Control of anthracnose (caused by *Colletotrichum gloeosporioides s.l.*) on mango in Senegal by fungicides and biofungicides.

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Keywords: mango, *Mangifera indica*, anthracnose control, *Colletotrichum siamense*, azoxystrobin, thiophanate methyl, Serenade, Sonata, sodium molybdate, ITS, Apn2/Mat, Senegal

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Academic ABSTRACT

Senegal ranks second for mango production among West African countries and has the potential to competitively produce mangoes for the European market. *Colletotrichum gloeosporioides* Penz. and Sacc. has historically been considered the causal pathogen of anthracnose of mango and other fruits, but is now known to represent a species complex. Field trials to examine the efficacy of fungicides and biofungicides were conducted in southern and northern production regions of Senegal. In three southern trials, Sonata (*Bacillus pumilus* strain QST 2808), Serenade Optimum (*Bacillus subtilis* strain QST 713), and sodium molybdate provided 63%, 67% and 76% control of disease severity, respectively, whereas thiophanate methyl and azoxystrobin provided 77% and 78% control, respectively. Disease severities of all treatments were significantly lower than that of the control. Thiophanate methyl and azoxystrobin produced more disease-free mangoes (64 and 62%, respectively) than Serenade (49%), sodium molybdate (44%), and Sonata (38%). Differences within each trial were not statistically significant, but when all trials were combined, all treatments resulted in significantly more disease-free mangoes than the control. Among treatments, thiophanate methyl and azoxystrobin resulted in significantly more disease-free mangoes than Sonata. No results were obtained in the northern orchards due to an absence of disease development. Sequencing of the ITS gene region of 30 *Colletotrichum* isolates from mangoes from different regions in Senegal indicated that all belonged to the *C. gloeosporioides* species complex. Sequencing of the ApMat intergenic region identified all of them as most closely resembling *C. siamense*.

Keywords: mango, *Mangifera indica*, anthracnose control, *Colletotrichum siamense*, azoxystrobin, thiophanate methyl, Serenade, Sonata, sodium molybdate, ITS, Apn2/Mat.

General ABSTRACT

Senegal ranks second for mango production among West African countries and has the potential to competitively produce mangoes for the European Market. The fungal disease anthracnose of mango can cause serious pre- and post- harvest crop losses, especially in southern Senegal, where intensive rainfall, usually over 1,500 mm per year, occurs from late May to October during the mango ripening season and produces conditions very favorable for anthracnose development; in the north, the average rainfall is below 400 mm per year, and anthracnose is serious in only some years. Effective anthracnose control measures are needed in both regions, but especially in the south. The first objective of this study was to examine the efficacy of a fertilizer and two biological control products against anthracnose on mango in Senegal and compare their efficacy with those of standard fungicides. In the south, Sonata (*Bacillus pumilus* strain QST 2808), Serenade Optimum (*Bacillus subtilis* strain QST 713), and sodium molybdate (a fertilizer) provided control of disease severity that was significantly different from the untreated control, however, they were less effective than two chemical fungicides (thiophanate methyl and azoxystrobin). Chemical fungicides also produced a somewhat higher percentage of disease-free mangoes than the biofungicides and fertilizer. No results were obtained in northern orchards due to an absence of disease development in 2015. The second objective was to determine which specific fungus species causes anthracnose disease in Senegal. A number of biologically different species have been distinguished in other part of the world by molecular analysis, but cannot be identified reliably based on symptoms or fungal morphology. Thirty isolates of *Colletotrichum* were obtained from anthracnose lesions of mango in Senegal. Molecular analysis indicated that all isolates most closely resembled *C. siamense*.

Keywords: mango, *Mangifera indica*, anthracnose control, *Colletotrichum gloeosporioides*, *Colletotrichum siamense*, azoxystrobin, thiophanate methyl, Serenade, Sonata, sodium molybdate, ITS, Apn2/Mat.

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Chapter 1: Introduction and Literature Review

Introduction

The mango tree (*Mangifera indica*) is native to the Indo-Burma region, where its history goes back over 4,000 years (Nakasone and Paull, 1998). It was introduced to Senegal in 1824 (Rey *et al.*, 2004), and currently represents the most important fruit produced in Senegal. The national production of mango has increased significantly over the past 20 years, doubling from 2005 to 2011, from 61,646 to 120,000 metric tons/year respectively (FAOSTAT, 2014). It currently generates 40,000 jobs in Senegal (FAOSTAT, 2014). Late-maturing varieties of mango used for export benefit from highly remunerative prices and are subject to increasing use in the Senegalese food industry where they are valued for juice and jam, but more importantly, the sales of fresh mangoes to the European market have increased (CTA 2007). Advantages for Senegal include a high potential for production, product quality, long export season from May to October, and short transit duration (6 days by boat and 6 hours by air) (González, 2014).

However, mango production is exposed to intensive rainfall during the rainy season, from June to September in the north, and especially in the South with high temperature and moisture levels conducive to anthracnose disease development. Anthracnose affects leaves, flowers and fruit, and inoculum is present year-round throughout the canopy (Ploetz, 2003). Fruits may drop from trees prematurely (Nelson, 2008). Ripe fruits affected by anthracnose develop sunken, prominent, dark brown to black decay spots before or after picking. As a consequence, much or all of the mango crop may be destroyed.

A management strategy for anthracnose is needed to protect mango production, especially in wet southern regions of Senegal, but also in the north where anthracnose causes damage in some years.

Systemic fungicides (azoxystrobin and thiophanate methyl) have been tested and have been used with success in Senegal (Diedhiou *et al.*, 2014), but only growers in the north are using them in their orchards. In the past, no biological control agents against anthracnose on mangoes were used or have been tested in Senegal. However, in the north, there are many organic orchards, and knowledge on biological control agents or other alternatives to standard synthetic fungicides can help these organic growers to control anthracnose on mango in Senegal.

Objective

The main overall objective of my thesis is to develop a strategy of mango anthracnose management adapted to the agro-ecological environment in Senegal, which allows the efficient and sustainable management of anthracnose of mango for improved competitiveness in the European market through good phytosanitary quality. Once completed, it would contribute to increased incomes for stakeholders in the mango industry by creating availability of a technology package enabling production of fruits of good sanitary quality that allows mangos to be stored longer and be competitive in export markets.

The specific objectives of this work can be broken down as follows:

- Identify effective fungicides and biological control agents against *C. gloeosporioides* (*sensu lato*) taking into account the biology of the pathogen under southern and northern production conditions in Senegal.
- Identify the species of *Colletotrichum* that cause(s) mango anthracnose in Senegal

Literature Review

Mango, along with banana, grapes, orange, apple and plantain, is one of the most commonly produced fruits in the world. Its cultivation covers a worldwide area of 3,699,434 hectares with an annual production of 35 million metric tons (UNCTAD, 2009). In Senegal, the arboricultural sector has grown considerably as reflected by the increase of harvested areas and quantities produced. The total production of mango has recently been estimated at 118,950 metric tons (ASEPEX, 2012).

Mango (*Mangifera indica* L.) fruit hosts a large number of pathogens, most of them fungi, which are also responsible for rot after the harvest. In Senegal, in addition to the devastating effects from the oriental fruit fly (*Bactrocera dorsalis*), fungal agents play an important role in fruit rot. Fungal agents involved are *Colletotrichum gloeosporioides s.l*, the agent of anthracnose, *Alternaria alternata* and *A. tenuissima* causing alternaria blight, *Botryodiplodia theobromae* and *Dothiorella spp*. responsible for peduncular rot, *Phoma mangiferae*, *Aspergillus niger*, etc. (Mbaye, 2006), (Dodd, Prusky, and Jeffries, 1997), (Okigbo and Osuinde, 2003) and (Arauz, 2000).

Anthracnose is the main disease of mango, and fruit infection occurs during its development, but remains latent until the concentration of antifungal components in the fruit declines at the time of ripening (Prusky, 1996), and the fungus is stimulated by the production of ethylene by the ripening fruit (Freeman, *et al.* 1998). Losses can be enormous especially in condition of high relative humidity (Diedhiou *et al.*, 2007; Arauz, 2000; Estrada, 1994). The microflora responsible for anthracnose of the fruits is primarily a function of climate in the production area (Arauz, 2000).

Management strategies for anthracnose have predominantly relied on four approaches: sanitation, resistant cultivars, biological control agents, and chemical application. In this study, we will focus on two major strategies in order to control anthracnose: Fungicide application and biological control agents.

For the management strategies, chemical application is the most common and successful control for anthracnose. Fungicide application focuses on reducing damage to inflorescences and fruit. The choice of fungicides used therefore depends on the intended destination of the exported fruit. The development of various predictive models of the risk of anthracnose in many cases allowed a reduction of the treatments to the strict minimum needed while maintaining optimal efficiency, as in the Philippines where a fungicide program based on a predictive model replaced 4-6 treatments (Akem, 2006).

Several systemic fungicides have become available that can control infections as well as protect against their establishment (Kumar *et al.*, 2007). Preharvest sprays of these materials can prevent the establishment of latent infections before harvest and reduce disease develoment, thereby considerably improving the effectiveness of postharvest fungicide treatments. The effectiveness of preharvest fungicide treatments to control postharvest diseases depends on the ability to time a limited number of sprays to inactivate inoculum present during the period of crop susceptibility. In some cases, a few well-timed sprays of a systemic fungicide can prevent infection inoculum of *Colletotrichum* responsible for anthracnose. These systemic fungicides have the ability to inhibit development of latent infections of *Colletotrichum* spp. and to penetrate through the host cuticle to reach infection hyphae inside the fruit tissue. Several classes of fungicides have been developed to control anthracnose: especially, demethylation inhibitors, benzimidazoles, and strobilurins (Quinone outside inhibitors, QoIs).

Currently, benzimidazoles and strobilurins are the dominant fungicides; they provide a protective barrier on the plant, permeate into the plant, and move upward in the plant's xylem (acropetal systemic activity). Among fungicides used for controlling anthracnose in Senegal, these fungicides have both protective activity including in new growth, and good curative activity (Diedhiou *et al.*, 2014a). Infection of the flower could be effectively reduced by two sprays of carbendazim (0.1%) at 15-day intervals. Leaf infection can be managed by two sprays of copper oxychloride (0.3%), while latent infection of the pathogen on the fruit could be reduced by spraying before harvest with thiophanate methyl (0.1%). Thiophanate methyl was as effective as benomyl, as both thiophanate methyl and benomyl break down to methyl 2-benzimidazole-carbamate (MBC).

The benzimidazole or methyl benzimidazole carbamate (MBC) fungicides all act by inhibition of nuclear division via inhibition of microtubule assembly during mitosis by binding to β-tubulin subunits and thiophanate methyl is one of this class. The benzimidazoles, primarily thiophanate methyl, provided excellent anthracnose control in the field on mangoes in Senegal (Diedhiou *et al.*, 2007; Diedhiou *et al.*, 2014a).

QoI fungicides inhibit mitochondrial respiration by binding to the Quinone outside site of cytochrome b. This, in turn, blocks electron transfer between cytochrome b and cytochrome c1 and halts ATP production (Bartlett *et al.*, 2002; Sundravadana *et al.* 2007). They have broad spectrum activity against the four major groups of plant pathogenic fungi, the including Ascomycota, Basidiomycota, Deuteromycota, and Oomycota. Azoxystrobin is one among the strobilurin class of systemic fungicides which disrupts some vital stages of fungal development (Hsiang *et al.*, 2004). Sundravadana *et al.* (2006) reported that azoxystrobin inhibits 100 % of mycelial growth of *C. gloeosporioides*. There are many reports on efficacy of azoxystrobin against anthracnose on mangoes (Diedhiou *et al.*, 2007; Diedhiou *et al.*, 2014a; Sundravadana, 2006).

However, overuse of fungicides can lead to development of resistance. Kumar (2007) showed that *Colletotrichum gloeosporioides* in India was moderately resistant to thiophanate-methyl. In addition, Meng-Jun Hu (2015) showed that *Colletotrichum siamense* from peach and blueberry in South Carolina, USA was resistant to azoxystrobin and thiophanate methyl.

In addition to the potential for development of resistance to some fungicides, environmental safety concerns have provided another impetus for development of alternative approaches (resistant cultivars, cultural practices, and biofungicides) to control diseases without using synthetic chemicals (Conway *et al.*, 1991; Sugar *et al.*, 1997; Wilson *et al.*, 1997; Janisiewicz *et al*., 2002; Hewajulige *et al*., 2010). Reports of efficacy in the field have been limited until Govender et *al.* (2006) demonstrated that *Bacillus licheniformis* controls anthracnose of mango. Senghor *et al.* (2007) showed that *B. subtilis* significantly reduced anthracnose incidence in ripening fruits. Moreover, Peralta (2004) showed that Virtuoso 10 AS (*Bacillus subtilis* QST 713 strain) had excellent activity in suppressing or reducing the severity of anthracnose on mango.

Some fertilizers have been used as fungicides, and some of them have been found effective as well for controlling anthracnose. According to Dutta *et al.* (1981); Miller *et al.* (1983); and Júnior *et al.* (2004), molybdate reduced the intensity of several diseases on bean and tomato. In addition, Polanco et *al.* (2014) stated that molybdate application decreased the area under the disease progress curve (AUDPC), could be because of its direct effect on *C. lindemuthianum*, thus increasing yield. However, overall, as many diseases are are not affected as are reduced, and some may be increased at certain molybdate levels (Graham and Stangoulis 2005).

The fungal species responsible for mango anthracnose has been called *Colletotrichum gloeosporioides* for many years. However, with the advent of molecular methods for pathogen identification, the number of reports naming other species of Colletotrichum infecting mangoes has increased. These species are often morphologically virtually indistinguishable. *Colletotrichum gloeosporioides* has recently been epitypified with a living strain (Cannon *et al.*, 2008). Subsequent to this, Phoulivong *et al.* (2010) showed that the newly epitypified *C. gloeosporioides* (*sensu stricto*) was not a common pathogen on fruits in the tropics and this has been confirmed with DNA sequence data (Phoulivong *et al.*, 2010; Lima *et al.*, 2013; Sharma *et al.*, 2013). Other species that have been reported as infecting mango fruit are *Colletotrichum asianum, Colletotrichum fructicola, Colletotrichum boninense, Colletotrichum tropicale* and *Colletotrichum karstii, Colletotrichum dianesei, Colletotrichum horii, Colletotrichum kahawae* (Phoulivong *et al.*, 2010; Damm *et al*., 2012; Lima *et al*, 2013; Ismail *et al*., 2015), and *Colletotrichum siamense* (Weir *et al*, 2012; Udayanga *et al*, 2013). Molecular methods have been used successfully in differentiation between species of *Colletotrichum* because of analysis of the nucleotide sequence of the internal transcribed spacer (ITS) of the ribosomal DNA (rDNA), glyceraldehyde–3–phosphate dehydrogenase (GPDH), histone 4 (*his4*), glutamine synthase (GS), b–tubulin 2 (TUB 2) (Prihastuti *et al*., 2009; Lima *et al*., 2013; Sharma *et al*., 2013; Vieira *et al*., 2014; Pardo-De la Hoz *et al*., 2016). However, ITS sequences alone are insufficient to resolve the biological and genetic relationships within the broad *C. gloeosporioides* clade. Weir *et al.* (2012) said that TUB2 or GS are the best to distinguish *C. aenigma* (*C. populi*) from *C. alienum* and some *C. siamense* isolates. More recently, the Apn2/MAT locus has been used by Silva *et al.* (2012) to improve phylogenetic resolution of the *Colletotrichum gloeosporioides* species complex. Moreover, Liu *et al.* (2015) showed that the combination of the loci ApMat (intergenic region of Apn2/MAT) and GS in a phylogenetic analysis was able to resolve all currently accepted species in the *C. gloeosporioides* species complex.

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Chapter 2: Control of anthracnose of mango in Senegal by fungicides and biocontrol

Abstract

Mango production in the South of Senegal is exposed to intensive rainfall from late May to October, with high temperature and moisture levels. These conditions are conducive for the development of anthracnose caused by *Colletotrichum gloeosporioides* (*sensu lato*) and produce a need for adequate control measures. Anthracnose disease causes pre- and postharvest fruit spots, fruit rot, and premature fruit drop. The purpose of this study was to examine the efficacies of several fungicide alternatives (fertilizers and biological control agents) against anthracnose of mango in Senegal and compare with those of standard fungicides. In three southern trials, Sonata (*Bacillus pumilus* strain QST 2808), Serenade Optimum (*Bacillus subtilis* strain QST 713), and sodium molybdate provided 63%, 67% and 76% control of disease severity, respectively, whereas thiophanate methyl and azoxystrobin provided 77% and 78% control, respectively. Disease severities of all treatments were significantly lower than that of the control. Thiophanate methyl and azoxystrobin produced more disease-free mangoes (64 and 62%, respectively) than Serenade (49%), sodium molybdate (44%), and Sonata (38%). Differences within each trial were not statistically significant, but when all trials were combined, all treatments resulted in significantly more disease-free mangoes than the control. Among treatments, thiophanate methyl and azoxystrobin resulted in significantly more disease-free mangoes than Sonata. No results were obtained in the northern orchards due to an absence of disease development.

Keywords: mango, *Mangifera indica*, anthracnose control, *Colletotrichum gloeosporioides*, azoxystrobin, thiophanate methyl, Serenade, Sonata, sodium molybdate

2.1. Introduction

Mango producing areas in Senegal are mainly in the north (Niayes area) and the south (Casamance). The Niayes area (the coastal area between Dakar, Thies, and Saint-Louis) is the main producer of mangoes for export (Mbaye *et al*, 2006) accounting for 80% of export volume. Casamance (Ziguinchor, Sedhiou, and Kolda) is the second main producer of mango for export. This crop is commercialized in the international market that is increasingly competitive and must comply with increasingly high standards. In order to meet demands of consumers who want fruit without flaws, healthy, tasty, and beautiful, but without pesticide residues, growers must resolve conflicting issues. They need to produce fruit of the desired quality, in accordance with regulations and specifications, without neglecting the yield needed to guarantee profitability. Despite the production potential in Senegal, environmental conditions that favor pathogens in the field and after harvest interfere with the ambitious goal to produce a high quality crop, which satisfies stringent export quality standards, in substantial tonnages.

Diseases of mango are often caused by fungi. The early stages of infection occur in the field, with resulting fruit rot developing during ripening and storage. In Senegal, fungi play an important role in post-harvest rot of mangoes and generate significant financial losses (Mbaye *et a.l*, 2006; Diedhiou *et al*., 2007). Among the fungal diseases causing fruit rots, anthracnose (Figure 1.1) is the most important mango disease. Depending on the agro-climatic zones and the

occurrence of rainfall, the disease can lead to very heavy losses (Diedhiou *et al.*, 2014b). The consequences of the disease are a shortening of the export window, and high risks of the rejection of cargo at the port of the importing country; therefore, this disease can cause large immediate financial consequences as well as a long-term loss of markets.

Several systemic fungicides have become available that can control infections as well as protect against their establishment (Kumar *et al.*, 2007). Preharvest sprays of these materials can prevent the establishment of quiescent infections before harvest and reduce disease pressure, thereby improving considerably the effectiveness of postharvest fungicide treatments. There are many reports on the efficacy of azoxystrobin and thiophanate methyl against anthracnose on mangoes (e.g., Diedhiou *et al*., 2007; Diedhiou *et al.* 2014a; Sundravadana, 2006).

However, because of health and environmental concern, some alternative approaches (resistant cultivars, cultural practices, and biofungicides) have been developed to control diseases without using chemical methods (Conway *et al.*, 1991; Sugar *et al.*, 1997; Wilson *et al.*, 1997; Janisiewicz *et al*., 2002; Hewajulige *et al*., 2010). Reports of efficacy in the field have been limited until Govender *et al* (2006) demonstrated that *Bacillus licheniformis* at 10^7 cfu mL^{-1} controls anthracnose of mango. Senghor *et al* (2007) showed that *B. subtilis* reduced anthracnose incidence in ripening, bagged fruits significantly (56.4% in 2003 and 51% in 2004). Moreover, Peralta (2004) showed that Virtuoso 10 AS (*B. subtilis* QST 713 strain) had excellent activity in suppressing or reducing the severity of anthracnose on mango.

The purpose of this study is to examine the efficacies of several fungicide alternatives (fertilizer and biological control agents) against anthracnose disease on mango in Senegal and compare their efficacies with those of standard fungicides.

2.2. Materials and Methods

2.2.1. Protective application laboratory experiment in Virginia, 2015: In this experiment, an isolate was used that was obtained from anthracnose-affected mangoes from the Homestead area in Florida which were generously provided by Dr. Jerry Bartz's lab at the University of Florida. Mango fruits from Mexico were bought from a local grocery store. Half of the surface of each fruit was wounded with a scalpel to a depth of approximately 10 mm, with approximately 50 punctures per fruit. After wounding, mangoes were sprayed with Sonata (*Bacillus pumilus* strain QST 2808, 1.17 ml/100 ml of sterile deionized water) or Serenade (0.25 g/100 ml) and see table 2.1 for company information. The non-treated control mangoes were sprayed with sterile deionized water. The mangoes were incubated in plastic boxes at room temperature for 2 days. They were then inoculated with the Florida isolate of *Colletotrichum sp.* by spraying with a spore suspension from a 7-day old culture on PDA, and determined to contain 27 x 10^3 spores/ml using a hemacyometer. They were incubated again in the plastic boxes with high humidity maintained by spraying sterile water. Four mangoes were used for each treatment, and there were three replications. The same experiment was conducted on non-wounding mangoes and disease incidence and severity was recorded every 2 days.

2.2.2. Effect of sodium molybdate on conidial germination and mycelial growth: In order to determine whether sodium molybdate has a direct effect on germination or mycelial growth of the mango anthracnose pathogen, two in-vitro experiments were carried out. A conidial

suspension (35 x 10^4 conidia per ml) was prepared and mixed 1:1 with a solution of 400 µg/ml sodium molybdate, and after 1 or 60 min of incubation, 0.1 ml of the mixed suspension was spread on a PDA plate. Spore germination was viewed after overnight incubation at room temperature. In addition PDA plates amended with 200 µg/ml sodium molybdate were prepared, and after they had solidified, mycelial plugs were placed in the center of five replicate plates per treatment; unamended PDA was used as a control. Colony growth was recorded daily by taking two measurements at right angles for each plate, until growth reached the edge.

2.2.3. Post-infection application laboratory experiment in Senegal, summer 2015: Mangoes from different cities were treated with the same fungicides, biofungicides, and fertilizer as were used in field trials (Table 2.1). One hundred ml spray material was prepared at the concentrations reported in Table 2.1. Five mangoes per tree per treatment were used with three replications. The mangoes had been exposed to the natural inoculum prior to harvest because they were harvested early in August, two months into the rainy season. Some of the mango fruits already showed lesions, but only mangoes without visible lesions were selected for the experiment. Treatments were applied by spraying with motorized sprayer. After treatment, the fruit were allowed to dry for 2 hours, and incubated in the lab at ambient temperature. In order to distinguish coalesced lesions from individual ones, lesion numbers were recorded every 2 days after treatment.

After 8 days, the pathogen was isolated for identification with the following technique: the mangoes were first soaked in a 10% bleach solution for 5 minutes, and two crossed incisions in the form of a V were made at the edge of a lesion with a sterile scalpel. A piece of flesh under the peel was taken and placed in a Petri dish containing potato dextrose agar and incubated at room temperature. Two days later, the mycelium growing out of the mango flesh was transferred into new Petri dishes to obtain pure cultures of the fungi.

2.2.4. Experimental fields in summer 2015: Studies were conducted in two regions of Senegal. In the North (Thies area), the experiments were set up in three orchards. Each orchard is more than 100 ha of commercial mango cultivars (Kent and Keitt). These orchards are modern operations, with mango trees planted at high density and trees kept small through the use of rootstocks and intensive pruning (Figure 2.2). All three orchards are certified (GlobalG.A.P.) organic (no synthetic pesticides are used), and only biofungicide and fertilizer treatments were tested**.** The treatments were begun before the start of the rainy season (usually, June to October). Sprays were applied twice at a 16-day interval, on June 9th and June 25th 2015.

In the South (Ziguinchor area), the trials were also set up in three orchards. These three orchards are considered traditional where mango, citrus, cashew, and papaya trees are grown mixed together in no particular pattern (Figure 2.3). All three orchards are conventional, and all treatments listed in Table 2.1 were tested. The treatments were begun before the start of the rainy season. Sprays were applied twice at a 16-day interval. The first treatment was applied on June 16th and the second on July 2nd 2015. Between the two treatments, the rainfall was 18 mm, with measurable rainfall on 5 days. From the second treatment to the harvest time, the rainfall was 339 mm, with measurable rainfall on 12 days. This rainfall was recorded at the Senegalese Institute of Research in Agriculture (ISRA), within 1 km from the three orchards.

In the south, light rain (0.5 mm), probably lasting no more than 30 minutes, occurred the night after the first treatment. However, the treatment materials had time to dry since the treatment was

applied early in the morning and the rain started at night. A motorized sprayer (mist blower) was used for the applications (Figure 2.4). The motorized sprayer released a mist of small droplets, which reached approximately 6 m high (Figure 2.5). Therefore, although the entire tree was sprayed in the North, in the South only the bottom 6 m was sprayed; the top of the tree was not covered with treatments.

2.2.5. Experimental design and treatment rates: The experimental design was completely randomized design for all six locations. An individual tree (surrounded by untreated trees) was the experimental unit, and there were three replications. Treatment rates were calculated based on the assumption of 121 trees/ha in the north or 100 trees/ha in the south (Table 2.1).

Table 2.1: Field treatments and rates applied in the north and the south.

Treatment	Formulation and company information	a.i./hectare	a.i. in 10 L water per 3 trees in the south	a.i. in 10 L water per 3 trees in the north
Azoxystrobin	Ortiva 250 SC (Syngenta)	425 ml	13 ml	11 ml
Thiophanate methyl	Fongsin 450 SC (Savana)	1,134 ml	36 ml	30 ml
Bacillus pumilus QST 2808	Sonata (Bayer)	4,732 ml	142 ml	117 ml
Bacillus subtilis QST 713	Serenade Optimum (Bayer)	992 g	30 g	25 g
Sodium molybdate	(Alfa Aesar)	90 g	3 g	2 g

[a] Azoxystrobin and thiophanate methyl treatments were not used in the north.

2.2.6. Sampling, disease evaluation, and statistical analysis: Mango fruits were collected at maturity or 27 days after the second treatment application (July 22nd in the north and July 29th in the south**)**, 5 fruits per tree, selected arbitrarily, placed in plastic bags and transported to the laboratory. At the laboratory, the fruits were maintained in a clean and ventilated area at room temperature (26-29° C) on a benchtop (Figure 2.6). The numbers of anthracnose lesions were recorded every 2 days to distinguish coalesced lesions from individual ones, for a total of 8 days. The same evaluation procedure was used for the summer laboratory experiment with naturally infected mangoes. Each treatment consisted of three replicates with a total of 15 fruits, and the means of number of lesions for each replication were used for statistical analysis.

For both the two laboratory experiments and the field trials, the mean disease lesion count per fruit and number of disease-free fruits per treatment were analyzed with the generalized linear

mixed model procedure (PROC GLIMMIX) of SAS version 9.4 (Cary, NC, USA). For the number of disease-free fruits, a negative binomial distribution was assumed, and for the disease lesion counts, a Poisson distribution was assumed. Once the effect of treatment was found to be significant, Fisher's least square difference (LSD) was used for the mean separation.

2.3. Results

2.3.1. Protective application laboratory experiment in Virginia, 2015: When the effect of wounding and treatment were examined with ANOVA, the results showed that although non-wounded fruits had more lesions per fruit, the effect of wounding was not significant ($F_{1,4} = 6.19$ $P = 0.07$) with 95% confidence level, but the effect of treatment was significant ($F_{3,15} = 21.70$ $P < 0.01$). The mean number of lesions per fruit in the inoculated control was significantly higher than the other treatments while the other treatments did not differ from each other (Table 2.2). The ANOVA results also indicated that there was no significant effect of wounding ($F_{1,4} = 1.09$ $P = 0.36$) or treatment ($F_{3,15} = 0.80$ $P = 0.51$) on the number of diseased fruit, which was probably due to low sample number (four per replication).

Table 2.2: Mean number of lesions on wounded and non-wounded Mexican mango fruit-halves inoculated with a Florida isolate, 8 days after treatment in the laboratory.

	Mean number of lesions per fruit			Number of diseased fruit[a]		
Treatment	Wounded	Non-wounded	Mean[b]	Wounded	Non-wounded	Mean
Control (inoculated)	77	201	138 A	3	3	5
Sonata	15	15	15 B	3	2	2.5
Serenade	5	20	12 B	3	2	2.5
Control (non-inoculated)	0	0	0 B	0	0	0

[a] n=4 mangoes/treatment

[b] Treatments followed by the same letter are not significantly different ($P < 0.05$). A generalized linear mixed model was used for ANOVA (PROC GLIMMIX, SAS 9.4), and Fisher's LSD was used for the mean separation.

2.3.2. Effect of sodium molybdate on conidial germination and mycelial growth: Conidia that had been incubated in a solution of 200 µg/ml sodium molybdate for 1 or 60 minutes, and were then plated on PDA, germinated at near-100%, indistinguishable from the control. Germ tube lengths were 76 and 71% of those in the control for conidia incubated 1 or 60 minutes, respectively, in sodium molybdate solution (significantly different from the control at $P<0.05$) Radial mycelial growth on PDA with 200 µg/ml sodium molybdate averaged 13.1 mm per day, whereas on unamended PDA it was 12.7 mm per day (difference not significant at P=0.28).

2.3.3. Post-harvest application laboratory experiment in Senegal, summer 2015: The ANOVA results for the post-harvest treatments showed that the treatment effect was significant on both the mean number of lesions per fruit ($F_{5,70} = 13.60$ $P < 0.01$) and the number of disease-

free fruits ($F_{5,70}$ = 3.23 P = 0.01). The non-treated treatment resulted in significantly higher mean lesion numbers than the other treatment (Table 2.3). Among the other treatments, thiophanate methyl and azoxystrobin resulted in significantly more disease-free fruits than Sonata (Table 2.3). The non-treated treatment resulted in significantly lower number of disease-free fruit than the other treatment (Table 2.3), but none of the other treatments were different from each other.

Table 2.3: Effect of post-harvest treatments on anthracnose control in naturally infected mangoes after 8 days of incubation in the lab.

Treatment	Mean number of lesions per mango[a]	Disease-free fruits (%)[ab]
Control	140 A	0 A
Sonata	55 B	53 B
Sodium molybdate	43 BC	47 B
Serenade	31 BC	67 B
Azoxystrobin	23 C	60 B
Thiophanate methyl	20 C	67 B

[a] Treatments followed by the same letter are not significantly different (P < 0.05). A generalized linear mixed model was used for ANOVA (PROC GLIMMIX, SAS 9.4), and Fisher's LSD was used for the mean separation.

[b] n=15 mangoes/treatment

2.3.4. Field experiments in summer 2015: In the orchards in the north, no disease developed because mango fruit was harvested before significant rain fell, and the pathogen requires warm and humid conditions to infect. Therefore, all results reported are from the southern region.

When the ANOVA was conducted to examine the effect of location, treatment and their interactions on the number of lesions per fruit among the three orchards, only treatment effect was significant (Table 2.4). Thus, there was no significant difference among locations and no significant interaction between location and treatment, indicating that differences among treatments across three locations were very similar (Table 2.5). The non-treated fruit had significantly higher mean lesion numbers than other treatments (Table 2.5).

For the number of disease-free fruit, both location and treatment were significant (Table 2.4). The lack of significant effect of the location and treatment interaction effect indicated that while there were differences among locations, the relative differences among treatments within a location were similar in all three locations. Badjo's location had a significantly lower number of disease-free fruit (P < 0.05) than the other two locations. The differences in the number of disease-free fruit among treatment are shown in Table 2.5. The non-treated fruit had significantly higher mean lesion numbers than the other treatment. Among the other treatments, thiophanate methyl and azoxystrobin resulted in significantly more disease-free fruits than Sonata (Table 2.5).

Table 2.4: ANOVA table for the effect of location, treatment, and their interaction on the mean number of lesions per fruit and the number of disease-free fruit

			Mean number of lesions per fruit		Number of disease-free fruit	
Effect	Num DF[a]	Den DF[a]	F[b]	*P*-value[b]	F[b]	*P*-value[b]
Location	2	4	0.55	0.61	12.91	0.02
Treatment	5	10	21.74	<0.01	9.80	<0.01
Location*Treatment	10	20	0.58	0.81	1.09	0.41

[a] The numerator and denominator degree of freedoms
[b] F statistics and *P*-value from a generalized linear mixed model (PROC GLIMMIX, SAS 9.4)

Table 2.5: Disease levels in mangoes from anthracnose control field trials in three orchards in southern Senegal after 8 days post-harvest incubation.

	Mean number of lesions per fruit				Number of disease-free fruits[a]			
Treatment	Badjo	Jean	Toure	Mean[b]	Badjo	Jean	Toure	%
Control	143	123	132	133 A	0	0	0	0 A
Sonata	54	63	31	49 B	2	6	9	38 B
Serenade	34	46	53	44 B	5	9	8	49 BC
Sodium molybdate	38	26	33	32 B	2	8	10	44 BC
Thiophanate methyl	37	30	27	31 B	6	11	12	64 C
Azoxystrobin	34	30	22	29 B	6	11	11	62 C

[a] n=15 mangoes/treatment.
[b] Treatments followed by the same letter are not significantly different ($P < 0.05$). A generalized linear mixed model was used for ANOVA (PROC GLIMMIX, SAS 9.4), and Fisher's LSD was used for the mean separation.

2.4. **Discussion**

Two laboratory assays and six field trials in Senegal were carried out in 2015 to investigate the efficacy of fungicides, biofungicides, and a fertilizer on the development of anthracnose on mango. We were unable to obtain data from three field trials in the north of Senegal due to lack of precipitation before harvest, but results from three field trials in the southern region showed good control of mango anthracnose with all treatments. The efficacies of these biocontrol (Sonata and Serenade Optimum) and fertilizer (sodium molybdate) products were close to those

of systemic fungicides (azoxystrobin and thiophanate methyl), despite of intense rainfall in the region.

Our lab study also showed that both Sonata and Serenade resulted in significant reduction in the mean number of lesions per fruit, when they were applied prior to the inoculation. Moreover, results from the lab experiment on post-infection application of these treatments revealed that all treatments resulted in significantly fewer lesions per fruit, as well as significantly more disease-free (= marketable) fruits.

The control obtained with azoxystrobin and thiophanate methyl was not similar to results from earlier reports, but these two fungicides were better than the others. For example, Diedhiou *et al.* (2014a) found that azoxystrobin (96% of fruits not infected) and thiophanate methyl (100% of fruits not infected) provided excellent control of mango anthracnose in Senegal. Thiophanate-methyl (benzimidazoles) was more effective than non-systemic fungicides in controlling mango anthracnose (McMillan, 1984). Sundravadana *et al.* (2006) reported that azoxystrobin at 1.0, 2.0, and 4.0 ml/l controlled the development of panicle and leaf anthracnose in the field trials.

Although controlling anthracnose on mango has relied heavily on the use of fungicides, the use of the benzimidazoles and strobilurins has come into question due to development of resistance by *Colletotrichum* species. According to Kumar *et al.* (2007), *Colletotrichum gloeosporioides* was moderately resistant to thiophanate-methyl in Andhra Pradesh, India. In addition, Hu *et al.* (2015) showed that resistance to azoxystrobin and thiophanate methyl existed in *Colletotrichum siamense* from peach and blueberry in South Carolina.

Our results suggest that growers can use Serenade, Sonata, or sodium molybdate in addition to, or in rotation with, or as a substitute for, systemic fungicides. Two commercial products with the active ingredient of *Bacillus* spp. were used in these experiments: *Bacillus pumilus* QST 2808 (Sonata) and *Bacillus subtilis* QST 713 (Serenade Optimum). In both field and lab studies, the efficacy of Serenade tended to be somewhat higher than that of Sonata. Thus, Serenade might be a better choice as a biological agent.

Anthracnose control by *Bacillus* species has been reported in several studies. Our results agree with the observation of Senghor *et al.* (2007) that *B. subtilis* LB5 provided good control (51 to 56.4%) of anthracnose on mango in Taiwan. In addition, Korsten *et al.* (1994) showed that *B. subtilis* B246 controlled anthracnose on avocado. Moreover, Douville *et al.* (1992) showed that anthracnose disease incidence and severity on alfalfa seedlings were significantly reduced by *B. subtilis*.

Our results from field and lab experiments suggested that efficacy of sodium molybdate was close to that of azoxystrobin and thiophanate-methyl. Plants require a minimum amount of molybdenum (0.1–1.0 ppm) in the soil to help nitrogen assimilation (Mengel *et al*., 2001). Drosdoff (1972) stated that in Africa, some soils are deficient in molybdenum, zinc and boron. Molybdenum has a role in stimulating the hormonal biosynthetic pathway, and in activating the enzymes nitrate reductase and nitrogenase (Graham and Stangoulis, 2007), and it is possible that a molybdenum deficiency interferes with plant defense through an unknown mechanism. According to Dutta *et al.* (1981); Miller *et al.* (1983); and Jesus Jr *et al.* (2004), molybdate reduced the intensity of leaf spot in common bean, and *Verticillium* wilt on cotton and tomato. It

has also been suggested that the effect of molybdenum on some plants diseases might be a direct effect. Contact of *Colletotrichum* conidia with sodium molybdate solution might inhibit their viability, germination, and germ tube development, and thus decrease anthracnose severity (Polanco *et al.*, 2014). In addition, "the application of sodium molybdate decreased the area under the disease progress curve (AUDPC) possibly because of its direct effect on *C. lindemuthianum*, thus increasing yield (Polanco *et al.*, 2014)." Our *in vitro* experiments with sodium molybdate indicated that neither conidial germination nor mycelial growth was inhibited by the concentration used, although germ tubes were slightly shorter after incubation with sodium molybdate, suggesting perhaps a slight delay in germination. However, this does not rule out a possible effect on production of a toxin or enzyme or other effector produced by the pathogen.

In the three orchards in the South, sanitation practice was very limited. Regular pruning of trees and removal of fallen plant debris were not practiced in these orchards. Sanitation of orchards is commonly recommended for the control of anthracnose of mango (Arauz, 2000). Rotting dead leaves and debris become a favorable environment for development of *C. gloeosporioides* (Nelson, 2008). Other cultural treatments also can contribute to controlling anthracnose on mangoes; Van Deventer (2011) and Senghor *et al.* (2007) reported that bagging mangoes before harvest and a 10-min hot water treatment (52-55°C) reduced anthracnose infection by 83%. Nelson (2008) discussed other treatments that reduce anthracnose development under post-harvest conditions: Keep mangoes in the refrigerator at 50°F (10°C), and steam heat for 3–6 hours at various temperatures.

Treatments should be repeated in a second season to confirm or disprove the first year's results. Combination of fertilizer and fungicides or between fertilizers might have increased efficacy for the management of anthracnose. These products, including sodium molybdate would then have to be registered in Senegal to allow growers to have access. Growers should be trained on how to use these products wisely, especially in the south where growers are not educated.

2.5. **Conclusion**

Pre-harvest treatments in the south of Senegal showed that all of the products tested in this study provided a good control of anthracnose of mango. Sonata and Serenade and sodium molybdate are promising choices of material to control anthracnose in Senegal, especially in organic orchards, where few alternatives are available. But even in conventional orchards, these materials can be used as rotation or mixing partners for synthetic fungicide such as azoxystrobin and thiophanate-methyl, which should reduce the selection pressure towards resistance to those fungicides. Also the same materials may be useful as post-harvest treatments to reduce the development of anthracnose in storage.

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Figures

Figure 2.1: Anthracnose on mangoes

Figure 2.2: Modern orchard in the Niayes region, northern Senegal

Figure 2.3: Traditional orchard (mango trees mixed with cashew and palm tree)

Figure 2.4: Motorized sprayer used in the field trials

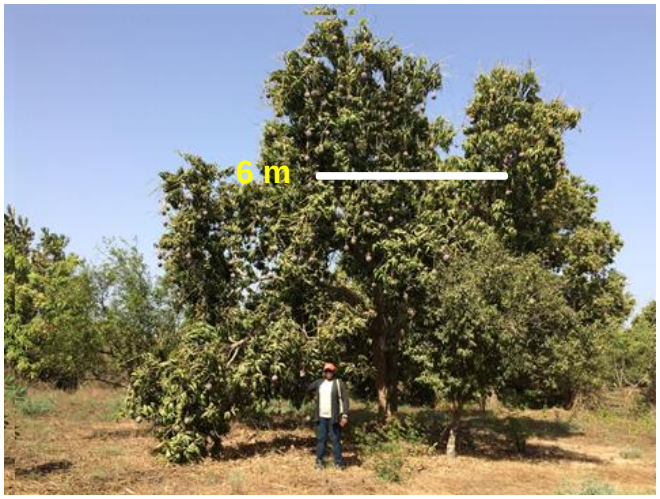

Figure 2.5: Spray mist can cover trees up to 6 m high.

Figure 2.6: Mango fruits on the benchtop during the 8-day incubation period

Chapter 3: Characterization of *Colletotrichum* species on mangoes from Senegal

Abstract
A number of species in the *Colletotrichum gloeosporioides* complex, as well as several additional *Colletotrichum* species, have been reported as causal agents of anthracnose on mango. Although morphological characters can contribute to the identification of some species, they are overlapping and variable, so species identification has relied on molecular characteristics in recent years. Thirty isolates of *Colletotrichum* were obtained from anthracnose lesions of mango from several regions in Senegal. Isolates were identified and grouped based on colony morphology, and size and shape of conidia and appressoria. Several color variations were examined among these isolates, with small differences in spore shape, spore size, and presence of black pigmentation in the colony. Sequence analysis of the rDNA internal transcribed spacer region indicated that all 30 isolates belonged to the C. *gloeosporioides* complex. Based on sequence analysis of the ApMat intergenic region identified all of them as most closely resembling *C. siamense*.

3.1. Introduction

Senegal ranks second for mango production among West African countries, and has the potential to competitively produce mangoes for the European Market, which offers good opportunities for the export of tropical fruits and vegetables. However, mango is attacked by several pests, among which the fungal disease anthracnose is the most important. Anthracnose causes both pre- and post- harvest fruit rot. *Colletotrichum gloeosporioides* Penz. and Sacc., the anamorph of *Glomerella cingulata*, has historically been named as the causal pathogen of anthracnose on mango and other fruits. It has more than 600 synonyms and many morphological and physiological variations (Von Arx, 1957), and seven formae speciales were described by Sutton (1992), who showed that *C. gloeosporioides* is a heterogeneous group with much variation in morphology.

Some studies have shown that morphological characters are useful for distinguishing some species within *C. gloeosporioides* group (Johnson *et al*., 1997). However, many of these morphological characters can change under different growing conditions (growth media, temperature, light, etc.), or they can change with repeated subculturing. Cox and Irwin (1988) used the shape and the size of the conidia and the shape of the appressoria as morphological criteria to separate groups within the *C. gloeosporioides* species complex. Conidial morphology, appressorial formation, color of colonies, and presence or absence of setae are traditional morphological characteristics used for differentiating *C. gloeosporioides*.

More recently, molecular methods have allowed further resolution of *Colletotrichum gloeosporioides* and it is now considered a species complex (Damm *et al*., 2010; Weir et al. 2012). At least nine different species within this complex have been reported as causal agents of mango anthracnose, but additional species of *Colletotrichum* in other groups can cause anthracnose on mango as well (Table 3.1, Lima *et al*. 2013; Phoulivong *et al*., 2010). Molecular methods have been used successfully to differentiate species of *Colletotrichum*, and the following loci have been used for this purpose: internal transcribed spacer (ITS) of the ribosomal DNA (rDNA); glyceraldehyde–3–phosphate dehydrogenase (GPDH); histone 4 (his4); glutamine

synthase (GS); b–tubulin 2 (TUB 2) (Prihastuti et *al*., 2009; Damm *et al*., 2010; Lima *et al*., 2013; Sharma *et al*., 2013; Vieira *et al*., 2014; Pardo-De la Hoz *et al*., 2016; Liu *et al*., 2016). The ITS locus has been proposed as a universal barcode, but, for Colletotrichum, is too evolutionarily conserved (Cannon *et al*., 2012), and, although helpful in distinguishing between Colletotrichum species complexes, does not resolve species within *C. gloeosporioides* s.l. (Weir *et al.* 2012). . Weir *et al.* (2012) discussed secondary barcode loci for the *C. gloeosporioides* species complex, and indicated that the TUB2 or GS loci are best for distinguishing *C. aenigma* (syn: *C. populi*) from *C. alienum* and some *C. siamense* isolates. In addition, the Apn2/MAT locus has been used by Silva *et al.* (2012) to improve phylogenetic resolution of the *Colletotrichum gloeosporioides* species complex. Moreover, Liu *et al.* (2015) showed that the combination of the ApMat (intergenic region of Apn2/MAT locus) and GS loci in a phylogenetic analysis is able to resolve all currently accepted species in the *C. gloeosporioides* species complex.

In Senegal, anthracnose of mango has been attributed to *C. gloeosporioides* (Mbaye *et al*., 2006; Diedhiou *et al*., 2014) and the identities and relative representation of the species according to modern criteria are unknown because no molecular characterization of *Colletotrichum* species associated with mango has been carried out. Therefore, the purpose of this study was to characterize isolates of *Colletotrichum* from orchards and markets in several production areas and cities in Senegal based on DNA sequence analysis.

Table 3.1: *Colletotrichum* species reported to cause anthracnose on mango

Complex	Species	Reference	Loci Analyzed
C. gloeosporioides	*C. asianum*	Prihastuti *et al*., 2009 Silva *et al*., 2012 Lima *et al*., 2013 Sharma *et al*., 2013 Vieira *et al*., 2014 Pardo-De la Hoz *et al*., 2016	ITS ACT ApMAT CAL GAPDH GS TUB2
C. gloeosporioides	*C. cliviae*	Vieira *et al*., 2014 Pardo-De la Hoz *et al*., 2016	ITS ACT GAPDH TUB2 ApMAT IGS
C. gloeosporioides	*C. dianesei*	Lima *et al*., 2013 Vieira *et al*., 2014 Pardo-De la Hoz *et al*., 2016	ITS ACT CAL GAPDH GS TUB2 ApMAT

C. gloeosporioides	*C. endomangiferae*	Vieira *et al*., 2014 Pardo-De la Hoz *et al*., 2016	ITS ACT GAPDH TUB2 ApMAT
C. gloeosporioides	*C. fructicola*	Prihastuti *et al*., 2009 Silva *et al*., 2012 Lima *et al*., 2013 Sharma *et al*., 2013 Vieira *et al*., 2014 Pardo-De la Hoz *et al*., 2016	ITS ACT ApMAT CAL GAPDH GS TUB2
C. gloeosporioides	*C. gloeosporioides*	Prihastuti *et al*., 2009 Weir *et al*., 2012 Silva *et al*., 2012 Lima *et al*., 2013 Sharma *et a.l*, 2013 Vieira *et al*., 2014 Pardo-De la Hoz *et al*., 2016	ITS ACT ApMAT CAL GAPDH GS TUB2
C. gloeosporioides	*C. horii*	Vieira *et al*., 2014 Pardo-De la Hoz *et al*., 2016	ITS ACT GAPDH TUB2 ApMAT
C. gloeosporioides	*C. siamense*	Prihastuti *et al*., 2009 Silva *et al*., 2012 Lima *et al*., 2013 Sharma *et al*., 2013 Pardo-De la Hoz *et al*., 2016 Liu *et al*., 2016	ITS ACT ApMAT CAL GAPDH GS TUB2
C. gloeosporioides	*C. tropicale*	Weir *et al*.. 2012 Lima *et al*., 2013 Sharma *et al*., 2013 Vieira *et al*., 2014 Pardo-De la Hoz *et al*., 2016	ITS ACT ApMAT CAL GAPDH GS TUB2
C. acutatum	*C. acutatum*	Shivas and Tan 2009. Damm *et al*, 2012. Pardo-De la Hoz *et al*., 2016	ITS β-tub GAPDH CHS-1 HIS3 ACT TUB2

C. acutatum	*C. fioriniae*	Shivas and Tan, 2009 Damm *et al*, 2012 Pardo-De la Hoz *et al*., 2016	ITS β-tub GAPDH CHS-1 HIS3 ACT
C. acutatum	*C. simmondsii*	Shivas and Tan, 2009 Pardo-De la Hoz *et al*., 2016	ITS β-tub
C. boninense	*C. karstii*	Damm *et al.* 2012b Lima *et al*, 2013 Vieira *et al*., 2014 Pardo-De la Hoz *et al*., 2016	ITS ACT CAL GAPDH GS TUB2
C. truncatum (falcate spores)	*C. capsici*	Damm *et al*., 2009	ITS ACT GAPDH TUB2

3.2. Materials and Methods

3.2.1. Sampling and fungal isolation: Mangoes were collected from orchards and markets in several cities in Senegal in July and August of 2015 (Table 3.2), both in the North (Pout, Notto Gouye Diama), and in the South (Kolda, Sedhiou, and Ziguinchor). Samples were brought to the laboratory in plastic bags and mangoes were held in bags at room temperature for 8 days at which time lesions were cultured for recovery of Colletotrichum.

In order to isolate the pathogen, the mangoes were first soaked in a 10% household bleach solution (0.8% sodium hypochlorite) for 5 minutes, and two crossed incisions in the form of a V were made at the edge of the lesion with a sterile scalpel. A piece of flesh was taken from under the peel and placed in a Petri dish containing potato dextrose agar (PDA), which was incubated at room temperature. Two days later, the mycelium growing out of the mango flesh was transferred into new Petri dishes to obtain pure culture of the fungi. The isolates were then incubated for 4 to 10 days to allow sporulation. The presence of Colletotrichum was confirmed based on morphological characteristics (i.e. mycelium color, conidial size and shape, and appressoria size and shape). Thirty Senegal isolates were sent to Virginia Tech for further characterization. At Virginia Tech, fungal isolates were subcultured on PDA and grown at 27 °C for 7 days.

Table 3.2: Description of *Colletotrichum* isolates from Senegal, collected in summer 2015

Locations	Code	Variety	Source
Notto Gouye Diama (North)	1A	Keitt	Orchard
	1B		
	1C	Keitt and Kent	Orchard
	1D		
	1E	Keitt	Market
Pout (North)	2A	Kent and Keitt	Market
	2B		
	2C		
	2D		
	2E		
Kolda (South)	3A	Keitt	Orchards and market
	3B		
	3C		
Sedhiou (South)	3D	Keitt	Orchards and market
	3E		
Ziguinchor (South)	4A	Keitt and Kent	Orchards and market
	4B		
	4C		
	4D		
	4E		
Badjo (Djibelor, South)	5A	Keitt	Orchard
	5B		
	5C		
	5D		
	5E		
Jean (Djibelor, South)	6A	Keitt	Orchard
	6B		
	6C		
	6D		
	6E		

3.2.2. Morphology of isolates: A total of 30 *Colletotrichum* isolates were collected from mango in eight cities that are in the principal mango production areas, and were characterized morphologically. Morphological traits observed were colony color, conidial size and shape, and size and shape of appressoria. Colony pigment was determined after colonies were grown 7 or 8 d on PDA at 27^0C in constant black light (F15T8-BLB lamp). Conidia were placed on a slide in a drop of lactic acid and fifty conidia were measured.

Appressoria were produced using a slide culture technique (Johnston and Jones, 1997). A small piece of PDA was placed on a microscope slide and covered with a cover slip. After 1 or 2 days

the cover slip was removed and placed in a drop of lactic acid on a microscope slide, and twenty appressoria were observed under 400X magnification in bright field.

3.2.3. DNA extraction: Thirty *Colletotrichum* isolates from anthracnose on mango fruit rots in Senegal were also used in the phylogenetic analysis. Isolates were grown on PDA and incubated for 7 days at 27°C. Fifty milligrams (50 mg) of mycelium were scraped from the surface of agar and placed into a tube containing three ceramic beads. The mycelium was homogenized using a FastPrep-24 homogenizer (MP, Santa Ana, CA) for 30 sec, or up to 60 sec, at a speed of 4 m/s. The Biosprint 15 DNA plant kit (Qiagen, Valencia, CA) was used for genomic DNA extraction, according to the manufacturer's protocol.

3.2.4. PCR amplification and DNA sequencing: The ITS region was amplified by PCR using the universal primers ITS5 forward (5' GGA AGT AAA AGT CGT AAC AAG G 3') and ITS4 reverse (5' TCC TCC GCT TAT TGA TAT GC 3') (Prihastuti *et al.* 2009).

The PCR reactions were carried out in a Mastercycler® pro (Eppendorf, Hamburg, Germany) with the following cycling parameters: 10 min denaturation step at 95°C; 35 cycles of 95°C for 30 s, 60°C for 35 s and 72°C for 50 s; and a final extension step of 10 min at 72°C. The PCR reaction mixture consisted of: 8.9 µl nuclease free water, 12.5 µl ImmoMix (2X) (Bioline, Taunton, MA), 1.3 µl each primer (10 µM) and 1 µl genomic DNA. PCR products were separated by electrophoresis on 1.5% agarose gel. GelRed™ (Biotium, Hayward, CA) was added to the gel to stain the nucleic acid and bands were visualized under UV light. Band sizes were estimated by comparison to a 100 bp molecular ladder.

PCR products were cleaned in preparation for sequencing with shrimp alkaline phosphatase/exonuclease I (SAP/Exo I) (Affymetrix, Santa Clara, CA) and the reaction mixture was: 5.0 µl nuclease free water, 1.0 µl shrimp alkaline phosphatase (1 unit/ µl), and 0.1 µl exonuclease I (10 units/µl). These reactions were placed in the mastercycler that was programmed as follows: 30 minutes at 37ºC, 20 minutes at 65ºC. PCR products were sequenced in both directions. Sequencing was performed by Eurofins Genomics (Huntsville, AL).

Two loci on the Apn2/MAT genes were also sequenced to further resolve the isolates. The ApMat intergenic region (713 bp) was amplified by PCR using primer pair AM [AM-F (5'-TCATTCTACGTATGTGCCCG-3')/AM-R (5'-CCAGAAATACACCGAACTTGC-3')] and the 5' end of the Apn2 gene (Apn25L, 883 bp) was amplified by PCR using primer pair A5L [A5L-F (5'-CAAGCGACGAAGTATACGAG-3')/A5L-R (5'-GCATCACGGGAATAACTAGG-3')] primers [Silva *et al*. (2012)]. The ApMat locus was amplified for all 30 isolates and Apn25L for ten isolates. Conditions were the same as those used for the ITS genomic region except for the annealing temperature, which was 60°C for both the AM and A5L primer pairs. Reactions were cleaned as described previously for the ITS region and sequenced in both directions by Eurofins Genomics.

Amplification of the Apn25L region of the 10 Colletotrichum isolates generated a fragment of approximately 1,000 bp for each isolate (Figure 3.2). However, the sequencing results were not satisfactory because there were many overlapping peaks in the chromatogram. Only the ApMat results will be reported.

3.3. Results

Amplification of the ITS region of the 30 Colletotrichum isolates generated a fragment of approximately 600 bp for each isolate (Figure 3.1). Chromatograms and sequences were aligned, viewed and manually edited using Lasergene (DNASTAR, 13) and consensus sequences were created. The consensus sequences of all the isolates were identical. The consensus sequence was compared with nucleotide sequences in the NCBI database using BLAST. The query sequence had 100% similarity to several sequences from isolates belonging to the *C. gloeosporioides* species complex, i.e. *C. siamense* (accession numbers HM131511; JX010245) or *C. populi* (JX010243; JX010244).

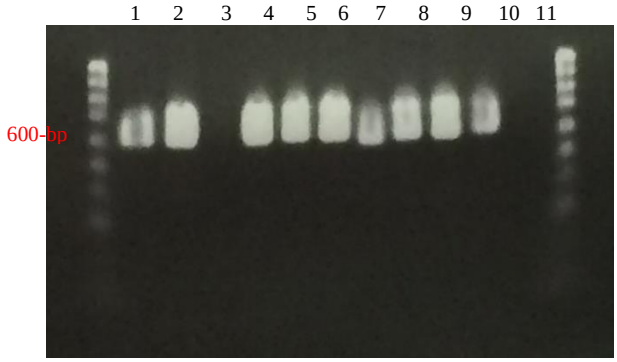

Figure 3.1: Products from amplification of the ITS region
100 bp ladder
1: Isolate 1D;
2: Isolate 1E;
3: Isolate 2A failed to amplify;
4: Isolate 2C;
5: Isolate 2D;
5: Isolate 3E;
7: Isolate 4C;
8: Isolate 5B;
9 Isolate 6B;
10: Isolate 6D;
11: Negative control
100 bp ladders.

PCR products produced for the ApMat intergenic locus of the 30 isolates were approximately 1,000 bp (Figure 3.2). Chromatograms and sequences were aligned, viewed and manually edited using Lasergene (DNASTAR, 13) and consensus sequences were created. The consensus sequences of all the isolates were identical. The consensus sequence was compared with nucleotide sequences in the NCBI database using BLAST. The query sequence had 99% similarity and 0.0 for the E-values to several sequences from isolates belonging to *C. siamense* (accession codes: KC790678; KC790694; KC790694.1; KJ954494; KJ954494.1; KJ954503.1; KJ954504.1; KJ954508; KJ954508.1; FR718813; HE657304; JQ899288).

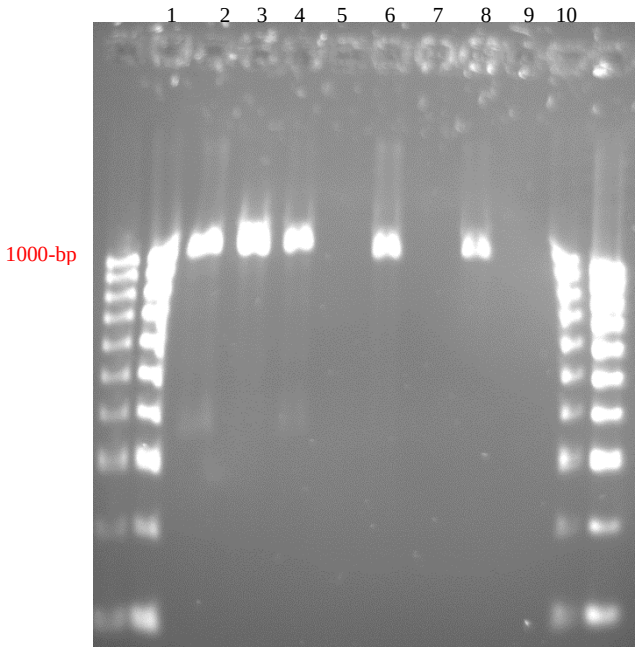

Figure 3.2: Products from amplification of the ApMat locus
100 bp ladders at 0.4µl;
1: 100 bp ladders at 0.5µl;
2: Isolate 2B;
3: Isolate 3A;
4: Isolate 3C;
5: Control;
6: Isolate 2B;
7: Isolate 3A;
8: Isolate 3C;
9: Negative control;
10: 100 bp ladders at 0.4µl;
100 bp ladders at 0.5µl;

Colletotrichum species complexes are characterized in part based on the size and shape of the conidia and appressoria. In order to determine whether the molecular species characterization

matched the morphological characteristics, the variation among the 30 isolates was studied. Initially, four color variations were distinguished. Morphology 1 was distinguished by the production of a black pigment in most of the reverse side of PDA cultures. Morphology 2 produced white mycelial colonies without black pigment. Morphology 3 had a central round area of black pigment , and morphology 4 produced a central black area, of irregular shape (Figure 3.3). After several additional transfers, morphologies 1 and 2 were still present, although sometimes in different isolates (i.e., some isolates shifted from one morphology to another), but morphologies 3 and 4 changed completely to another color, namely, white mycelium with many orange conidial masses all over the PDA plates (Figure 3.4).

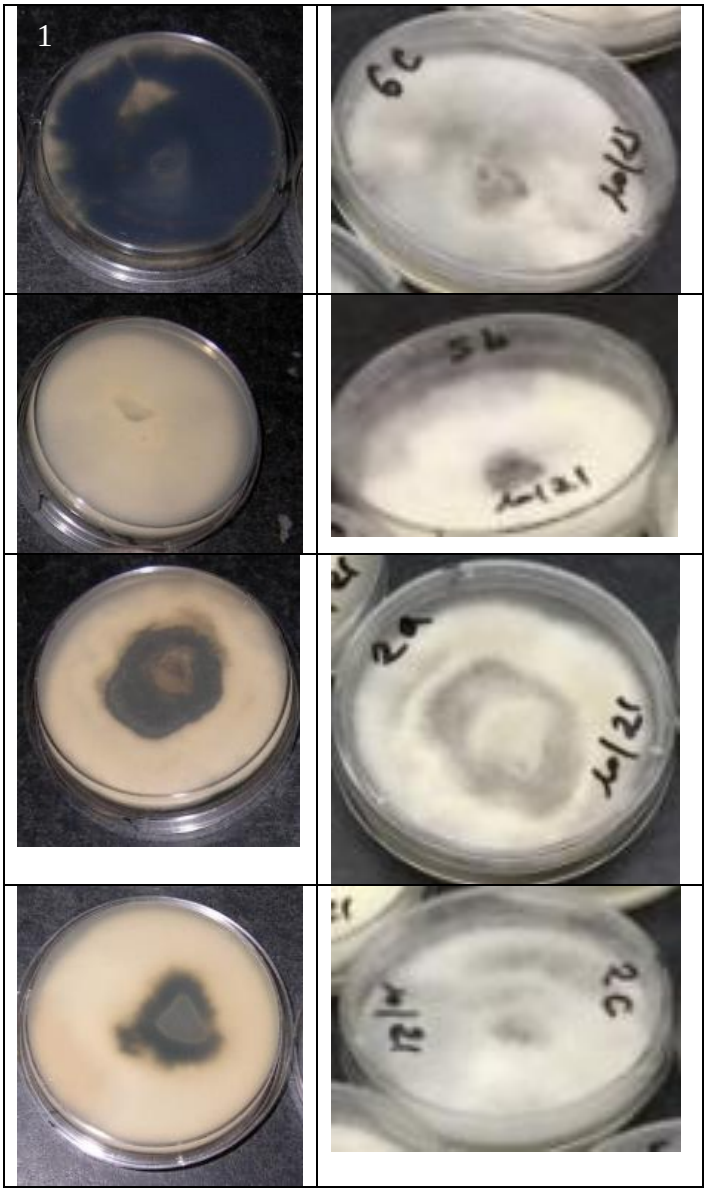

Figure 3.3: Morphology (1 to 4) found among isolates of *Colletotrichum* spp. isolated from symptomatic mango fruit collected in several orchards and markets in Senegal. Upper (right) and reverse side (left) of plate.

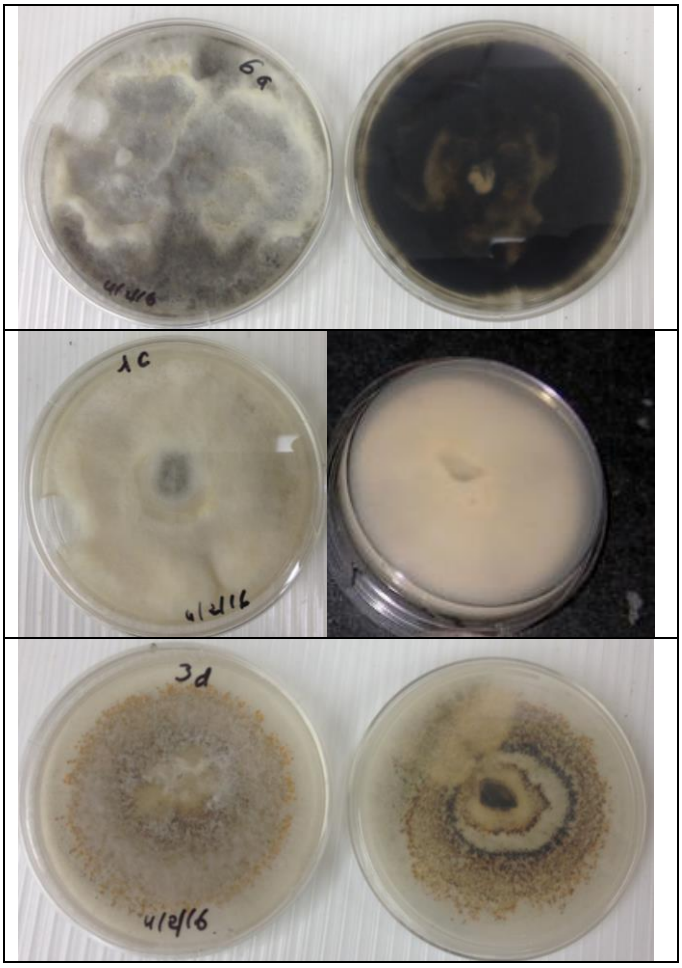

Figure 3.4: New morphology (1 to 3) found among isolates of *Colletotrichum* spp. isolated from symptomatic mango fruit collected in several orchards and markets in Senegal. Upper (left) and reverse side (right) of plate.

The *Colletotrichum* isolates in this study showed only slight variation in conidial size and shape (Table 3.3). Spore shapes of most of the isolates were cylindrical with rounded ends (Figure 3.4A), typical of *C. siamense* conidia, but some isolates also produced some conidia that were cylindrical with broadly rounded ends (Figure 3.4B). Conidial length was considerably more variable than width (Table 3.3). The length/width (L/W) ratio of the conidia of the isolates ranged from 2.4 to 2.8.

Table 3.3: Summary of morphological characteristics of Colletotrichum isolates grown on potato dextrose agar (PDA)

Morphology	Color	Length (µm)	Width (µm)	Isolates	Shape	Conidia
1	Reverse side of colony entirely black on PDA	14-15	5-6.25	6A; 6B; 6C; 6D; 6E; 5C; 5D; 4A; 4B; 2E; 4C; 4D; 5E	Cylindrical with rounded end	
2	White mycelial colonies	13-16	5-6.25	5A; 4E; 3A; 3D; 3E; 1D; 5B	Cylindrical with broadly rounded end	
3	Black color with round shape on the center of plate	14-18	5-6.25	3C; 2A; 1A; 1B; 1C; 2E	Cylindrical with rounded end	
4	Black irregular shape on the center of plate	13-16	5-6.25	3B; 2B; 2C; 1E	Cylindrical with rounded end	

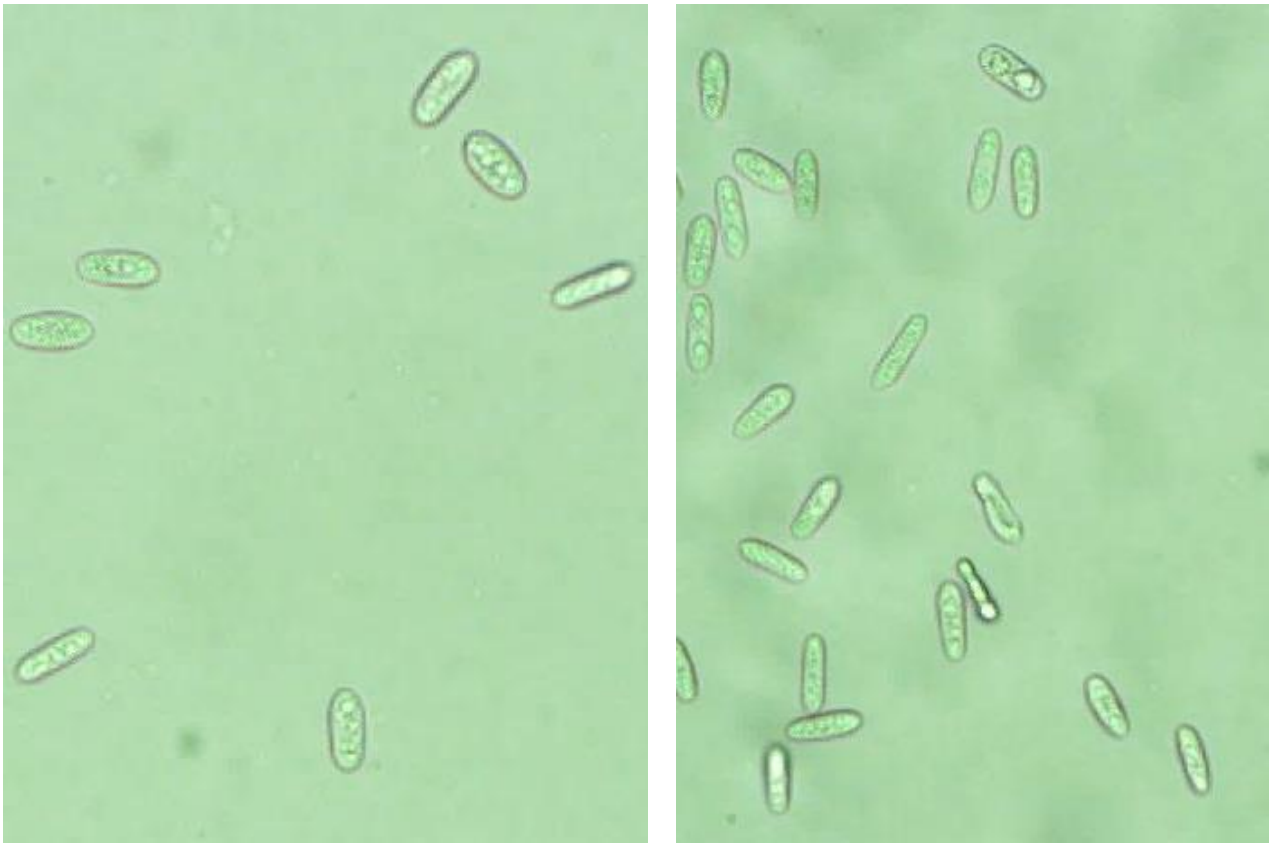

Figure 3.4A: Cylindrical conidia with rounded ends. 3.4B. broadly rounded end

Appressoria were produced within 24 hours for all isolates, and were light to dark brown (Figure 3.5). Appressoria varied in size with 5-7.5 µm to 3-5 µm for all *Colletotrichum* isolates.

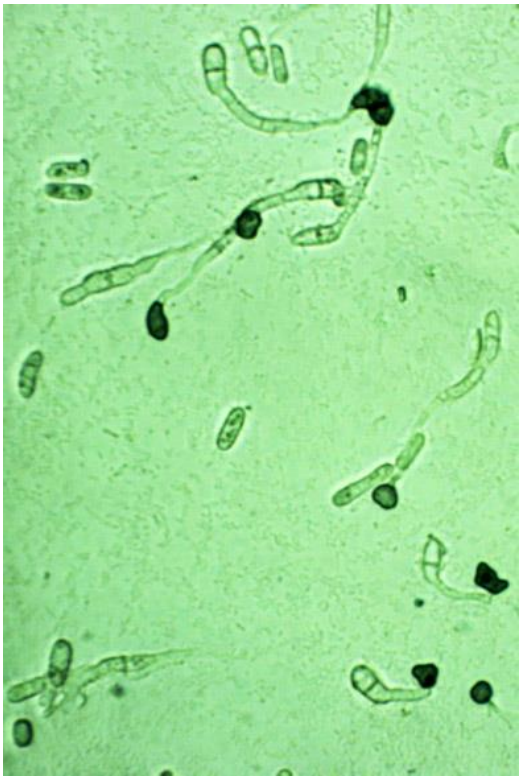

Figure 3.5: The appressoria appeared light to dark brown

3.4. Discussion

The advent of molecular taxonomy and its application to the genus *Colletotrichum* has demonstrated that what were formerly considered species are actually species complexes with members that are very difficult or impossible to differentiate and identify based on morphological characteristics (Cannon et al., 2012). Our study represents the first molecular characterization of *Colletotrichum* species from mango in Senegal. Both molecular and morphological characteristics were used to characterize 30 Colletotrichum isolates from anthracnose-diseased mangoes in Senegal. The morphological characters, while matching the characteristics of C. *gloeosporioides* s. l. and of *C. siamense*, were not useful in resolving to species. The ITS sequence confirmed that the isolates belong to the C. *gloeosporioides* complex. The results from analysis of sequences of the ApMat locus indicated that all 30 isolates were closest to *C. siamense.*

Many authors have stated in the past that *C. gloeosporioides* is the common pathogen that infects more than 1000 plant species (Phoulivong *et al.*, 2010). In order to be able to obtain molecular data, Cannon *et al.* (2008) obtained a culture from a necrotic spot of a living leaf of *Citrus sinensis* in Italy, the same host and location where the originally described type isolate was obtained, and designated it as ex-epitype culture of *C. gloeosporioides*. When 25 isolates from eight tropical fruits were compared with the epitype, none of them was *C. gloeosporioides* (Phoulivong *et al.*, 2010). Several studies since then have shown that the newly epitypified *C.*

gloeosporioides sensu stricto is not a common pathogen on tropical fruits (Phoulivong *et al*., 2010; Sharma *et al*., 2014).

The ITS sequence was the first barcode used (Cannon *et al*., 2012) and supports a close relationship of the 30 isolates, confirming that they all belong to the genus *Colletotrichum*, and placed them all in the *C. gloeosporioides* complex. ITS sequencing analyses have been suggested by many authors to distinguish one *Colletotrichum* species complex from another (Prihastuti *et al*., 2009; Weir *et al*., 2012; Damm *et al*., 2012; Lima *et al*., 2013; Sharma *et al*., 2013; Vieira *et al*., 2014; Liu *et al*., 2015; Pardo-De la Hoz *et al*., 2016). Based on the ITS sequence, the common causal agent of mango anthracnose in the main growing areas of Senegal belongs to the *C. gloeosporioides* species complex.

Silva *et al*. (2012) showed that the ApMat marker is helpful to separate species within the *C. gloeosporioides* complex. Sharma *et al.* (2013) used 6 genes (*act, cal, chs1, gapdh,* ITS and *tub2*), followed by ApMat sequence-analysis and wrote that 39 *Colletotrichum* isolates from India were separated into nine lineages based on ApMat, namely *C. fragariae sensu stricto*, *C. fructicola, C. jasmini-sambac*, *C. melanocaulon* and five undesignated groups. Going further, Sharma *et al.* (2015) concluded that *C. siamense* is a species complex based on ApMat marker analysis, and distinguished seven species within this complex (*C. hymenocallidis, C. jasmini-sambac, C. dianesei, C. endomangiferae, C. murrayae, C. siamense sensu stricto*, and *C. communis sp.*). They advised use of the ApMat marker as an efficient marker, to not only save time, but also the cost, compared with sequencing 5-8 gene loci. However, most recently, Liu *et al.* (2016) disagreed and argued that *C. siamense s. l.* is single species rather than a species complex. These authors compared sequence results for eight loci (CAL, GAPDH, GS, ITS, TUB2, ApMat, Apn25L, MAT1-2-1) and also conducted morphological analysis and mating experiments; they concluded that the individual phylogenetic trees obtained for the different characters and loci did not agree, and did not support recognition of separate species according to the Genealogical Concordance Phylogenetic Species Recognition concept. *C. siamense sensu stricto, C. jasmini-sambac*, and *C. hymenocallidis* have high similarity and they could not be distinguished in phylogenetic analysis by Wikee *et al.* (2011) and Weir *et al.* (2012).

Based on our ApMat marker analysis, *C. siamense sensu lato* is the probable causal agent of anthracnose on mango in Senegal. Additional evidence could be obtained by sequencing the GS locus (Liu *et al.* (2015) or other loci. Many researchers have shown the presence of *C. siamense sensu lato* in many plants hosts (Weir *et al*. 2012; Lima *et al*. 2013; Sharma *et al*. 2013; Udayanga *et al*. 2013; Vieira *et al*. 2014; James *et al.* 2014; Chowdappa *et al.* 2015; Ye *et al*. 2016). Weir *et al*. (2012) found that *C. siamense* is biologically and geographically diverse and this species can be in many hosts across many tropical and subtropical regions. Sharma *et al.* (2013) showed that *C. siamense sensu stricto* is one of the causal agents of anthracnose on mango in India. In addition, Udayanga *et al.* (2013) showed that *C. siamense sensu lato* was the most common *Colletotrichum* species in tropical wild fruits in northern Thailand.

Based on the conidial shape and the size, 50 conidia for each isolates were measured and they were not significantly different for both in length and width of conidia. The shape of these conidia was two types, rounded ends and broadly rounded end. Similar results have been

reported by Prihastuti *et al*. (2009) saying that conidia of *C. siamense* from coffee berries in northern Thailand are fusiform with slightly rounded ends. In addition, Sharma *et al.* (2015) wrote that *C. siamense* shape is fusiform to cylindrical. The length/width ratios of conidia were in agreement with the observations of Prihastuti *et al*. (2009), in which the isolates of *C. siamense* from coffee berries were 2.3-4.2.

Appressorial size and shape did not show consistent differences among the four morphologies. Similar results about the length and width of appressoria of *C. siamense* from coffee berries were obtained by Prihastuti *et al.* (2009). Than *et al*. (2008a) showed that *Colletotrichum* species from chilli cannot be distinguished by the morphology of appressoria.

These morphological characters may not be reliable for identification within *Colletotrichum*, but a combination of molecular methods with morphological characters is a reliable approach for studying *Colletotrichum* species complexes (Prihastuti *et al.*, 2009).

3.5. Conclusion

This study shows that molecular techniques are very useful and important tools. They can be used to complement identification based only on morphological characters. This study helps us to know which kind of pathogens cause anthracnose diseases in Senegal and compare what others have in their countries. This work is relevant because it can contribute in the application of disease control measures more effectively. Moreover, it allows us to look for biological differences because if a variety is resistant to this pathogen in other countries, we can grow this variety in my country to control the disease.

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