties of *C. arabica* such as Typica, Borbón and Caturra, among others. Gene S_H3 apparently comes from *C. liberica* W. Bull.: Hiern, and genes S_H6 to S_H9 have been introgressed from *C. canephora* into some introductions of Timor Hibrid (HDT). All derivatives of HDT have one or more of these resistance genes (Bettencourt and Rodrigues 1988; Eskes *et al.* 1990; Várzea & Marques 2005).

For more than 50 years, the HDT and its derivatives have been used worldwide as the main source of rust resistance. This is a self-fertile tetraploid (2n=44 chromosomes) of a spontaneous hybrid between *C. canephora* and *C. arabica* (Bettencourt and Norhona-Wagner 1971; Bettencourt 1973; Eskes 2005). In Colombia, high coffee cup qualities have been achieved through the unique cultivation of *C. arabica* varieties such as Borbon, Typica and Caturra. Breeding programs have relied on the development, and continued selection, of composite cultivars of CLR resistant arabica coffee. Such successful commercial CLR resistant varieties (F_5 and F_6 selections) are: var. Colombia (Castillo and Moreno 1988; Moreno and Alvarado 2000), var. Tabi (Moreno 2002) and var. Castillo® (Alvarado *et al*. 2005). These varieties are derived from crosses between *C. arabica* var. Caturra or var. Typica/Borbon (CLR susceptible) and the HDT/1343.

Although the CLR problem is mostly under control in Colombia, limited attention has been given to the exploration of genetic resistance to other coffee diseases in the country. One of these is the Ceratocystis Stem Canker (CSC) disease commonly

referred to as “llaga macana”, or trunk canker, attributed to infection by *Ceratocystis colombiana* Van Wyk & Wingf. and *Ce. papillata* Van Wyk &Wingf. (Van Wyk *et al*. 2010). Coffee plant infection by *Ceratocystis* species occurs through fresh stem wounds arising from mechanical injuries, such as pruning, but mainly wounds made to the tree bases by workers supporting themselves against the stems on the steep slopes on which coffee is grown in the country (Castro and Montoya 1997; Castro 1999). Losses of between 20-40% have been recorded for CSC, on all commercial coffee varieties planted in Colombia (Castro *et al*. 2003).

Management of CSC has relied on a combination of cultural and chemical control (Castro and Montoya, 1994; Castro and Zuluaga 2012). A line of *C. arabica* var. Borbon known as Brm, resistant to CSC, was identified in Colombia (Fernández 1964). Brm has been crossed with var. Caturra and F_5 progenies were released as CSC resistant, but they are susceptible to CLR (Castro and Cortina 2009). In robusta coffee (*C. canephora*) and in *C. liberica, "*immunity*"* to CSC has been mentioned by Echandi and Fernández (1961) and by Izquierdo (1988).

Two routes can be followed to obtain commercial *C. arabica* varieties combining the resistance against both CLR and CSC. Firstly, by introgression of *C. canephora* or *C. liberica* genes into *C. arabica,* and secondly by double introgression of CSC resistance genes from Bourbon (Brm) and CLR resistance from HDT. For the second route, crosses of (F_3 Brm x Caturra) with HDT/1343 were made, selecting in F_2 and F_3 progenies with double resistance against both CLR and CSC (Cenicafé- unpublished). Following the second route, the aim of this study was to select advanced progenies (F_5) resistant to both CLR and CSC. This was achieved by the establishment of a field experiment, where the resistance to CLR and Ceratocystis canker were assessed. Agronomic performance, yield and bean quality were also evaluated in the genotypes studied.

2. MATERIALS AND METHODS

2.1. Plant material and field trials

Eight F4 coffee progenies derived from a cross between F3 (Brm x var. Caturra) and HDT/1343, encoded as (DF x HDT-PL) were used in the study. These progenies were selected from seed-generated plants with CLR and CSC resistance in previous generations (unpublished data). Two controls, *C. arabica* var. Colombia (CLR resistant, but CSC susceptible) and var. Caturra (susceptible to both pathogens), were included.

The experiment was established in a plot having a flat topography at the Cenicafé Central Experiment Station (04°58' NL, 75°39' W, 1381 m) in 2006. The site has an annual average precipitation of 2556 mm, 1816 hours/year of sunshine, RH of 78%, mean temperature of 20.8°C, minimum of 16.4°C and maximum of 26.8°C (Cenicafé 2012). The progenies were planted in a completely randomized design. The experimental unit was a row of 12 plants with a distance of 1.30 m between plants and 1.50 m between rows and included five replicates. Standard agronomic management was applied.

2.2. Coffee leaf rust (CLR) evaluation

Incidence of *H. vastatrix* was assessed from January 2007 to August 2010, at the peak of CLR outbreaks, as determined by Sierra *et al.* (1991). The scoring scale of Eskes and Braghini (1981) was used to evaluate plants. This scale grades the whole plant as a unit of observation on a visual scale (0 to 9), where 0 = absence of sporulating lesions; 1= presence of one diseased branch; 2 to 8= gradual increase in number of diseased based branches with sporulating lesions; and 9= maximum disease incidence. At each evaluation, the number of plants with rust was scored and grouped into three categories: uninfected plants (grade 0) or complete resistance; resistant plants with low levels of rust (graded 1 to 4), and susceptible plants with high levels of infection (graded 5 to 9). The maximum grade reached by each plant was noted along with the frequencies of plants in each grade. Progeny with ≥ 70 % of CLR (graded 0-4) were selected as resistant, following the criteria of Alvarado and Cortina (1997) and Moreno and Alvarado (2000).

2.3. Ceratocystis stem canker (CSC) evaluation

When F_4 coffee plants reached four years of age, they were inoculated with two isolates of *Ceratocystis*, *Ce. colombiana* (CMW5768) and *Ce. papillata* (CMW10844). Fungal isolates were obtained from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa, and represented the original isolates used by Van Wyk *et al*. (2010). The fungi were prepared following the technique described by Marin *et al*. (2003). Drops of inoculum (70µl), containing 5.0 x 10^4 ascospores/ml^{-1}, were placed into inverted U-shaped wounds, approximately 2.0 cm in diameter. Isolate CMW5768 was inoculated on the lower part of the trunk (~15.0 cm above soil line) and isolate CMW10844 was inoculated in the upper part of the trunk (~160.0 cm above soil line). The inoculum was inserted under the bark and covered with moistened cotton wool and sealed with Parafilm® (Pechiney Plastic Packaging, Chicago, IL) to reduce desiccation. Ten days post-inoculation, the cotton wool was removed and pathogen colonization was evaluated.

Inoculated plants were monitored monthly for external symptoms such as yellowing, wilting or death. Seventeen months after inoculation, disease susceptibility was evaluated as described by Castro and Cortina (2009). External and internal lesions were assessed by peeling the bark from each plant in order to view the cambial surface. Three measurements were made for each plant, including stem circumference (SC), width of necrotic lesion (WNL) and lesion length (LL) for both inoculation points. The circumference of the stem affected (CSA) by the discoloration was expressed as percentage (WNL/SC x 100). Analysis of variance (ANOVA) was performed for CSA and LL data ($p=0.05$), using Duncan's test ($p=0.05$). Resistant genotypes were selected as those where the average CSA was less than 50% of the trunk circumference for both points of inoculation, and with LL smaller than controls, using the same criterion of Castro and Cortina (2009).

2.4. Evaluation of agronomic traits

All coffee plants were evaluated for plant height (cm) at 24 months of age in 2008. Yield data (Kg of fresh cherries/plant) were recorded from 2007 to 2009. The

percentage of bean defects such as peaberry, triangle and deformed beans were also evaluated at the peak of harvest in 2007 and 2008. The size of dry beans (Supreme type), determined as the percentage of husked beans (green coffee) retained in a mesh of 17/64 inches, were recorded following the methods of Castillo and Moreno (1988) and consistent with accepted coffee quality standards (FEDERACAFE 1988). ANOVA was calculated for plant height, bean characteristics, annual production and average yields for the cumulative harvest was also assessed. When statistical differences were found, Dunnett´s test was used to compare with var. Caturra data. All analyses were made using SAS (SAS Statistical Software 2010). Average of agronomic traits equal to, or better than those of the variety Colombia (Castillo and Moreno 1988) was used as the criterion to select promising plants for future use.

3. RESULTS

3.1. Coffee leaf rust (CLR) evaluation

High rust levels were observed in the Caturra controls in 2007, with an average of 88.0% of the plants affected in grade >4. In comparison, all F_4 progenies and var. Colombia controls had low levels of rust incidence (grades 0 to 4). Rust incidence declined slightly in 2008, with 60% of incidence >4 in var. Caturra. The maximum level of CLR was noticed in the second semester of 2010 (Figure 1) when the outbreak reached a 90% incidence (grade >4) in the var. Caturra control. In contrast, var. Colombia had only 18% incidence >4, and all F_4 progenies evaluated had rust incidence grades between 0 and 4.

3.2. Ceratocystis stem canker (CSC) evaluation

Symptoms of yellowing and wilting were noted six months after inoculation with *Ceratocystis* species in some of control plants. Eighty percent of these plants, as well as some plants of the F_4 progeny, died within a year of inoculation (data not shown). Seventeen months after inoculation, all control plants had died. These plants had discolored lesions, characteristic of Ceratocystis canker, that had advanced either upwards or downwards in the phloem tissues of the plants, at one or both points of inoculation (Figures 2a and 2b). Plants that survived in the F_4 progenies, with their stems not girdled at the time of evaluation, had evident lesions, but some exhibited

callus covering parts of the lesions, (Figure 2c). Resistant plants had small lesions (<50% of stem circumference) and extensive callus formation (Figures 2d, 2e). In some of these plants the lesions were long, but callus formation appeared to offset plant death (Figure 2f).

There was variability in the resistance reactions against *Ce. colombiana* and *Ce. papillata* in the progenies tested. Statistical differences in CSA and LL were noticed among progenies and controls caused by both species of *Ceratocystis* (Figures 3A and 3B). *Ceratocystis colombiana* (CMW5768), inoculated in the lower stem, caused average WSA of 50.7 to 86% ($F= 3.4$; $p>0.0003$) and LL between 10.0 to 21.6 cm ($F=2.11$; $p>0.0002$). Duncan´s test showed that progenies 603- HT- PL 17; 604- HT- PL 18 and 604- HT- PL 32 were the most susceptible, while the remaining progenies displayed some level of resistance. Progeny DF 608 HT- Pl 30 was the most resistant.

Ceratocystis papillata (CMW10844), inoculated in the upper stem, resulted in an average CSA of 55.0 to 70.0% ($F=4.3$; $p>0.0001$) and LL ranging between 9.0 and 15.9 cm ($F=1.9$; $p>0.005$). Duncan's test showed that progenies 603- HT- PL 17; 603- HT- PL 18; 603- HT- PL 32; 603- HT- PL 14 and 603- HT- PL 32 were less susceptible to *Ce. papillata* than *Ce. colombiana*, while the remaining progenies exhibited a similar level of WSA (Figures 3A and 3B). Progeny DF 608 HT- Pl 30 was the most resistant to infection by Ce. *papillata*.

Following the evaluation criteria used, progeny DF 608 HT- Pl 30 was selected as the most resistant to both *Ce. papillata* and *Ce. colombiana.* Plants that exhibited small lesion size (CSA<50% and LL between 2.0 to 20 cm), and callus covering completely the lesions were selected as resistant to both *Ceratocystis* species.

3.3. Evaluation of agronomic traits

The height of progenies ranged between 1.27 to 1.45 m. Although statistical differences were noted ($F=17.6$, $p<0.0001$) with the controls Caturra (140 cm) and var. Colombia (143 cm), all progenies were within the desired height of the dwarf type (Castillo and Moreno 1988) and as acceptable by coffee growers.

In bean quality (Table 1), some progenies had peaberry values statistically different from controls. The frequency of triangle and deformed beans was low in all progenies and within the acceptable range (Castillo and Moreno 1988). With respect to the large bean size (Supreme type), all progenies, except progenies DF 604 x H T-Pl 17 and DF 609 x HT-Pl 14, had greater sizes than Caturra (50.5%), and even larger than the var. Colombia control (72.5%). No statistical differences in yield were noted among the progeny and control varieties. Therefore, attributes such as bean size and yield were close to the Colombian commercial varieties (Moreno and Alvarado 2000).

Based on its resistance to infection by *H. vastatrix* and infection by *Ce. colombiana* and *Ce. papillata,* progeny DF 608 HT- Pl 30 was considered as the most promising progeny for commercial use. This genotype also had acceptable agronomic characteristics and bean size.

4. DISCUSSION

Most coffee breeding research globally, including Colombia has been focussed on achieving resistance against CLR, due largely to its economic importance in C. arabica varieties (Van der Vossen 2005; Rivillas *et al*. 2011; Cressey 2013; ICO 2013). Considering the increasing economic importance of CSC, caused by two *Ceratocystis* species in Colombia, this study aimed to develop *C. arabica* genotypes combining resistance against both CLR and CSC. Using a C. arabica var. Bourbon genotype resistant to CSC, and the HDT/1343 (CLR resistant), we successfully identified one selection with such simultaneous resistance against both diseases and acceptable agronomic characteristics.

In this study, the eight F_4 progenies tested exhibited acceptable resistance to CLR. In some cases, this was even higher than the one found in the previously selected CLR resistant var. Colombia controls. This could mean that although there are rust races compatible with the resistant gene combinations present in the progenies, apparently these races are not predominant in the field, resulting in disease values between levels 1 to 4. In contrast, the incidence (18%) graded >4 in the var. Colombia control, implies that although this variety shares the same resistant parents (HDT/1343), its resistant gene combinations (genes S_H6 to S_H9) can be different than the resistace gene

combinations in the F_4 progenies. In this variety, the increased incidence of rust is also an indication that the population of compatible virulent races is increasing in the field, threatening resistance durability, as it has been reported by several authors (Alvarado 2005; Prakash *et al*. 2005; Varzea & Marques 2005; Silva *et al*. 2006; Diniz *et al*. 2012; Rozo *et al*. 2012). It must be taken into consideration that var. Colombia performs in the field as a composite variety (35 lines) with different arrangements of resistant genes conferred by the HDT/1343 (Castillo and Moreno 1988). However, rust levels in grades >4 affect the production and require chemical treatments (Leguizamon and Arcila 1991; Sierra *et al*. 1995; Avelino *et al*. 1999).

The high levels of susceptibility in the commercial varieties Caturra and Colombia to infection by *Ce. colombiana* and *Ce. papillata* was confirmed in this study. However the resistance reactions noted in the progenies studied were not entirely satisfactory. The resistance against *Ce. fimbriata s.l.* discovered in a Borbon line (Fernández 1964), the parental line of these progenies, had clearly been passed to some progenies (F_5) derived from the crossing of this line with *C. arabica* var. Caturra, (Castro and Cortina 2009). On the other hand, in the previous breeding selections (progenies F_3), with inoculatios of *C. fimbriata s.l.,* more than 80% of plants (in eight progenies selected) survived, exhibiting small lesions (Castro, 2010, unpublished data). On the contrary, the resistance levels found in seven of theese eight F_4 progenies included in the present study displayed a decreasing resistance to CSC. This could be explained by either the segregation in these plant generations or to the fact that more aggressive isolates of *Ce. colombiana* and *Ce. papillata* were used for the inoculations in this study. In the line selected as reistant to CSC, the callus formation displayed confirms the resistance conferred by the parental Borbón line.

The Bourbon and Caturra varieties considered in this study have been recognized by their high productivity and cup quality (Moreno 2002). Bourbon exhibits smaller bean size (Castillo 1978). Currently the coffee market prefers larger grains, similar to those found in the Castillo® variety. Hence, our study allowed the selection of genotypes with larger bean size (82% of Supreme type), even larger than the var. Colombia control (72%) and Caturra (50%). On yield characteristics, similar results in the F_4 progenies compared to Caturra and Colombia control varieties were obtained. However, it is important to consider that such production was evaluated during 2007 to 2009, before

the peak of the rust epidemic occurred in 2009. Therefore, reduction in yield was unnoticed among rust resistant progenies and var. Caturra susceptible to CLR. Overall, this study is the first to select a *C. arabica* variety resistant to both CLR and CSC. Although only one such genotype was found, it represents an important step towards more sustainable coffee cultivation and provides a foundation for future breeding developments.

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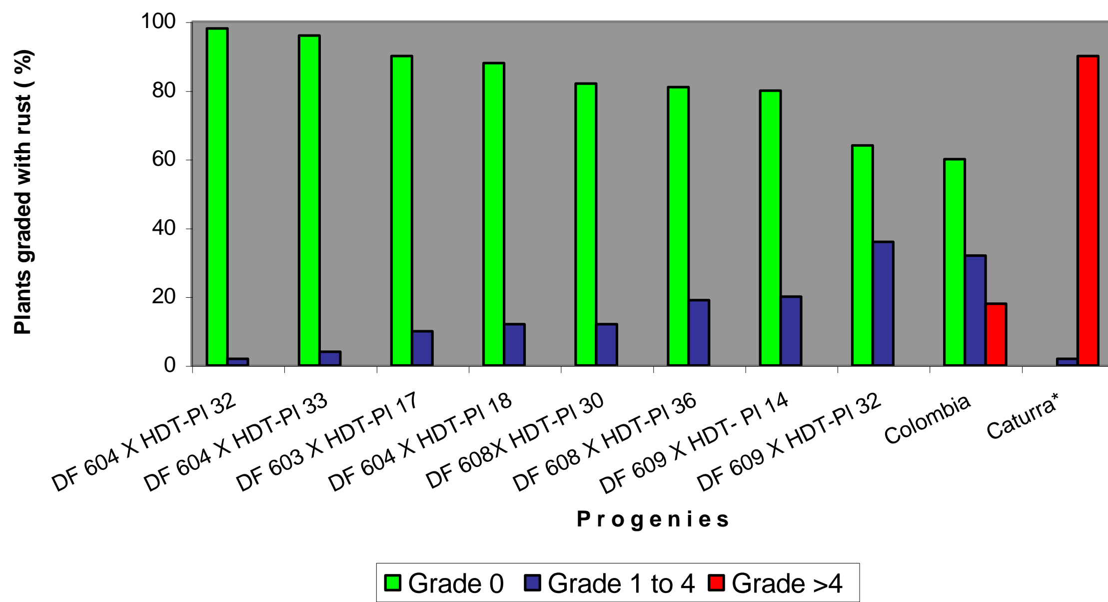
Figure 1: Maximum level of CLR (%) observed in the second semester of 2010 in progenies (F_4 DF x HDT). According Eskes and Braghini scale (0 to 9). Green bars represent uninfected plants (grade 0). Blue bars represent plants graded 1 to 4. And red bars represent plants graded 5 to 9.



Plants graded with rust (%)
100
80
60
40
20
0
DF 604 X HDT-PI 32
DF 604 X HDT-PI 33
DF 603 X HDT-PI 17
DF 604 X HDT-PI 18
DF 608X HDT-PI 30
DF 608 X HDT-PI 36
DF 609 X HDT- PI 14
DF 609 X HDT-PI 32
Colombia
Caturra*
Progenies
Grade 0
Grade 1 to 4
Grade >4

Figure 2. Different aspects of susceptibility/resistance reactions to *Ceratocystis colombiana* and *C. papillata* observed in progenies studied. Bark and phloem necrosis girdling the stem in susceptible controls of Vr. Caturra and Var. Colombia, characterized by the absence of any visible callus (a). Long lesion in the upper point of plant inoculation (b). Plant which displayed attempts of callus formation, but there was discoloration advance (c). Complete resistance reactions, with strong callus which completely replaced necrotized tissues and short size lesion (d - e). Long but narrow lesion caused by the pathogen surrounded by strong callus preventing the girdling on the stem (f).

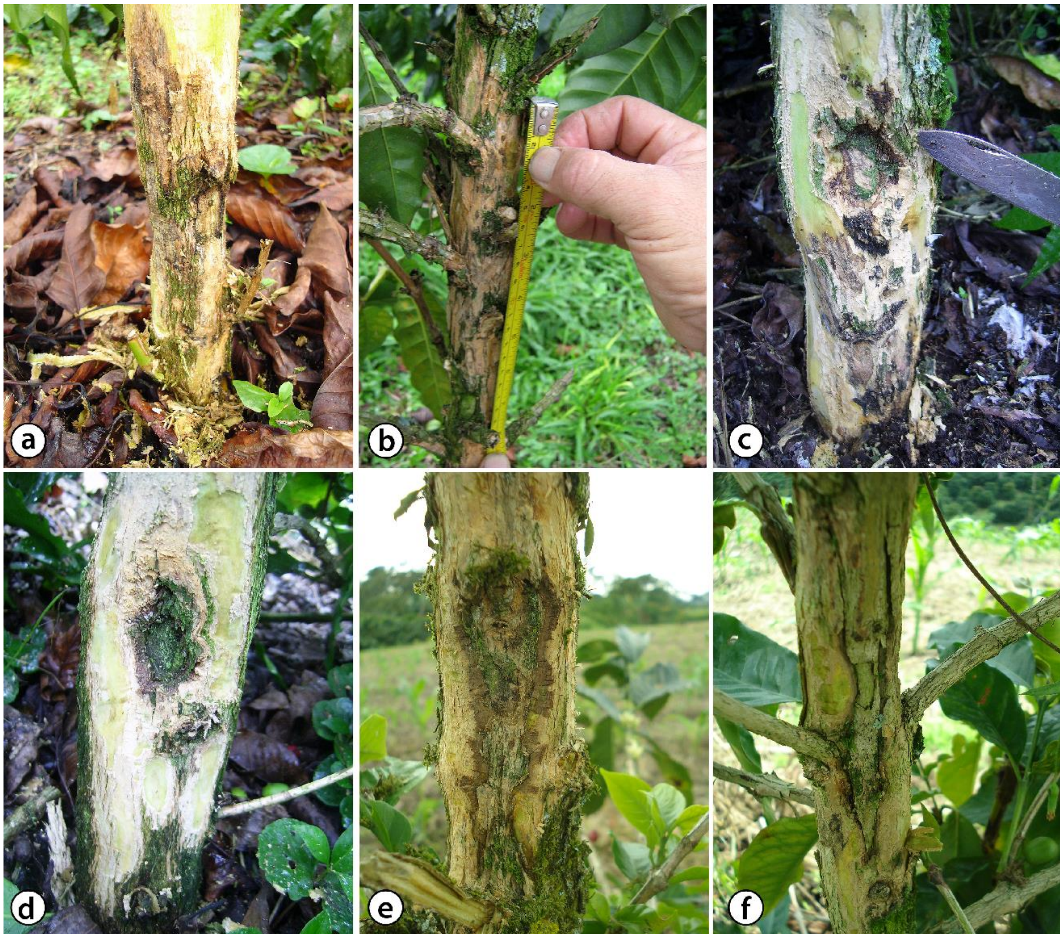
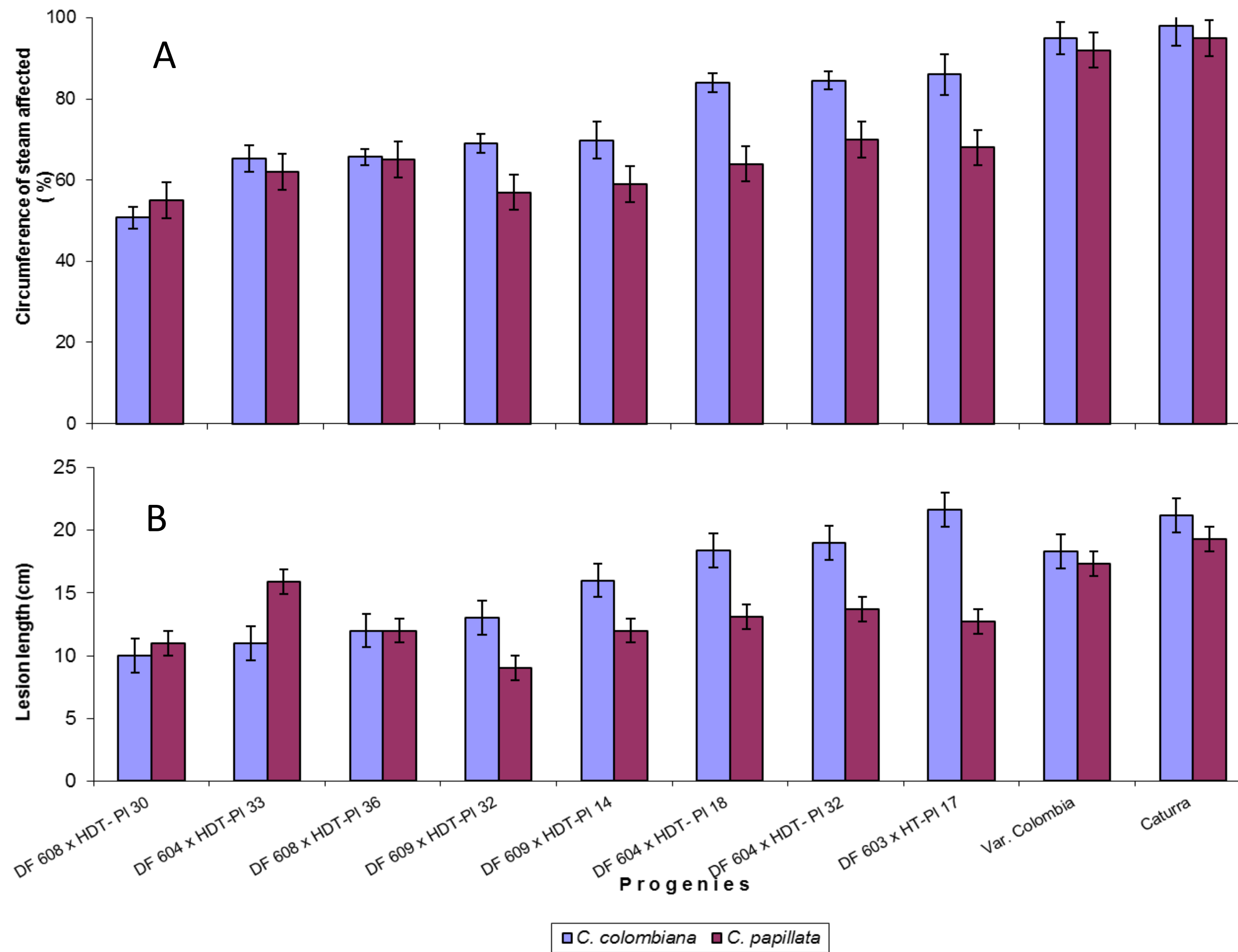

a
b
c
d
e
f

Figure 3. Resistance/susceptibility levels of coffee progenies to infection by *Ceratocystis colombiana* and *Ceratocystis papillata.* Means (%) of circumference of the stem affected (CSA) (A). And lesion lengths (LL) in cm. (B). Seventeen months after inoculation. Duncan's test (p=0.05), and bars represent the standard error (F= 0.05%).



A
B
Circumference of steam affected (%)
Lesion length (cm)
100
80
60
40
20
0
25
20
15
10
5
0
DF 608 x HDT- PI 30
DF 604 x HDT-PI 33
DF 608 x HDT-PI 36
DF 609 x HDT-PI 32
DF 609 x HDT-PI 14
DF 604 x HDT- PI 18
DF 604 x HDT- PI 32
DF 603 x HT-PI 17
Var. Colombia
Caturra
Progenies
C. colombiana
C. papillata

.

Table 1. Average of bean characteristics and yield (kg of berry/plant/year).

Progenies	Grain defects (%)[1]			Supreme type[d] (%)[1]	Yield Average kg
	Peaberry[a] (Mean ± SD)	Triangle[b] (Mean ± SD)	Deformed[c] (Mean ± SD)	(Mean ± SD)	(Mean ± SD)
DF 604 x HT-Pl 33	6.1 ± **2.6 ***	0.6± **0.5 ***	0.0	87.5 ± **6.2 ***	5.2 ± **1.4**
DF 604 x H T-Pl 32	5.7 ± **1.8 ***	0.8±**0.5 ***	0.1± **0.1**	81.2 ± **7.6 ***	5.2 ± **1.5**
DF 609 x HT-Pl 14	9.4 ± **3.4**	1.3± **0.9 ***	0.2± **0.1**	63.8 ±**12.2**	6.4 ± **2.0**
DF 604 x H T-Pl 17	8.8 ± **3.3**	1.3±**0.8 ***	0.1± **0.2**	69.6 ± **15.9**	5.1 ± **1.4**
DF 608 HT- Pl 30	10.8 ± **2.2**	1.3± **0.5 ***	0.5± **0.6**	81.0 ± **3.5 ***	4.5 ± **1.3**
DF 608 x H T-Pl 36	9.5 ± **2.5**	1.4± **0.7 ***	0.5±**0.3**	83.2 ± **10.4 ***	6.6 ± 1.6
DF 604 x HT- Pl 18	6.4 ± **1.7***	0.5± **0.5 ***	0.2± **0.2**	83.3 ±**8.9 ***	6.5 ± **1.5**
DF 609x HT-Pl 32	9.0 ± **1.8**	1.5± **0.7 ***	0.6± **0.7**	70.2 ± **9.3 ***	5.3 ± **1.6**
Var. Caturra	10.2 ± **1.5**	3.9 ± **1.8**	0.1±**0.2**	50.5 ± **13.4**	6.6 ± **1.8**
Var. Colombia	8.9 ± **2.8**	1.1± **0.5 ***	0.4± **0.1**	72.5 ± **18.5 ***	6.8 ± **1.9**
F-value	18.3	15.8	11.2	34.4	0.4
p	0.0001	0.0001	0.0001	0.0001	0.9320

[a] Peaberry : Average percent in three samples of 400 dry parchment beans of two harvest peaks.
[b] Triangle: Average percent in three samples of 400 dry parchment beans of two harvest peaks.
c Deformed: Average percent in three samples of 400 dry parchment beans of two harvest peaks.
[d] Supreme: Average percent of three samples of 100 g of husked beans (green coffee) retained by a 17/64 inch screen.
* Statistical differences (0.05) according Dunnett's test are indicated for each character

Summary

Diseases caused by fungal pathogens are the most prevalent in areas of the world where several varietiesof mainly *Coffea arabica* and *C. canephora* are planted. Among these, coffee leaf rust (CLR) caused by *Hemileia vastatrix* is the most important. In Colombia, 60% of coffee plantations have genetic resistance against the CLR, but other diseases such as root rot and stem canker caused by species of *Rosellinia* and *Ceratocystis* have been recorded causing serious yield reduction. Research for this thesis was aimed at studying primarily the latter two pathogens. Special emphasis was placed on the genetic resistance against Ceratocystis canker in coffee genotypes. A literature review was conducted covering all elements of coffee cultivation and also presenting information pertaining to diseases, especially those caused by species of *Rosellinia* and *Ceratocystis.* A study including DNA sequence analysis was conducted to identify isolates of *R. bunodes* and *R. pepo* found infecting both coffee and other crop species in the Colombian coffee area and showed that both species are important pathogens of coffee in Colombia. Several accessions of the diploid species *C. liberica*, *C. canephora* and the tetraploid Timor Hibrid (HDT)/1343 were tested for resistance to *Ceratocystis colombiana* and *Ce. papillata* . High resistance was exhibited by all the diploid species and moderate grades of resistance was noticed in the HDT. Following this study, combined resistance against CLR and *Ceratocystis* canker was evaluated in two populations of (F_2 and F_3) progenies derived from crosses of *C. arabica* var. Caturra with accessions of *C. canephora* backcrossed to Caturra. Five of these progenies were selected with high resistance to both pathogens as well as with acceptable agronomic characteristics to continue the research in order to obtain commercial genotypes. This led to an evaluation of resistance against CLR and Ceratocystis canker in eight advanced progenies derived from the double crossing between a Ceratocystis canker resistant line of *C. arabica* var. Borbon with *C. arabica* var. Caturra, crossed with the HDT/1343. One genotype was obtained exhibiting acceptable resistance against both pathogens, with acceptable agronomic characteristics and bean quality to be considered as a commercial genotype. The development of advanced coffee genotypes with resistance to both *H. vastatrix* and to Ceratocystis canker, derived from distinct resistance sources, promises to advance integrated disease management of two of the most severe biotic problems affecting Colombian coffee production.