Colletotrichum spp. Associated with Anthracnose Disease on Coffee in Vietnam and on Some Other Major Tropical Crops

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Doctoral Thesis
Swedish University of Agricultural Sciences
Alnarp 2010

Acta Universitatis Agriculturae Sueciae
2010:39

Cover: Landscape of coffee plantation in Vietnam, symptoms of coffee anthracnose, *Colletotrichum* colony, phylogenetic tree, coffee flowers and DNA sequences (photos: Erland Liljeroth)

ISSN 1652-6880
ISBN 978-91-576-7452-4

Print: SLU Service/Repro, Alnarp 2010

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Abstract

The genus *Colletotrichum* consists of many economically important pathogenic fungi on a broad range of host plants world-wide. They cause significant economic losses to tropical crops: fruits, cereals, grasses, vegetables, etc., due to diseases at different stages of plant development. Several species of *Colletotrichum* cause anthracnose on coffee and other major crops, which are valuable trade commodities in Vietnam and Thailand. However, populations of these pathogens have been poorly studied so far.

This thesis aims to identify species of *Colletotrichum* that are associated with anthracnose diseases on tropical crops, particularly coffee in Vietnam, and to characterise the populations of these pathogens. Studies on morphological, cultural and biochemical characteristics, pathogenicity, genetic diversity and population structures of the pathogens were employed. Random amplified polymorphic DNA (RAPD), unanchored/anchored microsatellite primed PCR (MP/AMP-PCR) and DNA sequence analysis of the mating type genes, the internal transcribed spacer region of nuclear ribosomal DNA (rDNA) and a portion of mitochondrial small subunit rRNA gene were used for diversity studies and in assisting species identification to complement the morphological data.

Colletotrichum gloeosporioides and *C. acutatum* were identified from diseased citrus, grape, asparagus, mango, durian, etc. originating from Vietnam and Thailand. In Vietnam, *C. gloeosporioides, C. actutatum, C. capsici, C. boninense* and several *Colletotrichum* isolates of unknown species were found to be associated with infected coffee leaves, berries, roots and twigs in different coffee growing areas. No evidence was found of the presence of *C. kahawae* in Vietnam. The majority of Vietnamese isolates belonged to *C. gloeosporioides* and they were more pathogenic on detached green berries than isolates of any of the other species. The isolates of *C. gloeosporioides* mainly grouped in accordance with geographical origin based on both RAPD and MP/AMP-PCR markers. High genetic variation in populations of *C. gloeosporioides* from different locations and different coffee tissues was observed. Moderate gene differentiation was found between the populations of northern and southern Vietnam. However, within the regions there was low and no differentiation between locations and host tissues, respectively, indicating significant gene flow. This thesis provides better insights into the *Colletotrichum* populations that may play an important role for future disease management strategies in sustainable coffee production in Vietnam.

Keywords: Arabica coffee, Vietnam, genetic diversity, pathogenicity, phylogeny, vegetative compatibility, mating type genes.

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Dedication

To my beloved FAMILY!

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Nguyen, T.H.P., Vinnere Pettersson, O. & Fatehi. J. Identification of *Colletotrichum* species on tropical crops in Vietnam and Thailand (manuscript)

II Nguyen, T.H.P., Vinnere Pettersson, O., Olsson, P. & Liljeroth, E. (2010). Identification of *Colletotrichum* species associated with anthracnose disease of coffee in Vietnam. *European Journal of Plant Pathology* 127 (1), 73-87

III Nguyen, T.H.P., Säll, T., Bryngelsson, T. & Liljeroth, E. (2009). Variation among *Colletotrichum gloeosporioides* isolates from infected coffee berries at different locations in Vietnam. *Plant Pathology* 58(5), 898-909

IV Nguyen, T.H.P., Vinnere Pettersson, O., Nguyen, T.H. & Liljeroth, E. Diversity of *Colletotrichum gloeosporioides* populations on different tissues of Arabica coffee in southern Vietnam (manuscript)

The contribution of Phuong Thi Hang Nguyen to the papers included in this thesis was as follows:

I Did all experimental work, analysed data and wrote manuscript together with co-authors.

II Collected the samples, did all experimental work; planned the study, analysed data and wrote the manuscript in cooperation with co-authors.

III Collected the samples, did all experimental work; planned the study, analysed data and wrote the manuscript in cooperation with co-authors.

IV Collected the samples, did large parts of experimental work; planned the study, analysed data and wrote the manuscript in cooperation with co-authors.

1 Introduction

The genus *Colletotrichum* consists of several economically important plant pathogenic fungi, occurring predominantly in tropical and subtropical regions on a wide range of crops. The genus is the best represented on diseased tissues of leaves, flowers, fruit, stems and crowns in warm moist environments encountered in the humid and sub-humid tropical zones (Waller, 1992). Diseases caused by *Colletotrichum* are particularly troublesome on perennial crops and also frequently cause significant economic losses in annual crops such as cereals, grasses, vegetables, etc. at all stages of their development, i.e. from seedlings to mature plants and seeds (Dodd *et al.*, 1992; Lenné, 1992; Waller, 1992). Multiple species of *Colletotrichum* can be jointly associated with anthracnose on a single host. For example, several *Colletotrichum* species can cause disease on coffee (Freeman *et al.*, 2000a). *Colletotrichum kahawae* Waller & Bridge is the causal agent of coffee berry disease (CBD) (Waller *et al.*, 1993; Hyde *et al.*, 2009a). This fungus, which is endemic in Africa, causes severe lesions on green berries and can result in yield losses of up to 80% (Varzea *et al.*, 2002; Chen *et al.*, 2005). *Colletotrichum gloeosporioides* (Penz.) Sacc. and *C. acutatum* Simmonds are mainly saprophytic on coffee, but can also produce minor disease by infecting ripening berries (Masaba & Waller, 1992). Coffee and some other economically important crops grown in both tropical and subtropical climates are considered the major foreign exchange earners that contribute significantly to the economic income of developing countries (Chomchalow, 2004; Doan, 2005; Soytong *et al.*, 2006; Waller *et al.*, 2007; Than *et al.*, 2008a). In Vietnam, coffee is the second most important agricultural commodity after rice. Vietnam is one of the world's largest producers of Robusta coffee, with an export value of US$ 570 million (Doan, 2005). Apart from coffee, other tropical crops such as mango, durian, citrus, grape, etc. are regarded as major crops and play an economically

important role in exports from Vietnam and Thailand (Chomchalow, 2004; Soytong *et al.*, 2006). Vietnam and its neighbour, Thailand, are situated in the central part of South-east Asia. Their geographical features are quite similar, with a typical warm and humid tropical climate. Thailand is one of the largest producers and exporters of fruits such as longan, durian, mangosteen, lychee, mango, pomelo and rambutan (Vichitrananda & Somsri, 2008).

However, in Vietnam problems with anthracnose of *Coffea* spp. have occurred in recent decades. The disease was first discovered in 1930 in Kon Tum and the Southern Highlands (Tran *et al.*, 1998). The symptoms somewhat resemble those of CBD, i.e. slightly sunken or brown blight lesions occur on green and ripening berries and can interfere with pulping during processing of the beans (Pinkert, 2004). The disease usually appears in the beginning of the rainy season (May) and causes damage in September during berry development and ripening (Tran *et al.*, 1998). It has mostly been observed on the Arabica cultivar 'Catimor' which is now commonly grown in Vietnam. The estimated yield losses caused by this disease range between 15-60% and vary between geographical zones (Tran *et al.*, 1998). Furthermore, premature berry shedding has been reported, as well as die-back and anthracnose on leaves and twigs (Tran *et al.*, 1998; Pinkert, 2004). The disease generally appears in the third year of coffee berry harvest. Anthracnose diseases of other tropical crops have also been reported in Vietnam (Chomchalow, 2004; Don *et al.*, 2007) and Thailand (Soytong *et al.*, 2006; Than *et al.*, 2008b).

There have only been a few studies on the causal agents of anthracnose disease on coffee outside Africa. Recently, *Colletotrichum* spp. has been found to be associated with diseases of coffee in Brazil, Papua New Guinea, Colombia and Thailand (Silva *et al.*, 2005; Kenny *et al.*, 2006; Prihastuti *et al.*, 2009; Rodrigues *et al.*, 2009). *Colletotrichum gloeosporioides* and *C. coffeanum* have been considered the causal agents of coffee diseases as previously reported by Paradela Filho and Paradela (2001). Orozco-Miranda (2003) indicated that *C. gloeosporioides* and *C. acutatum* are the major pathogens responsible for the diseases on different parts of coffee plants. *Colletotrichum gloeosporioides* and *C. acutatum* have been found to be associated with anthracnose disease of *Coffea arabica* in Papua New Guinea (Kenny *et al.*, 2006). In a recent study, Prihastuti *et al.* (2009) found three new species of *Colletotrichum*, namely *C. asianum*, *C. fructicola* and *C. siamense*, to be responsible for coffee anthracnose in Thailand. These new species could be differentiated from both *C. gloeosporioides* and *C. kahawae*.

To our knowledge, no thorough investigations of the causal agent of coffee anthracnose disease in Vietnam have ever been carried out. Therefore, studies of *Colletotrichum* species associated with diseases of coffee and some other tropical crops were addressed in this thesis. In addition, the genetic structure of *C. gloeosporioides* populations from different geographical regions and different coffee tissues was investigated.

I hope that the results of the present study are valuable in providing better insights into the biology and etiology of the pathogen and that these insights are helpful in the development of better disease management strategies and in the breeding of more resistant coffee varieties for sustainable coffee production in Vietnam.

2 Background

2.1 Coffee as a crop – general information

Coffee was first discovered in central Ethiopia and was taken to Yemen for cultivation during the 6th century AD (Kimani *et al.*, 2002). Coffee is now cultivated in more than 60 countries of the world, in both tropical and subtropical regions in Asia, Africa and South America at latitudes between 23° North and 25° South (Pinkert, 2004). Coffee products mainly come from South America (mostly Brazil and Colombia), Asia (mostly Vietnam, Indonesia and India) and Central America, representing about 40, 25 and 17% of the world's coffee production, respectively (Waller *et al.*, 2007). The remainder of the coffee produced originates from Africa (mostly Ethiopia, Tanzania, Kenya and Uganda). Three countries (Brazil, Vietnam and Colombia) are responsible for more than 50% of the world's coffee production (Waller *et al.*, 2007).

Coffee belongs to the genus *Coffea* in the family *Rubiaceae*, which consists of 500 genera and over 6000 species (Waller *et al.*, 2007). There are about 100 species belonging to the genus *Coffea* but only two main coffee species, Robusta coffee (*C. canephora*) and Arabica coffee (*C. arabica*), are commercially cultivated (Kimani *et al.*, 2002; Waller *et al.*, 2007). Arabica coffee is grown mostly in the tropical highlands of Africa and South America and makes up the bulk of global coffee, accounting for approximately 60% of world production (Kimani *et al.*, 2002). Robusta coffee is predominantly cultivated at low altitude in hot areas of Africa and Asia. Robusta coffee has a powerful taste, while Arabica coffee is a fine-flavoured, aromatic type with larger berries and beans than Robusta coffee. Robusta coffee is better adapted to the warm and humid equatorial climate and is more resistant to unfavourable conditions than Arabica coffee (Kimani *et al.*, 2002; Waller *et*

al., 2007). Arabica coffee is susceptible to coffee leaf rust (*Hemileia vastatrix*). However, 'Hybrido de Timor', a hybrid variety derived from natural crosses between *C. arabica* and *C. canefora*, has the phenotype of *C. arabica* but is resistant to coffee leaf rust. 'Catimor' is a contraction of the variety names 'Catura' and 'Hybrido de Timor' and is characterised by high yields and resistance to all known races of coffee leaf rust (Clarke & Vitzthum, 2001).

2.2 History of coffee and its cultivation in Vietnam

Vietnam is located in South-east Asia. The country is 1650 km long from north to south, and it is situated within the tropical and temperate zones of the Northern hemisphere. The Hai Van mountain pass, with an altitude of over 1000 m, separates the north and south of Vietnam into two different climatic zones (Doan, 2001). The south of Vietnam belongs to the hot and humid tropics and has two different seasons, i.e. a rainy season from May to October, and a dry season from November to April. The north of the country has four distinct seasons: a hot and wet summer, a dry autumn, a cold winter and a wet spring.

Coffee was introduced to Quang Binh and Quang Tri provinces of Vietnam in 1857 by French missionaries. Thirty years after coffee was first brought into Vietnam, large-scale coffee plantations were established in the midland provinces of the northern part, e.g. Coc Thon (Ha Son Binh) and Bavi (Ha Tay) and in the northern central regions of Vietnam, e.g. Van Du (Thanh Hoa) and Cao Trai (Nghe An). The total acreage reached approximately 10,000 ha in 1945, mainly in Dak Lac province in the Central Highlands (Doan, 2007). After the reunification of Vietnam in 1975, coffee production significantly increased as a result of collaboration with Russia, Germany, Bulgaria and Poland. The coffee development programme was mainly introduced in the Western Highland provinces, i.e. Dak Lac, Gia Lai, Kon Tum and Lam Dong and other provinces in the south. Later, it spread to the Northern provinces, such as Nghe An, Son La, Tay Bac, etc. (Doan, 2001; Doan, 2005). At present, coffee plantations (approximately 500,000 ha) are mostly concentrated in the Central Highland provinces, Dak Lak, Lam Dong, Gia Lai and Kon Tum, which account for 85% of the total coffee producing area in Vietnam. Dac Lak province is the main coffee producing region, with 260,000 ha of plantations representing 60% of the national coffee production and accounting for over 95% of local income (De Fontenay & Leung, 2004).

As mentioned above, coffee is the major income earner in Vietnam after rice, with an export value of US$ 570 million obtained from 900 thousand

tons of coffee and accounting for 12% of Vietnam's total exports in 2004 (De Fontenay & Leung, 2004; Doan, 2005). The dramatic boom in coffee production in the past 15 years has led to Vietnam overtaking Columbia to become the second largest coffee exporter in the world after Brazil. By 1999, Vietnam exceeded Indonesia and became the largest Robusta coffee producer in Asia (Greenfield, 2002; Doan, 2007; Waller *et al.*, 2007). Vietnamese coffee products are mainly exported and only 4% of the coffee produced is consumed domestically. In Vietnam, 85-90% of the coffee plantations belong to smallholders and the remaining area of cultivation is owned by state farms (De Fontenay & Leung, 2004). During the past 20 years Robusta coffee has gradually been replaced by Arabica coffee varieties, due to better quality and higher price advantages with Arabica coffee, as well as problems with coffee rust in many Robusta coffee plantations. Approximately 100,000 of 400,000 hectares of Robusta coffee have been replaced by Arabica coffee (Doan, 2005).

2.3 *Colletotrichum* as plant pathogens

Filamentous fungi of the genus *Colletotrichum* and its teleomorph *Glomerella* are among the most important plant pathogens world-wide. High yield losses due to both pre-harvest and post-harvest diseases caused by various species of *Colletotrichum* have been reported (Jeffries *et al.*, 1990; Bailey & Jeger, 1992). Sixty-six species of *Colletotrichum* recently described by Hyde *et al.* (2009a) can cause plant diseases. *Colletotrichum gloeosporioides* Penz is so far the most predominant *Colletotrichum* pathogen and can attack about 470 different host genera (Sutton, 1980; Dodd *et al.*, 1992; Cannon *et al.*, 2008). *Colletotrichum acutatum* Simmonds is also a major pathogen, with a world-wide distribution recorded from 34 host plant genera in 22 families (Walker *et al.*, 1991). Some species of *Colletotrichum* are more specific on a single host plant, e.g. *C. capsici*, *C. coccodes*, *C. falcatum, C. fragariae, C. kahawae*, *C. crassipes*, *C. graminicola, C. orbiculare*, *C. truncatum*, etc. (Waller, 1992; Waller *et al.*, 1993; Hyde *et al.*, 2009a). Different species can infect different parts of the same host plant, causing distinct diseases that occur successively during crop development (Waller, 1992; Freeman *et al.*, 2000b). For example, on legumes at least nine species of *Colletotrichum* have been found to be associated with diseases (Lenné, 1992).

The symptomatology of infection caused by *Colletotrichum* species varies depending on host plant and host tissue (Waller, 1992), e.g. infection of above-ground plant parts, leaves, young tissues and stems typically appears as depressed black lesions that are subcircular or angular in shape (commonly

known as anthracnose). More than 1000 plant species have encountered problems with anthracnose (Moriwaki *et al.*, 2002). *Colletotrichum* spp. are also known to cause branch die-back, root rot, leaf spot, defoliation and blossom blight and rot, as well as seedling blight (Jeffries *et al.*, 1990; Waller, 1992). The lesions enlarge, coalesce and destroy large areas, frequently around the edges of leaves, which cause leaf curling in cases of severe infection (Dodd *et al.*, 1992). Secondary leaf fall is considered to be the main problem of senescent rubber trees (Waller, 1992; Guyot & Omanda, 2005). Blossom infections of mango, citrus, avocado, coffee and other fruit crops caused by *Colletotrichum* have been reported (Jeffries *et al.*, 1990; Dodd *et al.*, 1992; Masaba & Waller, 1992; Waller, 1992). In blossom blight of mango, small brown or black spots on flowers first appear and can lead to the entire inflorescence blackening, and preventing the formation of fruit. Later infections produce lesions on young fruit that commonly result in fruit shedding (Jeffries *et al.*, 1990). At later stages of lesion expansion, a pinkish slimy mass of spores is generally produced on the plant surface as the underlying acervuli mature (Waller, 1992). Infections of several kinds of fruits usually do not develop further but become quiescent and later the lesion becomes dark when the fruit ripen during the post-harvest period, e.g. in banana, mango, avocado and papaya. Yield losses of mango due to post-harvest disease can be 20% (Waller, 1992). Higher economic losses (25%) are caused by quiescent infection, due to the expense of harvesting, transportation, storage and packing, compared with yield losses in the field (Almada-Ruiz *et al.*, 2003).

2.4 Diseases caused by *Colletotrichum* spp. on tropical crops

Diseases caused by *Colletotrichum* species occur on a broad range of crops and are predominantly found in tropical and subtropical regions (Waller, 1992). The warm and moist environmental conditions in the tropics and sub-tropics are suitable for the development of anthracnose diseases caused by *Colletotrichum*, in particular on perennial crops (Waller, 1992). Species of *Colletotrichum* cause tremendous losses by damaging the fruits, blossoms or other parts of the plants. Reduction in the quantity and quality of the harvested produce are often due to disease problems caused by *Colletotrichum* (Jeffries *et al.*, 1990).

Serious disease problems occur on some of the most important fruit crops world-wide, e.g. mango, coffee, avocado, papaya, citrus and banana (Jeffries *et al.*, 1990; Waller, 1992). *Colletotrichum* attack fruits during development in the field (pre-harvest) as well as during the post-harvest period. Infections at

the fruiting stage are common on these crops and cause the highest yield losses (Waller, 1992). Post-harvest diseases caused by *Colletotrichum* species are also a major problem on tropical crops as they lead to degraded fruit quality and can be troublesome for exported products (Jeffries *et al.*, 1990; Almada-Ruiz *et al.*, 2003).

Colletotrichum gloeosporioides is the most common pathogen on economically important tropical crops (Dodd *et al.*, 1992; Prusky & Plumbley, 1992; Waller, 1992). Besides *C. gloeosporioides,* other *Colletotrichum* species, e.g. *C. acutatum, C. capsici*, *C. falcatum, C. coccodes, C. dematium* etc.*,* can either infect the plant alone, or be jointly associated with *C. gloeosporioides* (Kimani *et al.*, 2002; Sharma *et al.*, 2005; Than *et al.*, 2008a; Than *et al.*, 2008b; Kang *et al.*, 2009; Mishra & Behera, 2009). Thus, these species can be either a primary pathogen or a secondary pathogen. The losses due to the diseases caused by *Colletotrichum* are reported to account for 10% of total pepper production in Korea (Kang *et al.*, 2009). *Colletotrichum acutatum*, *C. gloeosporioides* and *C. capsici* are considered the major causal agents of chili anthracnose, which causes severe infections and yield losses of up to 50% in Thailand (Than *et al.*, 2008b).

2.5 Diseases caused by *Colletotrichum* spp. on coffee

2.5.1 Coffee Berry Disease (CBD) in Africa caused by *C. kahawae*

Coffee berry disease (CBD) is an anthracnose of green and ripe coffee berries caused by *C. kahawae* Waller & Bridge (formerly referred to as a form of *C. coffeanum*) (Waller *et al.*, 1993; Waller & Bridge, 2000; Hyde *et al.*, 2009a). The fungus attacks all parts of the plant including flowers, berries and occasionally also branches and leaves (Waller *et al.*, 1993; Varzea *et al.*, 2002; Mouen Bedimo *et al.*, 2007; Waller *et al.*, 2007). The symptoms first appear as small dark sunken patches on the pericarp of green berries that can later coalesce rapidly to cover the whole berry surface and destroy the bean (Masaba & Waller, 1992; Mouen Bedimo *et al.*, 2007). The infected berries mostly shed at an early stage of infection or remain mummified on the stems (Waller, 1992). *Colletotrichum kahawae* also causes 'brown blight' symptoms in association with *C. gloeosporioides* on ripening berries (Waller *et al.*, 2007).

Coffee berry disease was first found in Western Kenya in 1922 (McDonald, 1926) and thereafter it was reported in Zaire in 1939, Cameroon in 1964 and later in Angola, Tanzania, Ethiopia, Malawi, Zimbabwe and Zambia (Masaba & Waller, 1992). The highest losses are due to premature berry shedding. This disease is the major threat to the

production of Arabica coffee in Africa. Yield losses caused by the fungus can be 50-80% without sufficient fungicide treatments and down to 10-15% in well-protected plantations (Griffiths *et al.*, 1971; Waller, 1985). Severe infection is particularly observed at high altitude (about 1600 m above sea level in Kenya) (Waller *et al.*, 2007). Waller & Bridge (2000) reported that the higher the altitude of the coffee cultivation, the higher the proportion of *C. kahawae* on diseased samples. It has been suggested that *C. kahawae* survives on vegetative organs such as flower buds, branch bark and remaining mummified berries and these can be regarded as the main source of inoculum for the infection of successive berries (Mouen Bedimo *et al.*, 2007).

The name *C. coffeanum* for *Colletotrichum* species found on coffee was applied in 1901 by Noak in Brazil, where CBD was not present (Waller *et al.*, 1993; Hyde *et al.*, 2009a). *Colletotrichum coffeanum* is regarded as a saprophyte or weak pathogen of coffee and has been described as being synonymous with *C. gloeosporioides* (Waller *et al.*, 1993; Hyde *et al.*, 2009a). Therefore, the use of *C. coffeanum* as the CBD pathogen was a misapplication in previous studies (Vermeulen, 1979; Sutton, 1980; Sutton, 1992). Waller *et al.* (1993) described the highly pathogenic strain causing CBD in Africa and renamed it *C. kahawae* based on morphology, growth rate and biochemical and ecological features. This species is very closely related to *C. gloeosporioides*, as indicated by genetic similarities (Sreenivasaprasad *et al.*, 1993; Cannon *et al.*, 2000). However, *C. kahawae* can be distinguished from *C. gloeosporioides* by its inability to utilise citric acid and ammonium tartrate as sole carbon sources, by its high pathogenicity on coffee and by molecular tools, e.g. AFLP, PCR-RFLP, VNTR-PCR and sequence analysis of multiple genes (Waller *et al.*, 1993; Martinez-Culebras *et al.*, 2003; Bridge *et al.*, 2008; Prihastuti *et al.*, 2009). Since *C. kahawae* causes a specific disease on coffee, there are practical reasons for considering it a distinct taxon (Bridge *et al.*, 2008). So far it has not been reported in areas outside Africa. However, it can still be regarded as one of the main threats to coffee production in Asia and South America (Waller *et al.*, 2007).

2.5.2 Diseases caused by *Colletotrichum* spp. on coffee in Vietnam and other countries outside Africa

Symptoms of diseases caused by *Colletotrichum* spp. on coffee are similar to those on other perennial crops and result in necrosis of flowers, fruits, branches and leaves. *Colletotrichum* infection on coffee is usually characterised by i) brown or black lesions on flowers and berries (brown blight

symptoms); ii) necrotic irregular spots on leaf margins and defoliation; and iii) blackened branches and die-back (Waller, 1992; Tran *et al.*, 1998; Sera *et al.*, 2007). Outside Africa, complex *Colletotrichum* infections on coffee have been reported in China, Papua New Guinea, India, Colombia, Costa Rica, Guatemala, and Brazil (Waller, 1992; Chen *et al.*, 2003; Kenny *et al.*, 2006; Sera *et al.*, 2007; Waller *et al.*, 2007; Rodriguez *et al.*, 2008).

In Vietnam, problems with anthracnose on coffee was first discovered in 1930 in Kon Tum and the Southern Highlands and more severe disease problems were reported in 1998 (Tran *et al.*, 1998). Diseases of coffee trees were investigated from 1995 to 1997 and the highest infection rate (51.4%) was found on *C. arabica*, where it caused premature berry shedding (Tran *et al.*, 1998). According to Pinkert (2004), coffee trees with a biennial bearing pattern can experience overbearing in some seasons, which results in physiological stress followed by die-back and low or no yield in the following year. This disease has been partly explained due to lack of basic agricultural knowledge in coffee cultivation in Vietnam.

Different species of *Colletotrichum,* e.g. *C. gloeosporioides, C. acutatum* and *C. coffeanum,* associated with anthracnose disease on coffee have been reported in Asia and South America (Chen *et al.*, 2003; Chen *et al.*, 2005; Silva *et al.*, 2005; Sera *et al.*, 2007; Prihastuti *et al.*, 2009). It has been reported that *C. gloeosporioides* is responsible for die-back disease of branches in China due to an excess of berries in the preceding seasons (Chen *et al.*, 2003). New species of *Colletotrichum* associated with diseases of coffee that could be distinguished from *C. gloeosporioides* and *C. kahawae*, i.e. *C. asianum, C. fructicola* and *C. siamense*, have been found in Thailand. According to morphological types, Tran *et al.* (1998) assumed that *C. coffeanum* and *C. capsici* could be the causal agent of coffee anthracnose in Vietnam. However, the species identification was not confirmed with molecular tools and there is still little knowledge about the *Colletotrichum* species associated with coffee anthracnose in Vietnam.

2.6 Systematics of *Colletotrichum*

The genus *Vermicularia*, former name of *Colletotrichum,* was first described by Tode in 1790 (Sutton, 1992; Hyde *et al.*, 2009a). Thereafter, the genus *Colletotrichum*, characterised by hyaline, straight or falcate conidia and setose acervuli, was established by Corda (1831). Later, von Arx (1957) studied the taxonomy of this genus carefully and reduced the number of described taxa from several hundred to 11 accepted species. Sutton (1992) increased the number of accepted species of *Colletotrichum* to 39. However, according to

the author, the taxonomic position of some species remained unclear. Several species of *Colletotrichum*, i.e. *C. gloeosporioides, C. acutatum*, *C. graminicola* and *C. dematium*, are broadly defined and considered to be species complexes or 'group species' (Sutton, 1992; Cannon *et al.*, 2000). Many species are now regarded as synonyms of *C. gloeosporioides* (Penz.) Sacc. There are about 600 synonyms to be cited for this species (von Arx, 1957). *Colletotrichum gloeosporioides* (teleomorph: *Glomerella cingulata* (Stoneman) Spauld. & H. Schrenk) is found on a broad range of host plants. The currently defined species boundaries are vague and relationships within some of these species complexes are not well-resolved (Sutton, 1992; Cannon *et al.*, 2000). In a recent study, Hyde *et al.* (2009a) described 66 species of *Colletotrichum* on the basis of morphology and sequence analysis of multiple genes.

Colletotrichum is an anamorphic genus with complicated mating behaviour. Only a few species are known to produce the *Glomerella* teleomorph. Within the same species of *Colletotrichum*, there are strains that are strictly homothallic or heterothallic, and at the same time there are strains exhibiting the phenomenon of unbalanced heterothallism due to compatible gene mutations in the self-recognition (homothallic) pathway (Vaillancourt *et al.*, 2000). Therefore the production of ascospores is unreliable, and conditions for ascospore formation are rather unpredictable. In the light of this, it is understandable that the taxonomy of the genus is based on the anamorph. The *Colletotrichum* anamorph, in its turn, shows a high plasticity of the morphological traits that are traditionally used for species identification and therefore morphological descriptions of different species overlap substantially (Sutton, 1992; Freeman *et al.*, 1998; Vinnere, 2004). In addition, there is large variation among and within *Colletotrichum* species in pathogenicity, culture appearance and uncertain relationships with host plants (Sutton, 1992). Therefore, these traits can make identification of *Colletotrichum* species difficult and inaccurate. Molecular systematics has been successfully used in studies of this problematic genus and has resulted in well-defined delineations of species (Mills *et al.*, 1992b; Freeman *et al.*, 1998; Cannon *et al.*, 2000; Guerber *et al.*, 2003; Abang *et al.*, 2006; Cannon *et al.*, 2008; Hyde *et al.*, 2009a). DNA sequencing of a variety of different genes and regions of the fungal genomes has been proven to be informative in assisting species identification (Sreenivasaprasad *et al.*, 1996a; Farr *et al.*, 2006; Cannon *et al.*, 2008; Cai *et al.*, 2009; Hyde *et al.*, 2009a) and is therefore used to complement the morphological data.

2.6.1 Traditional methods

Traditional approaches to identification of species belonging to the genus *Colletotrichum* as well as other filamentous fungi have always relied on morphological characteristics such as colony colour, size and shape of conidia, presence or absence of setae and teleomorph, and cultural criteria (Sutton, 1980; Van der Aa *et al.*, 1990; Gunnell & Gubler, 1992; Liyange *et al.*, 1992; Sutton, 1992; Agrios, 2005). Smith & Black (1990) have successfully used these features in differentiation between species of *C. fragariae*, *C. acutatum* and *C. gloeosporioides* associated with strawberry diseases. Growth rates and conidial morphology can be applied as criteria for delineation of *C. acutatum* from *C. gloeosporioides* (Simmonds, 1965; Vinnere *et al.*, 2002; Talhinhas *et al.*, 2005). However, to some extent, morphological features alone cannot be used as a reliable tool for accurate identification of *Colletotrichum* species, especially within species complexes that share similar morphology but are genetically different (Sutton, 1980; Sutton, 1992). *Colletotrichum* species grown in culture frequently produce intermediate forms of conidia and vary considerably in colony appearance (Sutton, 1992; Freeman *et al.*, 1998; Cannon *et al.*, 2000). Many morphological traits of the genus *Colletotrichum* are extremely plastic and variable and depend mostly on cultural and environmental conditions, which are rarely standardised (Sutton, 1992).

2.6.2 Vegetative compatibility grouping (VCG)

Vegetative compatibility is the ability of hyphae of two individuals to fuse and form a heterokaryon containing nuclei of both parent strains. This mechanism, which controls genetic isolation of fungal populations, was reviewed by Leslie (1993). Since hyphal anastomosis is a prerequisite for the exchange of genetic material, isolates belonging to the same vegetative compatibility group (VCG) are expected to be more similar to one another, therefore constituting a distinct genetic population (Freeman & Katan, 1997). Studies of VCG offer another tool to determine genetic relatedness among populations of asexual fungal pathogen populations (Correll *et al.*, 1987; Brooker *et al.*, 1991; Katan & Shabi, 1996; Varzea *et al.*, 2002; Abang *et al.*, 2004; Bridge *et al.*, 2008). This approach cannot be used for taxonomical classification of *Colletotrichum*, but is useful for characterisation of genetic diversity of *Colletotrichum* populations, which is valuable for insight into disease etiology, population structure, host specificity, geographical distribution and reproductive strategy (Freeman *et al.*, 1998; Katan, 2000; Varzea *et al.*, 2002; Abang *et al.*, 2004; Bridge *et al.*, 2008). Varzea *et al.* (2002) assumed that heterokaryon formation under *in vitro*

conditions was different from that in nature. Therefore, population diversity may be underestimated using this method (Varzea *et al.*, 2002; Abang *et al.*, 2004).

2.6.3 Molecular approaches

Molecular tools can be successfully applied for discrimination among species and genotypes of *Colletotrichum* derived from numerous hosts world-wide. Molecular methods that have been employed can be divided into two main groups. The first of these consists of PCR-based techniques, which are commonly used to characterise genetic diversity among *Colletotrichum* populations and closely related species. The most commonly used methods in this category include RAPD (random amplified polymorphic DNA) (Munaut *et al.*, 1998), MP-PCR (microsatellite-primed polymerase chain reaction) (Freeman *et al.*, 2000a; Weeds *et al.*, 2003), PCR-RFLP (restriction fragment length polymorphism PCR) (Martínez-Culebras *et al.*, 2000; Martinez-Culebras *et al.*, 2003), AFLP (amplified fragment length polymorphism) (O'Neill *et al.*, 1997), etc. The other group is based on DNA sequence comparisons of variable genetic regions, which have been widely used for identification and characterisation of *Colletotrichum* species (Sreenivasaprasad *et al.*, 1996a; Vinnere *et al.*, 2002; Guerber *et al.*, 2003; Lubbe *et al.*, 2004; Cannon *et al.*, 2008; Than *et al.*, 2008b; Damm *et al.*, 2009; Hyde *et al.*, 2009b).

2.6.3.1 Arbitrarily primed PCR (ap-PCR) markers

Ap-PCR markers are rapid, inexpensive and suitable for studying large amounts of samples. This approach only requires minimal amounts of DNA, can be applied without prior genetic information about the organism and are treated as dominant markers (Weising *et al.*, 2005). Ap-PCR markers are valuable tools in identifying variation in a wide variety of phytopathogens and can differentiate between closely related groups of pathogens (Assigbetse *et al.*, 1994; Freeman *et al.*, 1998). In general, these methods have been successfully used for discrimination and characterisation of inter- and intra-species variation in *Colletotrichum* (Freeman *et al.*, 1998; Martínez-Culebras *et al.*, 2000; Weeds *et al.*, 2003; Lu *et al.*, 2004; Abang *et al.*, 2006).

Random amplified polymorphic DNA (RAPD)

The technique employs single primers with 10 arbitrary nucleotide sequences and at least 50% GC content (Williams *et al.*, 1990). PCR products are separated on agarose gels and detected by staining in ethidium bromide. This approach has been applied mainly in molecular systematics at

the species level or for studying the genetic structure of populations (Weising *et al.*, 2005). However, the method is highly sensitive to non-stringent PCR conditions and therefore the reproducibility of banding patterns in independent assays can be troublesome (Weising *et al.*, 2005).

Unanchored/anchored microsatellite-primed polymerase chain reaction (MP/AMP-PCR)

There are different acronyms existing for this technique, i.e. single primer amplification reactions (SPAR), inter-simple sequence repeat PCR (ISSR-PCR) and unanchored/anchored microsatellite–primed PCR (MP/AMP-PCR) (Weising *et al.*, 2005). The technique uses a single primer (16-25bp long) in PCR reaction that amplifies inter-microsatellite sequences at multiple loci throughout the genome. The primers are either unanchored or anchored at the 5' or 3' end of a repeat region and extend into the flanking region. The primers are designed based on di-, tri- and tetra nucleotide tandem repeats (Weising *et al.*, 1995; Weising *et al.*, 2005). More reproducible bands are yielded by this method than by the use of RAPD markers (Weising *et al.*, 2005). The PCR-amplified products are separated on either agarose or polyacrylamide gels that are visualised after ethidium bromide or silver staining, respectively.

2.6.3.2 Phylogeny and DNA sequence analysis of multiple genes

Phylogenetic analysis of various genes and regions of fungal genomes has been proven to be informative in assisting species delineation in *Colletotrichum*. Examples of such regions are the internal transcribed spacer within the ribosomal DNA array (ITS) (Sreenivasaprasad *et al.*, 1996a; Freeman *et al.*, 1998; Cannon *et al.*, 2000; Cannon *et al.*, 2008), a fragment of the mitochondrial small subunit rRNA gene (mtSSU) and a portion of the ß-tubulin gene (Vinnere *et al.*, 2002), a 200-bp intron of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, a partial sequence of the actin (ACT) gene and the chitin synthase 1(CHS-1) gene (Damm *et al.*, 2009; Hyde *et al.*, 2009b), as well as the high mobility group domain within the mating type gene (*MAT1-2* HMG). All above-mentioned genes are adequate regions for studying *Colletotrichum* species complexes and can provide much better insights into the relationships within the genus *Colletotrichum* (Cannon et al., 2000; Du et al., 2005; Cannon et al., 2008; Cai et al., 2009; Damm et al., 2009). The ITS rDNA region and a portion of the β-tubulin gene are the most commonly used for molecular systematics of *Colletotrichum* (Sreenivasaprasad *et al.*, 1996a;

Freeman *et al.*, 1998; Cannon *et al.*, 2000; Vinnere, 2004; Cannon *et al.*, 2008; Than *et al.*, 2008b).

DNA sequence analysis of the ribosomal RNA (rRNA) genes and spacers

DNA sequence analysis of rRNA genes and spacers is known to be a valuable tool in modern fungal molecular systematics. Ribosomal RNA genes are present in multiple copies in the genome and include various regions with different rates of evolution (Hillis & Dixon, 1991). Four nuclear and two mitochondrial rRNA genes have been widely used as useful molecular probes in differentiation and diagnosis of fungi due to high levels of variation and a wide range of evolution rates (White *et al.*, 1990; Bruns & Szaro, 1992).

The nuclear rDNA array consists of genes coding for 18S, 5.8S and 28S and the internal transcribed spacer regions, ITS1 and ITS2 (White *et al.*, 1990). Due to high levels of conserved sequences of rRNA genes, 18S and 28S, universal primers have been constructed to allow DNA amplification and sequencing of interesting regions for characterisation of many fungal species (White *et al.*, 1990; Bruns *et al.*, 1991; Hillis & Dixon, 1991; Gardes & Bruns, 1993). The ITS (non-coding) region evolves fastest and varies among species and populations within a genus (Gerbi, 1985; Bruns *et al.*, 1991; Hillis & Dixon, 1991). Therefore, it has been found to be informative for characterisation of closely related species of *Colletotrichum* (Sreenivasaprasad *et al.*, 1996a; Cannon *et al.*, 2000; Lubbe *et al.*, 2004; Farr *et al.*, 2006; Cannon *et al.*, 2008). These regions are suitable for detection of recent evolutionary divergence within *Colletotrichum* due to less conservation than those in the 18S and 28S genes (Sreenivasaprasad *et al.*, 1996a; Cannon *et al.*, 2000; Freeman, 2000; Sreenivasaprasad & Talhinhas, 2005; Cannon *et al.*, 2008). In addition, species-specific primers have been designed on the basis of the sequence variation within the ITS region. They can be used in combination with conserved universal primers in species identification of *Colletotrichum* (Sreenivasaprasad *et al.*, 1992; Sreenivasaprasad *et al.*, 1996b; Freeman, 2000).

Mitochondrial rRNA genes have also been successfully used for numerous phylogenetic studies because of their relatively high evolution rates. They evolve much more rapidly than the nuclear rDNA (Brown *et al.*, 1979; Gerbi, 1985; Hillis & Dixon, 1991) and can therefore potentially offer a useful tool in classification and phylogenetic reconstruction of genetically closely related species (Bruns & Szaro, 1992; Hong *et al.*, 2002). Bruns & Szaro (1992) reported that the evolution rate of the mitochondrial small subunit (mtSSU) is 16 times faster than that of the small subunit nuclear

gene 18S but less variable than that of the ITS rDNA. Analysis of partial sequences of mtSSU amplified by universal primers allows differentiation of fungal strains or populations at genus or species level (White *et al.*, 1990; Li *et al.*, 1994; Hong *et al.*, 2002). This can also be a reliable probe for diagnosis of different fungal species including *Colletotrichum* (Li et al., 1994; Vinnere et al., 2002). Sequence analysis of the mtSSU region, in addition to ITS, can provide better resolution in phylogenetic analyses of *Colletotrichum* (Vinnere *et al.*, 2002).

Sequences of high mobility group domain within the mating type genes (MAT1-2 HMG)

Mating type (*MAT*) genes have a faster rate of evolution compared with many other sequences in the genome, and *MAT* sequences exhibit high genetic diversity among species and low diversity within species of filamentous fungi (Turgeon, 1998). Sequencing of the high mobility group domain within the mating type gene (*MAT1-2* HMG) is therefore considered a useful tool for phylogenetic studies of *Colletotrichum* and differentiation among closely related groups belonging to *Colletotrichum* species complexes (Du *et al.*, 2005; Moriwaki & Tsukiboshi, 2009). Phylogenetic analysis based on the *MAT1-2* genes shows better resolution among the various lineages of the *Colletotrichum* species than the trees generated from the ITS sequences due to a higher variation of the *MAT1-2* genes compared with the ITS region (Du *et al.*, 2005; Moriwaki & Tsukiboshi, 2009).

3 Objectives of the study

The overall aim of this thesis was to study the morphology, pathogenicity, phylogeny and genetic diversity of *Colletotrichum* spp. associated with diseases, mainly on coffee in Vietnam but also on some other major tropical crops in the region. Specific objectives were to:

1) Identify and characterise *Colletotrichum* spp. on coffee in Vietnam and some economically important crops in Vietnam and Thailand.

2) Investigate whether these isolates were pathogenic on green berries and hypocotyls of coffee and could therefore be the causal agent of the disease in Vietnam.

3) Investigate whether the CBD pathogen is present in Vietnam by comparing Vietnamese *C. gloeosporioides* isolates and reference isolates of the CBD pathogen, *C. kahawae,* on the basis of pathogenicity and molecular, morphological, cultural and biochemical traits.

4) Characterise the genetic structure and diversity among *C. gloeosporioides* populations from various geographical areas in Vietnam.

5) Study genetic variations among *C. gloeosporioides* populations from different coffee plant tissues and determine whether there is tissue specificity within *C. gloeosporioides* populations on coffee plants in Vietnam.

4 Summary of results and general discussion

4.1 Disease symptoms and survey of disease severity in coffee growing areas of Vietnam

According to our own observations and interviews with coffee researchers Tran Kim Loang (Western Highland Institute) and Bui Thi Tram (Ba Vi) and several local coffee farmers, the following symptoms were observed (see Fig. 1): Slightly sunken dark lesions or brown blight lesions were commonly found on green and ripening berries in different geographical areas of Vietnam. The fungus penetrated and colonised the pericarp but in most cases it did not penetrate and destroy the bean. Berry shedding usually occurred as infected berries ripened. Furthermore, anthracnose symptoms on leaves and twigs as well as die-back were often observed on different coffee plantations. Leaf anthracnose first appeared as necrotic and brown lesions on leaf margins and later concentric rings developed in which visible acervuli could occur. Anthracnose of coffee leaves was often observed during coffee development, and it caused defoliation and die-back in severe cases. On coffee twigs, the lesions were usually dark brown or black and formed girdles surrounding the stem. In senescent branches and trees, the disease could be so severe that they yielded no berries and defoliation occurred. The disease appeared during the whole year and it is claimed that it can also cause quiescent infections on twigs (Tran Kim Loang and Bui Thi Tram, personal communication). High infection rates of leaves, twigs and berries are reported to occur simultaneously during the fruiting stage in the beginning of the rainy season and cause severe damage during mature stages of the berries (Tran *et al.*, 1998). These symptoms have mostly appeared on the Arabica cultivar 'Catimor', which is now commonly grown and has

Figure 1. Symptoms of coffee anthracnose on berries, leaves and twigs in Vietnam.

gradually replaced Robusta coffee in Vietnam during the past 20 years. Levels of disease vary among coffee plantations and geographical areas. More severe infection often occurs on coffee plantations that have been exposed to environmental stress conditions due to low financial investment in coffee cultivation and poor cultural practices in particular (Tran Kim Loang and local coffee farmers, personal communication). Among the coffee farmers, there is a knowledge gap regarding cultivation for sustainable coffee production and this is promoting disease spread and infection severity.

Figure 2. The locations of coffee growing plantations where samples for *Colletotrichum* isolation were collected in Vietnam, indicated by round dots.

Surveys of the disease severity on berries, leaves and twigs were carried out in fields of Arabica coffee (cv. Catimor) in Lam Dong (Don Duong and Lam Ha) and Dac Lak (Ma Drac) provinces in 2009 (see Fig. 2). Since we suspected that the infection rate might depend on the age of the coffee plants, the age of plants in the visited fields was noted with the assistance of local farmers. The plant age of the inspected fields ranged between 2-12 years. At each location, 7-9 coffee fields were carefully inspected (Table 1).

Table 1. *Survey of coffee anthracnose caused by Colletotrichum spp. in southern Vietnam in 2009.*

Location (district)		Age of coffee plants (years)	Infected tissues (%)		
Commune	Village name		Berries	Leaves	Twigs
Ma Drac					
Earieng	18	4	1.0	0.5	24.0
Earieng	18	6	0.5	0.5	24.0
Earieng	7	12	7.5	0.5	10.0
Earieng	16	7	0.5	4.0	10.0
Earieng	1	6	1.0	0,5	1.0
Earieng	1	6	4.0	3.0	16.0
Earieng	1	6	2.5	2.0	6.0
	Mean		*2.4*	*1.6*	*13.0*
	SD		*2.6*	*1.5*	*8.8*
Don Duong					
Da Ron	3	7	40.0	15.0	55.0
Da Ron	3	4	22.5	3.5	20.0
Da Ron	3	3	5.0	0.5	15.0
Da Ron	3	4	40.0	40.0	60.0
Da Ron	2	5	17.5	10.0	40.0
Da Ron	2	12	40.0	1.0	40.0
Da Ron	2	2	10.0	0.5	5.0
Hiep Thach	Hiep Thach	5	2.5	2.5	15.0
Hiep Thach	Hiep Thach	10	25.0	70.0	60.0
	Mean		*22.5*	*15.9*	*34.4*
	SD		*15.1*	*23.9*	*21.3*
Lam Ha					
Phuc Tho	Phuc Tan	12	15.0	4.0	65.0
Phuc Tho	Phuc Tan	12	7.5	4.0	35.0
Phuc Tho	Phuc Tan	3	2.0	0.5	10.0
Phuc Tho	5	6	35.0	4.0	65.0
Phuc Tho	5	6	2.0	1.0	15.0
Phuc Tho	4	8	2.0	0.5	7.5
Phuc Tho	4	4	10.0	0.5	30.0
Phuc Tho	3	4	2.5	1.0	30.0
Tan Van	Tan Van	6	20.0	0.5	65.0
	Mean		*10.7*	*1.8*	*35.8*
	SD		*11.2*	*1.7*	*23.8*

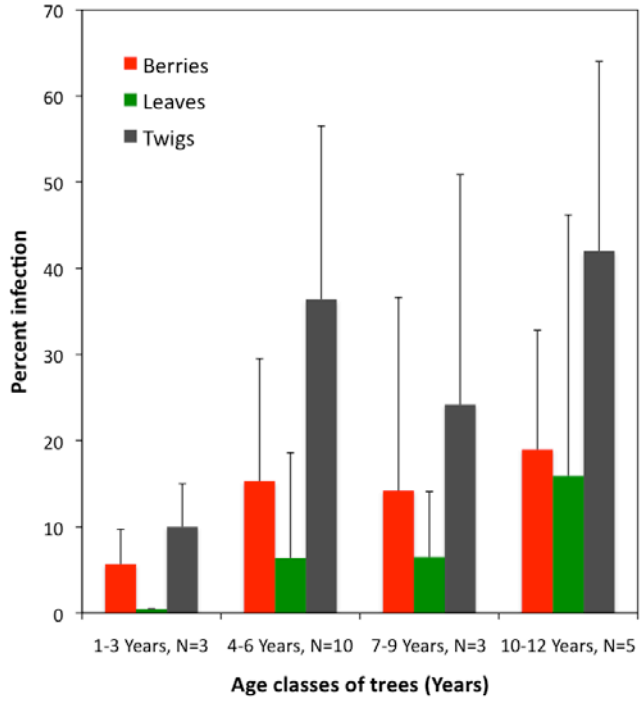

Figure 3. Infection rates on berries, twigs and leaves of coffee trees of different ages in the south of Vietnam in 2009; bars represent standard deviation; N: Number of coffee fields.

In each field, the average percentage infection was estimated, i.e. percentage of infected berries and leaves as well as branch length with anthracnose symptoms. Ten to 15 coffee tree canopies at 5-10 metre intervals following a line between two opposite ends of the field were scored. The distance between the fields inspected at each location was generally 3-5 km. Infection rates of berries, leaves and twigs varied substantially between plantations, probably due to differences in management practices and fertilizer inputs among coffee smallholders. In general, higher levels of infection were found on older trees (Table 1, Fig. 3). The symptoms were most severe on trees that were older than five years, probably due to physiological stress after overbearing in earlier seasons (Fig. 3). The disease infection rates on berries, leaves and twigs were higher in Lam Ha and Don Duong than in Ma Drac. For example, on average the percentage of infected berries was 22% in Don Duong and 11% in Lam Ha, while in Ma Drac only 2.4% of the berries were infected. However, to some extent the infection rates on berries might have been an underestimate of the disease severity, since the numbers of fallen berries were not counted. Assessment of the disease severity on berries is often complicated since survey data only express disease as a percentage of infected berries present on the tree, while

considerably numerous amounts of infected berries are not taken into account in the assessment due to berry shedding (Waller *et al.*, 2007).

4.2 Identification of *Colletotrichum* species on tropical crops (Paper I)

Fifty *Colletotrichum* isolates, including 33 from Thailand and 17 from Vietnam, were obtained from diseased parts of *Coffea arabica*, *Citrus aurantifolia, Vitis vinifera, Mangifera indica,* etc. Morphological examination of these *Colletotrichum* isolates revealed a great level of variation, as reported before by Sutton (1992, 1998). The majority of the isolates had straight cylindrical conidia that overall fitted into the commonly accepted description of *C. gloeosporioides* (Mordue, 1971; Sutton, 1980). However, among those isolates there were several which had conidia that were acuminate at one end and therefore had features of both *C. gloeosporioides* and *C. acutatum*. Growth rate tests were able to clearly separate isolates of the group with cylindrical conidia from the group with fusiform conidia. Differences in growth rate between *C. gloeosporioides* and *C. acutatum* have been reported by several scientific groups to be a reliable diagnostic feature able to separate these two species even if they have intermediate morphology (Simmonds, 1965; Smith & Black, 1990; Vinnere *et al.*, 2002). Similarly to previous studies, the isolate with fusiform conidia grew more slowly than those with cylindrical conidia.

The ITS1 & ITS2 regions and 5.8S ribosomal RNA gene from nuclear rDNA and a part of the mtSSU rRNA gene were PCR-amplified in order to identify the isolates obtained at species level. The PCR products from eight selected representative isolates of the fungal collection obtained in this study were sequenced and subjected to phylogenetic analysis together with sequences of reference isolates of several established species of *Colletotrichum*. Our morphological grouping was fully in agreement with the molecular data. Isolates with straight, cylindrical conidia were identified as *C. gloeosporioides* and isolates with fusiform conidia as *C. acutatum*.

However, we encountered problems with the identification of isolates with falcate conidia. After morphological examination, we preliminarily assigned those isolates to the *C. dematium* complex. The isolates were morphologically similar to our reference strains of the latter taxon (kindly identified by E. Mordue, IMI; see Vinnere *et al.* 2002 for details). However, sequencing and phylogenetic analysis showed that these isolates were not closely related, which was supported by both high bootstrap and posterior probability values. Comparisons with ITS sequences of several species with

falcate conidia obtained from the GenBank listed in Tables 1 and 2 (Paper I) gave a confusing pattern, probably due to incorrect identification of several of those specimens (data not shown). *Colletotrichum dematium* has been designated as the same taxon as *C. capsici* (von Arx, 1957). However, according to Mordue (1971), *C. dematium* has been described as a saprophytic species and has mainly been found in temperate regions (Sutton, 1980). In contrast, *C. capsici* is regarded to be a pathogenic fungus on a wide range of host plants in the tropics and subtropics (Sutton, 1980; Sutton, 1992; Than *et al.*, 2008b). *Colletotrichum truncatum* is known to be pathogenic strictly to legumes (Sutton, 1998). Therefore, we assumed that these Vietnamese isolates are closely related to *C. capsici*.

It was interesting to observe that mango can be infected by both *C. gloeosporioides* and *C. acutatum* in Vietnam. Similarly, coffee plants in a nursery in Ha Tay province (Ba Vi) of Vietnam were also infected by two different species of *Colletotrichum*, namely *C. capsici* and *C. gloeosporioides*. Interestingly, *C. gloeosporioides* strains VNBR5 and VNR15 were isolated from diseased roots of coffee trees. That is quite uncommon for *Colletotrichum,* which is usually restricted to aboveground parts.

4.3 *Colletotrichum* spp. associated with coffee diseases (Paper II)

Sampling was carried out during berry development (September) in 2004, 2005 and 2007 in different coffee growing areas of northern and southern Vietnam (Fig. 2).

Approximately 550 Vietnamese isolates of *Colletotrichum* spp. were obtained, based on morphological characters previously described in the literature (Mordue, 1971; Sutton, 1980; Sutton, 1992). The frequencies of species from the collection of Vietnamese *Colletotrichum* isolates, on the basis of spore type/cultural characteristics derived from infected coffee trees, are shown in Figure 4. *Colletotrichum gloeosporioides* was the dominant species, with an overall frequency of 87% and on berries it was even higher (92%; unpublished data).

Forty-six *Colletotrichum* isolates from the collection, representing different morphological types, were chosen for molecular studies (Paper II). Thirty-nine of these isolates had cylindrical conidia rounded at one or both ends, five isolates had fusiform conidia, and two isolates had falcate conidia. According to morphological and cultural traits as well as sequence analysis, four *Colletotrichum* species, namely *C. gloeosporioides*, *C. acutatum*, *C. capsici* and *C. boninense*, were identified. In addition, several isolates of unknown

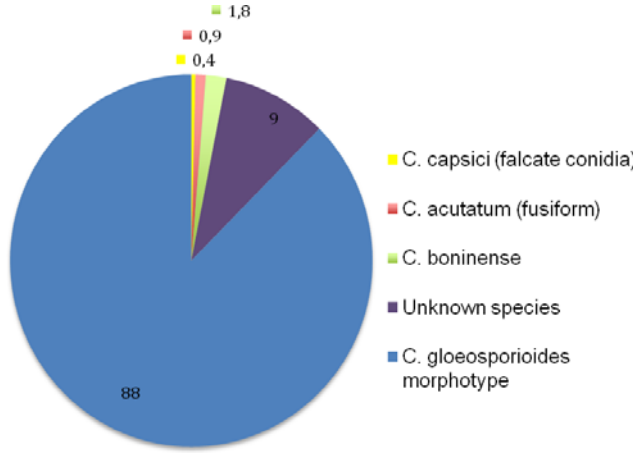

Figure 4. Frequencies of *Colletotrichum* spp. isolated from anthracnose diseases of coffee.

species were found. The conidia of the Vietnamese *Colletotrichum* varied considerably in length and width. Large conidia may be an artefact as fungi are grown on rich culture medium compared with direct examination on the plant material. Munaut *et al.* (2001) found that the length of conidia could be up to 29.5 µm among isolates of *C. gloeosporioides* associated with *Stylosanthes* spp. in Mexico. Twenty-three Vietnamese isolates of the 39 with cylindrical conidia and five isolates with fusiform conidia were identified as *C. gloeosporioides* and *C. acutatum* based on the results of PCR amplification with species-specific primers. These results were in agreement with phylogenetic analysis based on DNA sequence data from the ITS and a portion of mtSSU rDNA.

Some isolates having somewhat broader cylindrical conidia than *C. gloeosporioides* were identified as *C. boninense* (Moriwaki *et al.*, 2003). This species was first isolated in Japan and has been described as a new species in the *C. gloeosporioides* complex (Moriwaki *et al.*, 2003). Thereafter, it has been reported from South America, China, Australia, Zimbabwe and Brazil (Lu *et al.*, 2004; Lubbe *et al.*, 2004; Farr *et al.*, 2006; Tozze Jr *et al.*, 2009). *Colletotrichum boninense* has been found on a broad range of hosts, i.e. mono/dicotyledonous, herbaceous and woody plants (Farr *et al.*, 2006). We found the teleomorph in pure cultures in almost all Vietnamese *C. boninense* isolates, as it was *C. boninense* isolates derived from other host plants reported by Lu *et al.* (2004). To our knowledge, the presence of the teleomorphic stage has not been reported in other studies of *C. boninense* with the exception of Lu *et al.* (2004).

In addition, several isolates had cultural and morphological features that were considerably different in morphology from *C. gloeosporioides* and other *Colletotrichum* species described by Sutton (1980; Sutton, 1992). They had dark grey colour of colony, abundant sclerotia and larger cylindrical conidia than any of the previously described species of *Colletotrichum*, e.g. in isolates BMT25(L3) and LD16(L2) conidial length varied in the range 25.4-40.6 µm and width in the range 8.4-11.7 µm. These isolates were grouped into clades distinctly different from the other *Colletotrichum* species in the phylogenic trees. According to sequence analysis of mtSSU, isolates of the unknown species fell into three clades with high bootstrap support, which was higher than in the ITS tree. Clades 3a and 3b (Fig. 4 in Paper II) derived from subclades within clade 3 of the ITS tree and therefore we considered them as unknown species. Interestingly, a BLAST search against the GenBank nucleotide database resulted in close hits (99% similarity and E-values=0) to strains of endophytes of non-identified species of *Colletotrichum* from tropical plants such as *Coffea* spp., *Musa* spp., *Orchis* spp., etc. from previous studies (AY438553 and AY442184; Lu *et al.* (2004)) and (AY 26644404; Photita *et al.* (2005)).

Moreover, two other isolates of unknown species (LD33(L1) and LD11(L3) belonged to a separate clade (clade 6) in our phylogenetic trees (Figs. 3 and 4 in Paper II). Morphologically, they were slightly different from *C. gloeosporioides,* e.g. they had dark grey colonies with a felt-like surface and almost no aerial mycelium. The ITS sequences of these isolates gave high BLAST scores to sequences deposited by Farr *et al.* (2006) representing strains of unknown species of *Colletotrichum* originating from orchid genera *Cattleya* and *Dendrobium*.

Similarly to the results presented in Paper I, two isolates of *Colletotrichum* with falcate conidia derived from coffee berries were identified as *C. capsici* based on the phylogenetic analysis. The ITS sequences of our isolates showed 99% identity to the *C. capsici* strain (Ccmj7) associated with anthracnose disease on *Capsicum* spp. previously described by Than *et al.* (2008b). Isolates with morphological characters like those of *C. capsici* have previously been found on infected coffee in Vietnam (Tran *et al.*, 1998). However, the taxonomic difference between *C. capsici* and *C. dematium* seems to be unclear, partly due to the lack of holotype material of species with falcate conidia. Lubbe *et al.* (2004) reported that *C. capsici* grouped together with *C. dematium* according to sequence analysis of either the ITS region or partial sequences of the β-tubilin gene.

4.4 Diversity of *C. gloeosporioides* populations on coffee in Vietnam (Papers III & IV)

4.4.1 *C. gloeosporioides* populations on coffee berries in southern and northern Vietnam (Paper III)

The patterns generated from the cluster analyses revealed a difference between the northern and the southern populations. Overall, we found a higher genetic variation (H=0.31 & 0.34) in the northern population of *C. gloeosporioides* compared with the southern population (H=0.26 & 0.19), according to the RAPD and MP-PCR markers respectively, which is also indicative of a difference between the two populations. Corresponding levels of variation have commonly been reported in *C. gloeosporioides* populations from other crops in tropical regions, e.g. in a study on yam (*Dioscorea* spp.) in different agroecological zones in Nigeria, where Abang *et al.* (2006) found genetic diversities in the range 0.23-0.27. The lower genetic diversity observed in the south of Vietnam may be due to evolution in the *Colletotrichum* population having led to some specialisation for coffee as the host plant. In the southern regions coffee has been grown continuously and widely for more than a hundred years, while in the north coffee has been a large-scale crop only during the past two decades. A pattern corresponding to geographical origin is not always found in studies of *Colletotrichum* populations. Munaut *et al.* (1998) found among 29 studied isolates of *C. gloeosporioides* from *Stylosanthes* that they grouped into two main clusters, corresponding to two pathotypes, while only partial relations were found to geographical origin. However, 37 *C. graminicola* isolates of the sorghum (*Sorghum bicolor*) anthracnose pathogen were divided in accordance with geographical origin in Brazil, as reported by Valério *et al.* (2005).

The gene differentiation (G_{ST}) values between the north and south of Vietnam were about 0.1, while very low values were found within the north and the south populations (Table 2 in Paper III). Thus, the results also indicate gene flow among locations, but limited flow between north and south. Abang *et al.* (2006) studied genetic variability in *C. gloeosporioides* associated with foliar diseases on water yam (*Dioscorea alata*) based on MP-PCR and found a moderate or low genetic differentiation among populations from different hosts, i.e. yam species (G_{ST} 0.1) and agroecological zones (G_{ST} 0.04), indicating significant gene flow. Those authors postulated that the moderate genetic differentiation between hosts in their study was possibly due to cross-infection occurring in nature. No genetic differentiation was found between isolates of *C. acutatum* from

anemone (*Anemone coronaria* L.) and the two vegetative compatibility groups (VCGs) of *C. acutatum* from strawberry, probably due to the ability of the fungus to cross-infect those plant species (Freeman *et al.*, 2000a).

We did not investigate the presence of the sexual state in the fields, but several of the strains produced ascospores in pure culture. The four-gamete test performed on both RAPD and MP-PCR data demonstrated the existence of sexual recombination and showed that it was higher in the southern population than in the northern one. McDonald and Linde (2002) proposed that plant pathogens undergoing recombination attain increased genetic diversity. This may play an important role in adaptation to host plants and in the formation of new pathogenic races. Abang *et al.* (2006) observed the presence of the sexual stage of *C. gloeosporioides* on severely diseased yam in Nigeria and concluded that there was potentially frequent recombination in the pathogen population. The fact that the southern population in Vietnam, which had a lower overall level of variation, showed a relatively higher level of recombination is highly intriguing. It has been reported that *C. gloeosporioides* isolates from the same host can generally mate, while isolates from different hosts are more seldom compatible (Prusky *et al.*, 2000).

4.4.2 *C. gloeosporioides* populations on coffee tissues in southern Vietnam (Paper IV)

Colletotrichum gloeosporioides was frequently isolated from diseased samples of coffee leaves (67%), berries (92%) and twigs (78%) from southern Vietnam in 2007 (unpublished data). We assessed the genetic variation among *C. gloeosporioides* populations from different coffee plant tissues originating from different geographical locations by employing MP/AMP-PCR markers, *MAT1-2* sequence analysis and studies of vegetative compatibility.

No major differences between the *C. gloeosporioides* populations on the three different plant organs were found based on either cluster analysis of MP/AMP-PCR data or the *MAT1-2* sequence analysis. However, the genetic diversity among *C. gloeosporioides* isolates from leaves (H=0.26) was lower than among isolates from twigs (H=0.38) and berries (H=0.36). Furthermore, isolates of *C. gloeosporioides* from coffee leaves tended not to spread into different clades of the phylogenetic trees to the same extent as the isolates from berries and twigs. Studies of genetic structures of *Colletotrichum* species associated with anthracnose diseases on different strawberry tissues have been carried out by different scientific groups (Gunnell & Gubler, 1992; Sreenivasaprasad *et al.*, 1992; Sreenivasaprasad *et al.*, 1996b; Freeman & Katan, 1997; Buddie *et al.*, 1999; Ureña-Padilla *et al.*,

2002; Jelev *et al.*, 2008). The latter observed tissue specialisation of *Colletotrichum* species on strawberry, since *C. acutatum* was responsible for fruit rot while *C. gloeosporioides* caused crown rot.

Lower genetic diversity of the *C. gloeosporioides* population in the Ma Drac area and the separation between this population and the other populations based on the MP/AMP-PCR analysis were in agreement with previous results (Paper III) and are discussed in Paper IV. The other two locations belonging to Lam Dong province, Lam Ha and Don Duong, did not differ from each other according to either the molecular analysis or VCGs. These two locations are relatively close (see Fig. 1 in Paper IV), with altitude higher than that of Ma Drac (approx. 1000 m above sea level compared with 400-500m). The differences between the populations may be due to climate, agricultural practices, age of plantations, etc.

Seven VCGs were found among the isolates of *C. gloeosporioides* investigated but most of the isolates belonged to VCG1. *Colletotrichum gloeosporioides* populations responsible for anthracnose of yam yielded a high VCG diversity, as isolates derived from the same lesion were assigned to different VCGs (Abang *et al.*, 2004). Correll *et al.* (1993) assumed that VCG diversity in a population of *C. gloeosporioides* varied depending on host plant. In this study, no relationship between VCG and geographical origin was observed, as VCG1 isolates were present at all locations.

One lineage of the Vietnamese *C. gloeosporioides* studied here grouped distantly from the remaining isolates according to analysis of the MP/AMP-PCR markers and *MAT1-2* sequences. The similarity between these groups was low (37%), based on the MP/AMP-PCR analysis. However, ITS sequences were highly similar (99%) between two isolates representing these two different lineages of Vietnamese *C. gloeosporioides* (data not shown). As already mentioned in section 2.6.3.2, sequences of the *MAT1-2* region are informative in differentiating between closely related lineages of *Colletotrichum* species complexes. Based on this, we can also speculate that isolates from the distantly related clade in our study might represent another species that is morphologically similar to, but genetically distinct from, *C. gloeosporioides*.

Epidemiological investigations of *Colletotrichum* pathogens in the tropics indicate that anthracnose epidemics on coffee and other perennial and tropical crops are due to disease transmission from different tissues of the tree canopy (Dodd *et al.*, 1992; Waller, 1992). Most data about *Colletotrichum* infections of coffee are from studies of *C. kahawae* (the causal agent of CBD), which is closely related to *C. gloeosporioides*. Waller *et al.* (2007) suggested that the immature bark of coffee twigs could be regarded as the

initial inoculum source of the CBD epidemic. Likewise, Mouen Bedimo *et al.* (2007) considered leaves and twigs to be the primary inoculum source for CBD. Our results concur, in indicating that twigs and mummified berries within the coffee tree canopy are probably the main source of inoculum for berry infection.

4.5 Pathogenicity tests of Vietnamese *Colletotrichum* spp. and distinguishing between Vietnamese *C. gloeosporioides* and *C. kahawae,* the CBD pathogen in Africa (Papers II, III & IV)

To some extent, the symptoms of infected berries in Vietnam, described by Tran *et al.* (1998), resemble those of CBD and it has been hypothesised that CBD might be present in Vietnam. To address this, comparisons between Vietnamese *Colletotrichum* isolates and *C. kahawae* reference isolates were carried out on the basis of morphological characters, colony growth rates, assessment of pathogenicity, ability for ammonium tartrate utilisation and *MAT1-2* sequence analysis (Papers II, III & IV). According to our results, the Vietnamese *C. gloeosporioides* could be distinguished from *C. kahawae* on the basis of growth rate, tartrate utilisation, pathogenicity and *MAT1-2* gene analysis.

In pathogenicity test on hypocotyls, the symptoms caused by Vietnamese isolates ranged from scabs to brown to deeper black slightly sunken lesions on the surface, commonly in the grade from 1-9 according to the scale described by van der Vossen (1976) (Paper III). However, symptoms caused by the reference isolate of *C. kahawae* (CBS 396.67) were much more severe (Fig. 5). Five of the 32 Vietnamese isolates tested caused clear symptoms with an infection grade from 7 to 9, while the infection grade of the CBD isolate (*C. kahawae*) was 11-12. Isolates causing slight infections were found at all locations in Vietnam.

The results of the pathogenicity tests for different species of Vietnamese *Colletotrichum* on detached green berries indicated that some of the Vietnamese isolates were virulent (Papers II & III). However, none of the isolates of *C. acutatum*, *C. capsici* or *C. boninense* produced symptoms on non-wounded green berries. The CBD isolate (IMI 357057, *C. kahawae*) was the most pathogenic, as indicated by the high infection percentage (100%) of both wounded and non-wounded green berries (Table 3 in Paper II). Conversely, most of the Vietnamese *Colletotrichum* isolates caused only moderate damage, mainly on wounded berries, with less severe and less sunken lesions than those caused by the reference CBD isolate (*C. kahawae*). However, several of the Vietnamese isolates also produced clear disease

symptoms on non-wounded green berries, although at a lower rate. Six of the isolates belonging to *C. gloeosporioides* and one from an unknown species were able to induce lesions on non-wounded berries. One *C. gloeosporioides*

5a

Figure 5. Pathogenicity tests. Symptoms on (a) hypocotyls and (b) detached green berries after inoculation with Vietnamese *C. gloeosporioides* (right) and the CBD fungus, *C. kahawae* (left).

isolate, 119b(1), induced symptoms on both hypocotyls and berries (Papers II & III). In our tests, *C. kahawae* caused deeply sunken lesions on the berry surface, then invaded and destroyed the bean as reported in previous studies by Chen *et al.* (2005) and Masaba and Waller (1992). The Vietnamese *C. gloeosporioides* isolates seldom infected the coffee beans, although they produced sunken lesions on the pericarp. Although the other species were not pathogenic by themselves, they may be involved in enhancing infection on the skin of the berries. *Colletotrichum gloeosporioides* has previously been reported to be responsible for die-back disease and defoliation of coffee (Chen *et al.*, 2003; Waller *et al.*, 2007). One of the isolates of unknown species also infected non-wounded berries and therefore it would be valuable to describe this putative species and characterise its pathogenic abilities further. No relationship between pathogenicity and genotype was found among the *C. gloeosporioides* isolates, in agreement with previous studies (Valerio *et al.*, 2005; Abang *et al.*, 2006).

Apart from five isolates, approximately 400 Vietnamese *Colletotrichum* isolates tested were able to use ammonium tartrate as a sole carbon source. Inability to utilise ammonium tartrate by the five Vietnamese isolates was consistently observed in many repeated tests. None of the three reference isolates of *C. kahawae* was able to utilise this carbon source. However, according to ITS and mtSSU phylogeny trees (Paper II), the five Vietnamese isolates that could not metabolise tartrate included two isolates of *C. boninense*, one isolate of unknown species and only two isolates of *C. gloeosporioides*. Waller *et al.* (1993) also reported that *C. gloeosporioides* isolates occasionally could not metabolise tartrate or citrate. Therefore, to distinguish these isolates from *C. kahawae,* the tartrate test had to be combined with other analysis.

A significant difference in growth rate between *C. gloeosporioides* and *C. kahawae* was found at 25 and 30 $^{\circ}$C in our study (Fig. 2 in Paper III). This correlated to previous reports by Waller *et al.* (1993) and Varzea *et al.* (2002). In contrast to *C. kahawae,* the Vietnamese isolates grew rapidly at high temperature.

All isolates of *C. gloeosporioides* and the reference isolate of *C. kahawae* (IMI357056) fell into the main group of *C. gloeosporioides* (clade 3) with 100% support based on both ITS and mtSSU sequence analyses (Figs. 3 and 4 in Paper II). This is in agreement with earlier studies (Sreenivasaprasad *et al.*, 1993; Cannon *et al.*, 2008). This included the two Vietnamese isolates that were unable to utilise tartrate. However, according to the *MAT1-2* sequence analysis, the three reference isolates of *C. kahawae* grouped separately from the groups of Vietnamese *C. gloeosporioides* isolates, well

supported by a high bootstrap value (Fig. 3 in Paper IV). It had previously been reported that *C. kahawae* can be separated from *C. gloeosporioides* by mitochondrial, ribosomal DNA restriction fragment length polymorphism and phylogenetic analysis based on multiple genes (Waller *et al.*, 1993; Martinez-Culebras *et al.*, 2003; Bridge *et al.*, 2008; Prihastuti *et al.*, 2009).

The CBD fungus (*C. kahawae*) in Africa is sometimes considered as a specialised form of *C. gloeosporioides* and low levels of genetic diversity have been reported in populations of this fungus (Derso & Waller, 2003; Bridge *et al.*, 2008). In contrast, high genetic variation, as was found here in Vietnamese *C. gloeosporioides* populations (Papers III & IV), is typical for *C. gloeosporioides* populations on different hosts (Mills *et al.*, 1992a; Freeman & Rodriguez, 1995; Freeman & Katan, 1997; Ureña-Padilla *et al.*, 2002; Abang *et al.*, 2006).

5 Conclusions and future prospects

5.1 Conclusions

Several species of Colletotrichum were found on coffee in Vietnam and on other tropical crops.

Apart from *C. gloeosporioides*, which was the dominant species, *C. acutatum, C. capsici, C. boninense* and several unknown species associated with coffee anthracnose were found.

Many Colletotrichum isolates belonged to unknown species.

According to morphological and phylogenetic analysis based on the ITS and mtSSU regions, 10 *Colletotrichum* isolates were assigned to (at least three) unknown species and their taxonomic position remains unresolved. More than 30 isolates of those morphological types were obtained. In addition, within the *C. gloeosporioides* aggregate, one lineage of the isolates studied was morphologically similar to *C. gloeosporioides,* but grouped distantly from the remaining isolates according to analysis of the MP/AMP-PCR markers and the *MAT1-2* sequences. They might also be considered another species.

Isolates of C. gloeosporioides were more pathogenic than isolates of the other species and we did not find any evidence of the presence of C. kahawae in Vietnam.

Some of the *C. gloeosporioides* isolates and one isolate of unknown species produced slightly sunken lesions on green berries resembling CBD symptoms, although they rarely destroyed the bean. *Colletotrichum acutatum*, *C capsici* and *C. boninense* produced no symptoms on detached non-wounded green berries. No relationship between pathogenicity and genotype based on MP-PCR/RAPD analysis was found among the *C.*

gloeosporioides isolates. Vietnamese *C. gloeosporioides* could be distinguished from *C. kahawae* based on ammonium tartrate utilisation, fungal growth rate, pathogenicity, and sequence analysis of the *MAT1-2* genes.

High genetic variation was observed in the populations of Vietnamese C. gloeosporioides.

Our results indicate higher genetic variation in the northern Vietnamese population of *C. gloeosporioides* compared with the southern population, although high variation was also found in some plantations in the south (Don Duong and Lam Ha). Among the populations from different locations in Vietnam, we found the lowest genetic diversity of *C. gloeosporioides* in Ma Drac. The genetic diversity among *C. gloeosporioides* isolates from leaves was lower than that among isolates from the other tissues. There was little or no adaptation or specialisation of *C. gloeosporioides* to certain tissues.

Vietnamese C. gloeosporioides isolates were mainly grouped in accordance with their geographical origin.

According to RAPD and MP-PCR/AMP-PCR analysis, we found moderate gene differentiation (G_{ST}=0.1) between populations from the north and the south, indicating a difference between the northern and southern populations. However, there was low or no differentiation between locations and host tissues within the south and north regions, indicating significant gene flow.

5.2 Future prospects

The taxonomic position of several *Colletotrichum* isolates remains unresolved. One of the isolates of unknown species also infected non-wounded berries. Therefore, taxonomical clarification of the putative unknown species has to be taken into consideration and their pathogenic abilities need to be further evaluated.

The results of the present study provide a better understanding of the *C. gloeosporioides* populations on coffee in Vietnam. The low gene differentiation among different tissues and different locations indicates that transmission of the disease may occur both at short distances, i.e. within the tree, and over long distances. To improve our understanding of disease etiology, it is necessary to assess whether cross-infection occurs among populations from different parts of the coffee tree canopy, which would require extensive pathogenicity tests on *C. gloeosporioides* isolates from different tissues.

Colletotrichum gloeosporioides can have wide host ranges, so individuals from other non-cultivated indigenous host plants growing adjacent to the coffee fields may infect coffee. Several crops are grown as shade trees or mixed crops or integrated into mixed cropping systems with coffee in Vietnam. Therefore, it would be valuable if diseased samples from these crops at the same location could be diagnosed and identified. Potential cross-infections between pathogens of coffee and other host plants need to be further studied.

In Vietnam, coffee is grown primarily as a single cultivar in monoculture over large areas, and there is a lack of knowledge regarding sanitation in coffee cultivation, i.e. pruning infected parts of the coffee tree, removal of mummified berries and using shade trees. These cultural practices can limit pathogen transmission during disease epidemics. Poor agricultural practices promote disease spread and severity among coffee trees, which in turn lowers the yield in the following year. It seems likely that the cultural practices used in coffee growing areas elsewhere need to be carried out in Vietnam and such knowledge can form the basis for good recommendations to smallholders and state farms about controlling anthracnose disease of coffee and other perennial crops in Vietnam.

Understanding of the infection process caused by *Colletotrichum* on coffee berries, leaves and twigs is not yet complete. Therefore, it would be valuable to microscopically investigate the infection process on Vietnamese coffee plants and compare it with that of the CBD fungus, *C. kahawae*.

Many studies report resistance of coffee variaties to the CBD pathogen, *C. kahawae,* which occurs in Africa. However, very little published work on resistance to *C. gloeosporioides* in coffee can be found. Recently, Sera *et al.* (2007) reported variation in partial resistance to berry necrosis caused by *Colletotrichum* spp. among coffee cultivars in Brazil, showing that breeding for this trait should be possible. It is of great importance to develop coffee cultivars with at least partial resistance to these types of anthracnose diseases.

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Acknowledgements

It is now a wonderful feeling for me since my PhD study is ending happily after a long and difficult journey. This is a suitable time to express all of my appreciation to those people who contributed significantly to my PhD education.

There is not enough space for me to individually acknowledge everyone who helped me during all my years of education abroad. However,

I would like to express my deepest thanks to my supervisors for accepting me as a student, without whom I do not think this study would be completed in good way. Firstly, to my main supervisor, **Erland Liljeroth**, thank you for being a great supervisor, your significant contributions and efforts to guide me in my studies and exhausted field trips to coffee fields in Vietnam as well as discussions about papers. I am deeply thankful for your supervision in my PhD work, for always finding solutions to various things that allowed my studies to be completed in a good way. I will never forget your endless encouragement in the work or your help with other practical things for a foreign student like me whenever I needed it. Secondly, I would like to express my appreciation to my co-supervisor, **Olga Vinnere Pettersson**. Thank you, Olga, for being a great mentor, showing me the interesting subject of *Colletotrichum* for almost 10 years with your great ideas and discussions. It was great for me to get your supervision in this study also. First I thought it would not be so easy when you were not here in Alnarp and but I felt confident as you were always available when I needed it. I also would like to thank **Tomas Bryngelsson**, my co-supervisor for nice discussions when I started my PhD. I would like to acknowledge my co-authors, *Torbjorn Säll*, *Peter Olsson* and *Nguyen Thanh Ha* for their great contributions to my papers. My special thanks to **Le Thi Anh Hong** for giving me the chance to be PhD in Sweden; I will always appreciate that. I

also give my thanks to the management at the Institute of Agricultural Genetics and Biotechnology in Hanoi, who supported my PhD extension.

I wish to express my special thanks to The Swedish International Development Agency (Sida/SAREC) for financial support for my PhD study and for facilitating my studies in Vietnam and Sweden. I would like to thank *Gity Behravan* (Sida) and *Ivar Virgin* (SEI) for your great support. *Benita Forsman* (SEI), thank you for your quick response when I needed your help in collecting documents for my visa applications.

I am deeply grateful to my technician *Ann-Charlotte Strömdahl* for being available and for assistance and kind help when I was overloaded with work. Thanks also for your comprehension and having such nice chats with me when I was overstressed during the latter part of my studies. My grateful thanks also to my former technicians, *Britt Green* and *Therese Bengtsson*, who valuably guided and helped me in technical and practical things. *Therese*, thanks for being so nice to a foreigner like me in working time and free time. We had so much fun and many memorable things happened during my education time. Thanks also for solving problems related to contamination in molecular works. My sincere thanks to *Helén Lindgren* and *Annelie Ahlman* for being available for me when I needed help with practical things that I could not do by myself. I would like to express my appreciation to Lars Hagtorn and Göran Olsson for fixing my computer problems and to Jonas Hansson and Rita Larsson for administrative work they did for me. My thanks also go to *Helena Persson Hovmalm, Asayas Aga* and *Mulatu Geleta* for nice and valuable discussions on molecular aspects. Ann-Sofie Fält, your nice talks, concern with warm heart made me feel better when I was far from my home country, thanks for good cakes in coffee times. *Tintin*, thanks for the nice talks, warm hugs when we were in the lab as well as your taking care.

My grateful thanks also go to *Barbara Richie* and *Paul Cannon for* valuable meetings and fruitful discussions in the UK. *Suraparedy Sreenivasaprasad*, thanks for giving me the chance to be in the *Colletotrichum* workshop and your kind supply of reference *Colletotrichum* isolates for my study. *Hans Vermeulen*, thank you for interesting hours with discussions in Holland about the Coffee Berry Disease problem in Kenya in the 1960s, as well as about coffee diseases in Vietnam at the beginning of my PhD studies.

I am very grateful to my colleagues, *Nguyen Thanh Ha* for having funs with me when we were doing experimental work together in Vietnam as well as in Sweden. You assisted me in samplings during field trips. Thanks for always being with me whenever I needed. My thanks also go to *Hoang Thi Ngat* for valuable help with the pathogenicity tests and for putting so

much effort into doing experimental works with me during my study time in Vietnam. I acknowledge *Nguyen Thu Ha* for help with arrangement of travel costs. Thanks to all members of my home department who welcomed me back and created good conditions for me when I did some parts of the study there.

I would like to thank to *Tran Kim Loang* and *Bui Thi Tram* for their fruitful discussions related to coffee cultivation and coffee diseases in Vietnam. Thanks for your warm welcoming during our visits and for arrangements for coffee field trips during my education. Special thanks to *Loang*, for your great contribution in correction of the thesis summary in Vietnamese. I highly appreciate Mr *Tu*, Mr *Thu* and Mr *Dung* in Don Duong, Mr *Huyen*, Mr *Ky* and their co-workers in Ma Drac, and Mr *Dung* and *Mr Son* in Lam Ha for being so kind in arranging valuable field trips and for accompanying us to visit coffee fields. Thanks for sharing your experiences, I learnt a lot from you. I also had so much fun with you under hot and rainy conditions.

(Tôi xin bày tỏ lòng biết ơn của mình ơn đến chị Loang và chị Trâm về sự quan tâm giúp đỡ nhiệt tình của các chị trong những chuyến công tác thực địa của chúng tôi cũng như sự đóng góp có ý nghĩa lớn lao về mặt khoa học giúp cho tôi hoàn thành tốt nghiên cứu luận văn của mình. Tôi muốn gửi lời cảm ơn chân thành đến anh Huyên, anh Kỳ và các anh chị thuộc nông trường 715A (Ma Drac), anh Tú, anh Thu anh Dũng (Đơn Dương), anh Dũng và anh Sơn (Lâm Hà) đã tạo mọi điều kiện thuận lợi giúp tôi trong những chuyến công tác khảo sát và thu thập mẫu bệnh cà phê)

I would like to express my thanks to my Vietnamese friends here, *Thuy*, *Cuong* and *Toan* for nice company in Alnarp during the PhD period. I felt much better when I spoke Vietnamese with you whenever I missed home. To Thuy, I always think about the moments of sadness and happiness that we shared together in Sweden, I think we will never forget that memory. Thanks to my friends in Lund, Tu, Tam, Thuoc, etc. for nice party and nice foods we had during Xmas, New Year and weekends. Mats, thank you for your warm hospitality during my visits to Uppsala for discussions of my study with Olga. Thanks also to my colleagues and friends in Sweden, Eva, Per, Ylva, Ramune, Carlos, Salla, Jens, Åsa, Anna, Isabel, Dickson, Svetlana, Pooja, Susanne, Linus, Johannes, Sergey, etc. for nice chats, good company, moral support, encouragement and nice help when I needed you. I am highly thankful to all people in the "H house" in Alnarp for accepting me as an "H house" member and I had happy and great working times with you during my stay in Sweden. I will miss you all and the "Swedish" sounds I got used to hearing from you in corridors or coffee room.

Finally, from my deepest and warmest heart I would like to express my special thanks to my beloved family, my relatives and friends; to my dearest parents to whom I owe a lot; to my great brothers, Quang and Ha and to all members of their families, Le, Hien, Huy, Linh, and Lam for your endless encouragements to my study and all your strong love for me. You are always present in my heart. Communications with you gave me energy to cope with difficulties in such cold and bad weather in the Swedish Winter. I will be back home soon to compensate for the things I could not fulfil during my PhD study.

(Tôi xin bày tỏ lòng biết ơn sâu sắc đối với gia đình thân yêu, người thân và bạn bè; đặc biệt tới bố mẹ kính yêu, anh trai và em trai, Quang và Hà và tất cả các thành viên khác trong gia đình của tôi, chị Lê, Hiên, Huy, Linh và Lâm. Sự khích lệ động viên của gia đình là nguồn động lực giúp cho tôi có thêm ý chí và nghị lực để có thể vượt qua những giai đoạn khó khăn trong suốt thời gian sống và học tập xa quê hương)

Popularized summary in Vietnamese

Nấm *Colletotrichum* bao gồm nhiều loài gây hại có ý nghĩa kinh tế trên rất nhiều loài cây trồng trên thế giới. Đối với các cây có nguồn gốc nhiệt đới như cây ăn quả, ngũ cốc, cỏ dại, rau..., chúng gây hại nghiêm trọng ở tất cả các giai đoạn sinh trưởng của cây. Một số loài *Colletotrichum* gây bệnh thán thư trên cây cà phê và một số cây trồng chủ lực khác có giá trị thương mại tại Việt Nam và Thái Lan. Tuy nhiên, cho đến nay các nghiên cứu về các loài *Colletotrichum* gây bệnh thán thư trên những loại cây trồng trên tại Việt Nam rất ít và không có hệ thống.

Mục đích nghiên cứu của đề tài tiến sĩ này là chẩn đoán và nhận dạng loài *Colletotrichum* gây bệnh thán thư trên một số cây trồng nhiệt đới, đặc biệt là trên cây cà phê, đồng thời mô tả quần thể của tác nhân gây bệnh này. Các nghiên cứu về hình thái học, đặc điểm sinh trưởng, sinh hóa, tính gây bệnh, sự đa dạng về mặt di truyền và cấu trúc quần thể của các tác nhân gây bệnh đã được tiến hành đánh giá. Đề tài đã sử dụng một số phương pháp nghiên cứu sinh học phân tử để nghiên cứu sự đa dạng di truyền cũng như phối hợp phương pháp nghiên cứu sinh học phân tử với nghiên cứu hình thái học để phân loại các loài *Colletotrichum* đã phân lập được.

Đề tài đã phân lập được các loài *C. gloeosporiodes* và *C. acutatum* trên cam, quýt, nho, măng tây, xoài, sầu riêng, vv có nguồn gốc từ Việt Nam và Thái Lan. Ở Việt Nam, đã phát hện được loài *C. gloeosporioides*, *C. actutatum*, *C. capsici*, *C. boninense* và một số loài *Colletotrichum* sp. chưa được định danh khác gây hại trên lá, quả, rễ và cành của cây cà phê. Kết quả nghiên cứu của đề tài cho thấy không có sự xuất hiện của *C. kahawae* tại Việt Nam, đây là tác nhân gây bệnh chính trên quả cà phê Arabica tại Châu Phi.

Phần lớn các chủng nấm *Colletotrichum* phân lập được ở Việt Nam thuộc loài *C. gloeosporioides,* chúng có khả năng gây bệnh trên quả cà phê chưa chín cao hơn so với các loài khác. Các chủng của loài *C. gloeosporioides* chủ yếu được phân nhóm về mặt di truyền theo nguồn gốc địa lý. Kết quả nghiên cứu cho thấy sự đa dạng lớn về mặt di truyền trong quần thể *C. gloeosporioides* ở phía Bắc và

phía Nam Việt Nam. Tuy nhiên không có sự khác nhau rõ rệt của quần thể nấm thu được từ các bộ phận trên cây cà phê như thân, lá và quả cũng như ở các vùng trồng cà phê khác nhau. Điều này chứng tỏ rằng bệnh có thể lan truyền giữa các bộ phận trên cây cà phê và giữa các vùng trồng cà phê. Kết quả nghiên cứu cũng đã chỉ ra sự khác nhau về mặt di truyền giữa quần thể *C. gloeosporioides* ở phía Bắc và phía Nam Việt Nam.

Kết quả nghiên cứu của đề tài cung cấp một số thông tin cho các nhà nghiên cứu bệnh cây cũng như người nông dân có cái nhìn rõ nét hơn về nấm *Colletotrichum*, tác nhân gây bệnh trên cây trồng nói chung và cây cà phê nói riêng, góp phần quan trọng trong việc xây dựng chiến lược quản lý bệnh hại trong sản xuất cà phê bền vững tại Việt Nam.