Université catholique de Louvain

Faculté d'ingénierie biologique, agronomique et environnementale

Earth and Life Institute

Pole of Applied Microbiology

Laboratory of Mycology

Biodiversity and antifungal properties of endophytes from the Madagascan medicinal plants *Centella asiatica* (L.) Urb. and *Catharanthus roseus* (L.) G. Don

Thèse de doctorat présentée par **Rakotoniriana Erick Francisco** en vue de l'obtention du grade de Docteur en Sciences agronomiques et ingénierie biologique

Promoteurs	: Prof. S. Declerck (UCL)
	Prof. J. Quetin-Leclercq (UCL)
Membres du Jury	: Prof. J. Mahillon (UCL), président du Jury
	Prof. A.-M. Corbisier (UCL)
	Prof. P. De Vos (LM-UGENT)
	Prof. M. El Jaziri (ULB)
	Dr F. Munaut (UCL)

Louvain-la-Neuve, août 2012

Acknowledgments

I am very grateful to the “Administration de la Relation Internationale / Université catholique de Louvain, ADRI / UCL” for offering me a grant to accomplish this thesis project.

I would like to express my deep gratitude to Prof. Anne-Marie Corbisier for introducing me to the world of microbial endophytes, for opening the door in the laboratory and for being my supervisor during the first part of my thesis.

My deepest gratitude is addressed to my supervisor Prof. Joëlle Quetin-Leclercq for her generous guidance, encouragement and sincere support during the course of my PhD and for extending facility to work in her institute.

I am also greatly indebted to my co-supervisor Prof. Stéphane Declerck for accepting to take over the guidance of this project after the retirement of Prof. Anne-Marie Corbisier. I am thankful for the valuable comments and proposals for improvement, encouragement and support during the course of this project and for providing excellent scientific working facilities.

I would like to thank the “Coopération Universitaire pour le Développement (CUD)” for the financial support to the project “Biodiversity and

Biotechnology in Madagascar". My profound gratitude goes to the key persons in charge of the project: Prof. Mondher El-Jaziri, Prof. Anne-Marie Corbisier, Prof. Jacques Mahillon, Prof. Suzanne Urveg-Ratsimamanga, Dr Kiban Check and Dr Christian Rabemanantsoa who gave me the opportunity to be part of the team of that project. The knowledge and expertise acquired during the project have paved the way to this "dream come true" PhD project.

My special thanks go to Dr Françoise Munaut for her endless encouragements, constructive comments and complete commitment throughout this thesis.

My sincere gratitude also goes to Prof. Paul De Vos, Prof. Jacques Mahillon, Prof. Mondher El Jaziri, Prof. Anne-Marie Corbisier, and Dr Françoise Munaut for participating in the examination committee.

I am thankful to all of MUCL/MBLA members who have assisted me either directly or indirectly throughout the duration of my project, especially to Dr Cony Decock, Pascale Massart and Pierre Evrard for initiating me into the identification of molds, fungi and yeast; Stéphanie Huret and Céline Bivort for the technical assistance in the molecular part of this research; François Van Hove, Jonathan Scauflaire, Stéphane Dethine, Philippe Charue, Olivier Devroye and Isabelle Charue for their availability and readiness to help. I also thank the Laboratory of Food and Environmental Microbiology (UCL, Belgium) for providing technical assistance and facilities in bacterial identification.

I wish to thank the technical staff at the "Laboratoire de Pharmacognosie, Louvain Drug Research Institute, UCL", especially to Marie Christine Fayt, Jean-Paul Vanhelleputte, Ramazan Colak, Françoise Gelders, Mary Yvonne

Tassin and Alphons Brams for a kind assistance in the laboratory and for friendly working atmosphere. My special thanks go also to Dr Eldra Delonnay and Dr Gabrielle Chataignée for the numerous runs in the electrospray ionization mass spectrometric experiments and help in analyses of NMR spectra.

I owe great gratitude to "Institut Malgache de Recherches Appliquées (IMRA)" for providing the necessary facilities and enabling me to complete this thesis. My gratitude goes also to my colleagues at IMRA, especially to Dr Guy Raoelison, Dr Denis Randriamampionona, Dr Mamy Rafamantanana, Tiana Rakotomalala and Jacob Rajaonarivelo for helping me with my experiments in field, greenhouse and laboratory.

I would like to express my appreciation to other research groups for their kind cooperation, sharing their time and knowledge: Dr Van Trappen (BCCM/LMG) for her excellent help in the molecular identification of the *Streptomyces* sp. strain TS3RO; Dr Michel Fréderich (CIRM, Université de Liège) for running the NMR analysis; Prof. Didier Lambert for providing facility to perform the infrared spectrum analysis and Prof. Jacqueline Marchand for providing facility to carry out the semi-preparative HPLC.

I would also like to take this opportunity to express my gratitude to some person who played an important part during my all stay in Belgium. To my landowners Paule & Eric Boulangé and Cathérine & Jacques Bavey: my homesickness is mitigated thanks to your warm hospitality and friendship. To Lucie Van Esbroek: many thanks for your continuous support and all the moments of relaxation.

My fellow countrymen and friends: Denise Sissan, Mamy Botralahy, Herilala Razafimbahiny, Lova Randria and José Ratsimbason are thanked for great time and lots of fun.

Last but not least I could not have finished this thesis without the love, support, understanding and patience of my family from the beginning to the end of my PhD project. This thesis is yours!

E. Francisco Rakotoniriana

Louvain-la-Neuve

Table of contents

Summary

In the recent years, endophytes from medicinal plants have attracted increasing attention. In particular because they represent a rich source of new and useful compounds of pharmaceutical and agricultural interests and are a good niche for the discovery of novel microorganisms. Nowadays, very scanty information is available on endophytic microorganisms of the Madagascar flora. Although this island is rich of approximately 12000 species, of which more than 80% are endemic and potentially having medicinal virtues.

The present work investigated the diversity and antifungal activities of microbial endophytes from two Madagascan medicinal plants, *Centella asiatica* (L.) Urb. and *Catharanthus roseus* (L.) G. Don. The study also outlined the interactions of some endophytic bacteria against *Colletotrichum higginsianum*, a causal agent of anthracnose disease on several plants, using *in vitro*-produced *C. asiatica* plantlets. Endophytes were isolated by microbiological methods following surface disinfection of plant tissues and plating onto appropriate culture media. The microorganisms were further identified using molecular tools.

A new species of *Colletotrichum*, named *Colletotrichum gigasporum* sp. nov., from *C. asiatica* leaves and a new subspecies within the *Streptomyces cinnamoneus* group from *C. roseus* stems were described based on a combination of phenotypic properties and phylogenetic analysis. The sexual stage of *C. gigasporum* was obtained in laboratory cultures when the

anamorph was plated onto culture media supplemented with sterilized leaf powder of *C. asiatica*.

A number of beneficial endophytes were isolated from *C. asiatica* with biological control properties. In particular, *Bacillus subtilis* strain BCA31 was found to confer enhanced resistance to the *in vitro* plants of *C. asiatica* and significantly reduced disease incidence and severity caused by *C. higginsianum.* Also, the xylariaceous non sporulating species NSS1, the most frequently isolated fungal endophyte from *C. asiatica* leaves markedly inhibited the growth of *C. higginsianum* in dual culture experiments.

Some of the isolated endophytes produced bioactive compounds which are of high interest to the pharmaceutical and agricultural sectors. In particular, *Streptomyces cinnamoneus* strain TS3RO was highly inhibitory against a large number of human and plant pathogenic fungi. Strain TS3RO is a good polyene macrolide antibiotic producer. Structural elucidation of the bioactive compound was obtained via LC/MS spectrometry, UV-visible spectra and NMR data.

These findings provide additional insights on the high potential of endophytes from medicinal plants as a considerable source of new microbial species and useful bioactive compounds.

List of abbreviations

AmB : amphotericin B
ATCC : American Type Culture Collection
BCCM : Belgian Co-ordinated Collections of Micro-organisms
BLAST : Basic Local Alignment Search Tool
BuOH : butanol
CBS : Centraalbureau voor Schimmelcultures
CTAB : Cetyltrimethylammonium bromide
CUD : Coopération Universitaire pour le Développement
DCM : dichloromethane
DSE : dark septate root endophytes
DSI : disease severity index
EMBL-EBI : European Molecular Biology Laboratory- European Bioinformatics Institute
GPS : General Positioning System
HPLC : High performance liquid chromatography
IF : isolation frequency
IMRA : Institut Malgache de Recherches Appliquées
ITS : internal transcribed spacer
LMG : Laboratorium voor Microbiologie
MA2 : 2% malt agar
MBA : modified Bennett's agar

MEAC : malt extract agar supplemented with chloramphenicol
MEB : malt extract broth
MeOH : methanol
MS : mass spectrometry
MUCL : Mycothèque de l'Université catholique de Louvain
NaOCl : sodium hypochlorite
NCBI : National Center for Biotechnology Information
NMR : nuclear magnetic resonance
NSS1 : non-sporulating species 1
OA : oatmeal agar
PDA : potato dextrose agar
SEM : scanning electron microscopy
TLC : thin layer chromatography
TSA : tryptic soy agar
UCL : Université catholique de Louvain
ULB : Université Libre de Bruxelles

GENERAL INTRODUCTION

General introduction

Of the 250000 to 500000 plant species that exist on Earth (Borris, 1996), Madagascar, the fourth largest island in the world, shelters approximately 12000 species. Interestingly, more than 80% of these plants are endemic to the island (Robinson, 2004). Owing to environmental degradation, increasing deforestation, slash and burn agriculture in primary forest, this unique patrimony is threatened to extinction. Nowadays, only 9% of the original areas are preserved, while the country belongs to the 25 most critical regions for life protection in the world (Myers *et al*., 2000). To counter the rapid rate of plant species extinction, ongoing investigations should be conducted in parallel to effective environmental preservation policies.

In 2001, the project entitled "Biodiversity and Biotechnology in Madagascar", funded by the "Cooperation Universitaire belge pour le développement" (CUD), was initiated and joined research groups at the "Université Libre de Bruxelles" (ULB), "Université catholique de Louvain" (UCL) and "Institut Malgache de Recherches Appliquées (IMRA). The project is in line with the sustainable development policy in Madagascar and aims to preserve the medicinal plant resources of the island and its associated endophytic microorganisms. Ever since, a collection of various medicinal plants and micropropagated plants along with a collection of endophytic microorganisms have been maintained at IMRA.

Microbial endophytes have attracted considerable interest and attention since they have been reported to confer a wide range of beneficial effects on host plants such as protection against herbivore (Afkhami & Rudgers, 2009), growth promotion (Khan & Doty, 2009) and elicitation of defense mechanisms (Gao *et al*., 2010). In the current context, where public opinion and environmental stakeholders are concerned by the preservation of a healthy environment, the use of chemical herbicide to control weeds or insecticides becomes more and more restrained. Chemical products are often costly, require repeated applications and represent a real risk for the environment and human health. Endophytes are a good alternative as biological control agents of plant pathogens (Melnick *et al*., 2008; Mejía *et al*., 2008), due to their intimate association with plant tissues, occupying an ecological niche similar to that of phytopathogens. In addition, the need for new active antimicrobial compounds is constantly growing to cope with the increased incidence of multidrug-resistant bacteria, the emergence of life-threatening infectious diseases, in particular in immuno-compromised patients. The screening of antimicrobial compounds from endophytes have recently shown promising results against methicillin-, penicillin- and vancomycin-resistant *Staphylococcus aureus* strains, suggesting the potential of endophytic secondary metabolites in medicine (Arivudainambi *et al*., 2011).

During their relatively long co-evolution, microbial endophytes and their host plants have evolved together in a balanced continual process within a particular ecological niche where they can interact biochemically and/or genetically. This long co-evolution could explain why some endophytes produce the same or similar bioactive compounds as those found in the host plants. An excellent review on this topic was reported by Zhao *et al*. (2011). For instance, taxol, an anticancer drug, that was originally extracted from the bark of the Pacific yew (*Taxus brevifolia*) (Wani *et al*., 1971), was found to

be produced by many endophytic fungi associated with *Taxus* species or related plants (Stierle *et al*., 1993; Strobel *et al*., 1996. Li *et al*., 2008; Zhang *et al*., 2009). This finding was very interesting because, apart from the fact that endophytes would be an alternative source to produce taxol, the development of methods for efficiently producing taxol through microbial fermentation processes and genetic engineering would preserve in the long term the over-exploitation of *Taxus* spp.

In the recent years, several attempts have been made to isolate and identify secondary metabolites from endophytes associated with medicinal plants. Weber *et al*. (2004) found a novel antibiotic, phomol, in the fermentation broth of *Phomopsis* species, endophytes of the medicinal plant *Erythrina crista-galli*. Shen *et al*. (2006) also found that the endophytic fungi *Myrothecium roridum* isolated from the Chinese medicinal plant *Artemisia annua* produced three novel macrolides, myrothecine A-C. Two novel antimalarial benzoquinones were extracted from *Xylaria* sp., an endophytic fungus of the Thai medicinal plant *Sandoricum koetjape* (Tansuwan *et al*., 2007).

Ecological and environmental conditions are important factors leading to the diversity of the secondary metabolite profiles of microbial products. Some useful criteria for plant selection were proposed by Strobel & Daisy (2003) who stipulated that plants from unique environment, endemics, having ethnobotanical uses and growing in areas of great biodiversity are likely to harbour novel endophytes and potentially to produce new compounds.

In Madagascar, medicinal plants are often used as the first resort for the treatment of diverse diseases. The practice of traditional medicine is well-embedded in the lifestyle of the eighteen indigenous tribes of this island. Screening surveys have already shown some biological activities such as

antiplasmodial (Rasoanaivo *et al*., 2004), antiviral (Hudson *et al.,* 2000) and cytotoxic activities (Williams *et al*., 2006). Some Madagascan medicinal plants have been extensively studied leading to the discovery of very useful drugs. For example, the Madagascar Periwinckle also called *Catharanthus roseus* is one of the island's most famous plant, from which, the anticancer drugs, vinblastine and vincristine, were extracted. These secondary metabolites are used in the chemotherapy of various types of cancers such as Hodgkin's lymphoma (Seam *et al.*, 2009). Triterpenoids of *Centella asiatica* enter in many commercial drug formulations for skin care, anti-leprosy and anti-ulcerous (Kartnig, 1988).

The present thesis aims to study the microbial endophytes from these two Madagascan medicinal plants. The research project is divided in two major parts. In the first part, the isolation and identification of fungal and bacterial endophytes from *C. asiatica* are reported using microbiological method for the isolation of endophytes and molecular tools for their identification. The role played by antagonistic bacteria in enhancing the resistance of *C. asiatica* against *Colletotrichum higginsianum* is also discussed. Finally, a novel species of *Colletotrichum* isolated from *C. asiatica* leaves is described. The second part of the project is focused on the characterization of endophytic whorl-forming *Streptomyces* producing antifungal compounds from *C. roseus* stems. The results obtained allow a better understanding of the plant-endophyte-pathogen interaction.

CONTEXT OF THE STUDY

Overview on endophytes

Introduction

In contrast to epiphytic microorganisms that inhabit the outer surface of plants, the term "endophytes", in its broader definition, was defined by Petrini (1991) as "*All organisms inhabiting plant organs that at some time in their life can colonize internal plant tissues without causing apparent harm to the host*". Endophytic infections are inconspicuous; the infected host tissues are at least transiently asymptomatic (Stone *et al*., 2000). This definition implies that any microbe inhabiting the internal tissue of apparently healthy plants, for a short period or whole life cycle, can be referred as an endophyte. Microscopic fungi and bacteria are the most common endophytes that often coexist in plant tissues. However, prokaryotic microbes such as blue-green algae are also cited as potential endophytes (McInroy & Kloepper, 1995; Fisher *et al*., 1992) even though no evidence of their presence as endophytes has been reported yet.

Since the discovery of fungal endophytes in darnel by Freeman in 1904, endophytes have been isolated from all land plants investigated to date throughout the world from both temperate and tropical regions (Arnold *et al*., 2000; Fröhlich *et al*., 2000) demonstrating their ubiquitous distribution. Some aquatic plants such as algae (Stanley, 1992), mosses and ferns (Swatzell *et al*., 1996) were also reported to host endophytes. Microbial endophytes could colonize any parts of the plant (leaves, petioles, twigs, bark, flowers and roots), above or below ground (Stone *et al*., 2000). A great number of economically important crops, for instance maize (Fisher *et al*.,

1992), rice (Stoltzfus *et al*., 1997), soybean (Pimentel *et al*., 2006), coffea (Vega *et al*., 2005) and banana (Photita *et al*., 2001) have been reported to host endophytes.

In recent years, studies on endophytes isolated from medicinal plants have received increasing attention because it is thought that plant with medicinal virtues provide a good niche for the discovery of novel microbes which could produce new secondary metabolites (Strobel *et al*., 2004). Bioprospection of secondary metabolites in endophytes extracted from medicinal plants has already revealed a wide variety of new and useful compounds of pharmaceutical interests possessing, for examples, antimicrobial, anticancer and antimalarial properties (Wiyakrutta *et al*., 2004) and antioxidant activity (Huang *et al*., 2007).

Evidence was reported that some endophytes may produce the same bioactive products as those found in their host plants. Stierle *et al.* (1993) demonstrated that *Taxomyces andreanae*, an endophyte of the Pacific yew, also produced taxol, an anticancer drug extracted from the bark of the plant. In *Catharanthus roseus*, a *Fusarium oxysporum* and a mycelia sterilia 97CY3 endophyte were reported to produce vincristine an anticancer drug which is the same as that produced by the plant (Tung *et al*., 2002, Yang *et al*., 2004). One mechanism proposed to explain why some endophytes produce the same bioactive compounds as their host plants is genetic recombination of the endophyte with the host in an evolutionary context (Stierle *et al*. 1993; Tan & Zou, 2001). However, due to the small amounts of endophytic organisms within plants, it is improbable that the compounds are entirely produced by the endophytes *in situ*. But when the endophytes were isolated and cultivated *in vitro*, the numbers of cells were increased far above the normal concentration in the plant and measurable substances were detected.

It is not uncommon to isolate a large number of endophytes from a single plant species. Typically, endophytic community is characterized by a normal distribution where one or few species are frequently isolated in parallel to numerous species with rare occurrence (Rodrigues & Petrini, 1997; Fröhlich *et al*., 2000). Most endophytes are ubiquitous being isolated from various host plants, even though some are restricted to a single host plant. This phenomenon, which is termed "host-specificity", is frequently encountered when studying endophytes from temperate ecosystems (Zhou & Hyde, 2001; Petrini *et al*., 1992) and should be reserved for organisms that will only grow in one host (Schulz & Boyle, 2005). Only scant information is available on the basic processes that lead to host-specificity by some endophytes. Leuchtmann (1992) suggested that host-specificity involved in grass/endophyte symbioses had a genotypic basis as specific isozyme genotypes were found on several host species. On the contrary, the level of host-specificity in tropical leaf endophytes is low because plant tissues of unrelated species were often infected by apparently similar endophytes (Cannon & Simmons, 2002). This differential pattern between temperate and tropical leaf endophytes in term of host-specificity is probably related to the type of forests which are characterized by one or a few dominant tree species in the temperate ecosystems while tropical forests are generally composed of a high diversity of tree species.

Endophytes disseminate within and among plants via vertical and horizontal transmission (Saikkonen *et al*., 1998). Endophytes that infect seeds and spread through the seedling via hyphae causing systemic infection in above-ground tissues are categorized as vertically transmitted. These seedborne endophytes never leave their host plant and can only reproduce by invading seed tissues of the plant. They are commonly encountered in foraging grasses and literally called grass endophytes or balansiaceous endophytes since they belong mainly to the ascomycetous genera *Epichloë*, *Balansia*,

and their anamorphs *Neotyphodium* and *Ephelis*. Conversely, endophytes that spread via spore dispersal by wind, rainfall or insects and colonize the internal tissue of plants after conidial attachment, germination and penetration (Toti *et al.*, 1992) are categorized as horizontally transmitted. Generally with these organisms, the level of endophytic infection increases overtime as the plant tissue ages (Wilson, 2000). For examples, leaves within the buds are endophyte-free or contain few endophytes in comparison to open leaves (Wilson & Carroll, 1994). In addition, the anatomy and morphology of the leaves also have a key role in the infection level. The parts of leaves where rainwater may be channeled, through the midrib for example, and where trichomes that are known to act as spore trap (Allen *et al*., 1991) are dense may give rise to higher infection (Wilson, 2000). Horizontal transfer is also observed in the rhizosphere via lateral root emergence or wounded roots (Kobayashi & Palumbo, 2000). The root endophytes can thereafter colonize root tissues inter- and intra-cellularly and even spread throughout the plant (Hallmann *et al.*, 1997).

Several studies have documented the beneficial effects of endophytes to the host plants. These benefits range from plant growth enhancement and fitness to increased resistance to herbivores and pathogens. In their mutualistic relationship, root endophytes can benefit host plants by enhancing plant growth based on the production of plant growth hormone such as indole acetic acid (Khan & Doty, 2009) and greater absorption of soil nutrient such as phosphorus and other inorganic elements. Jumpponen *et al*. (1998) showed that plants associated with the dark septate root endophytes (DSE), *Phialocephala fortinii*, extracted more phosphorus from the soil than those grown without the DSE. In addition, some nitrogen-fixing endophytes such as the *Acetobacter diazotrophicus* (Boddey *et al*., 1991) and a *Clostridium* sp. (Saito & Minamisawa, 2006) can promote plant growth by fixing atmospheric nitrogen. Under environmental stress conditions, it is reported

that the protection effects of endosymbionts are most pronounced. Endophyte-infected grasses showed increased tolerance to drought and to poor soil conditions (Kuldau & Bacon, 2008; Malinowski & Belesky, 2000). Increased plant resistance to herbivore insects has been associated to the presence of some endosymbionts producing-alkaloids such as peramine and lolines, which serve as antifeeding agent (Clay, 1989; Tanaka *et al*., 2005). Ergovaline and lolitrem B, on the other end, act as anti-vertebrate and are responsible on grazing animal eating toxic forage grasses of the toxic-syndrome called "fescue foot" and "ryegrass staggers" (Tor-Agbidye *et al*., 2001).

Endophytes-host plants interactions

Microbial endophytes may develop diverse forms of interactions with their host plants ranging from benign commensals to latent and quiescent pathogens passing through mutualistic microbes or even saprobes (Schulz & Boyle, 2005).

The asymptomatic colonization of endophytes within the plant tissues may be transient or extent throughout the plant's life cycle. The stability of the status of interaction or the switch from a type of interaction to another depends on factors such as the genetic disposition of the two partners, their developmental stages, nutritional status and environmental factors (Schulz & Boyle, 2006). According to Schulz (1999) the asymptomatic colonization is the result of a balanced antagonism between host and endophytes. As long as the microbial virulence and the plant defense are in balance, no disease symptoms occur and the plant continues to grow normally. When host plants are stressed by changes in environmental or nutritional conditions, or are attacked by pathogens or senesce, the interaction becomes imbalanced

resulting either in the appearance of disease symptoms or the death of the invading microbes.

It is common to isolate known pathogens as endophytes (McInroy & Kloepper, 1995; Bacon & Hinton, 1996). Whatever the stage of the development of the pathogens their presence inside the plant is inconspicuous until the symptoms of the disease appears. Asymptomatic colonization may correspond to a prolonged incubation period or a latency infection which is a type of tolerance of the host to certain pathogens (Sinclair & Cerkauskas, 1996). *Colletotrichum* spp., *Fusarium* sp., *Phomopsis* spp., *Alternaria alternata* are frequently reported in latent fungal infections of various host plants (Sinclair & Cerkauskas, 1996). Freeman & Rodriguez (1993) demonstrated that a mutation at a single genetic locus can change the pathogenic *Colletotrichum magne* to a non-pathogenic *Colletotrichum* endophytic organism with no effect on host. Latent-infecting endophytes may be responsible for the important losses in pre- and post-harvest of economically important crops (Hartman *et al*., 1986).

The term endophyte has also been used for saprophytic microorganisms found on dead or senescing tissues (Stone, 1987; Wong & Hyde, 2001). Promphutta *et al*. (2007) provided evidence of some endophytic species becoming saprotrophs when the host senesces or decays. Their phylogenetic study suggested that fungal colonizers of decaying leaves can have an endophytic origin. It is likely that some endophytic microorganisms have the ability to switch their lifestyle from endophytic to saprotrophic during host senescence suggesting their important ecological role in living plant and dead plant materials. The findings of Wilson (1993) and Faeth & Hammon (1997) supported the important role of fungal endophytes within leaves of trees to hasten the senescence and leaf abscission. The former observed that endophytic-free leaf discs of Oregon white oak remained green and intact for

over 18 months when planted out on PDA in a cold room whereas endophytes-infected leaf discs turned brown rapidly. While Faeth & Hammon (1997) found that Oak leaves with low endophytes presence, especially with the endophyte *Ophiognomonia cryptica* remained on the trees for up to an additional 7 months whereas those that had high endophytes infection presence were shed.

Endophytes represent thus a continuum of variable interactions (Schulz & Boyle, 2005). Like pathogens, endophytes possess the necessary tools such as exoenzymes and phytotoxic metabolites to cross plant barriers and to grow within plants (Castro & Fontes, 2005). In response to this invasion, plants respond with their constitutive and induced defense mechanisms. However, in contrast to pathogens, the phenotypic plasticity of endophytes allows them to establish asymptomatically inside plant tissue, intercellularly or intracellularly with more options such as local or also systemic colonization, mutualism, latent pathogens, or saprophytism (Schulz & Boyle, 2005).

Methods for assessing endophytes

Endophytes can be studied via three methods:

1. Histological method revealing the internal location of the endophytes, whether infection is intercellular or intracellular.
2. Genetic method by direct amplification of microbial genomic DNA from colonized plant tissue.
3. Microbiological method by isolation from surface disinfected tissue and plating onto appropriate growth media (Schulz & Boyle, 2005).

Microbiological method is so far, the most frequently practiced method for routine study of endophytes, and thus described in summary below

(for detailed information, see Bills, 1996). A typical isolation protocol can be resumed as follows: plant collection, transport of collected material to the laboratory, surface sterilization, dissection of tissue into small segments using aseptic techniques, plating of the plant tissue onto growth medium, isolation of endophytic colonies or hyphae emerging from the plant segments, microbial purification, conservation and identification.

Plant collection is one of the most important steps when surveying endophytes. The exact position of the vegetation should be recorded using for example a GPS (General Positioning System) to allow for repeated re-sampling. Healthy plant tissues without lesions or spots should be prioritized. In addition, conducting samplings on different parts of the plants, on plants of different ages, growing in different location and at different season allows gathering useful information on the distribution of the endophytes, community analysis, ecological diversity, and influence of abiotic factors on endophyte infections.

As far as possible, fresh plant material should be transported in good condition to the laboratory and surface sterilization should be done within 24 hours after moving from the collecting areas. During the transport, plant tissues should be maintained in proper aeration in order to prevent desiccation, tissue senescence and contamination by epiphytic fungi and bacteria. Processing plant materials at the collection site remains the most adequate to avoid hyper-contamination with epiphytes.

The surface sterilization procedure should be optimized for the isolation of endophytes from a given plant. The choice of sterilization times and concentration of disinfectant will be dictated by the thickness of the sample, the relative permeability and the texture of its surface (Bills, 1996). The aim

of the surface sterilization is to eliminate epiphytic microorganisms from the surface of the plant tissues, while preserving the endophytic microorganisms inside the tissues by the use of disinfectant or a strong oxidant. Prior to surface sterilization, plant material is cut in segments followed by thorough washing in running tap water and/or sonication in water with tween 80 to remove dust and propagules adhering on the surface or hidden in the trichome. Eventually, plant segments are rinsed in sterile distilled water.

Among the various surface sterilization procedures, the most commonly used is a three-step ethanol, sodium hypochlorite (NaOCl) and ethanol treatment (Fisher *et al*., 1986). Ethanol that acts as a wetting agent is commonly used at concentrations ranging between 70-95% while NaOCl is used at concentrations of 2 to 10% (Zhang *et al*., 2006). Calcium hypochlorite can be an alternative to sodium hypochlorite when assessing delicate tissue since it is believed to less penetrate the tissue (Boccon-Gibod, 1982). For larger diameter twigs and wood fragments, ethanol immersion followed by flaming has been used in some studies (Griffith & Boddy, 1990). Serial washing in sterile distilled water finishes ultimately the sterilization procedure to eliminate the penetrating and killing effects of sterilizing chemicals.

The effectiveness of the surface sterilization procedure must be checked by different techniques such as plating the last rinsing water, imprinting or rolling the sterilized fragments on the media before incubating the plates. The test could be validated if no colonies or hyphae appear after 3 weeks (Schulz *et al*., 1998). Then, surface sterilized fragments are either dissected into small segments and placed onto appropriate growth media for the isolation of filamentous fungi or ground between pestle and mortar with a little amount of water and a serial dilution of the triturated plant tissues are plated on the growth media for the isolation of endophytic bacteria. Plates

are then incubated at 25°C or at room temperature and are examined every day. When hyphae or colonies emerge from plant segments, they are removed and sub-cultured to another growth media. In order to recover the broadest range of internal endophytic communities, the use of selective media and media which favor the growth of slow-growing endophytes is needed to avoid that plate to be overgrown by rapid-growing ones. For example, if the goal is to isolate endophytic fungi, antibacterial antibiotics such as chloramphenicol, streptomycine sulphate, oxytetracycline are incorporated into the agar media either singly or combined along with low concentration of cyclosporin A, dichloran or rose bengal that restrict rapid radial growth of fungi. In contrast, for selective isolation of endophytic bacteria, fungitoxic agents such as cycloheximide and/or nystatin are added to the growth media.

Following serial subcultures to another growth media without antibiotic or fungitoxic agents, pure isolates are obtained and preserved prior to their identification. Preservation can be performed in short time period with plugs of fungal mycelial dipped in sterile distilled water or under paraffin oil, while lyophilization and freezing at ultralow temperature (e.g. -130°C) allowed a long-term conservation of the microbial isolates.

Microbiological method has many advantages. The materials for performing experiences are relatively inexpensive. The results are available in days and the procedure is quite simple. This method may also be useful for surveying spatial and temporal distribution of endophytic community within the plant. The method present however some disadvantages. The isolation of slow growing endophytes may be masked by the presence of more rapidly growing microbes or may be discredited by speculations that sterilization procedures were inadequate to eliminate all adhering surface contaminants.

Host plants

Every plant investigated to date was host to microbial endophytes. In this thesis project, two Madagascan medicinal plants, *Centella asiatica* and *Catharanthus roseus* were selected. The choice of *C. asiatica* and *C. roseus* was primarily based on criteria established during the project "Biodiversity and Biotechnology in Madagascar" including: plants of socio-economic importance, plants having medicinal virtue, plants being endangered, threatened, or rare and endemic to Madagascar.

Centella asiatica is not a threatened species, but has an economic impact for Madagascar and is largely overexploited. This plant is the second most exported medicinal plant in Madagascar (Péchard *et al*., 2005) to meet the huge demand from western countries for triterpenoids. These secondary metabolites are components for various medicinal drugs and are largely used in cosmetic preparations for skin care. Even though, *C. asiatica* is widely distributed in tropical and subtropical regions such as Asia, Australia, India, New Guinea, Malaysia, South Africa and Madagascar, *C. asiatica* from Madagascar is strongly requested because a comparative study supported that *C. asiatica* leaves from Madagascar contain 3 to 7 times higher asiaticoside content than those from India (Rouillard *et al*., 1997).

Catharanthus roseus is native and endemic to Madagascar but is now widely cultivated and naturalized worldwide, mainly in tropical and subtropical areas. Due to the practice of slash and burn agriculture in Madagascar, *C. roseus*, is a threatened plant in its natural habitat.

Centella asiatica

Botanical description

Centella asiatica (L.) Urb. also called hydrocotyle, gotu kola, Indian Pennywort or talapetraka in Madagascar is an evergreen perennial, herbaceous, creeper plant of the family Umbelliferae or Apiaceae. It is widely distributed in tropical and subtropical regions such as Asia, Australia, India, New Guinea, Malaysia, South Africa and Madagascar. It grows abundantly in moist areas. The plant is characterized by a long, slender petiole up to 30 cm in height, which brings a simple reniform-cordate leaf and propagated by means of horizontal stems called stolons interconnecting one plant to another.

Fig. 1. *Centella asiatica*.

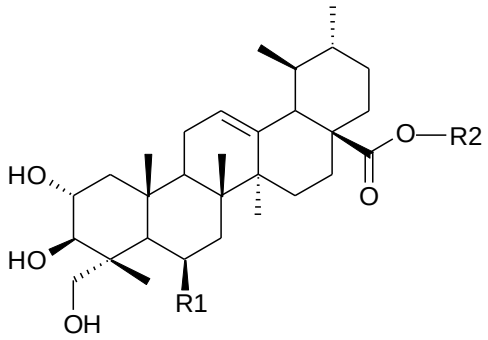
Uses in traditional medicine

C. asiatica is an ethnomedicinal herb used for ages in Ayurvedic medicine, Chinese and Madagascan traditional medicine. It was traditionally used for wounds healing, leprosy, skin tuberculosis, stomach

aches, arthritis, varicose veins, high blood pressure (Boiteau & Ratsimamanga, 1956). It is also claimed to increase performance in mentally retarded children (Appa-Rao *et al*., 1973). Other ethnomedicinal uses included the treatment of insomnia, depression, stress and epilepsy (Ganachari *et al*., 2004).

Main chemical constituents found in the plant and their pharmacological and biological properties

The major chemical constituents found in the plant are triterpenoids, flavonoids and an essential oil. *C. asiatica* contains mostly pentacyclic triterpenic glycosides and their respective aglycones. The four major triterpenes produced in the aerial parts of the plant are asiaticoside, asiatic acid, madecassoside and madecassic acid (Inamdar *et al*., 1996).



Asiatic acid	R1=H	R2=H
Madecassic acid	R1=OH	R2=H
Asiaticoside	R1=H	R2=Glu-Glu-Rha
Madecassoside	R1=H	R2=Glu-Glu-Rha

Fig. 2. Structure of main triterpenoids from *Centella asiatica*.

Many other triterpenes have been found in the plant but in a lower proportion such as brahmic acid, brahmoside, thankuniside, isothankuniside, madasiatic acid, centelloside, betulinic acid, cenellic acid, centic acid, indocentic acid (Zheng & Qin, 2007). Earlier, the BuOH fraction of *C. asiatica* was reported to contain four new triterpenoid glycosides named asiaticosides C, D, E and F (Jiang *et al*., 2005).

Triterpenoids from *C. asiatica* possess various biological activities providing strong support for its use in ethnomedicine. Kimura *et al*. (2008) confirmed that asiaticoside accelerated wound repairs by promoting angiogenesis process at the site of injury. However, another report showed that the claimed wound healing effect of *C. asiatica* seems to be attributed to madecassoside by involving various mechanisms including antioxidant activity (reduction of the nitric oxide levels and the malonaldehyde content in the burn skin), angiogenesis (proliferation of endothelial cell growth) and collagen synthesis in an experimental assay of burn injury in mice (Liu *et al*., 2008a).

The anti-inflammatory and anti-ulcerous activities of *C. asiatica* herbs and its active constituents are well established. Recent reports showed that madecassoside and asiaticoside inhibited the collagen-induced arthritis in mice in agreement with the use of *C. asiatica* herbs for rheumatoid arthritis cure (Liu *et al*., 2008b; Li *et al*., 2007). In addition, *C. asiatica* water extract and asiaticoside reduced the size of ulcers in acetic acid induced gastric ulcers in rats in a dose-dependent manner, with a concomitant attenuation of myeloperoxidase activity at the ulcer tissues (Cheng *et al*., 2004).

The *in vitro* growth inhibition of several tumour cells such as the human gastric adenocarcinoma (MK-1), human uterine carcinoma (HeLa), and murine melanoma (B16F10) cells by the MeOH extract from the aerial parts

of *C. asiatica* brought evidence for its antiproliferative effects (Yoshida *et al*., 2005). This anti-cancer property is likely due to asiatic acid which was shown to induce apoptosis and inhibit the growth of SW480 human colon cancer cells (Tang *et al*., 2009).

Asiatic acid has also been shown to possess neuroprotective properties in a mouse model of permanent cerebral ischemia suggesting its use for the treatment of cerebral ischemia (Krishnamurthy *et al*., 2009). Current research on Alzheimer's disease in animal model revealed that *C. asiatica* extracts may reduce neuropathological mechanisms involved in the disease by preventing cognitive deficits (Kumar & Gupta, 2003), decreasing in a long-term treatment the level of amyloid β peptide (Dhanasekaran *et al*., 2009) which accumulation into plaques in the brain parenchyma and cerebral blood vessel have been shown to play a significant role in the development and progression of Alzheimer's disease (Glenner & Wong, 1984).

Additionally, various flavonoids including quercetin, kaempferol, catechin, rutin and naringin were characterized from *C. asiatica* (Zheng & Qin, 2007). Some of them were thought to contribute to the antioxidative activity of the plant (Zainol *et al*., 2003). Petuletin, a flavonol, along with kaemferol 3-O-β-D-glucuronide were also present in the aerial parts of *C. asiatica*. These metabolites were reported to possess potent inhibitory activity on aldose reductase in rats (Matsuda *et al.*, 2001).

Besides terpenoids and flavonoids, *C. asiatica* also contains an essential oil whose constituents are mainly composed of β-caryophyllene, bicyclogermacrene, germacrene B and D, trans-β-farnesene and myrcene having broad spectrum antibacterial activities against Gram-positive and Gram-negative bacteria (Oyedeji & Afolayan, 2005).

Catharanthus roseus

Botanical description

Known as Madagascar Periwinkle and *Vinca rosea*, *Catharanthus roseus* (L.) G. Don is an herbaceous sub-shrub plant of the family Apocynaceae. Originally native of the south-eastern and eastern area of Madagascar, the plant is easily cultivated and now becomes naturalized in many parts of tropical and subtropical countries. It develops well in hot and humid environment. Attractive ornamental plants found commonly in gardens and homes, *C. roseus* is now grown commercially across the world because of its pharmaceutical interest. The plant has white or pink flowers comprising five petals, dark green oval to oblong leaves arranged in opposite pair and is up to 30 cm to 1 m in height. The genus *Catharanthus* contained eight species, seven of which are endemic to Madagascar (*C. coriaceus* Markgr, *C. lanceus* (Bojer ex A.DC.) Pichon, *C. longifolius* (Pichon) Pichon, *C. ovalis* Markgr, *C. scitulus* (Pichon) Pichon, *C. trichophyllus* (Baker) Pichon and *C. roseus* (L.) G. Don).

A B C

Fig. 3. *Catharanthus roseus*. A- var. *roseus*; B- var.*ocellatus*; C- var. *albus*

(From: http://www.scribd.com/doc/3547408/Catharanthus-alkaloids)

Uses in traditional medicine

Catharanthus roseus has a long traditional use in folk medicine from different areas in the world. Extracts are used in herbal medicine to treat diabetes, malaria, wasp sting, eye irritation, infection, dysmenorrhea, high blood pressure and as poultice to stop bleeding (Dobelis, 1986; Duke, 1985). Other virtues such as diuretic, antidysenteric, astringent, and cough remedy were reported from Chinese traditional medicine.

Main chemical constituents found in the plant and their pharmacological and biological properties

More than 130 alkaloids mainly belonging to the class of indolomonoterpenic alkaloids have been isolated from *C. roseus* some of which are of pharmaceutical importance (van der Heijden *et al*., 2004). Vinblastine and vincristine are being used in the chemotherapy of leukaemia and treatment of Hodgkin's disease (Noble, 1990; Seam *et al.*, 2009). These alkaloids bind to tubulin, thereby disrupting the microtubule formation in the mitotic spindle, resulting in a mitotic arrest at metaphase stage (Jordan *et al.*, 1985). They are formed by coupling of two monomeric indole alkaloids catharanthine and vindoline.

Due to the low content of vinblastine and vincristine in the plant, many efforts have been made with the *in vitro* culture of *C. roseus* cells to increase their production (Pietrosiuk *et al*., 2007). The effects of various elicitors to induce alkaloid accumulation under *in vitro* cell suspension of *C. roseus* were mostly investigated and resulted in higher level of ajmalicine,

catharanthine and vindoline compared to non-treated cultures (Vázquez-Flota *et al*., 2009; Ramani & Jayabaskaran, 2008).

Ajmalicine (adreno-sympatholytic) and serpentine (acetylcholinesterase inhibitor) are proved to possess antihypertensive and sedative activities respectively (van der Heijden *et al*., 2004). Later, Ara *et al*. (2008) demonstrated that *C. roseus* leaves extract had hypotensive effects on adrenaline induced hypertensive rats. Vincamine, another indole alkaloid from *C. roseus*, is a peripheral vasodilator that enhances blood flow to the brain. Its therapeutic efficacy to treat vascular dementia was confirmed by a clinical trial (Fischhof *et al*., 1996).

Anthocyanins belonging to the methylanthocyanidin were also found in the flowers of *C. roseus* (Filippini *et al*., 2003; Toki *et al*., 2008). Besides, several phenolic compounds were identified from the plant (Mustafa & Verpoorte 2007; Ferreres *et al*., 2008). Recently, the presence of 5-hydroxy flavone, a flavonoid compound, from the leaves of *C. roseus* with antifungal activity has been reported (Roy & Chatterjee, 2010). The aerial parts of the plant also contain numerous volatile compounds (De Pinho *et al*., 2009).

During this thesis project, we isolated from *C. roseus* stems an endophytic *Streptomyces* producing polyene macrolide antibiotics. To that purpose, a brief literature review of polyene macrolides from *Streptomyces* is provided in the following section.

Polyene macrolide antibiotics from *Streptomyces*

Polyene macrolide antibiotic are a class of natural product mainly produced by *Streptomyces* species, characterized by a lipophilic conjugated polyene unit and a hydrophylic polyol unit which may or not be a sugar (Bolard, 1986). Polyene macrolide antibiotics were classified according to the number of conjugated double bonds in the molecule into trienes, tetraenes, pentaenes, hexaenes, and heptaenes (Hamilton-Miller, 1973).

Polyene macrolides having current clinical uses

The main utilization of polyene macrolides is mostly related to their antifungal properties. The affinity of the molecules to interact with ergosterol, the principal sterol in fungal plasmic membranes, results in membrane perforation, leakage of ions and ultimately cell death (Bolard, 1986). Despite their fungicidal actions and their extremely rare incidence of resistant pathogens, only few polyene macrolide antibiotics found widespread clinical use including rapamycin (triene), nystatin (tetraene), and amphotericin B (heptaene), due to the relatively high toxicities for mammalian cells and low water solubility (Zotchev, 2003; Nosanchuk, 2006).

Rapamycin (**1**) was extracted from the mycelium of *Streptomyces hygroscopicus* isolated from an Easter Island (Rapa Nui) soil sample (Vézina *et al.*, 1975). Rapamycin, also termed sirolimus, is a triene macrolide

antibiotic with potent immunosuppressive activity, used in pediatric transplant population (Markiewicz *et al*., 2003; Ibáñez *et al*., 2005). Rapamycin seemed to extend lifespan in mice (Harrison *et al*., 2009) and can slow or block Alzheimer's disease progression in a mouse model of Alzheimer's (Spilman *et al*., 2010).

Nystatin (**2**), which was discovered by Hazen & Brown (1950) from a soil actinomycetes, *Streptomyces noursei*, is a tetraene macrolide mainly used for topical treatment of oral, gastro-intestinal and genital candidosis (Fjærvik & Zotchev, 2005). *Streptomyces fungicidicus* ATCC 27432 (Matsuoka, 1960) and *Streptomyces albulus* ATCC 12757 (Veiga *et al*., 1983) were also nystatin producer.

Amphotericin B (AmB) (**3**) was first isolated from cultures of *Streptomyces nodosus* (Vandeputte *et al*., 1955). Since its approval for use in the USA in 1957, AmB has long been the drug of choice for systemic mycoses (Nosanchuk, 2006). However, the prescription of AmB is limited by its various side effects, i.e. fever, hypotension, respiratory distress, toxicity (mainly nephrotoxicity) and by its intravenous administration. Recent formulations such as nanosuspension (Kayser *et al*., 2003), lipid-based formulation (Risovic *et al*., 2007) and lipid nanospheres (Amarji *et al*., 2007) for oral administration of AmB have shown promising results in reducing significantly the side effects and improving its oral bioavailability.

Other biological properties of polyene macrolide antibiotics

In addition to their antifungal properties, some polyene macrolides display diverse biological properties. For example, from the mycelium of *Streptomyces virdigreseus*, Thirumalachar & Menon (1962) isolated two

oxo-hexaene macrolides, dermostatin A and B (**4**), having high anti-proliferative activity against HIV in H9 cells (Pandey, 1992). Total synthesis of dermostatin A was first reported by Sinz & Rychnovsky (2002) and later by Zhang *et al*. (2011).

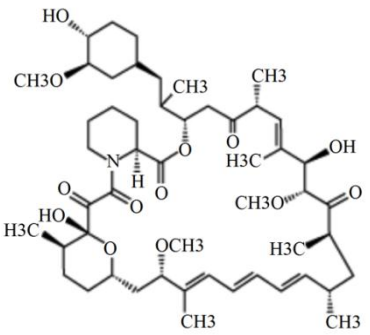
Filipin (**5**) is a pentaene macrolide antibiotic extracted from *Streptomyces filipinensis* (Whitfield *et al*., 1955). Filipin is a mixture of four molecules, justifying its appellation as filipin complex, with filipin III being the most abundant one (Bergy & Eble, 1968). The first total synthesis of filipin III was achieved by Richardson & Rychnovsky (1997). The strong affinity of filipin III for cholesterol explains that it is used as a histochemical stain for cholesterol in the clinical diagnosis of the type C Niemann Pick disease (Vanier, 2010).

Studies showed that polyene macrolide antibiotics having a large and rigid macrolide ring such as amphotericin B and aureofacin may enhance antiviral activity of acyclovir. However, the acyclovir potentiating activity were not observed with polyene antibiotics with small and rigid (pimaricin and filipin) or large but flexible (nystatin A1 and lienomycin) macrolide rings (Malewicz *et al*., 1984). Besides that, some derivatives of polyene macrolides such as hydrophosphoryl derivatives of mycoheptin (Belakhov & Shenin, 2007) and methyl ester of amphotericin B (Schaffner *et al.*, 1986) were described to possess good antiviral activity.

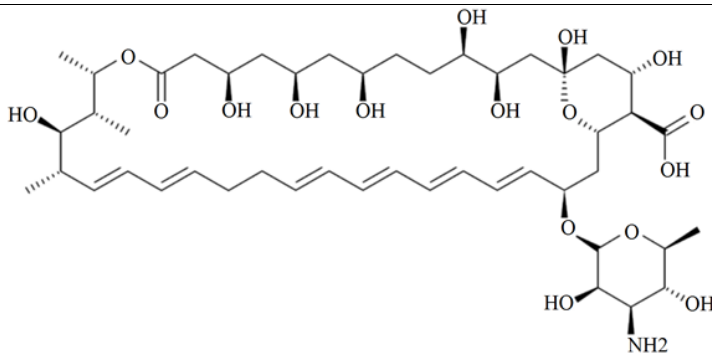
Darisipudi *et al*. (2011) demonstrated *in vitro* in dendritic cells isolated from mouse bone marrow that amphotericin B, nystatin, and natamycin directly activate the host's innate immunity by triggering interleukin-1β secretion in monocytes. Natamycin (**6**), also known as pimaricin, is a mycosamine-containing tetraene isolated from cultures of *Streptomyces natalensis* (Struyk *et al*., 1958) and related species, *S. chattanoogensis* and *S. gilveosporus*

(Hamilton-Miller, 1973). Natamycin (E 235) is used in food industry as a food additive (European Food Safety Authority, 2009).

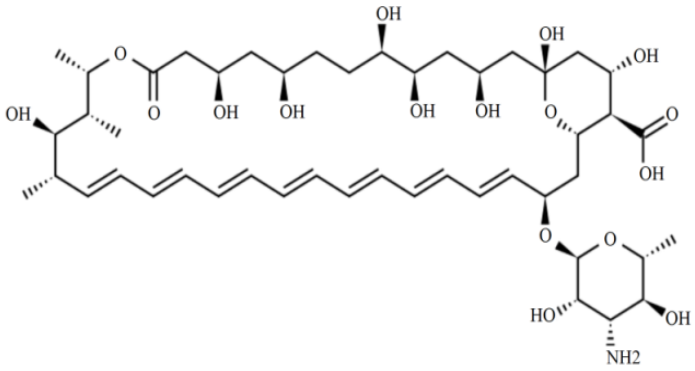
In contrast to many polyene macrolides, such as amphotericin B and nystatin, which have no antibacterial activity, faeriefungin, a pentaene macrolide, produced by *Streptomyces griseus* var. *autotrophicus* (Nair *et al.*, 1989) displayed a good antibacterial activity. It inhibited the growth of many gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* strains with a minimum inhibitory concentrations ranging from 8 to 64 µg mL^{-1}. However, faeriefungin was less active against gram-negative bacteria (Mulks *et al*., 1990). Similarly, pentamycin, also a pentaene macrolide antibiotic naturally produced by *Streptomyces pentaticus* (Umezawa *et al*., 1958), exhibited a broad spectrum of antimicrobial activity, justifying the clinical use of intravaginal pentamycin for the treatment of vaginal infections caused by *Trichomonas vaginalis*, *Candida albicans*or mixed microorganisms (Balmer, 2009). Pentamycin, also called fungichromin (**7**), was reported to occur in several *Streptomyces* species including *Streptomyces cinnamoneus* and *S. roseoluteus* (Hamilton-Miller, 1973), *S. griseus* (Pandey *et al*., 1980), *S. cellulosae* (Nogushi *et al*., 1988) and *S. padanus* PMS 702 (Shih *et al*., 2003). It was found to be effective in controlling *Rhizoctonia* damping-off of cabbage and tomato late blight (Shih *et al*., 2003) and was patented for the treatment of skin yeast infections (Hedgpeth, 2005).



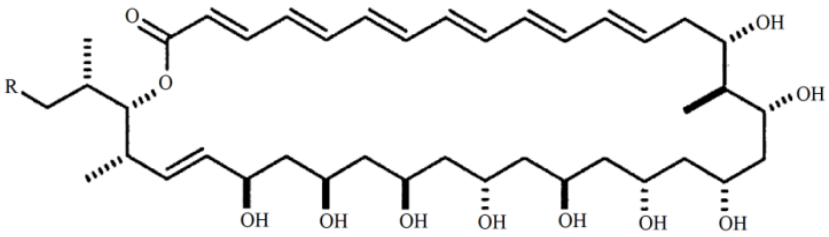
(1)



(2)



(3)



(4)

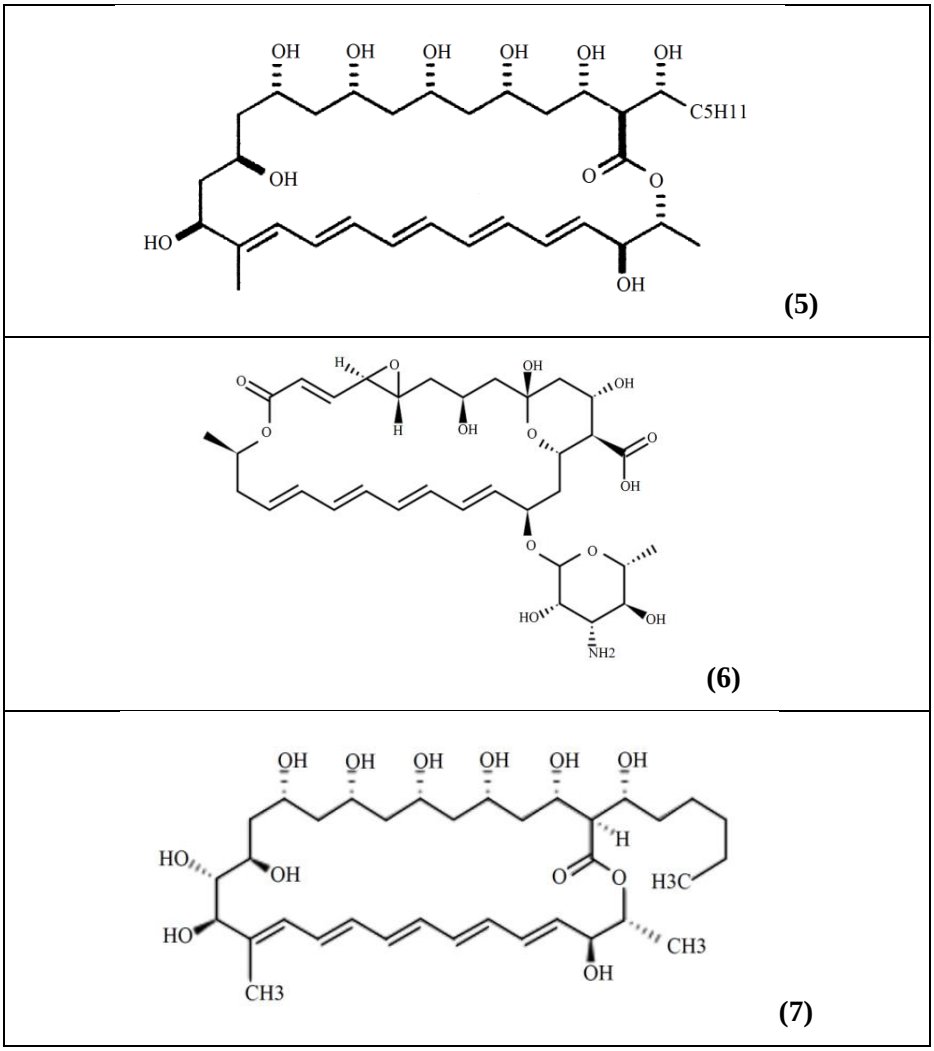


Fig. 4. Molecular structures of major representatives of polyene macrolide antibiotics. (**1**) Rapamycin; (**2**) Nystatin; (**3**) Amphotericin B; (**4**) Dermostatin A, R = H; Dermostatin B, R = CH_3; (**5**) Filipin III; (**6**) Natamycin; (**7**) Fungichromin.

RESEARCH OBJECTIVES
AND
OUTLINE OF THE THESIS

Research objectives and outline of the thesis

To date, natural products of microbial origin, especially from microbial endophytes are still unexplored and relatively unstudied in Madagascar. However, based on Bills' observations (1996), predicting that each vascular plant is host to at least two to four undescribed microbial species, specific to that plant species, the Madagascan endemic flora, with its 12000 species, provide opportunity to find numerous undescribed endophytic microorganisms. In addition, plants with medicinal virtues and growing from areas of great biodiversity are assumed to be a good niche for the discovery of endophytes that may produce bioactive compounds of pharmaceutical importance (Strobel & Daisy, 2003). Indeed, Madagascan medicinal plants fulfill the criteria of plant selection for the assessment of endophytes with its rich, diversified and unique ecosystem that has been developing over time, after the island broke away from the African continent more than 120 million years ago.

The present work addresses these above-mentioned assumptions and provides evidence that medicinal plants, not only shelter endophytic microorganisms capable of protecting their hosts against pathogens and producing antifungal compounds, but also harbour new endophytic species. The **main objectives of this thesis** were to assess the biodiversity of microbial endophytes from Madagascan medicinal plants and to characterize

their bioactive metabolites, especially their antifungal properties. In order to achieve the goals of the study, two medicinal plants (*Centella asiatica* and *Catharanthus roseus*) were selected and their microbial endophytes were investigated.

The core of the study could be divided into two parts. The first part of the study (chapters I, II and III) was devoted to microbial endophytes (culturable fungi and bacteria) from *C. asiatica*. It highlights the diversity of endophytic species associated with *C. asiatica* leaves collected in Mangoro, Madagascar. The second part of the study (chapter IV) concentrated on *Streptomyces* species isolated from *C. roseus* stems producing antifungal compounds. Isolation of endophytes in both plants was achieved by microbiological method following surface sterilization of plant tissues to eliminate epiphytes and incubation of disinfected samples on suitable media. Identification was made using molecular tools. Endophytes showing special features as new species, with biocontrol potential and as producer of antifungal compounds were further investigated. Figure 5 briefly outlined the different steps that were considered.

In chapter I, we performed community analysis of fungal endophytes from *C. asiatica* through the study of species composition, distribution and assemblage of endophytic fungi within leaves. The potential interactions between major endophytes were examined using dual cultures. This work was published in Antonie van Leeuwenhoek (2008).

The study of the biodiversity of microbial endophytes from *C. asiatica* was complemented in **chapter II** by the isolation and identification of its endophytic bacteria. The protection effects of the two most antagonistic bacteria against *Colletotrichum higginsianum* were assessed using co-inoculation assays in *in vitro C. asiatica* plantlets. The results showed that

they were able to significantly inhibit anthracnose disease symptoms caused by the hemibiotrophic plant pathogen *Colletotrichum higginsianum*. The results of this study were submitted to Antonie van Leeuwenhoek.

In **chapter III**, we described a new *Colletotrichum* species producing long straight conidia isolated from leaves of *C. asiatica*. A teleomorph was produced in laboratory cultures when the anamorph was cultivated in potato dextrose agar supplemented with sterilized powder leaf of *C. asiatica*. Based on morphological and phylogenetic evidence, the fungus was named *Colletotrichum gigasporum* sp. nov. The results of this study were accepted for publication in Mycological Progress.

Since a recent paper has already reported the study of endophytic fungi from *C. roseus* (Kharwar *et al*., 2008), in **chapter IV** we focused on the investigation of endophytic *Streptomyces* from this valuable medicinal plant. To our knowledge, we reported here the first endophytic whorl-forming *Streptomyces* sp. isolated from healthy stems of *C. roseus*, designated as *Streptomyces* sp. strain TS3RO. Direct bioautography on thin layer chromatography plate with *Cladosporium cucumerinum* was conducted throughout the purification steps for bioassay-guided isolation of the active antifungal compounds from the crude culture filtrated extracts. Finally the structure of the major antifungal compound was elucidated using spectroscopic techniques. This study was published in the Canadian Journal of Microbiology (2012).

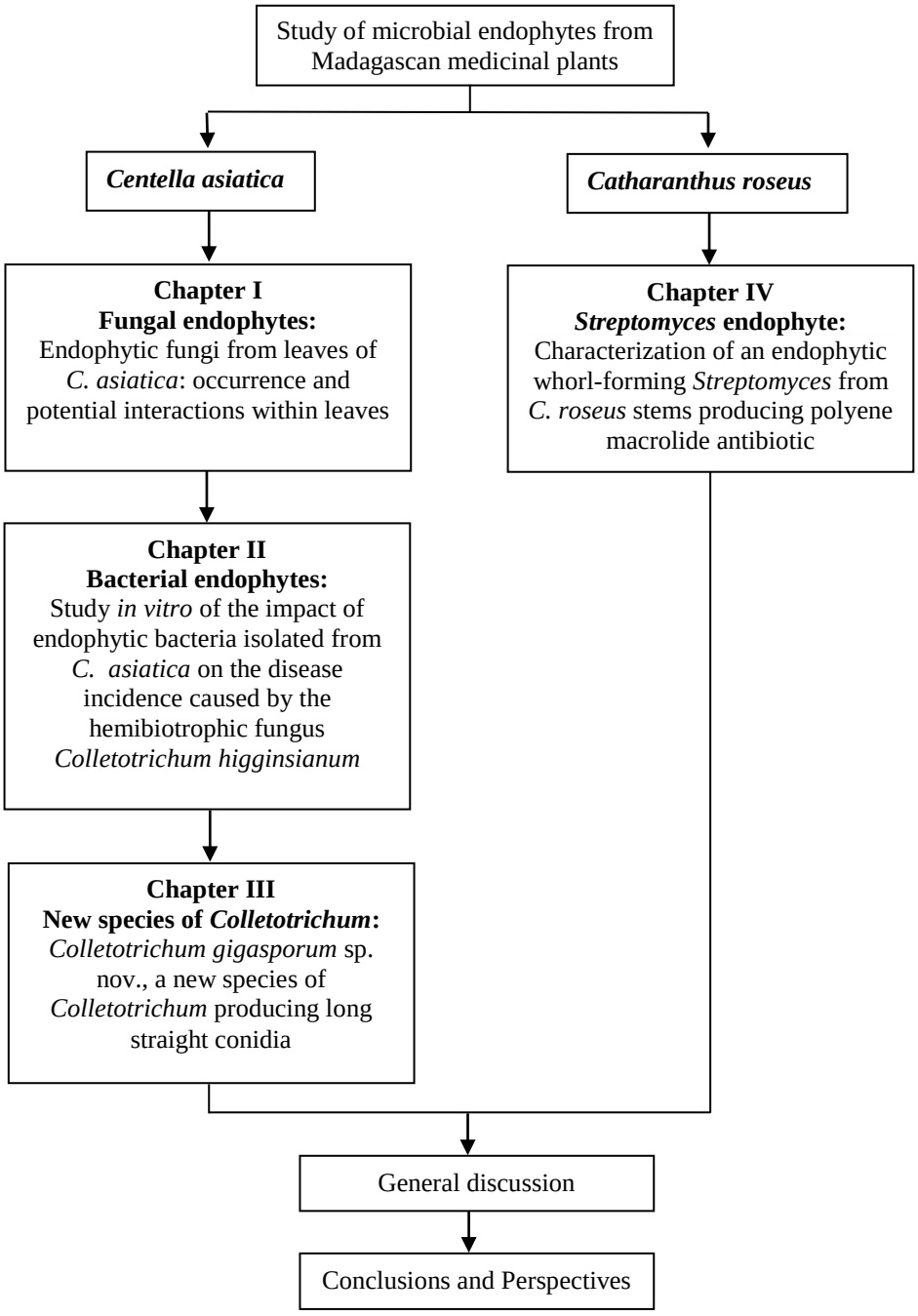


Fig. 5. Outline of the thesis.

CHAPTER I

ENDOPHYTIC FUNGI FROM LEAVES OF *CENTELLA ASIATICA*: OCCURRENCE AND POTENTIAL INTERACTIONS WITHIN LEAVES

This chapter has been published in Antonie van Leeuwenhoek, 2008, 93: 27–36.

Rakotoniriana EF, Munaut F, Decock C, Randriamampionona D, Andriambololoniaina M, Rakotomalala T, Rakotonirina EJ, Rabemanantsoa C, Cheuk K, Urveg-Ratsimamanga S, Mahillon J, El-Jaziri M, Quetin-Leclercq J, Corbisier AM

Preface

Centella asiatica (APIACEAE) is the second most exported medicinal plant in Madagascar (Péchard *et al*., 2005). The huge demands for *C. asiatica* from western countries can be explained by the presence in this plant of useful triterpenoids which are components of various medicinal drugs and are largely used in cosmetic preparations for skin care. Variability in triterpenoid content found in *C. asiatica* from geographically different regions has been reported. Rouillard *et al*. (1997) proved that *C. asiatica* leaves from Madagascar contained 3 to 7 time higher concentration in asiaticoside in comparison with those from India. Randriamampionona *et al*. (2007) observed a significant variability in triterpenoid content of *C. asiatica* samples collected from different locations in Madagascar. Interestingly, the authors found that the sample from Mangoro region contained the highest content in asiaticoside.

Because of the ability of many fungal endophytes such as *Arthrobotrys*, *Chaetophoma*, *Dematium* and *Colletotrichum* to convert betulinic acid, a triterpenoid found in various plants species including *C. asiatica* (Zheng & Qin, 2007), to many oxygenated derivatives (Bastos *et al*., 2007), endophytic fungi are likely to be involved in the variability of the triterpenoid content in *C. asiatica* samples. We therefore, explored the fungal endophytes of *C. asiatica* collected from Mangoro where the highest content of asiaticoside was recorded. The within-leaf distribution of the main endophytes was also assessed since terpenoids are well known to act as plant defense compounds and to determine the extent of fungal colonization (Mucciarelli *et al*., 2007). In this chapter, we demonstrated that the observed differential distribution patterns of the main endophytes on opposite areas in leaves of *C. asiatica* may be due to antagonistic interactions between those endophytic fungi.

1.1. Abstract

Fungal endophytes were isolated from leaves of *Centella asiatica* (Apiaceae) collected at Mangoro (Middle Eastern region of Madagascar, 200 km from Antananarivo). Forty- five different taxa were recovered. The overall foliar colonization rate was 78%. The most common endophytes were the non-sporulating species 1 (isolation frequency IF 19.2%) followed by *Colletotrichum* sp.1 (IF 13.2%), *Guignardia* sp. (IF 8.5%), *Glomerella* sp. (IF 7.7%), an unidentified ascomycete (IF 7.2%), the non-sporulating species 2 (IF 3.7%) and *Phialophora* sp. (IF 3.5%). Using sequences of the ribosomal DNA internal transcribed spacer (ITS) regions, major endophytes were identified as xylariaceous taxa or as *Colletotrichum higginsianum*, *Guignardia mangiferae* and *Glomerella cingulata*. Results from *in vitro* fungal disk experiments showed a strong inhibitory activity of the xylariaceous non-sporulating species 1 against *G. mangiferae* and *C. higginsianum* and of *C. higginsianum* against *G. mangiferae*. This can be explained by antagonism between dominant taxa.

Key words: *Centella asiatica*, *Colletotrichum higginsianum*, Endophytic fungi, Xylariaceae

1.2. Introduction

Endophytic fungi have been described as fungi that asymptomatically colonize healthy plant tissues, even though they may, after incubation or a latency period, cause diseases (Petrini, 1991; Stone *et al*., 2000). Diverse associations with host plants have been reported, ranging from mutualistic relationships (Schulz *et al.*, 2002) and cryptic commensalism (Deckert *et al.*, 2001) to latent and quiescent pathogens (Sinclair & Cerkauskas, 1996). The stability or the variability of the asymptomatic interaction depends on numerous factors such as environmental stress, senescence of the hosts, virulence of the endophytes and the host defense response (Schulz & Boyle, 2005). Endophytes may be beneficial to the hosts in conferring resistance to insects and herbivores (Clay, 1988), drought tolerance (West, 1994), protection against pathogens (White & Cole, 1985) and enhanced vegetative growth (Porter *et al.*, 1979).

Endophytic fungi have been encountered from any plant ever investigated. In contrast, endophytic fungi in medicinal plants, especially in tropical regions, are still poorly explored though they could represent a source of valuable new and bioactive compounds (Li *et al.*, 2001). Recently, endophytic fungi isolated from a Brazilian medicinal plant were shown to produce guignardic acid (Rodrigues *et al.*, 2001). Likewise, new bioactive metabolites were produced by a *Colletotrichum* sp., an endophytic fungus in *Artemisia annua*, a traditional Chinese medicinal herb (Lu *et al.*, 2000).

Centella asiatica (L.) Urban (Apiaceae, Umbelliferae), also known as Gotu kola or Indian pennywort, is a small, annual, slender, creeping entwined herb that grows near swamps on damp ground. It is mainly found in tropical regions of India, Madagascar, Sri Lanka, China, Indonesia, Australia and

South Africa. It is used for various medical treatments (Boiteau & Ratsimamanga, 1956; Pointel *et al.*, 1987).

Reports have shown that terpenoid-producing plants such as *Sequoia sempervirens* develop differential activity on foliar endophyte growth (Espinosa-García *et al*., 1996). Moreover, a recent study using an improved HPLC method has revealed the presence of six triterpenes in *C. asiatica* extracts (Schaneberg *et al.*, 2003). However, the endophytic community of this potentially important pharmaceutical medicinal herb has not yet been investigated. Here, we describe the diversity of endophytic fungi in leaves of *C. asiatica*. We also assess the within-leaf distribution of and possible interactions between major fungal endophytes. Our research is part of an ongoing project on the conservation of medicinal plants and their associated microorganisms in Madagascar. It may contribute to the discovery of novel bioactive compounds probably deriving from endophytes of these medical plants.

1.3. Materials and Methods

1.3.1. Sampling

Centella asiatica plants were collected in Mangoro (18° 52' 20 South, 48° 06' 25 East, 823 m a s l, middle eastern region of Madagascar, 200 km from Antananarivo), a collection site of IMRA (*Institut Malgache de Recherches Appliquées*) in May 2003 after the rainy season. Five apparently healthy about 5 months old and 10 to 15 cm long shoots were randomly selected, pulled up with their rhizomes, placed into a cooled bag and sent immediately to BCCM/MUCL (Belgium). On arrival, two days later, the samples were dipped in a container filled with tap water to refresh them and processed within four days.

1.3.2. Surface sterilization procedures

Prior to surface sterilization, leaves were individually removed from shoots and thoroughly washed in running tap water to remove dust particles on leaf surfaces, then shaken in a flask containing 200 mL of sterile distilled water with the addition of 2 drops of Tween 80 (Sigma Chemical Co., St. Louis, Mo, USA). Thereafter, the samples were soaked in 75% ethanol for 1 min, then in 3% calcium hypochlorite (VWR international, Leuven) for 10 min and again in 75% ethanol for 30 s. Finally, the leaves were rinsed three times in sterile distilled water and dried with sterile paper toweling.

In order to test the effectiveness of surface sterilization, 20 mL of the last rinsing water were centrifuged for 10 min at 3000 rpm. The supernatant was removed, leaving 500 µL in the centrifugal tube, which were homogenized with a vortex; 100 µL of this volume were then plated onto a medium containing 2% malt extract agar supplemented with chloramphenicol to suppress growth of bacteria (MEAC: 20 g malt extract, 20 g glucose, 1 g

peptone and 15 g agar in 1 L distilled water and 200 mg L^{-1} chloramphenicol). In addition, sterilized leaves were imprinted onto MEAC before incubating the plates at room temperature for three weeks. The test was validated if no mycelial growth occurred (Schulz *et al.*, 1998).

1.3.3. Isolation of endophytic fungi

After surface sterilization, five leaves were cut up into very small segments of approximately 2 × 2 mm using a flame-sterilized scalpel. About 600 leaf pieces were placed in 30 Petri dishes (90 mm) containing MEAC, with an average of 20 pieces per dish. The scalpel was flame-sterilized anew for each of the 600 pieces to avoid the contamination of the following piece of leaf by the endophyte of its predecessor. The plates were sealed with cellophane and incubated at room temperature. Fungi growing out from the plant tissue fragments were subcultured onto 2% malt extract agar without antibiotics by transferring a plug of agar with the piece of leaf under which hyphal tips emerged. The emerging hyphae were then transferred with the plant material to avoid that the growing mycelium masked the growth of the others and to ensure to yield the whole endophytic communities, because such a minute plant square could still contain another type of hyphae of slow-growing fungi (Gamboa & Bayman, 2001).

The overall colonization rate (CR) was calculated as described by Petrini *et al*. (1982):

$$\frac{\text{Total number of leaf segments in a sample yielding} \geq 1 \text{ isolate}}{\text{Total number of leaf segments}} \times 100$$

The isolation frequency (IF) was determined by the following formula:

$$\frac{\text{The sum of isolation number per leaf of a given taxon}}{\text{Total number of leaf segments}} \times 100$$

Pure isolates were subcultured onto different media for morphological identification. After three weeks of incubation at 25°C in two 12-hour cycles, one of cool white fluorescent light followed by one of darkness, most isolates sporulated on either 2% malt agar (MA2: 20 g malt extract, 15 g agar and 1 L distilled water), oatmeal agar (OA: 10 g rolled oats, 15 g agar in 1 L distilled water) or potato dextrose agar (PDA: 4 g potato extract, 20 g glucose, 15 g agar and 1 L distilled water) and could be identified at genus level. The remaining isolates that failed to produce reproductive structures on PDA, MA and OA after two months were again subcultured onto 2 percent water agar in a Petri dish containing a strip of autoclaved *C. asiatica* leaf and the cultures were subjected to near-UV light (366 nm). Cultures that did not sporulate after four months or more after these treatments were considered as sterile mycelium and separated into taxonomic groups according to cultural characteristics (Fröhlich *et al.,* 2000). Representative strains were deposited in the BCCM/ MUCL collection.

1.3.4. Molecular identification of major endophytes

The main endophytic fungi encountered were identified by using molecular tools. Genomic DNA was extracted from ground mycelium by the method of Lee *et al.* (1988) and purified from solution using the GENECLEAN® III Kit (Qbiogene, Inc., Carlsbad, California) according to the manufacturer's instructions, to yield a total volume of 50 µL purified DNA. The regions of ribosomal DNA (rDNA) were amplified using the primers NS7 and ITS4 (White *et al.,* 1990). The amount of purified DNA acquired was adjusted to 10 ng μL^{-1} using a BioPhotometre (Eppendorf) at 260 nm. First, 10 µL of fungal DNA containing 10 ng μL^{-1} were transferred to the reaction tube. Then, 40 µL of master mix consisting of 5 µL buffer (100 mM Tris HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.1% Bovine serum albumin), 3 µL $MgCl_2$ (50 mM) (Invitrogen SA, Merelbeke, Belgium), 1 µL deoxynucleotide triphosphates dNTP mix (10 mM)

(Fermentas GMBH, Germany), 2 µL primers (10 µM) (Invitrogen SA, Merelbeke, Belgium), 0.5 µL Taq DNA polymerase (5 U μL^{-1}) (Invitrogen SA, Merelbeke, Belgium) and 28.5 µL of sterile distilled water were added. Tubes were placed in a DNA Thermal Cycler 480 (Perkin Elmer Life and Analytical Sciences, Inc., Milano, Italy) and PCR was performed applying the following programme: initial denaturation for 5 min at 94°C followed by 30 cycles, each consisting of 90 s denaturation at 94°C, 90 s annealing at 55°C and 3 min extension at 72°C. At the end, a final extension step of 7 min at 72°C was included. The PCR products were cooled down to 4°C before purification, using QIAquick® PCR Purification Kit (QUIAGEN Inc., Hilden, Germany), according to the manufacturer's protocols.

The purified PCR products were processed for reverse and forward sequencing, using a CEQ DTCS Quick Start kit (Beckman Coulter, Inc., Fullerton, CA). Reaction products were run on CEQ 2000XL capillary automated sequencer (Beckman Coulter). Sequence similarities were obtained from GenBank using the BLAST® programme.

1.3.5. Assessment of interactions between main endophytes using dual cultures

Bioassays were performed using the fungal disk technique (Fuhrmann, 1994) to survey the interactions between endophytes. Pairs of fungal disks were placed 5 cm from each other on opposite sides of a Petri dish containing malt extract agar. Plates were incubated at room temperature and the radiuses of the centripetally spreading parts of the two colonies were measured every two days, starting seven days after incubation. The control dishes contained only a disk of each strain in the centre. Assays were stopped when mycelial control reached the edge of the plates. The

experiments were performed in triplicate. The ratios between mycelium growth radiuses on tested strains were calculated with the following formula:

$$\text{Growth reduction (\%)} = 100 - \frac{T \times 100}{C}$$

where, C: control growth radius

T: tested strains growth radius

1.4. Results

1.4.1. Diversity of fungal endophytes from leaves of *C. asiatica*

About 600 leaf fragments of 4 mm^2 were generated from five leaves collected at random and cut up completely. Mycelium emerged from 468 out of 600 segments, yielding an overall colonization rate of 78%. In most cases (465 leaf segments out of 468), a single fungal strain emerged from a leaf segment. In a decreasing order of frequency, the non-sporulating species NSS1 (IF 19.2%), *Colletotrichum* sp.1 (IF 13.2%), *Guignardia* sp. (IF 8.5%), *Glomerella* sp. (IF 7.7%), an unidentified ascomycete ASCO1 (IF 7.2%), the non-sporulating species NSS2 (IF 3.7%) and *Phialophora* sp. (IF 3.5%) represented the most frequently isolated species (Table 1). Other species occurring with an IF of less than 1% belonged to the genera *Phoma*, *Nodulisporium*, *Physalospora*, *Pestalotiopsis*, *Colletotrichum*, *Phomopsis*, *Curvularia* and *Cladosporium* (Table 1).

Overall, 475 isolates were recovered and were classified into forty-five different taxa according to morphological characters (Table 2). All isolates belonged to the Ascomycetes. Teleomorphs were produced in 151 isolates in culture and represented five distinct taxonomic groups belonging to five genera: *Glomerella* Spauld & H. Schrenk, *Guignardia* Viala & Ravaz, an unidentified ascomycete (ASCO1), *Leptosphaerulina* McAlpine and *Physalospora* Niessl.

Anamorphs only were encountered in 125 isolates, representing eleven different taxa in nine genera (six coelomycetous, five hyphomycetous): *Colletotrichum* Corda (three species), *Phomopsis* (Sacc.) Bubák, *Phoma* Sacc., *Pestalotiopsis* Steyaert, *Phialophora* Medlar, *Penicillium* Link,

Nodulisporum Preuss, *Curvularia* Boedijn and *Cladosporium* Link. 199 isolates remained sterile and were grouped into twenty-nine distinct morphologies (NSS1-29).

Table 1. Frequency of endophytic fungi isolates from *Centella asiatica* leaves.

Taxon	Isolation frequency (%)
Xylariaceae sp.1 (NSS1)	19.2
Colletotrichum higginsianum	13.2
Guignardia mangiferae	8.5
Glomerella cingulata	7.7
Unidentified ascomycete (ASCO1)	7.2
Non-sporulating species N°2 (NSS2)	3.7
Phialophora sp.	3.5
Leptosphaerulina sp.	1.3
Penicillium sp.	1.2
Other sterile mycelium	10.3
Rare isolates [a]	3.5

[a]Less than 1% of isolation frequency for each species: *Phoma* sp., *Nodulisporium* sp., *Physalospora* sp., *Pestalotiopsis* sp., *Colletotrichum* sp.2, *Colletotrichum* sp.3, *Phomopsis* sp., *Curvularia* sp. and *Cladosporium* sp.

Table 2. Number of isolates per leaf.

Taxon	Group*	Leaf 1	Leaf 2	Leaf 3	Leaf 4	Leaf 5	Total
Xylariaceae sp.1 (NSS1)[a]	NSS_1	27	24	8	32	24	115
Non-sporulating species N°2 (NSS2)	NSS_2	3	2	7	4	6	22
Other non-sporulating species	NSS_{3-29}	21	9	14	6	12	62
Colletotrichum higginsianum[1]	AC_1	10	28	11	12	18	79
Colletotrichum sp.2	AC_2	1	1	1	-	-	3
Colletotrichum sp.3	AC_3	1	-	-	-	-	1
Pestalotiopsis sp.	AC_4	1	-	1	-	1	3
Phomopsis sp.	AC_5	1	-	-	-	-	1
Glomerella cingulata[a]	T_1	15	2	12	10	7	46
Guignardia mangiferae[a]	T_2	4	9	18	8	12	51
Unidentified ascomycete (ASCO1)	T_3	2	4	20	5	12	43
Leptosphaerulina sp.	T_4	4	4	-	-	-	8
Physalospora sp.	T_5	-	2	1	-	-	3
Phialophora sp.	AH_1	4	5	6	3	3	21
Penicillium sp.	AH_2	-	2	-	3	2	7
Phoma sp.	AH_3	-	-	2	2	1	5
Nodulisporium sp.	AH_4	2	1	-	-	-	3
Curvularia sp.	AH_5	-	-	-	1	-	1
Cladosporium sp.	AH_6	-	-	-	-	1	1
Total of isolates		96	93	101	86	99	475
Total of leaf segments[b]		120	120	120	120	120	600

[*] Forty-five different morphologies grouped in 29 distinct non-sporulating species (NSS_m), 5 anamorph coelomycetous (AC_n), 5 teleomorphs (T_p) and 6 anamorph hyphomycetous (AH_x). Footnotes in each group specify each individual species.

[a]ITS sequence analysis of the representative isolate of the taxon was performed.

[b]Leaf segments: a square of 4 mm^2 piece of leaf.

1.4.2. Sequence analysis of major endophytes

ITS sequences of the four main endophytes (NSS1, *Colletotrichum* sp.1, *Glomerella* sp. and *Guignardia* sp.), submitted to EMBL-EBI, were compared with published sequences using BLAST searches at GenBank.

The search using the 579 bp fragment of NSS1 (accession number AM403716) demonstrated homology with members of the Xylariaceae. *Xylaria longipes* (GenBank accession number AF163038) appeared to be most similar. It differed by 46 bp from the sequence of NSS1 (91% similarity), followed by various other xylariaceous taxa of the genera *Xylaria* Hill ex Schrank, *Rosellinia* De Not., *Nemania* Gray and *Kretzschmaria* Fr.

Sequence similarities of 500 bp of the ITS regions of *Colletotrichum* sp.1 (accession number AM411029) revealed an identity score of 99% (498/500) with *Colletotrichum higginsianum* (GenBank accession number AB0422303) and with *Colletotrichum destructivum* (GenBank accession number AB0105959), the first taxa proposed by the BLAST search. Morphological identification of *Colletotrichum* sp.1 showed immersed, light brown mycelium on PDA. Setae were present. Appressoria were pale to medium brown and ovoid. Conidia formed in whitish masses were hyaline, aseptate, straight or slightly curved, guttulate, measuring 13–18 µm × 3–4 µm (measurements were performed on 50 conidia). These cultural characters closely resembled those of *C. higginsianum* according to Sutton (1992) and the species is therefore identified as such.

Complete ITS sequences of *Glomerella* sp. (accession number AM403718) and *Guignardia* sp. (accession number AM403717) showed an identity score of 100% to *Glomerella cingulata* (accession number AY423474) and *Guignardia mangiferae* (accession number AY277717).

1.4.3. Within leaf distribution patterns of major endophytes

The within-leaf distribution patterns of the most commonly isolated endophytes (IF > 7 %) are presented (Fig. 6). The distribution showed a disjointed pattern. NSS1 was mainly isolated from the distal part of leaf 1 (23 out of 27 isolated) while *G. cingulata* (11/15) and *C. higginsianum* (8/10) were mostly recorded nearer to the leaf base. In leaf 2, NSS1 (20/24) mostly occupied the right side, while *C. higginsianum* (25/28) occupied the left. In leaf 3, *G. mangiferae* (17/18) was found on the right side, while the unidentified ascomycete ASCO1 (18/20) was observed on the opposite side. In leaf 4, no particular pattern was noticed. In leaf 5, ASCO1 (8/12) was mostly scored on the left side, while *C. higginsianum* (12/18) and *G. mangiferae* (8/12) were located on the right side. These observations led us to check for interactive relationships between these major leaf endophytes of *C. asiatica* by using a fungal disk technique.

1.4.4. Dual cultures between main endophytes

Several tested fungi showed inhibitory effects on the growth of the others (Fig. 7). NSS1 and *C. higginsianum* repressed the growth of ASCO1 by 27.3% and 9.1%, respectively (Fig. 7A). NSS1 and *C. higginsianum* markedly reduced the growth of *G. mangiferae* by 33.3% and 70.8%, respectively (Fig. 7B). *Colletotrichum higginsianum* was clearly inhibited by NSS1 and ASCO1 by 35% and 31.2%, respectively (Fig. 7C). The growth of NSS1 was slowed down by ASCO1 and *G. mangiferae* by 17.5% (Fig. 7D). *G. mangiferae* slightly stimulated the growth of ASCO1 by 9%.

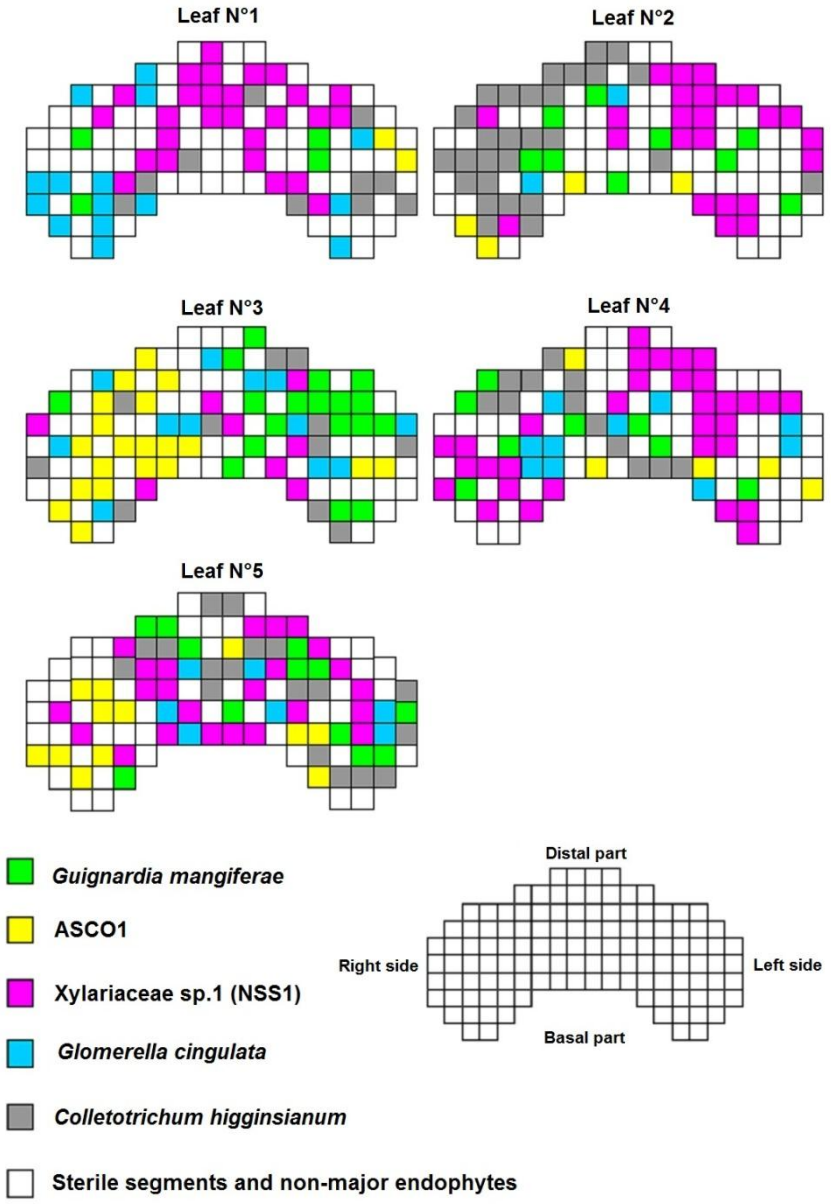


Fig. 6. Diagram showing the within-leaf distribution patterns of most commonly isolated endophytes of *Centella asiatica* leaves.

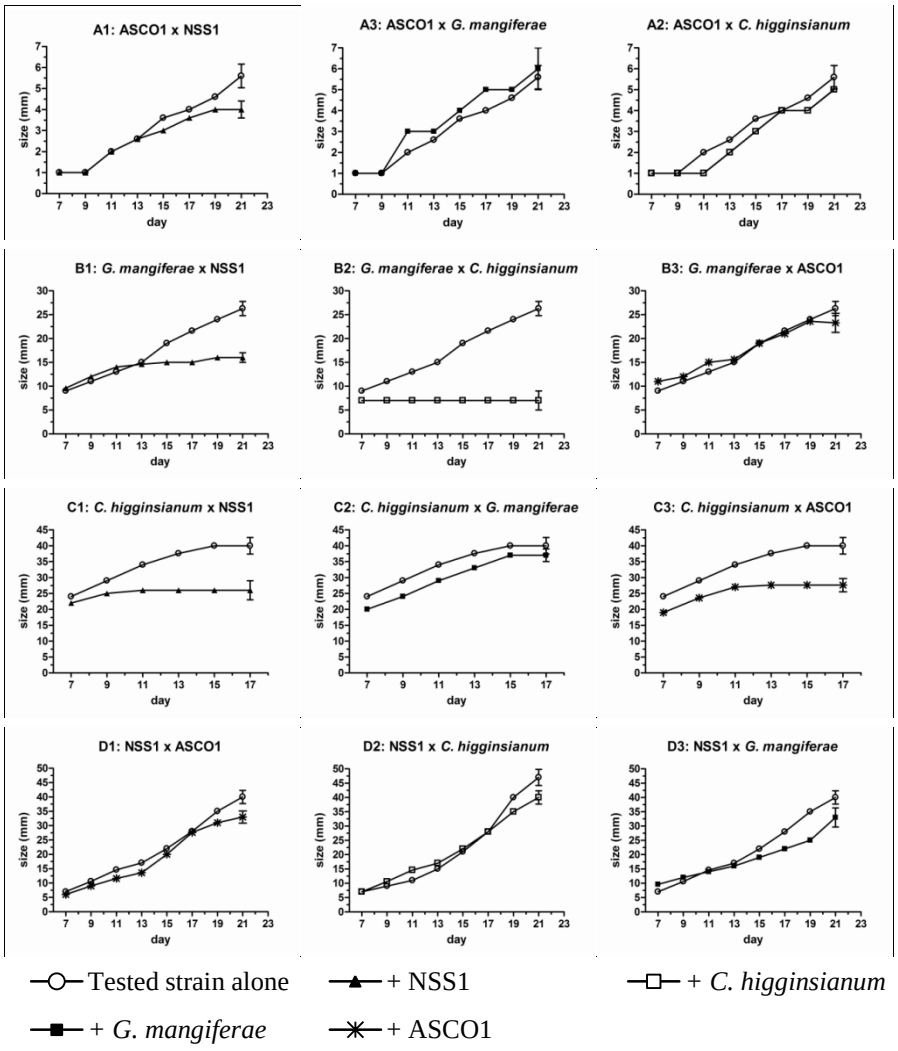


Fig. 7. Growth curves of major endophytes in the presence of other endophytes. ASCO1 in the presence of NSS1 (A1), *C. higginsianum* (A2) and *G. mangiferae* (A3). *G. mangiferae* in the presence of NSS1 (B1), *C. higginsianum* (B2) and ASCO1 (B3). *C. higginsianum* in the presence of NSS1 (C1), *G. mangiferae* (C2) and ASCO1 (C3). NSS1 in the presence of ASCO1 (D1), *C. higginsianum* (D2) and *G. mangiferae* (D3).

1.5. Discussion

We preferred the use of calcium hypochlorite (Rodrigues, 1994; Arnold *et al.*, 2001) as a surface disinfectant because it damages the tissue less than sodium hypochlorite (Boccon-Gibod, 1982). We showed that our surface sterilization procedure is sufficiently effective. Therefore, we conclude that all isolated fungi are endophytes.

The colonization rate of *C. asiatica* leaves by endophytes reached 78%. High colonization rates ranging from 81% to 89% were reported in palms in Brunei and Australia (Fröhlich *et al.*, 2000) and up to 95% to 98% in leaf fragments of *Guarea guidonia* (Meliaceae) in Puerto Rico (Gamboa & Bayman 2001). On the other hand, lower rates (29% to 67%) were recorded in other tropical hosts such as banana (Brown *et al.*, 1998) and 21% to 30% in the palm *Euterpe oleracea*, in Brazil (Rodrigues 1994). Our results were near the upper range. A possible explanation of the relatively high overall colonization rate noted in the present study could be due to the position of *C. asiatica* leaves close to the soil, facilitating the penetration and the colonization of endophytes. Moreover, samples were collected after rainy season, offering favorable spore dispersal.

All 45 distinct morphological taxa that we isolated were ascomycetes. About 58% produced either a teleomorph or an anamorph but 42% of isolates remained sterile. This confirms Petrini's findings (1986) that fungal endophytes mainly belong to the ascomycetes. Fifteen morphologically distinct taxa could be identified to genus according to microscopic examinations of any sporulation and reproductive structures including asci, ascospores, conidia, conidiophores and conidiomata. Twenty nine taxonomic groups remained sterile among which NSS1 was the most common taxon. In order to capture maximum diversity among non-sporulating isolates, minor

variations in culture were taken into account to distinguish near-identical strains and assign them to different taxonomic groups.

Molecular characterization of NSS1 showed that this species most likely belongs to the Xylariaceae. Although we did not observe any xylariaceous teleomorph in culture during this survey, endophytic community in *C. asiatica* was dominated by the xylariaceous non sporulating species NSS1. In addition, *Nodulisporium*, a Xylariaceae anamorph, was isolated from two leaves with less than 1% of isolation frequency. This is in agreement with earlier studies showing that xylariaceous fungi were the most commonly isolated endophytes in tropical regions but very seldom form teleomorph in culture (Rodrigues, 1994; Rodrigues & Petrini, 1997). Also, various proportions of mycelia sterilia have been reported from investigations of endophytes from different hosts and an attempt to identify them have shown that they belonged mainly to the Xylariaceae (Guo *et al.*, 2003).

The second most frequently isolated endophyte of *C. asiatica* was *C. higginsianum*. This species can cause an anthracnose leaf spot disease on a wide range of cruciferous plants (Higgins, 1917). It displays intracellular hemibiotrophic colonization strategies with a transient asymptomatic biotrophy followed by a rapid necrotrophic phase (Narusaka *et al.*, 2004). *Colletotrichum higginsianum* has never been described as an endophyte. It is possible that we have encountered this species during its symptomless, latent infection stage.

The endophytic community in *C. asiatica* was characterized by two most frequently isolated endophytes (the xylariaceous NSS1 and *C. higginsianum*) and a numerous species isolated with rare occurrence (Table 1). Typical distribution has also been reported from studies on endophytic mycobiota in plants (Petrini, 1991; Rodrigues & Petrini 1997, Fröhlich *et al.*, 2000). For

species with less than 1% of isolation frequency were cosmopolitan species such as *Phoma* sp., *Phomopsis* sp., *Pestalotiopsis* sp., *Curvularia* sp., *Physalospora* sp. and *Cladosporium* sp. and have been isolated as endophytes from a wide range of different host plants (Kumaresan & Suryanarayanan, 2001; Cannon & Simmons, 2002).

The observation that the main endophytes were found on opposite sides on *C. asiatica* leaves suggested antagonistic effects between those endophytic fungi. The results of our dual culture tests clearly indicate that the differential distribution patterns could be the result of competition or any other interactions between fungi. Similar patterns have been attributed to competitive interactions (Hata *et al.*, 2002). These observations suggest that some endophytic species in *C. asiatica* could synthesize compounds that inhibit the growth of the others and were responsible for the observed distribution patterns. Xylariaceous fungi are a rich source of diverse secondary metabolites (Whalley & Edwards, 1995). The competitive ability of xylariaceous NSS1 and specifically its strong inhibitory effect on other tested fungi may be attributed to production of inhibitory compounds. Xylariaceous NSS1 may be beneficial to the host for protection against pathogens and should be considered for further investigation as potential source of bioactive compounds.

1.6. Conclusion

Centella asiatica leaves harboured a high diversity of fungal species with forty-five different morphological taxa, of which the xylariaceous non-sporulating species NSS1 and *C. higginsianum* were the most frequently isolated species. Within leaf distribution of most frequently encountered endophytes showed an asymmetric pattern that may be ascribed to interaction or competition between fungi as shown by *in vitro* dual cultures.

Acknowledgements

This work is part of the research program at IMRA (*Institut Malgache de Recherches Appliquées*, Madagascar) on Biodiversity and Biotechnology. We wish to thank the Belgian *Commission Universitaire pour le Développement* (CUD) for contributing financially to this research. Erick Francisco Rakotoniriana is grateful to ADRI/UCL (*Administration de la Relation Internationale / Université catholique de Louvain, Belgique*) for the doctoral fellowship enabling him to continue this research. Cony Decock and Françoise Munaut gratefully acknowledge the financial backing received from the Belgian Federal Office for Scientific, Technical, and Cultural affairs (OSTC, contract BCCM/MUCL C2/10/007), and thank the directors of MUCL and MBLA for their constant encouragement and the provision of research facilities. We also thank Dr Geraldine Van der Auwera and Dr Aro Vonjy Ramarosandratana for critically reading manuscript.

CHAPTER II

STUDY *IN VITRO* OF THE IMPACT OF ENDOPHYTIC BACTERIA ISOLATED FROM *CENTELLA ASIATICA* ON THE DISEASE INCIDENCE CAUSED BY THE HEMIBIOTROPHIC FUNGUS *COLLETOTRICHUM HIGGINSIANUM*

Submitted to Antonie van Leeuwenhoek

Rakotoniriana EF, Rafamantanana M, Randriamampionona D, Rabemanantsoa C, Urveg-Ratsimamanga S, El Jaziri M, Munaut F, Corbisier AM, Quetin-Leclercq J, Declerck S

Preface

In chapter I, we observed that various species of *Colletotrichum* can occur endophytically inside *C. asiatica* leaves. This group of fungi is known to have diverse ecological roles as dormant saprobes (Promputtha *et al*., 2007), latent pathogens (Photita *et al*., 2004), and mutualists (Redman *et al*., 2001). According to Schulz (1999) the asymptomatic colonization of endophytes is the result of a balanced antagonism between host plant and endophytes, while Sinclair & Cerkauskas (1996) considered this symptomless stage of latent pathogens infection as "*a type of tolerance of the host to certain pathogens which find the conditions of the internal environment, usually during the early growth stages of the plant, unsuitable for completion of its life cycle*". Thus, the symptoms will appear when environmental or nutritional conditions change, or when the host tissues reach maturity or senescence or are damaged by any cause. Besides that, Freeman & Rodriguez (1993) demonstrated that a mutation at a single genetic locus can change the pathogenic *Colletotrichum magne* to a non-pathogenic *Colletotrichum* endophytic organism with no effect on host.

Anthracnose is a plant disease caused by species of *Colletotrichum*. It is responsible for considerable losses on economically important crops worldwide (Bailey & Jeger, 1992; Yang *et al*., 2009; Nguyen *et al*. 2010). Various parts of the plant can be affected by the pathogen, causing fruit rot, bloom blight, defoliation and crow root rot (Phoulivong, 2011). Typical leaf symptoms initially begin as tiny, irregular yellow, brown or black spots. Then the spots merged, expanded to the affected area and became necrotic (Sergeeva *et al*., 2008). *Colletotrichum higginsianum*, in particular, is a known plant pathogen affecting various cruciferous plants and the model plant *Arabidopsis thaliana* (Higgins, 1917; Narusaka *et al*., 2004). However, this fungus was isolated at relatively high frequency in healthy leaves of *C.*

asiatica grown in the wild (chapter I). Preliminary inoculation of *C. higginsianum* into endophytes-free leaves of *C. asiatica* grown *in vitro* resulted in apparition of typical anthracnose symptoms three days after inoculation (Personal observation). Based on this observation, we aimed to study in chapter II whether endophytic bacteria from *C. asiatica* may inhibit the growth of *C. higginsianum* and can delay or suppress the expression of its virulence. To verify this assumption, we first performed dual cultures to select the most antagonistic bacteria that inhibited the mycelium growth of *C. higginsianum*. Then *in vitro* plants of *C. asiatica* were used as culture systems to evaluate the impact of co-inoculation between the fungal pathogen and the two most active bacteria.

2.1. Abstract

Thirty-one endophytic bacteria isolated from healthy leaves of *Centella asiatica* were screened *in vitro* for their ability to reduce the growth rate and disease incidence of *Colletotrichum higginsianum*, a causal agent of anthracnose. Isolates of *Cohnella* sp., *Paenibacillus* sp. and *Pantoea* sp. significantly stimulated the growth rate of *C. higginsianum* MUCL 44942, while isolates of *Achromobacter* sp., *Acinetobacter* sp., *Microbacterium* sp., *Klebsiella* sp. and *Pseudomonas putida* had no influence on this plant pathogen. By contrast, *Bacillus subtilis* BCA31 and *Pseudomonas fluorescens* BCA08 caused a marked inhibition of *C. higginsianum* MUCL 44942 growth by 46 and 82%, respectively. Cell-free culture filtrates of *B. subtilis* BCA31 and *P. fluorescens* BCA08 were found to contain antifungal compounds against *C. higginsianum* MUCL 44942. Inoculation assays on *in vitro*-cultured plants of *C. asiatica* showed that foliar application of *B. subtilis* BCA31, three days before inoculation with *C. higginsianum* MUCL 44942, significantly reduced incidence and severity of the disease. The role of endophytic bacteria in maintaining the apparent inactivity of *C. higginsianum* MUCL 44942 in *C. asiatica* grown in the wild is discussed.

Keywords: antifungal activity, *Centella asiatica*, *Colletotrichum higginsianum*, endophytic bacteria, *in vitro* plants

2.2. Introduction

It is not uncommon, when studying endophytes to isolate known plant pathogenic fungi, which inhabit in symptomless manner the living internal tissue of their host plant for a short period or whole life cycle. These latent-infecting fungi meet the broad definition of microbial endophytes, as stated by Petrini (1991): "*All organisms inhabiting plant organs that at some time in their life can colonize internal plant tissues without causing apparent harm to the host*".

Species of *Colletotrichum* (teleomorph *Glomerella*) are causal agents of anthracnose that affect many economically important crops worldwide (Bailey & Jeger, 1992; Yang *et al*., 2009; Nguyen *et al*., 2010). However, various species were also repeatedly isolated as endophytes from a wide range of host plants (Bussaban *et al*., 2001; Lu *et al*., 2004; Photita *et al*., 2005; Rojas *et al*., 2010). This group of fungi is known to have diverse ecological roles as dormant saprobes (Promputtha *et al*., 2007), latent pathogens (Photita *et al*., 2004), and mutualists (Redman *et al*., 2001). Freeman & Rodriguez (1993) demonstrated that a mutation at a single genetic locus can change the pathogenic *Colletotrichum magne* to a non-pathogenic *Colletotrichum* endophyte with no effect on host. According to Sinclair & Cerkauskas (1996) the symptomless stage of latent pathogens infection is considered as a type of tolerance of the host to certain pathogens which find the micro environment conditions of the internal tissue of the plant inappropriate for their development. While Schulz *et al*. (1999) assumed that the asymptomatic colonization of endophytes is the result of a balanced antagonism between host plant, environment and endophytes. Thus, the symptoms will appear when environmental or nutritional conditions change, or when the host tissues reach maturity or senescence or are damaged by any cause.

Numerous studies have reported that some endophytic bacteria are able to inhibit the activity of plant pathogens and thereby can delay or suppress the appearance of disease symptoms. For example, an endophytic *Streptomyces* isolated from surface-sterilized banana roots significantly reduced the Fusarium wilt disease severity in banana plantlets grown in a greenhouse under natural lighting (Cao *et al*., 2005). Using *Catharanthus roseus*, Lacava *et al*. (2007) showed, under greenhouse conditions, that the endophyte *Curtobacterium flaccumfaciens* decreased citrus-variegated chlorosis symptoms, a disease of the sweet orange caused by *Xylella fastidiosa*. Chili seeds treated with *Pseudomonas fluorescens* endophytes displayed low incidence of pre- and post-emergence damping off caused by *Pythium aphanidermatum* (Muthukumar *et al*., 2010).

Centella asiatica (L.) Urb (Apiaceae) is an ethnomedicinal plant, native to India, Madagascar, Sri Lanka, China, Indonesia, Australia and South Africa. The major chemical constituents found in the plant are triterpenoids (Zheng & Qin, 2007). Previous studies on microbial endophytes from *C. asiatica* mainly focused on fungi. A new fungal species, named *Echinosphaeria macrospora*, teleomorph of *Vermiculariopsiella endophytica*, was discovered from the stems of *C. asiatica* (Puja *et al*., 2006). Krishnamurthy *et al*. (2008) reported that *Chaetomium globosum* was the most frequently isolated fungal species within healthy leaves of *C. asiatica* collected from the Malnad region (Southern India). Inoculation of the growth-promoting endophytic fungus *Piriformospora indica* to roots of *C. asiatica in vitro* cultures resulted in the rapid enhancement of root and shoot biomass of the host plant and an increase in asiaticoside production in leaves (Satheesan *et al.*, 2012). To the contrary, very little information is available on the endophytic bacterial community of *C. asiatica*. Recently, Rafat *et al*. (2012) showed that *Pantoea agglomerans* AR-PSBH2, an endophytic bacteria associated with *C. asiatica*, had high antioxidant potential.

In the course of a study on fungal endophytes from leaves of *C. asiatica*, we observed that leaves of this plant were host to the plant pathogen *Colletotrichum higginsianum* without showing any signs of disease. However, the fungus was isolated at an isolation frequency of 13% in the leaves (Rakotoniriana *et al*., 2008). In the present study, we demonstrated that some antagonistic endophytic bacteria from *C. asiatica* may play a major role in inhibiting or delaying the expression of the virulence of *C. higginsianum* in *C. asiatica* plants under *in vitro* culture conditions. We first isolated culturable endophytic bacteria colonizing *C. asiatica* leaves grown in the wild in Mangoro (Madagascar). Then we investigated their potential to inhibit the growth of *C. higginsianum* by means of dual culture method and finally, we evaluated the impact of the most antagonistic bacteria on *C. higginsianum* on *in vitro* produced plants of *C. asiatica*.

2.3. Materials and Methods

2.3.1. Isolation of endophytic bacteria

Ten healthy leaves of *C. asiatica* randomly collected at a site located at Mangoro (18°52′20″ South, 48°06′25″ East) in the eastern region of Madagascar were surface sterilized according to Rakotoniriana *et al.* (2008). The effectiveness of the disinfection process was checked by blotting the sterilized leaves tightly onto tryptic soy agar (TSA: 15 g casein peptone, 5 g soya peptone, 5 g sodium chloride, 15 g agar in 1 L distilled water; pH 7.3) medium and also by plating aliquots of the sterile distilled water used in the final rinse onto TSA. The test was validated if no colonies were apparent after incubating the Petri dishes at 25°C for 15 days (Kuklinsky-Sobral *et al*., 2004). Surface sterilized leaves were then triturated with 5 ml of 0.02 M-phosphate buffer at pH 7 using autoclaved mortars and pestles. One ml of the resulting suspensions was spread-plated on 4 Petri dishes containing TSA medium supplemented with 100 mg L^{-1} cycloheximide in order to inhibit the growth of endophytic fungi. The Petri dishes were incubated under the same condition as above. Bacterial colonies, morphologically distinguishable were picked after 72 h incubation at 25°C and subsequently transferred to the same TSA medium but without cycloheximide. Following successive subcultures, pure colonies were obtained and stored at -80°C in 20% glycerol solution prior to identification.

2.3.2 Identification of bacterial isolates

The bacteria recovered from the plant samples were grouped on the basis of phenotypic characteristics including colony colour, form, margin, elevation, opacity, surface and diameter as well as biochemical properties such as Gram coloration, catalase and oxydase reactions. Thirty-one

distinctive colonies were selected for further identification by sequence analysis of the 16S rRNA gene using primers Univ 1 (5'-ACTCCTACGGGAGGCAG-3') and Bact 4 (5'-GGCGTGTGTACAAGGCCCGG-3'). Bacterial suspensions were then prepared for each colony in 10 µL of sterile Ringer solution. PCR was made up of 1 µL of the bacterial suspension and 49 µL of the PCR mixture consisting of 5 µL of 10X high fidelity PCR buffer with $MgCl_2$, 2.5 µL of each primer, and 0.5 µL of deoxynucleoside triphosphate mix 20 mM, 0.5 µL of Taq polymerase and 38 µL of sterile distilled water. PCR program consisted of an initial denaturation step of 5 min at 98°C followed by 30 cycles of 1 min denaturation at 95°C, 1 min annealing at 50°C and 1 min extension at 72°C. At the end, a final extension step at 72°C for 10 min was included. The DNA amplification was visualized by electrophoresis on a 0.8% (w/v) agarose gel (Sambrook *et al*., 1989). The PCR products were purified from gel band using GFX PCR DNA and gel band purification kit (Amersham Biosciences) according to manufacturer's instruction. The DNA sequencing was performed with an ABI Prism 377 DNA sequencer (PE Applied Biosystems Inc., Foster City, Calif.) using the primers Univ 1 and Bact 4. The partial nucleotide sequences of the16S rRNA gene were subjected to BLAST analysis (www.ncbi.nlm.nih.gov/BLAST) for similarity searches. For a more robust identification, identities of closely related microorganisms from BLAST searches were retained when their nucleotide sequence accession numbers were proved to have a link to SILVA rRNA database, known for its quality checked rRNA sequence data (Pruesse *et al*., 2007). The nucleotide sequences generated in this study were deposited in the GenBank (Table 3).

2.3.3 Source of the hemibiotic phytopathogen *C. higginsianum*

The fungal strain *C. higginsianum* MUCL 44942 (GenBank accession number AM411029) used in this study was initially isolated from healthy leaves of *C. asiatica* grown in the wild in Mangoro, Madagascar in May 2003 as endophytes (Rakotoniriana *et al*., 2008). The strain was preserved on agar slants in sterile water and under oil in screw cap tubes or lyophilized at the MUCL (http://bccm.belspo.be/about/mucl.php, Belgium) prior to use.

2.3.4. *In vitro* propagation of *C. asiatica* and isolation of microbial endophytes from *in vitro*-produced *C. asiatica* plantlets

In vitro plants of *C. asiatica* were produced according to the procedure described by Randriamampionona *et al*. (2007). Briefly, *C. asiatica* apical buds were surface sterilized by immersion in 5% calcium hypochlorite solution for 5 min, followed by washing four times in sterile distilled water. The explants were cultured in 50 ml Falcon tubes containing 10 ml of half-strength MS medium (Murashige & Skoog, 1962) (Duchefa, The Netherlands) without vitamin and solidified with 0.2% of phytagel (Sigma Chemicals, USA). Cultures were grown in a growth room at 23±2°C under a 16/8 h light/dark photoperiod (70 µmol m^{-2} s^{-1}, Cool-White fluorescent lamp, Osram).

Isolation of microbial endophytes was also carried out from *in vitro* produced plants of *C. asiatica*. Leaves were directly crushed without undergoing the step of surface-sterilizing with 5 mL of 0.02 M-phosphate buffer at pH 7 using autoclaved mortars and pestles. One ml of the resulting suspensions was spread-plated on two Petri dishes containing TSA for

bacteria and potato dextrose agar (PDA: 4 g potato extract, 20 g glucose, 15 g agar and 1 L distilled water; pH 5.6) for fungi without selective agents. The Petri dishes were incubated under the same conditions as above.

2.3.5. Virulence of *C. higginsianum* MUCL 44942

The *in vitro* produced plants of *C. asiatica* were screened for the virulence of *C. higginsianum* by adding 40 µL of the spore suspension of *C. higginsianum* adjusted to 10^6 conidia per ml to the upper surface of leaves. Typical anthracnose symptoms appeared on inoculated leaves within 3 days following inoculation as irregular yellow or dark-brown spots. The spots extended to the whole leaves and darkened as they aged.

2.3.6. Dual cultures of endophytic bacteria and *C. higginsianum* MUCL 44942

The effect of the identified bacteria (see Table 3) on the growth of *C. higginsianum* was evaluated using dual cultures on Petri dishes (90 mm diam.) following Przybył & Żłobińska-Podejma (2000) with some modifications. A 5-mm-diam disc of the fungus isolated from a 7-day old culture grown on PDA was placed at a distance of 3 cm from the edge of the Petri dish containing one-tenth-strength TSA plus 0.2% of sucrose adjusted at pH 6. Five µL of a bacterial suspension containing 10^8 CFU mL^{-1} from a 24-hour-old bacteria culture grown on TSA was spotted at a distance of 3 cm from the disc. In the control Petri dishes, sterile distilled water was spotted instead of the bacterial suspension. Following an incubation period at 25°C in the dark, the test was stopped when the mycelium of the fungus in the control dishes reached the point where distilled water was placed. Each test was carried out in six replicates. Experiments were repeated three times. The growth rate of the fungus was expressed as the colony radial growth of dual

or separated cultures during the test. The radial growth inhibition of *C. higginsianum* was calculated using the following formula:

$$\text{Radial growth inhibition (\%)} = \frac{(\text{Rc} - \text{Ri}) \times 100}{\text{Rc}}$$

where, Rc = Radial growth in the control Petri dishes

Ri = Radial growth in the bacterial-inoculated Petri dishes

2.3.7. Determination of the presence of antifungal compounds in the cell-free culture filtrates of the most potential antagonistic bacteria

Two bacteria (BCA08 and BCA31 – see Table 3) showing the most pronounced inhibition effect on the growth of *C. higginsianum* MUCL 44942 were selected. Five colonies from a 2-day-old culture grown on TSA at 25°C were used to inoculate a 50 mL malt extract broth (MEB: 17 g malt extract and 1 L distilled water; pH 4.8) and incubated on a rotary shaker for 48 h at 25°C. Bacterial suspension was then adjusted to 10^8 CFU mL^{-1} and added to 450 mL of MEB. Cultures were grown for 7 days at 25°C on a rotary shaker at 105 rpm. At the end of the incubation period, liquid cultures were centrifuged at 12,000 g for 20 min in centrifuge type K70 (Engels orf, Germany). The supernatant was filter-sterilized through a 0.22 µm Millipore (Bedford, MA) and incorporated into molten malt extract agar (MEA: 30 g malt extract, 3 g peptone from soymeal, 15 g agar and 1 L distilled water; pH 5.6) at 45°C to give concentrations of 20, 40, 60 and 80% in 20 ml final volume of culture media per Petri dishes. For example for BCA08 on MA20%, 4 mL of cell-free culture filtrates of strain BCA08 were added to 16 mL molten MEA to give a final concentration of 20%. In the control Petri dishes, sterile distilled water was added to MEA to give the above-mentioned concentrations. A 5-mm-diam disc of *C. higginsianum* MUCL 44942 from a 7-day old culture grown on PDA was placed at the centre of

the 9-cm Petri dishes. The diameter of the mycelium was measured after 7 days following incubation at 25°C in the dark. Each test was carried out in four replicates. The experiment was repeated three times.

2.3.8. Inoculation of *in vitro* plants of *C. asiatica* with putative antagonistic bacteria and the hemibiotroph *C. higginsianum* MUCL 44942

The two most active bacteria BCA08 and BCA31 against *C. higginsianum* MUCL 44942 (see Table 3) were cultivated respectively in liquid King B medium and nutrient broth (Oxoid) on a rotary shaker at 150 rpm overnight. Using a densicheck (Vitek®2 Densicheck, Biomerieux), the cultures were diluted to 10^7 CFU mL^{-1} in sterile distilled water added with 0.001% wetting agent Tween 20 (v/v) (Mahadtanapuk *et al*., 2007). To promote entry of bacterial cells through stomata, the *in vitro* produced plants of *C. asiatica* were placed in plastic bags containing sterile water and closed for 45 min to maintain high humidity and thereby enhance stomatal opening (Kangatharalingam *et al*., 2003). The plants were removed from the bag and using a micropipette, 40 µL of the endophytic bacterial suspensions (10^7 CFU mL^{-1}) was applied on the entire surface of the adaxial face of each leaves. A set of plants inoculated with a mixture of suspensions of BCA08 and BCA31 (1:1; vol:vol) was also included. Inoculated *in vitro* plants were returned to the growth room. Control plants were treated with sterile distilled water following the same procedure.

Conidial induction from *C. higginsianum* MUCL 44942 was obtained on PDA from 10-day-old culture incubated at 25°C in the dark. Fungal spore suspension was adjusted to 10^6 conidia per mL using a haemacytometer. Three days after bacterial inoculation, 40 µL of the fungal suspension was applied to the upper surface of each leaf. The disease severity was

subsequently recorded using a rating scale from 1 to 5, according to the percent of leaf area with lesions (1 = 0%; 2 = 1–25%; 3 = 26–50%; 4 = 51–75%; and 5 ≥ 75% of leaf area with lesions) (Shiomi *et al*., 2006). The disease severity index (DSI) of each treatment was obtained from the mean disease severity of the whole leaves within each treatment (Douville & Boland, 1992). The disease incidence was evaluated 10 days after inoculation with the fungal suspension and was expressed in terms of percentage of leaves with anthracnose symptoms compared to the total number of leaves. The experiment was performed twice and each experiment contained eight sets of ten eight week-old micropropagated plants providing three to four leaves per tube (set CA: *C. asiatica* inoculated with sterile distilled water as negative control; set BCA08: *C. asiatica* inoculated with BCA08 alone; set BCA31: *C. asiatica* inoculated with BCA31 alone; set BCA08-31: *C. asiatica* inoculated with the mixture of BCA08 and BCA31 (v/v); set COL: *C. asiatica* inoculated with *C. higginsianum* as positive control*;* set BCA08COL: *C. asiatica* inoculated with BCA08 and *C. higginsianum*; set BCA31COL: *C. asiatica* inoculated with BCA31 and *C. higginsianum*; and set BCA08-31COL: *C. asiatica* inoculated with BCA08, BCA31 and *C. higginsianum*).

2.3.9. Statistical analysis

Descriptive statistics of variables measured were presented as mean values ± standard deviation. Homogeneity of variances was first assessed by Levene's test. Mean values were compared by Tukey test for independent samples assuming unequal variances. The effects of the media concentration and the culture filtrate on the growth of *C. higginsianum* MUCL 44942 were analyzed using one-way ANOVA at 5% level of significance. The software used was STATISTICA version 10.

2.4. Results

2.4.1. Isolation and identification of endophytic bacteria

No microbial growth was noticed after two-week incubation on TSA either when the final set of washes used to rinse the tissues was plated onto the medium or when the ten sterilized leaves were blotted on the agar surface. This indicated the effectiveness of the surface-disinfection procedure used.

A total of 31 bacterial colonies were selected from surface-disinfected crushed *C. asiatica* leaves based on phenotypic characteristics of colonies and sequence analysis. These bacteria could be classified into eleven different genera of which six were affiliated with the phylum *Proteobacteria* (four *gammaproteobacteria*: *Acinetobacter* sp., *Klebsiella* sp., *Pantoea* sp. and *Pseudomonas* spp., and two *betaproteobacteria*: *Achromobacter* sp. and *Delftia* sp.), four were assigned to the phylum *Firmicutes* (*Bacillus* spp., *Cohnella* sp. *Lysinibacillus* sp. and *Paenibacillus* sp.) and one belonged to the phylum *Actinobacteria* (*Microbacterium* sp.).

The 16S rRNA gene sequences of most isolates displayed 99% to 100% identity with sequences deposited in the NCBI data bank, except for isolate BCA29 which showed 97% similarity with *Cohnella luojiensis* HY-22R^T (GenBank accession number GQ214052) (Table 3). But *C. luojiensis* HY-22R^T differed by 30 bp from the sequence of BCA29, suggesting that the isolate BCA29 was likely an undescribed species within the genus *Cohnella*. It was not possible to determine accurately to species level the remaining isolates since we only performed preliminary biochemical tests and sequenced only 16S rRNA gene.

The search for microbial endophytes from *in vitro* produced plants of *C. asiatica* revealed that no fungal endophytes grew on PDA even after 6 week incubation period whereas a single bacterial flora of *Microbacterium* sp. occurred on TSA.

2.4.2. Antagonistic effects of endophytic bacteria against *C. higginsianum* MUCL 44942 in dual cultures on Petri dishes

Bioassays were carried out using dual cultures to assess the potential effects of the isolated endophytic bacteria on the growth rate of *C. higginsianum* MUCL 44942. Various responses were recorded ranging from absence of effects to growth stimulation and inhibition (Table 3). Out of the 31 endophytic bacteria isolated from *C. asiatica* leaves, eleven isolates of *Acinetobacter* sp., *Achromobacter* sp., *Klebsiella* sp., *Microbacterium* sp. and *Pseudomonas putida* had no effect on the growth rate of *C. higginsianum*, while five isolates of *Cohnella* sp., *Pantoea* sp. and *Paenibacillus* sp. slightly stimulated (up to 5–6%) the growth of the pathogen. *Pseudomonas fluorescens* BCA08 displayed the highest inhibitory effect (82%) on the mycelial growth of *C. higginsianum* MUCL 44942. All *Bacillus* isolates found in our study were effective in inhibiting the growth of *C. higginsianum* MUCL 44942. In a decreasing order of activity, *B. cereus* (BCA02) was found highly active against the pathogen with 56% of fungal growth inhibition, followed by isolates of *B. subtilis* (26 to 46% of fungal growth inhibition), and *B. pumilus* (12 to 20% of fungal growth inhibition). However, the antagonistic activity of *B. cereus* BCA02 was not obvious since the bacteria spread toward the mycelium forming a barrier effect.

Table 3. List of endophytic bacteria isolated from *C. asiatica* and their effects on the growth of *C. higginsianum* MUCL 44942 in dual cultures on Petri dishes.

Isolate	Accession no	NCBI closest match (strain/GenBank accession no)[b]	% identity (nucleotide comparisons)	% growth of *C. higginsianum* MUCL 44942[a]
BCA08	HE716877	*Pseudomonas fluorescens* (LMG 5167^T/GU198104)	100 (1233/1233)	-82.06*
BCA02	HE716871	*Bacillus cereus* (Hs2-17/JF899264)	99 (1241/1242)	-56.86*
BCA20	HE716889	*Pseudomonas fluorescens* (LMG 5167^T/GU198104)	100 (1233/1233)	-56.30*
BCA31	HE716900	*Bacillus subtilis* (DSM10^T/AJ276351)	100 (1242/1242)	-42.80*
BCA26	HE716895	*Bacillus subtilis* (DSM10^T/AJ276351)	100 (1242/1242)	-26.86*
BCA14	HE716883	*Bacillus subtilis* (DSM10^T/AJ276351)	100 (1242/1242)	-26.66*
BCA30	HE716899	*Bacillus pumilus* (ATCC 7061^T/GQ911554)	99 (1240/1242)	-20.00*
BCA25	HE716894	*Bacillus pumilus* (ATCC 7061^T/GQ911554)	99 (1239/1242)	-16.86*
BCA04	HE716873	*Bacillus pumilus* (ATCC 7061^T/GQ911554)	99 (1239/1242)	-14.46*
BCA01	HE716870	*Bacillus pumilus* (ATCC 7061^T/GQ911554)	99 (1240/1242)	-13.73*
BCA19	HE716888	*Delftia* sp. (P-144/AM412119)	100 (1324/1324)	-12.96*
BCA06	HE716875	*Lysinibacillus sphaericus* (13651U/EU741100)	100 (1243/1243)	-12.96*

(Table 3 continued)

Isolate	Accession no	NCBI closest match (strain/GenBank accession no)[b]	% identity (nucleotide comparisons)	% growth of *C. higginsianum* MUCL 44942[a]
BCA16	HE716885	*Bacillus pumilus* (ATCC 7061^T/ GQ911554)	100 (1241/1241)	-12.80*
BCA03	HE716872	*Bacillus pumilus* (ATCC 7061^T/ GQ911554)	100 (1241/1241)	-12.43*
BCA05	HE716874	*Lysinibacillus sphaericus* (13651U/ EU741100)	100 (1243/1243)	-11.86*
BCA27	HE716896	*Achromobacter* sp. (AO22/EU696789)	99 (1225/1227)	-2.43
BCA21	HE716890	*Pseudomonas putida* (M5TSA/GQ504714)	100 (1233/1233)	-1.30
BCA11	HE716880	*Pseudomonas putida* (M5TSA/GQ504714)	100 (1233/1233)	-0.93
BCA07	HE716876	*Acinetobacter* sp. (2EBS15/ FJ984619)	100 (1234/1234)	-0.76
BCA09	HE716878	*Microbacterium* sp. (WPCB136/FJ006901)	100 (1233/1233)	-0.56
BCA17	HE716886	*Pseudomonas putida* (M5TSA/GQ504714)	100 (1233/1233)	-0.56
BCA24	HE716893	*Pseudomonas putida* (WH3/FJ262368)	100 (1235/1235)	-0.56
BCA10	HE716879	*Microbacterium* sp. (YT0620/AB376082)	99 (1230/1232)	-0.20
BCA15	HE716884	*Acinetobacter* sp. (2EBS15/FJ984619)	100 (1234/1234)	+0.16
BCA18	HE716887	*Klebsiella* sp. (PB31/EU360123)	99 (1227/1232)	+0.36

(Table 3 continued)

Isolate	Accession no	NCBI closest match (strain/GenBank accession no)[b]	% identity (nucleotide comparisons)	% growth of *C. higginsianum* MUCL 44942[a]
BCA28	HE716897	*Klebsiella* sp. (PB31/EU360123)	99 (1227/1232)	+0.53
BCA13	HE716882	*Paenibacillus chibensis* (JCM 9905^T/AB073194)	99 (1232/1242)	+5.36*
BCA12	HE716881	*Pantoea* sp. (y2/GQ395336)	99 (1228/1232)	+5.73*
BCA23	HE716892	*Pantoea* sp. (y2/GQ395336)	99 (1228/1232)	+5.90*
BCA22	HE716891	*Pantoea* sp. (y2/GQ395336)	99 (1228/1232)	+6.16*
BCA29	HE716898	*Cohnella luojiensis* (HY-22R^T/GQ214052)	97 (1105/1135)	+6.83*
COL	AM411029	*Colletotrichum higginsianum*		

[a] + growth stimulation; - growth inhibition

[b] All accession numbers retained in the study have a link to SILVA database (Pruesse *et al*., 2007) except for JF899264 (*Bacillus cereus*)

For each bacteria, data with * indicate that growth inhibition or stimulation differed significantly to the control standard value (COL= 30 mm) at $p < 0.05$ (Tukey)

2.4.3. Antifungal activity of cell-free culture filtrates of the most potentially antagonistic bacteria

The capacity to produce antifungal compounds *in vitro* was evaluated with two isolates, *P. fluorescens* BCA08 and *B. subtilis* BCA31, which showed the highest antagonistic activity against *C. higginsianum* MUCL 44942 in the dual culture tests. *P. fluorescens* BCA08 and *B. subtilis* BCA31 were found to produce diffusible antifungal compounds in the media as indicated by the drastic inhibition of the mycelium growth of *C. higginsianum* MUCL 44942 (Fig. 8). The filtrates of both bacteria were highly inhibitory to the growth of *C. higginsianum* MUCL 44942, even at the lowest concentration with a growth inhibition of 67% and 90% for BCA08 and BCA31 respectively. At higher concentrations of BCA31 culture filtrate, no growth of the fungus was recorded after an incubation period of 7 days.

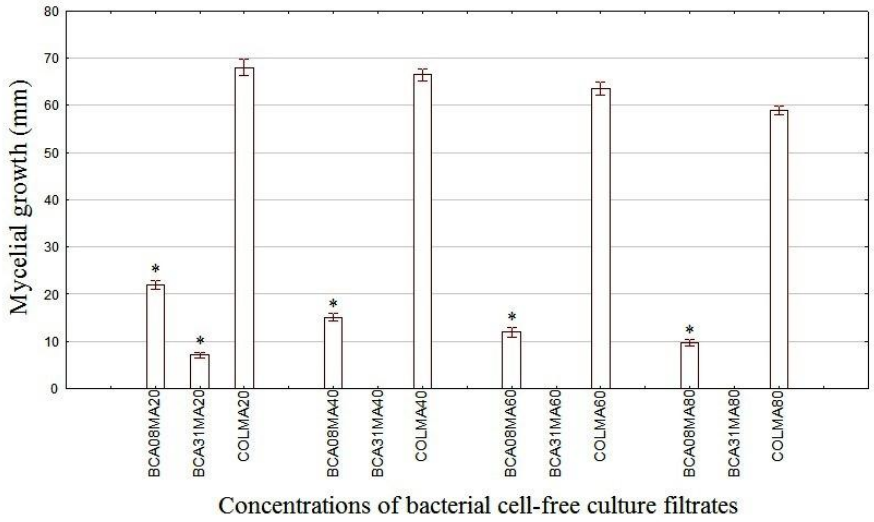


Fig. 8. Effects of different concentrations of *P. fluorescens* BCA08 and *B. subtilis* BCA31 culture filtrates on the mycelial growth of *C. higginsianum* MUCL 44942 after 7 days. A 5-mm-diam disc of *C. higginsianum* MUCL 44942 was placed at the centre of the 9-cm Petri dishes containing BCA08MA20, BCA08MA40, BCA08MA60 and BCA08MA80: malt extract agar supplemented with *P. fluorescens* BCA08 cell-free culture filtrate to give a final concentration of 20, 40, 60, and 80% respectively; BCA31MA20, BCA31MA40, BCA31MA60 and BCA31MA80: malt extract agar supplemented with *B. subtilis* BCA31 cell-free culture filtrate to give a final concentration of 20, 40, 60, and 80% respectively; COLMA20, COLMA40, COLMA60 and COLMA80: control malt extract agar supplemented with sterile distilled water instead of bacterial culture filtrates to give a final concentration of 20, 40, 60 and 80%. The diameter of the mycelium was measured after 7 days incubation at 25°C in the dark. *Significant difference at $P \leq 0.01$ by Student's t test compared to control. Bar charts represent means ± standard deviation.

2.4.4. Antagonistic effects of endophytic bacteria against *C. higginsianum* MUCL 44942 in *in vitro*-produced *C. asiatica* plantlets

The results of the inoculation assays showed that the applications of *P. fluorescens* BCA08 and *B. subtilis* BCA31 on leaves of *in vitro* produced plants of *C. asiatica* significantly reduced the anthracnose disease severity caused by *C. higginsianum* MUCL 44942, with a DSI varying from 4.2 in the *C. higginsianum* treatment to 3.4 and 1.4 respectively in the bacterial treatments, that accounted for a control level of 19% for BCA08 and 66% for BCA31 (Table 4; Fig. 9). *Bacillus subtilis* BCA31 was also the most effective in reducing anthracnose development in leaves with a disease incidence rate of 32.2%. Even though, *P. fluorescens* BCA08 showed the most prominent activity against *C. higginsianum* MUCL 44942 in dual cultures assays (Growth inhibition up to 82%), its performance in the inoculation assays on *in vitro* produced plants of *C. asiatica* was less pronounced with a disease incidence rate of 90.3%. No significant reduction was observed as regards to disease severity or incidence when BCA31 was tested singly or concurrently with BCA08 against *C. higginsianum* MUCL 44942 on *in vitro* plants of *C. asiatica*, suggesting an absence of additive action between the two bacteria against *C. higginsianum* MUCL 44942.

Table 4. Number of leaves showing anthracnose symptoms relative to disease score after co-inoculation with endophytic bacteria and *C. higginsianum* MUCL 44942 on *in vitro* plants of *C. asiatica*.

	Score (% of leaf areas with lesions)	CA (n=64)	BCA (n=64)	COL (n=64)	BCA08COL (n=62)	BCA31COL (n=62)	BCA08-31COL (n=60)
Disease severity	1 (0%)	64	64	0	6	42	36
	2 (1-25%)	0	0	4	4	16	16
	3 (26-50%)	0	0	14	22	4	8
	4 (51-75%)	0	0	12	18	0	0
	5 (≥75%)	0	0	34	12	0	0
Disease severity index (DSI)		1^{a}	1^{a}	4.2 ± 1^{b}	3.4 ± 1.1^{c}	1.4 ± 0.6^{d}	1.5 ± 0.7^{d}
Disease incidence (%)				100^{b}	90.3^{c}	32.2^{d}	36.3^{d}

CA: *C. asiatica* inoculated with sterile distilled water as negative control; BCA: *C. asiatica* inoculated with either BCA08 or BCA31; COL: *C. asiatica* inoculated with *C. higginsianum* MUCL 44942 as positive control*;* BCA08COL: *C. asiatica* inoculated with BCA08 and *C. higginsianum* MUCL 44942; BCA31COL: *C. asiatica* inoculated with BCA31 and *C. higginsianum* MUCL 44942; BCA08-31COL: *C. asiatica* inoculated with BCA08, BCA31 and *C. higginsianum* MUCL 44942. Means followed by the same letters are not significant at $p<0.05$ (Tukey test)

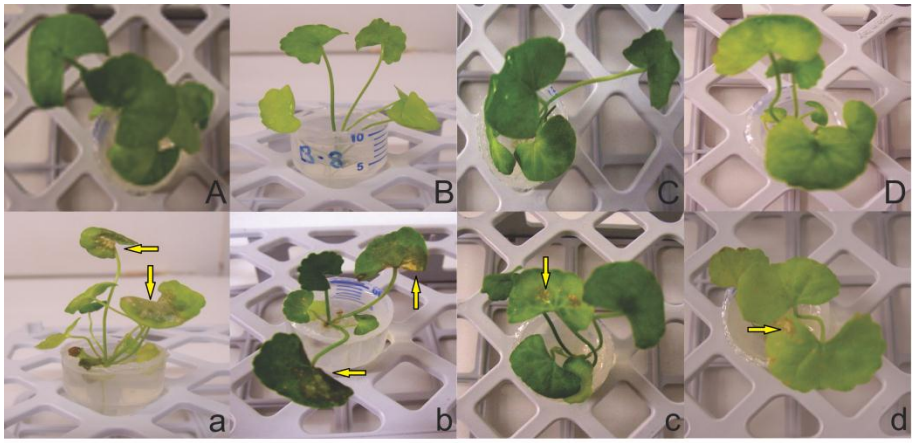


Fig. 9. *In vitro* inoculation assays. A: *C. asiatica* inoculated with sterile distilled water; a: *C. asiatica* inoculated with *C. higginsianum* MUCL 44942; B: *C. asiatica* inoculated with bacteria BCA08; b: *C. asiatica* inoculated with bacteria BCA08 and *C. higginsianum* MUCL 44942; C: *C. asiatica* inoculated with BCA31; c: *C. asiatica* inoculated with BCA31 and *C. higginsianum* MUCL 44942; D: *C. asiatica* inoculated with bacteria BCA08 and BCA31; d: *C. asiatica* inoculated with bacteria BCA08, BCA31 and *C. higginsianum* MUCL 44942. Anthracnose symptoms are indicated with arrows.

2.5. Discussion

Most of the bacteria that composed *C. asiatica* endophytic flora are frequently encountered in the rhizosphere. This may be explained by the fact that *C. asiatica* is an herbaceous creeper plant that grows abundantly in moist areas; accordingly the main source of bacterial inoculum for plant colonization is likely of rhizosphere-origin. Besides, endophytic bacteria from *C. asiatica* leaves were ubiquitous, being isolated from several plants such as soybean (Kuklinsky-Sobral *et al*., 2004), coffee (Shiomi *et al*., 2006), maize (Rai *et al*., 2007), poplar (Ulrich *et al*., 2008), rice (Mano & Morisaki, 2008), *Eucalyptus* spp. (Procópio *et al*., 2009), sugarcane (Magnani *et al*., 2010) and cacao (Melnick *et al*., 2011). However, the whole endophytic bacterial community of the plant may be underestimated by the isolation procedure adopted in the present study, since only bacteria that could grow on the medium used under cultural conditions were isolated. In addition, fast-growing bacteria may conceal slow growing ones and selection of colonies according to phenotypic properties may overlook near-similar morphological bacteria that were taxonomically different. In this study emphasis was given on the rapid screening and qualitative analysis of culturable bacteria from *C. asiatica*. Indeed, the combination of isolation method from plating plant segments on culture media to fingerprinting techniques and analysis of bacterial clone library can supply further information for exploring the whole community composition of endophytic bacteria (Reiter & Sessitsch, 2006; Ulrich *et al*., 2008).

Centella asiatica was found to host endophytic bacteria with various effects on the growth rate of the plant pathogen *C. higginsianum*. Isolates of *Pantoea* (BCA12, BCA22 and BCA23), *Paenibacillus* (BCA13) and *Cohnella* (BCA29) stimulated the growth rate of *C. higginsianum* MUCL 44942 whereas all species of *Bacillus*, *Lysinibacillus sphaericus* (BCA05

and BCA06), *Delftia* (BCA19) and *Pseudomonas fluorescens* (BCA08 and BCA20) showed antagonistic activity against *C. higginsianum* MUCL 44942. Similar results were reported by Lacava *et al*. (2004) on citrus plants. These authors showed that the growth rate of *Xylella fastidiosa*, the causal agent of citrus-variegated chlorosis was positively affected by *Methylobacterium extorquens* and inhibited by *Curtobacterium flaccumfaciens*, both were citrus endophytic bacteria. Besides, it appears that isolates of *Acinetobacter* sp., *Achromobacter* sp., *Klebsiella* sp., *Microbacterium* sp. and *Pseudomonas putida* had no effect on the growth rate of *C. higginsianum* (Table 3). These results contrasted with those obtained by some researchers on other *Colletotrichum* species. For instance, Sarode *et al*. (2009) demonstrated that the supernatant of *Acinetobacter* SCW1 from healthy wheat plant inhibited the growth of *Colletotrichum capsicum* by 35%. Under greenhouse experiments, *P. putida* NH-50 was shown to inhibit *in vitro* the mycelium growth of *Colletotrichum falcatum* by 65% (Hassan *et al*., 2010). A possible explanation of the discrepancy found between our findings and those of these studies could be due to the fact that the identification of the strains was restricted to the genus and that the isolates may belong to different species. Moreover, a high degree of intraspecific variations was often reported in the genus *Acinetobacter* (Ecker *et al*., 2006; Ko *et al*., 2007) and *Pseudomonas* (Raaijmakers & Weller, 2001; Costa *et al*., 2006).

It appeared that the susceptibility of *C. higginsianum* MUCL 44942 varied widely among isolates of the same species. This was observed for instance between BCA08 and BCA20, both identified as *P. fluorescens* with 82% and 56% of fungal growth inhibition respectively. The different activity was also noted between isolates of *B. subtilis*, BCA31 and BCA26 with 42% and 26% of fungal growth inhibition respectively. These observations led to the hypothesis that some isolates may be different strains of the same species

with differential antimicrobial activity, ecology, epidemiology, and pathogenicity. This finding is in agreement with those of Srivastava & Shalini (2008), who tested *in vitro* five isolates of *P. fluorescens* exhibiting varying levels of antifungal activity against some plant pathogens such as *Alternaria cajani, Curvularia lunata, Fusarium* sp., *Bipolaris* sp., and *Helminthosporium* sp. In addition, Lin *et al*. (2011) demonstrated that the observed different antifungal activity within *B. subtilis* isolates was linked to the production of both iturin A and surfactin by antagonistic strains whereas isolates that showed no inhibitory activity against the tested fungal pathogens, produced surfactin alone.

The hemibiotrophic *C. higginsianum* caused typical anthracnose leaf spots on a wide range of cruciferous plants and on the model plant *Arabidopsis thaliana* (Higgins, 1917; Narusaka *et al*., 2004) but *C. asiatica* leaves collected in Mangoro, Madagascar seemed to show a type of tolerance to this pathogenic fungus. Infection of *C. asiatica* by *C. higginsianum* MUCL 44942 did not result in visible damage or disease symptoms to the host in the wild, even though the infection rate by *C. higginsianum* MUCL 44942 was 13% (Rakotoniriana *et al*., 2008). In the present study we used *in vitro* produced plants of *C. asiatica* to assess the implication of some antagonistic bacteria in the observed reduced virulence of *C. higginsianum* MUCL 44942. Inoculation of *C. higginsianum* MUCL 44942 onto *in vitro C. asiatica* plantlets produced tiny, irregular yellow, brown or black spots on leaves after 3 days inoculation. Then the spots merged, expanded to the affected area and became necrotic. The infection process was in line with that described by Narusaka *et al*. (2004), who showed that *C. higginsianum* infection was initially characterized by a transient asymptomatic biotrophy followed by a rapid destructive necrotrophic phase. It appeared that the absence of beneficial endophytic bacteria in the *in vitro* produced plants of *C. asiatica* made them highly susceptible to the anthracnose disease caused

by *C. higginsianum* MUCL 44942. In the control *C. asiatica* plants, 100% of the leaves had anthracnose symptoms and 72% of them had a disease severity score above 4, i.e., more than 50% of the leaf area covered with lesions. To the contrary, the pathogenicity of *C. higginsianum* MUCL 44942 decreased when *B. subtilis* BCA31 was applied onto leaves of *in vitro C. asiatica* plantlets 3 days before inoculation with the pathogen. None of the tested *in vitro* plants challenged with *B. subtilis* BCA31 alone or in combination with *P. fluorescens* BCA08 had a disease score above 4. These observations suggest that strain BCA31 conferred enhanced resistance to the *in vitro* plants of *C. asiatica* against *C. higginsianum*. At least, the likely explanation would be the production of allelochemicals toxic to *C. higginsianum* MUCL 44942 and the induction of plant defense mechanisms.

The effectiveness of other *B. subtilis* strains in controlling anthracnose diseases caused by *Colletotrichum* spp. has also been successfully demonstrated in various plants. For example, Fu *et al*. (2010) showed that *B. subtilis* strain B106 was effective in controlling post-harvest anthracnose diseases of banana caused by *C. musae* with a level control of 48.6%. The combination of *B. subtilis* strain LB5 and fruit bagging significantly reduced anthracnose diseases caused by *C. gloeosporioides* on mango fruits grown in Taiwan by 56.4% (Senghor *et al*., 2007). A *B. subtilis* strain BD0310 isolated from tea leaves effectively controlled tea foliar anthracnose caused by *C. theae-sinensis* with a control level of 77.3% in glasshouse conditions (Kim *et al*., 2009). With regard to *B. subtilis* strain BCA31 our results were consistent with these previous findings. We observed a level of control of 66% against *C. higginsianum* MUCL 44942 on *in vitro* plants of *C. asiatica*.

On the other hand, *P. fluorescens* strain BCA08 which displayed the strongest antagonistic effect in the dual culture assays (82% of fungal growth inhibition), induced only moderate disease suppression (a control level of

19%) in the *in vitro* inoculation assays of *C. asiatica* plantlets. This clearly indicated that the disease suppression may be due to other factors than direct antagonism of fungal growth. Interestingly, Chen *et al*. (1994) suggested that the biocontrol effect of bacterial strains within plant tissues mainly resulted from enhanced host defense rather than from bacterial metabolites. This suggestion was later supported by ample evidence that endophytic *Pseudomonas* and *Bacillus* species functioned as elicitors of induced systemic resistance in plants (Kloepper & Ryu, 2006). Thus, it would be worth studying the impact of some endophytic bacteria found in this study on the defense mechanisms of *C. asiatica* against *C. higginsianum*, notably on the salicilic acid- (SA) and ethylene/jasmonate (ET/JA)-dependent defense pathways. The SA-dependent pathway is mainly involved against biotrophic pathogens, whereas ET/JA-dependent responses are essential against necrotrophic pathogens (Glazebrook, 2005). As the hemibiotrophic fungus *C. higginsianum* displayed both biotrophic and nectrotrophic lifestyles, it is likely that defense mechanisms related to these two pathways could be triggered in *C. asiatica* infection by *C. higginsianum*.

Recently, Kleemann *et al*. (2012) demonstrated that for effective colonization of *A. thaliana*, *C. higginsianum* secreted effector proteins necessary to maintain host viability during initial biotrophic growth and elicit host death for subsequent necrotrophic growth. Earlier, Narusaka *et al*. (2004) reported that various ecotypes of *A. thaliana* had differential responses to *C. higginsianum* infection, ranging from hypersensitive, ecotype Columbia (Col-0), to highly resistant ecotype Eil-0 whose resistance was conferred by a locus named RCH1. Whereas Chanda *et al*. (2008) showed that high levels of glycerol-3-phosphate in plants were linked to basal resistance to *C. higginsianum*. We hypothesized that some endophytic bacteria in *C. asiatica* may be involved either in prolonging the asymptomatic initial biotrophic stage of *C. higginsianum* or delaying the

necrotic stage by altering *C. higginsianum* gene regulation. Further investigations are needed to elucidate the immunological, cytological and genetic basis leading to the observed apparent inactivity of *C. higginsianum* MUCL 44942 in *C. asiatica*.

2.6. Conclusion

C. asiatica harboured endophytic bacteria behaving diversely towards the hemibiotrophic *C. higginsianum* MUCL 44942 from absence of effects to growth stimulation and inhibition. The role of *B. subtilis* BCA31 in reducing the disease incidence and severity caused by *C. higginsianum* was proved on *in vitro* plants of *C. asiatica*. Further studies should be carried out to analyse secondary metabolites produced by the promising isolates in the antagonistic interactions to uncover putative inhibitory compounds. Also, investigating the beneficial effects of *B. subtilis* BCA31 under field conditions would establish its real potential for bio-control over *Colletotrichum* species and other plant fungal pathogens.

Acknowledgements

Erick Francisco Rakotoniriana is grateful to ADRI/UCL (Administration de la Relation Internationale / Université catholique de Louvain, Belgium) for the doctoral fellowship allowing him to perform this research. We also thank the Laboratory of Food and Environmental Microbiology (UCL, Belgium) for providing technical assistance and facilities in bacterial identification. We wish to thank the Belgian Commission Universitaire pour le Développement (CUD) for contributing financially to this research.

CHAPTER III

COLLETOTRICHUM GIGASPORUM SP. NOV., A NEW SPECIES OF *COLLETOTRICHUM* PRODUCING LONG STRAIGHT CONIDIA

Accepted for publication in Mycological Progress

Erick Francisco Rakotoniriana, Jonathan Scauflaire, Christian Rabemanantsoa, Suzanne Urveg-Ratsimamanga, Anne-Marie Corbisier, Joëlle Quetin-Leclercq, Stéphane Declerck, Françoise Munaut

Preface

We isolated in chapter I an unusual long straight cylindrical conidia-producing *Colletotrichum* from leaves of *Centella asiatica* collected in Mangoro, Madagascar. Based on morphological characteristics and phylogenetic analysis, the fungus is described as a new species in the genus *Colletotrichum*. In addition, the teleomorph was induced in laboratory cultures yielding fertile perithecia with asci and atypical long ascospores up to 90 µm. This study describes the morphological characteristics of the novel species and discusses its phylogenetic relationships with closely related species using ITS and β-tubulin genes. In this chapter, we confirm earlier reports that microbial endophytes are potential sources for new species (Strobel & Daisy, 2003).

3.1. Abstract

A new species of *Colletotrichum* was described, based on morphology and phylogeny. The fungus was isolated in Madagascar from healthy leaves of *Centella asiatica*, in Mexico from wild native of *Stylosanthes guianensis* and in Colombia from *Coffea arabica*. The fungus differed from the currently related species in the genus by its longer and wider size of conidia. In potato dextrose agar medium supplemented with sterilized leaf powder of *C. asiatica*, the fungus produced fertile perithecia containing asci and unusual long ascospores measuring up to 90 µm. In addition to these morphological characteristics, the maximum parsimony analysis of the ITS region and β-tubulin gene placed the fungus in a distinct clade far from the currently valid *Colletotrichum* species. Based on the morphological and molecular characterization, *Colletotrichum gigasporum* sp. nov. was proposed as a new species in the genus *Colletotrichum* Corda.

Keywords: *Colletotrichum gigasporum*, new species, *Centella asiatica*, fungal endophytes, Taxonomy

3.2. Introduction

Colletotrichum Corda is an important fungal genus containing more than 700 species (*Index Fungorum* – www.index fungorum.org). Several of these species are responsible for the anthracnose diseases on a wide range of plants (Freeman & Katan 1997; Martín & García-Figueres 1999; Yang *et al*., 2009; Nguyen *et al*., 2010), while a few were repeatedly isolated as endophytes from apparently asymptomatic healthy plants (Bussaban *et al*., 2001; Lu *et al*., 2004; Photita *et al*., 2005; Promputtha *et al*., 2007). In the past, many *Colletotrichum* species were named solely on morphological characteristics and on host plants (Sutton, 1980, 1992; von Arx, 1957). The lack of reliable morphological criteria within this genus further resulted in doubtful identifications. It is likely that a number of the several hundred species described in this genus are synonyms (Sutton, 1980, 1992; von Arx, 1957). Thus, there is a need for taxonomic revision of many *Colletotrichum* species (Hyde *et al.*, 2009).

Recently, Cai *et al*. (2009) proposed to describe these fungi using a combination of multilocus molecular phylogenetic analysis with morphological and cultural characteristics, physiology and pathogenicity tests. Considerable progress has been made in species delimitation in this genus and many new species and designation of epitypes were reported following this polyphasic approach (Crouch *et al*., 2009; Damm *et al*., 2009; Moriwaki & Tsukiboshi, 2009; Prihastuti *et al*., 2009, Weir & Johnston, 2010; Wikee *et al*., 2011; Yang *et al*., 2011). In parallel, systematic revisions of major *Colletotrichum* species complexes, such as *C. acutatum*, *C. boninense* and *C. gloeosporioides sensu lato* have been proposed (Damm *et al.*, 2010, 2012).

Centella asiatica (L.) Urb (Apiaceae) is an ethnomedicinal plant, native to India, Madagascar, Sri Lanka, China, Indonesia, Australia and South Africa. Puja *et al*. (2006) described a new fungal species, *Echinosphaeria macrospora*, teleomorph of *Vermiculariopsiella endophytica* from the stems of *C. asiatica*, while Krishnamurthy *et al*. (2008) showed that *Chaetomium globosum* was the most frequently isolated fungus from living healthy leaves of *C. asiatica* collected from the Malnad region (Southern India). Recently, Rakotoniriana *et al*. (2008) isolated an unusual long straight conidia-producing *Colletotrichum* species (MUCL 44947), morphologically similar to the original description of *C. crassipes* (von Arx, 1957). The fungus was isolated as endophyte from *C. asiatica* in Madagascar. Single conidia grown on potato dextrose agar (PDA) supplemented with *C. asiatica* leaf powder resulted in the production of fertile perithecia. Preliminary genotypic comparisons using BLAST searches for ITS sequence similarities in GenBank yielded many *Colletotrichum* sequences identical or closely related to that of strain MUCL 44947. Interestingly, most of these isolates were sampled as endophytes from a wide range of host plants located in geographically distant tropical areas. However, the species identity of these *Colletotrichum* strains remains unclear. No formal or Latin diagnosis has been reported so far. They likely belong to undescribed species in the genus *Colletotrichum*.

Here we described a new species of *Colletotrichum*, i.e. *Colletotrichum gigasporum* sp. nov., based on morphology and molecular phylogeny. The morphological characteristics of the teleomorph were also described and discussed.

3.3. Materials and Methods

3.3.1. Fungal isolates

Isolate MUCL 44947 was isolated from healthy leaves of *C. asiatica* collected in the Middle Eastern region of Madagascar in 2003 following surface sterilization (Rakotoniriana *et al*., 2008). Isolates MUCL 42034, 42044 and 42077 were obtained as foliar endophytes from *Stylosanthes guianensis* in Nayarit state in Mexico in 1995 (Gama-López *et al*., 2007). The isolate MUCL 41702 referred to as *C. macrosporum* was recovered from *Bulbophyllum* sp. (Orchidaceae) collected at Bukit Timah Nature Reserve in Singapore in 1999. All isolates are deposited in the BCCM/ MUCL collection, Université catholique de Louvain, Belgium and preserved in vials either under sterile paraffin oil, lyophilized or frozen at -130°C. Four strains identified as *C. crassipes*, CBS 169.59, CBS 159.75, CBS 112984 and CBS 112988 obtained from the Centraalbureau voor Schimmelcultures (CBS), Netherlands, and a *Colletotrichum* sp. strain VEGA 508, an endophytic fungus from *Coffea arabica* (*Co. arabica*) showing identical ITS sequences to isolates from *C. asiatica* and *S. guianensis*, were also included. Overall, ten fungal isolates were examined for detailed study of the morphological and cultural characters.

3.3.2. Morphological and cultural studies

Conidial shape and size along with cultural characters including colony morphology (colony color, mycelial characteristics, presence of setae, and spore masses) were recorded from cultures derived from single germinated conidia on potato dextrose agar (PDA, Scharlau Chemie S.A., Barcelona, Spain) and incubated in the dark at 25°C for 2 weeks. Growth rate was calculated as the 7-day average of mean daily growth (mm per day)

(Photita *et al.*, 2005). Experiments were repeated three times, each experiment was performed in four replicates with two measurements per replicate.

Dilutions were made with conidia masses and drops of the suspension were placed on microscope slides and mixed with lactophenol/cotton blue (Shipton & Brown, 1962). Length and width of fifty conidia were measured with an Olympus BX 52 under 1250 × magnification. Appressoria were observed in slide cultures on PDA (Sutton, 1980) grown under the same conditions as above.

To induce the production of the teleomorph, single conidia isolates were grown on PDA supplemented with 2 g L^{-1} sterilized air-dried leaf powder of *C. asiatica* and incubated in the dark at 25°C for 10 days. Plates were further exposed constantly under near UV light combined with cool white fluorescent light at room temperature till the production of perithecia. A total of 20 perithecia were examined and measured (length × width), with the width of each perithecium measured at its widest point using an ocular micrometer. Length and width of fifty ascospores and asci from various perithecia stained with lactophenol/cotton blue were measured with an Olympus BX 52 under 600-1250 × magnification.

3.3.3. DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from lyophilized fungal mycelium derived from single conidia culture using a CTAB-based method (Abang *et al.*, 2002) adapted as follows. Fifty mg of dried mycelium were put in microtubes and ground to a fine powder with a MagNa Lyser (Roche Diagnostics Gmbh, Germany). Microtubes were filled with 700 µL of CTAB

extraction buffer. After incubation at 65°C for 1 h, 700 µL of chloroform/isoamylalcohol (24/1; vol/vol) were added to the homogenate and mixed by vortexing to form an emulsion. Following centrifugation for 10 min at 13,000 rpm, the aqueous top layer was transferred to a new microcentrifuge tube and incubated with RNase A (100 mg mL^{-1}) and proteinase K (10 mg mL^{-1}) for 30 min at 37°C. DNA was precipitated by adding 1.2 volume of isopropanol and incubating at room temperature for 10 min. Supernatant was discarded after centrifugation for 15 min at 13,000 rpm and the pellet was washed twice in 70% EtOH, air-dried and dissolved in 100 µL of milliQ water.

PCR amplifications were performed in DNA Thermal Cycler 480 (Perkin Elmer Life and Analytical Sciences, Inc, Milano, Italy) using primers ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) (White *et al*., 1990) to amplify the entire ribosomal internal transcribed spacer (ITS) region and primers Bt2a (5′- GGTAACCAAATCGGTGCTGCTTTC -3′) and Bt2b (5′-ACCCTCAGTGTAGTGACCCTT-3′) (Glass & Donaldson, 1995) for the β-tubulin nuclear gene. The PCR reaction mixture of 50 µL included 10 µL of fungal DNA containing 10 ng μL^{-1} and 40 µL of master mix consisting of 5 µL buffer, 3 µL $MgCl_2$ (50 mM), 1 µL dNTPs (10 mM), 1 µL of each primer (10 µM), 0.5 µL Taq DNA polymerase (5 U μL^{-1}) and 28.5 µL of sterile distilled water. PCR programs consisted of an initial denaturation step for 3 min at 94°C followed by 40 cycles of 90 s denaturation at 94°C, 90 s annealing at 55°C and 2 min extension at 72°C. At the end, a final extension step of 72°C for 10 min was included. PCR products were purified using MSB Spin PCRapace (Invitek, Berlin) according to the manufacturer's protocols. Primers ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′), ITS3 (5′-GCATCGATGAAGAACGCAGC-3′), ITS4 and ITS5 for ITS sequencing and primers Bt2a and Bt2b for β-tubulin sequencing were used to sequence

both strands of DNA molecule using a CEQ DTCS Quick Start kit (Beckman Coulter, Inc., Fullerton, CA). Reaction products were run on CEQ 2000XL capillary automated sequencer (Beckman Coulter Inc., USA).

3.3.4. Phylogenetic analysis

For the phylogenetic analysis of ITS region, 38 ITS sequences, of which 26 were from *Colletotrichum* spp. retrieved from EMBL databank, 11 were obtained from this study and one was from *Plectosphaerella cucumerina* NRRL 20430 used as outgroup (see Table 5). Sequences were aligned using the Clustal W2 software (Chenna *et al*., 2003). All the ITS sequences started at the first base of the ITS1 region and ended at the last base of the ITS2 region.

The final dataset for the phylogenetic analysis of ITS consisted of 32 sequences after selecting representatives of haplotypes from sequence comparison. The aligned ITS sequences matrix was imported into PAUP* 4.0b10 (Swofford, 2000) for phylogenetic analysis. Heuristic searches were run with parsimony as the optimality criterion. Alignment gaps were treated as a fifth character, and all characters were unordered and of equal weight. Starting trees were obtained via stepwise addition of 1000 random-addition sequences with tree-bisection-reconnection branch swapping algorithm and branches were collapsed if maximum branch length is zero. To estimate confidence in the clades of the resulting trees, maximum parsimony bootstrap values were generated from 1000 replicates. Consistency index (CI), homoplasy index (HI) and retention index (RI) were calculated to obtain the amount of homoplasy in the dataset.

For strains having the same ITS sequences as that of *C. gigasporum*, and of which the β-tubulin sequences were available in public database, a

phylogenetic analysis of the β-tubulin gene was performed using the same software with 1000 parsimony bootstrap replications.

Table 5. Origin of ITS and β-tubulin sequences from GenBank database or sequenced for this study (in bold) used for phylogenetic analyses.

Species	Isolate	Country	Host	GenBank no.	
				ITS	β-tubulin
C. acutatum	BBA 70339	The Netherlands	*Vaccinium corymbosum*	AJ301911	
C. boninense	MEP1554	China	*Pachira* sp.	DQ286160	
C. boninense	465	Portugal	*Citrus limon* var. *Lisboa*		FN611025
C. incarnatum	unknown	unknown	unknown	GQ369600	
C. coccodes	STE-U 5301	Zimbabwe	*Lycopersicon esculentum*	AY376528	
C. crassipes	CBS 169.59	The Netherlands	*Oncidium excavatum*	**FN557344**	
C. crassipes	CBS 159.75	India	Air and stored grains	**FN557345**	
C. crassipes	CBS 112984	Madeira	*Dryandra* sp.	**FN557347**	
C. crassipes	CBS 112988	Switzerland	*Dryas octopetala*	**FN557348**	
C. destructivum	CD-hz 03	China	Cowpea	EU070913	
C. dracaenophilum	MEP1537	China	*Dracaena* sp.	DQ286207	
C. fragariae	BBA 70340	unknown	*Fragaria* sp.	AJ301912	
C. fuscum	MAFF 238340	Japan	*Nemesia strumosa*	AB105966	

(Table 5 continued)

Species	Isolate	Country	Host	GenBank no.	
				ITS	β-tubulin
C. gloeosporioides	AR 4040	Panama	*Theobroma cacao*	DQ286194	
C. gloeosporioides	MCA 2499	Hungary	unknown	DQ286196	
C. higginsianum	IFO 6182	Japan	unknown	AB105957	
C. kahawae	STE-U 5295	Kenya	*Coffea arabica*	AY376540	
C. lagenarium	BBA 71046	Europe	*Cucumis sativus*	AJ301965	
C. lindemuthianum	BBA 65483	unknown	*Phaseolus* sp.	AJ301958	
C. lupini	BBA 70884	Ukraine	*Lupinus albus*	AJ301948	
C. macrosporum	MUCL 41702	Singapore	*Bulbophyllum* sp.	**FM163593**	
C. musae	BBA 62471	Germany	*Musa* sp.	AJ301904	
C. trifolii	BBA 70709	USA	*Trifolium* sp.	AJ301941	
C. gigasporum	MUCL 42034	Mexico	*Stylosanthes guianensis*	**AM982794**	**FN557443**
C. gigasporum	MUCL 42044	Mexico	*Stylosanthes guianensis*	**AM982795**	**FN599823**
C. gigasporum	MUCL 42077	Mexico	*Stylosanthes guianensis*	**AM982796**	**FN599824**
C. gigasporum	MUCL 44947	Madagascar	*Centella asiatica*	**AM982797**	**FN557442**

(Table 5 continued)

Species	Isolate	Country	Host	GenBank no.	
				ITS	β-tubulin
C. gigasporum	MUCL 52571	Madagascar	*Centella asiatica*	**FN557441**	**FN599825**
C. gigasporum	VEGA 508	Colombia	*Coffea arabica*	**FN557349**	**FN599822**
Colletotrichum sp.	3386	Panama	*Theobroma cacao*	GU994389	GU994480
Colletotrichum sp.	4801	Panama	*Theobroma cacao*	GU994386	GU994479
Colletotrichum sp.	E1249	Panama	*Trichilia tuberculata*	GU994388	
Colletotrichum sp.	Z31	China	*Taxus chinensis* var. *mairei*	JN198428	
C. yunnanense	AS3.9616	China	unknown	EF369491	
C. yunnanense	AS3.9617	China	unknown	EF369490	
Glomerella septospora	C040981	Korea	*Capsicum annuum*	GU935907	GU935912
Glomerella septospora	C07005	Korea	*Capsicum annuum*	GU935910	GU935916
Glomerella septospora	C07011	Korea	*Capsicum annuum*	GU935911	GU935915
Glomerella septospora	C07001	Korea	*Capsicum annuum*		GU935913
Glomerella septospora	C07002	Korea	*Capsicum annuum*		GU935914
Plectosphaerella cucumerina	NRRL 20430	Egypt	*Viola odorata*	AF176952	

3.4. Results

3.4.1. Induction of the teleomorph

Fertile perithecia were readily produced when monoconidial isolates of *Colletotrichum* sp. MUCL 44947 from healthy leaves of *C. asiatica* were grown on PDA supplemented with 2 g L^{-1} of *C. asiatica* leaf powder and exposed constantly under near UV light and cool white fluorescent light. Under these conditions, perithecia were consistently produced whereas this strain formed rarely the teleomorph on PDA alone. Perithecia grouped mostly near the inoculum point at the center of the plates (< 2 cm around), and were rarely found in the margin of the colony. The teleomorph was only produced in culture by the isolate MUCL 44947 from *C. asiatica* but it was not observed neither for isolates from *S. guianensis* (MUCL 42034, MUCL 42044 and MUCL 42077) nor for isolate from *Co. arabica* (VEGA 508), when grown under the same conditions.

3.4.2. Morphological comparison of *C. gigasporum* with related *Colletotrichum* species

Details of the morphological and cultural characters of the ten fungal isolates observed in the study were provided (Table 6). The mean length of the conidia from *C. asiatica* (MUCL 44947) was a bit longer than those from *S. guianensis* (MUCL 42034, MUCL 42044 and MUCL 42077) and from *Co. arabica* (VEGA 508) but the length range overlapped. Conidia from *C. gigasporum* (MUCL 44947, MUCL 42034, 42044, 42077 and VEGA 508) mostly bore resemblance to those identified as *C. crassipes* CBS 169.59. Conidia were hyaline, aseptate, straight, cylindrical and having somewhat similar size except that both apices were obtuse for isolates of *C. gigasporum* whereas conidial base was truncate in isolates identified as *C.*

crassipes. Observations showed that isolates CBS 112984 and CBS 112988 had considerably shorter conidia than isolates CBS 15975 and CBS 16959. Overall, *C. macrosporum* MUCL 41702 had the largest conidia measuring (28)31.5–38(40) × 9–11 µm.

3.4.3. Molecular phylogenetic analysis

The final alignment of the ITS sequences generated a total of 540 characters, 309 of which were constant, 99 were parsimony uninformative and 132 were parsimony informative. One of the eighteen parsimonious trees retained is shown (Fig. 10). The tree length was 485, the consistency index (CI) 0.7052, the homoplasy index (HI) 0.2948 and the retention index (RI) 0.8462. Isolates of *C. gigasporum* clustered in a clade with isolates identified as *Glomerella septospora*, *C. incarnatum* and several *Colletotrichum* sp. strains from various host plants, and their grouping was supported by high bootstrap of 99%. This clade was well separated from other clades containing known, valid *Colletotrichum* species. Isolates identified as *C. crassipes* were resolved into three different clades.

For β-tubulin gene alignment data ten taxa provided 499 characters, of these 411 characters were constant, 77 characters were parsimony uninformative and 11 characters were parsimony informative. The one parsimonious tree resulting from the analysis of the β-tubulin gene is shown (Fig. 11). The tree length was 91, the consistency index (CI) 0.9780, the homoplasy index (HI) 0.0220 and the retention index (RI) 0.8947. The phylogenetic tree of the β-tubulin gene placed isolates of *C. gigasporum* in a distinct clade with a bootstrap support of 86%.

Table 6. Morphological and cultural comparison between *C. gigasporum* and related taxa.

Species isolates	Colony diameter [a]	Aerial mycelium	Conidial shape	Conidial length × width (µm) Mean	Range	SD [b]
C. gigasporum						
MUCL 44947	65.9 ± 1.3	Gray to green	Cylindrical, both apices rounded	26.9 × 7.7	22–32 × 7–9	1.9 × 0.7
MUCL 42044	70.1 ± 1.7	Gray	Cylindrical, both apices rounded	22.1 × 6.8	18–26 × 6–8	1.8 × 0.6
MUCL 42034	67.5 ± 2.7	Gray	Cylindrical, both apices rounded	21.0 × 6.9	16–24 × 6–8	1.8 × 0.7
MUCL 42077	57.1 ± 1.9	Gray	Cylindrical, both apices rounded	20.3 × 6.9	18–24 × 6–8	1.4 × 0.7
VEGA 508	69.2 ± 1.5	Brown	Cylindrical, both apices rounded	22.7 × 7.8	20–28 × 7–9	1.7 × 0.7
Isolates identified as *C. crassipes*						
CBS 159.75	42.8 ± 1.4	Brown	Cylindrical, apex rounded, base truncate	18.2 × 8.2	14–20 × 7–9	0.6 × 0.7
CBS 169.59	77.2 ± 2.7	Brown	Cylindrical, apex rounded, base truncate	22.4 × 5.8	18–26 × 5–7	1.7 × 0.6
CBS 112984	47.6 ±1.7	Whitish	Cylindrical, apex rounded, base truncate	14.1 × 4.3	11–16 × 4–5	1.1 × 0.5
CBS 112988	49.2 ±1.3	Whitish	Cylindrical, apex rounded, base truncate	15.2 × 4.4	12–17 × 4–5	0.9 × 0.4
C. macrosporum						
MUCL 41702	73.2 ± 1.2	Dark green	Cylindrical, both apices rounded	34.4 × 10.5	28–39 × 9–11	2.3 × 0.5

[a] Colony diameter (mm) after 7 day-culture, except for *C. macrosporum* for which values were taken after 5 days due to its high growth rate.

[b] SD = Standard deviation

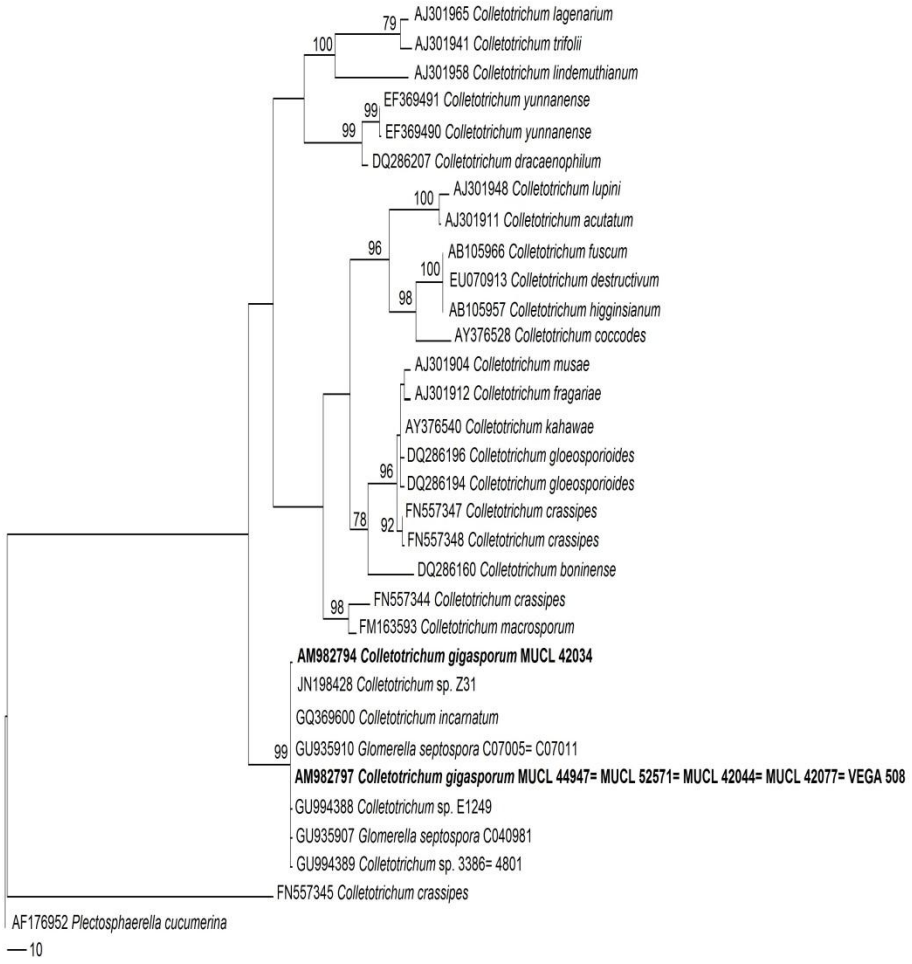


Fig. 10. Phylogenetic tree based on maximum parsimony analysis of the ITS sequences of *Colletotrichum gigasporum* and the closest relatives from GenBank. Percent of 1000 bootstrap replications greater than 70% are indicated at the internodes. *Plectosphaerella cucumerina* (accession number AF176952) was used as an outgroup.

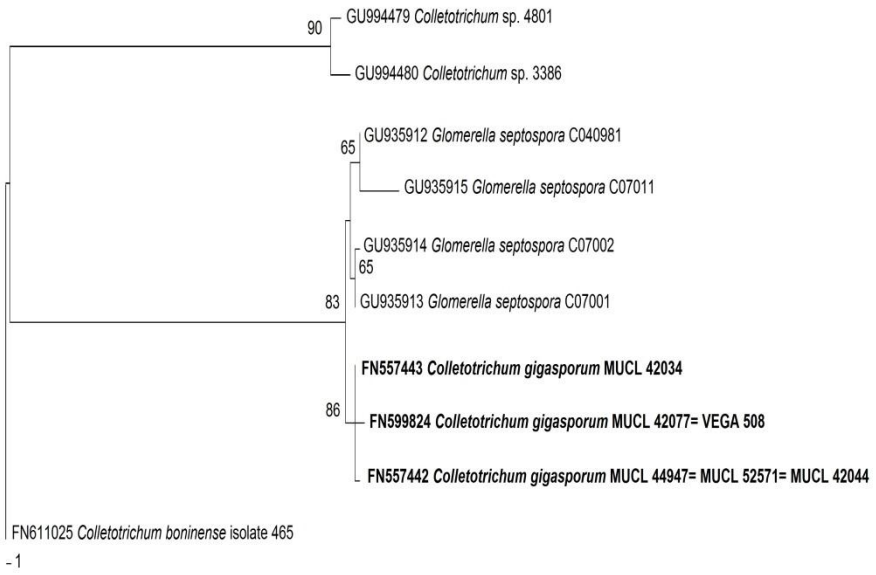


Fig. 11. Phylogenetic tree based on maximum parsimony analysis of the β-tubulin gene of *Colletotrichum gigasporum* and the closest relatives from GenBank. Percent of 1000 bootstrap replications are indicated at the internodes. *Colletotrichum boninense* (accession number FN611025) was used as an outgroup.

3.5. Taxonomy

Colletotrichum gigasporum E.F. Rakotoniriana & F. Munaut sp. nov.
(Fig. 12 a–i)

MycoBank no. MB 800175

Etymology. The epithet *gigasporum* refers to the large size of the conidia.

Holotype. MUCL 44947-H. MADAGASCAR. MANGORO (18°52′20″S, 48°06′25″E): isolated from the inner part of healthy leaves of *Centella asiatica*, May 2003, E.F. Rakotoniriana, dried culture. Ex holotype culture: MUCL 44947, deposited at BCCM/ MUCL, Louvain-la-Neuve, and preserved under sterile paraffin oil, lyophilized or frozen at -130°C.

Paratype. MUCL 52571-H. Dried culture with perithecia produced from single conidia of MUCL 44947 (ex holotype), June 2009, E.F. Rakotoniriana. Ex-paratype culture: MUCL 52571, deposited at BCCM/ MUCL, Louvain-la-Neuve, and preserved under sterile paraffin oil, lyophilized or frozen at -130°C.

Anamorph. Colonies on PDA at 25°C in complete darkness 6.6 cm diam in 7 days, greyish green margin regular. Aerial mycelium grey to pale green, short, reverse dark green. Sclerotia absent. Setae present, light to dark brown abundant, ruguous, septate, straight becoming narrower, tapered at the apex or rounded 90–140 × 3.5–4 µm. Conidioma acervular. Appressoria abundant, pale to medium brown with germ pore, clavate or irregular in shape with moderate crenate edges 14–16 × 10–12 µm, becoming complex. Conidiogenous cells cylindrical to clavate, hyaline or slightly brown, phialidic, cylindrical, 17–28 × 5–6 µm. In PDA (24 g L^{-1}, Duchefa) supplemented with 2 g L^{-1} *Centella asiatica* powder, under near UV,

sporulation abundant, with conidia formed in a pinkish masses, hyaline, aseptate, straight, cylindrical, guttulate, rounded at both ends, measuring (22)25–29(32) × (6)7–9 µm.

Teleomorph developed on PDA supplemented with 2 g L^{-1} sterilized *Centella asiatica* leaf powder. Ascoma an ostiolate perithecium, dark brown, obpyriform to subglobose, 400–640 µm high, 240–320 µm wide, rarely solitary to mostly confluent. Asci unitunicate, 8-spored, broadly cylindrical to clavate, truncate at apex, thin-walled, short-stalked, evanescent, 64–120 × 8–12 µm. Paraphyses septate. Ascospores hyaline, fusoid to cylindrical, mostly straight to slightly curved, rounded at both ends, aseptate, 1-septate at maturity, septum median, (56)60–84(92) × 5–7 µm.

Isolates examined. MADAGASCAR. MANGORO. Isolated from *Centella asiatica*, Mai 2003, E.F. Rakotoniriana, MUCL 44947 (EX HOLOTYPE); MADAGASCAR. MANGORO. Produced in laboratory culture from single conidia of MUCL 44947, June 2009, E.F. Rakotoniriana, MUCL 52571 (EX-PARATYPE); MEXICO. NAYARIT STATE. Isolated from *Stylosanthes guianensis*, Jan 1995, F. Munaut, MUCL 42034; MEXICO. NAYARIT STATE. Isolated from *S. guianensis*, Jan 1995, F. Munaut, MUCL 42044; MEXICO. NAYARIT STATE. Isolated from *Stylosanthes guianensis*, Jan 1995, F. Munaut, MUCL 42077; COLOMBIA. Isolated from *Coffea arabica*, July 2003, F.E. Vega, VEGA 508. A dried culture of each isolates was deposited at the herbarium of MUCL (as, MUCL 42034-H, MUCL 42044-H, MUCL 42077-H and VEGA 508-H), Louvain-la-Neuve, Belgium.

Notes: Isolates MUCL 42034, MUCL 42044, MUCL 42077 and VEGA 508 produce slightly shorter conidia (16)21–24(28) × (6)7–9 µm compared to those of the ex-holotype (MUCL 44947) but they are similar in shape, i.e.,

hyaline, aseptate, straight, cylindrical, guttulate and rounded at both ends. Teleomorph not produced.

Ascospore-derived colonies displayed the same cultural characters and conidial morphology as those from monoconidial isolates of the parental *Colletotrichum*.

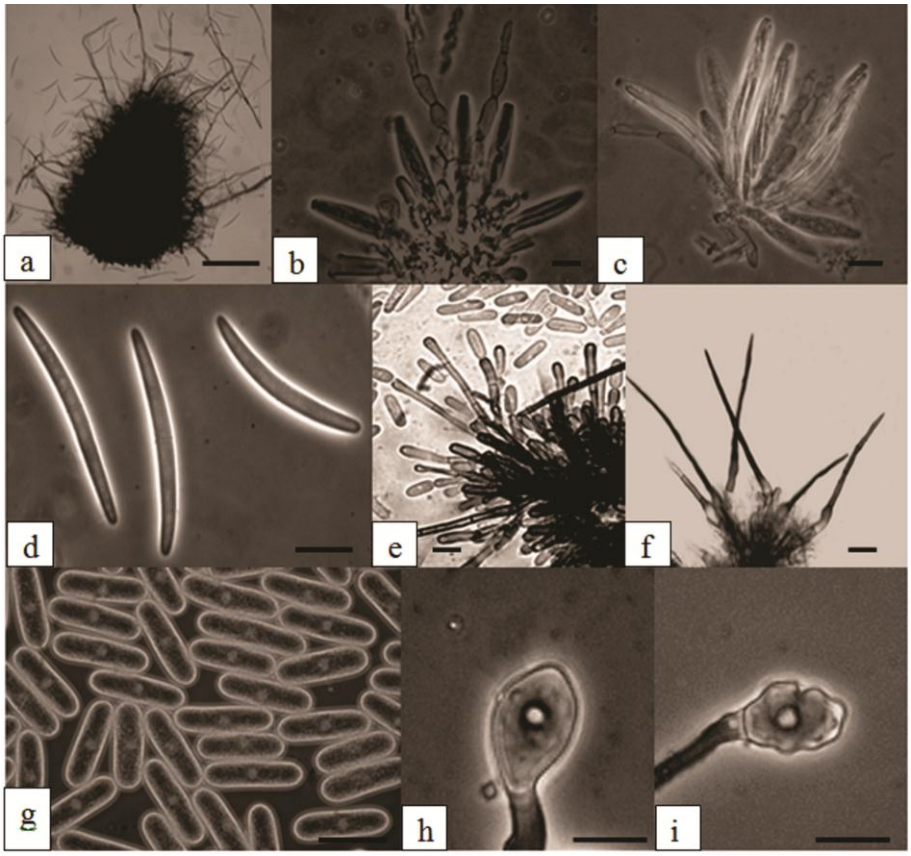


Fig. 12. *Colletotrichum gigasporum* and its teleomorph. **a** Perithecia formed in culture. **b** Immature asci with septate paraphyses. **c** Mature asci. **d** Ascospores. **e** Conidiophores of *C. gigasporum* produced in culture. **f** Setae of *C. gigasporum* formed on PDA. **g** Conidia of *C. gigasporum* formed on PDA at 25°C in the dark for 2 wk. **h**–**i** Appressoria of *C. gigasporum* formed on potato carrot agar slide culture at 25°C in the dark for 3 d. Bars: 1 = 200 µm; 2–7 = 20 µm; 8–9 = 10 µm.

3.6. Discussion

Conidial features (both size and shape) are important taxonomic criteria for morphological identification of *Colletotrichum* species. Sutton (1980) informally divided the genus into 2 groups based on the conidia shape: species with straight and species with falcate conidia. Recently, Crouch *et al.*, (2009) and Damm *et al*., (2009) investigated the falcate-spored *Colletotrichum* species from graminicolous and herbaceous hosts. Here, we report the description of a new *Colletotrichum* species, *Colletotrichum gigasporum*, based on morphological characters and phylogenetic analysis.

Single conidia cultures of *C. gigasporum* MUCL 44947 produced fertile perithecia with asci bearing 0 to 1-medianly septate, long ascospores measuring (56)60–84(92) × 5–7 µm. Typical common ascospores are usually shorter, less than 20 µm long and aseptate, but they may become 1-septate when old (Hanlin, 1990). The supplement of *C. asiatica* powder along with constant exposure to near UV and cool white fluorescent lights enhanced the production of the teleomorph by *C. gigasporum* MUCL 44947. Under these conditions, perithecia were consistently produced by this strain whereas it rarely formed the teleomorph on PDA alone. Colonies from single ascospores displayed the same cultural characters and conidial morphology as those from monoconidial isolates of the parental *Colletotrichum*, supposing that *C. gigasporum* may be homothallic. The sexual form of *C. gigasporum* produced ascospores that mostly resembled those of *Glomerella septospora* with respect to the ascospore size [60–95(100) × 5.0–7.5 µm]. However, *G. septospora* ascospores were mostly 3-septate and may become up to 6 or 8 septate when mature (Sivanesan & Hsieh, 1993). Given these atypical morphological characters the inclusion of *G. septospora* within the genus *Colletotrichum* was still regarded as doubtful (Hyde *et al*., 2009), even though evidence for its belonging to the genus *Colletotrichum* was earlier

demonstrated using maximum parsimony analysis of the nuclear small-subunit ribosomal DNA (18S rDNA) with sequences issued from the type strain (accession number U78779, strain ATCC90830, IMI353024) (Wanderlei-Silva *et al*., 2003).

Although *C. gigasporum* isolates MUCL 42034, MUCL 42044, MUCL 42077 and VEGA 508 failed to produce teleomorph under the cultural conditions used in this study, their belonging to *C. gigasporum* was proved on the basis of ITS and β-tubulin phylogenetic analysis. Indeed, it was previously demonstrated that MUCL 42034, MUCL 42044 and MUCL 42077 were conspecific, since they displayed very similar RAPD profiles with the random primer OPA-15 (Gama-Lopez *et al*., 2007). As the type strain of *C. gigasporum*, the isolate MUCL 44947, was morphologically similar to isolates MUCL 42044, MUCL 42034, MUCL 42077 and VEGA 508 and because the ITS and β-tubulin sequences were identical or near-identical (differed in one to two nucleotides position), isolates MUCL 42044, MUCL 42034, MUCL 42077, and VEGA 508 could consequently be referred to as *C. gigasporum*.

Morphologically, *C. gigasporum* differed from currently valid *Colletotrichum* species by its long conidia up to 32 µm (average length 26 µm). The search in the literature for previously identified taxa yielding similar conidial traits in shape and size revealed the existence of *C. macrosporum* (28–32 × 8–10 µm) (Saccardo, 1899) collected from leaves of an orchid in Brazil, *C. echinatum* (22–26 × 8–9 µm) (Saccardo & Trotter, 1913), and *C. vinosum* (20–30 × 6–8 µm) (Saccardo & Saccardo, 1906). But these taxa were considered as synonyms to *C. crassipes* by von Arx (1957). Compared to the species original description of *C. crassipes* by von Arx (1957), mainly based on the conidial size (22–31 × 6–8 µm) and the morphology of the appressoria with deeply lobed margins, some minor

morphological differences could be noted with *C. gigasporum*. This latter produced conidia with rounded apices at both ends and the appressoria edges were entire to crenate. Whereas conidia of *C. crassipes* were obtuse at the apex but truncate at the base, and the appressoria edges were deeply lobed. Since no ex type culture was currently available for *C. crassipes* (Hyde *et al.*, 2009) it would be difficult to distinguish closely related taxa to *C. crassipes* based only on morphological characteristics due to the wide range of the conidial size and lack of distinct morphological features. Indeed, further taxonomic and phylogenetic studies are needed to designate an epitype strain in *C. crassipes*, which will serve as the basis for an accurate identification in this species. Anyway, a high level of genetic dissimilarities was observed between *C. gigasporum* and isolates identified as *C. crassipes* obtained from the Centraalbureau voor Schimmelcultures (CBS), Netherland. For instance, the ITS region of *C. gigasporum* MUCL 44947 differed in about 32 bp nucleotides to strain CBS 159.75. Furthermore, morphological comparison (Table 6) combined with the maximum parsimony analysis of the ITS sequences (Fig. 10) provided ample evidence that *C. gigasporum* can be distinguished from *C. macrosporum* and the so-called *C. crassipes* isolates included in this study.

Similar types of conidia as long as those produced by *C. gigasporum*, even longer, were also reportedly produced by an unusual *Colletotrichum* named *C. taiwanense*. However, conidia of *C. taiwanense* may become multiseptate (0–3 septate conidia 22–35(45) × 5–8 µm, 4–5 septate conidia 50–58 × 5–8 µm) with age (Sivanesan & Hsieh, 1993) while those of *C. gigasporum* remained aseptate. Interestingly, the teleomorph of *C. taiwanense* (*Glomerella septospora*), appeared to have identical ITS sequence as that of *C. gigasporum* and gathered together in a clade distinct from known *Colletotrichum* species (Fig. 10). Inappropriately, due to the lack of ITS and β-tubulin sequences from the type strain of *G. septospora* (W.H. Hsieh

IMI353024, ATCC90830) isolated from stems of *Styrax formosanus* Matsum. in Taiwan, we used available sequences of *G. septospora* isolated from *Capsicum annuum* in Korea to perform the analysis. In any case, the β-tubulin phylogenetic analysis allowed their separation into two different clades with a bootstrap value of 83%, supporting the hypothesis that they belong to two different species (Fig. 11).

The BLAST searches for ITS sequence similarity also showed that *C. incarnatum* (GQ369600) and *C. gloeosporioides* (AY266402) had the same ITS sequence with *C. gigasporum*. But conidia of *C. gigasporum* were much longer than those of *C. incarnatum* (14–19 × 5 µm) (Zimmermann, 1901) and those of *C. gloeosporioides* (AY266402) [(12)16.1(20) × (3)4.6(6)] (Photita *et al*., 2005). The BLAST searches also revealed the occurrence of numerous *Colletotrichum* sp. that had similar ITS sequence as *C. gigasporum*, comprising endophytic isolates from various hosts, such as *Magnolia liliifera* in Thailand (Promputtha *et al*., 2007); *Taxus chinensis* var. *mairei* in China; *Theobroma cacao, Trichilia tuberculata* and *Virola surinamensis* in Panama (Rojas *et al*., 2010) as well as a *Colletotrichum* sp. isolate LD35b(B2) associated with anthracnose disease of coffee in Vietnam (Nguyen *et al*., 2010). The identity of these *Colletotrichum* isolates is not very clear at the species level. They likely belong to a taxon distinct from known and accepted *Colletotrichum* species.

Endophytism is an important factor in microbial speciation and biodiversity (Stone *et al*., 2000). Liu *et al*. (2007) reported a new *Colletotrichum* endophyte from *Buxus* sp. assigned to *C. yunnanense* with bacilliform or slightly clavate conidia, rounded at both ends, hyaline, measuring (14)16–21 × 5–6 µm. However, this latter was shorter and narrower than *C. gigasporum*. Hitherto, *Colletotrichum* isolates previously described as endophytes belong mainly to *C. acutatum*, *C. gloeosporioides*, and *C.*

boninense (Bussaban *et al*., 2001; Lu *et al*., 2004; Photita *et al*., 2005; Rojas *et al*., 2010). However, the conidial size of *C. gigasporum* were clearly longer and wider than that of *C. gloeosporioides* which measured 12–17(–23.5) × 4.5–6 µm in water and (10.5–)11–15 × 4.5–5.5 µm in lactic acid/cotton blue (Cannon *et al*., 2008) and that of *C. acutatum* 7–14 × 2.5–3.5 µm (Than *et al*., 2008). In addition, in *C. gloeosporioides* the conidial shape was generally ovoid to oblong and that of *C. acutatum* was often fusiform, whereas *C. gigasporum* produced cylindrical conidia. All *C. gigasporum* isolates reported in this study were found to be endophytes from various plant sources located from geographically distant areas. To some extent, *C. gigasporum* appeared to have a widespread distribution mostly in tropical regions, being isolated in Madagascar, as foliar endophyte of *C. asiatica*, in Mexico, from healthy leaves of *S. guianensis* and in Colombia, as endophyte of *Co. arabica*. Further isolates are required to define its host range and to clarify its true lifestyle if *C. gigasporum* could occur as epiphyte or pathogen in other plants.

Cai *et al*. (2009) suggested performing multi-gene phylogenetic analysis for a better resolution of the relationships among *Colletotrichum* species and for an accurate and reliable species diagnosis. Consistent with this, using a multi-gene phylogeny of a combined datasets of the partial actin, β-tubulin (tub2), calmodulin, glutamine synthetase, glyceraldehyde-3-phosphate dehydrogenase genes and the complete rDNA ITS1-5.8S-ITS2 regions, Prihastuti *et al*. (2009) was able to distinguish *C. gloeosporioides* from *C. kahawae* supported by high bootstrap support (100%). In this study, we only carried out sequencing of ITS region and β-tubulin gene since, the taxonomic position of *C. gigasporum* was well resolved among the main groups of straight conidia-producing *Colletotrichum* species in the phylogenetic tree of the ITS sequences. The subsequent phylogenetic analysis based on the β-tubulin gene clearly differentiated *C. gigasporum*

from other *Colletotrichum* species sharing identical ITS sequences. Notably, the β-tubulin gene sequences have also been successfully used to solve identification problems of closely related species of other fungal genera. Chung *et al*. (2006) reported that β-tubulin sequences separated *Ophiostoma breviusculum* from *O. piacea* and *O. canum*, while ITS sequence did not. Thus, based on the integration of the morphological and phylogenetic features, it is concluded that *C. gigasporum* is a distinct species in the genus *Colletotrichum*.

Acknowledgements

Erick Francisco Rakotoniriana is grateful to ADRI/UCL (Administration de la Relation Internationale / Université catholique de Louvain) for the doctoral fellowship allowing him to perform the study. The staff at the Mycothèque de l'Université catholique de Louvain is thanked for the logistical support. We wish to thank the Belgian Commission Universitaire pour le Développement (CUD) for contributing financially to this research.

CHAPTER IV

CHARACTERIZATION OF AN ENDOPHYTIC WHORL-FORMING *STREPTOMYCES* FROM *CATHARANTHUS ROSEUS* STEMS PRODUCING POLYENE MACROLIDE ANTIBIOTIC

This chapter has been published in the Canadian Journal of Microbiology, 2012, 58(5): 617–627

Rakotoniriana EF, Chataigné G, Raoelison G, Rabemanantsoa C, Munaut F, El Jaziri M, Urveg-Ratsimamanga S, Marchand-Brynaert J, Corbisier AM, Declerck S, Quetin-Leclercq J.

Preface

Catharanthus roseus was the second host plant selected for the investigation of microbial endophytes associated with Madagascan medicinal plants included in this thesis. Previous reports on microbial endophytes from *C. roseus* mainly focused on endophytic fungi (Kharwar *et al*., 2008; Krishnamurthy *et al*., 2008; Tung *et al*., 2002; Zhang *et al*., 2000). So, in this second part of the thesis, we explored the occurrence of streptomycetes endophytes in the aerial parts of *C. roseus* from Madagascar to determine whether it was as rare as that of *C. roseus* from India (Sharma *et al*., 2011).

Given the potential of these microorganisms to produce bioactive compounds with diverse bioactivities, we particularly addressed in this chapter the antifungal activity of an endophytic whorl-forming *Streptomyces* sp. strain TS3RO, isolated from the stems of *C. roseus*. The spectrum of antifungal activity of the crude filtered culture extract was evaluated on a wide range of human and plant pathogenic filamentous fungi. Then, the major active compound was purified and characterized by means of spectroscopic data. We also questioned the taxonomic identity of strain TS3RO using methods and media described by the International Streptomyces Project (ISP) (Shirling & Gottlieb, 1966) as well as phylogenetic analysis of the partial 16S rRNA sequences between strain TS3RO and closely relatives retrieved from GenBank.

4.1. Abstract

An endophytic whorl-forming *Streptomyces* sp. designated as TS3RO having antifungal activity against a large number of fungal pathogens including *Sclerotinia sclerotiorum, Rhizoctonia solani, Colletotrichum gloeosporioides, Cryphonectria parasitica, Fusarium oxysporum, Pyrenophora tritici-repentis, Epidermophyton floccosum* and *Trichophyton rubrum* was isolated from surface-sterilized *Catharanthus roseus* stems. Preliminary identification showed that *Streptomyces cinnamoneus* subsp. *sparsus* was its closest related species. However, strain TS3RO could readily be distinguished from this species using a combination of phenotypic properties, 16S rRNA gene sequence similarity and phylogenetic analyses. Thus, the whorl-forming *Streptomyces* sp. strain TS3RO is likely a new subspecies within the *Streptomyces cinnamoneus* group. Direct bioautography on thin layer chromatography plate with *Cladosporium cucumerinum* was conducted throughout the purification steps for bioassay-guided isolation of the active antifungal compounds from the crude extract. Structural elucidation of the isolated bioactive compound was obtained via LC/MS spectrometry, UV-visible spectra and NMR data. It revealed that fungichromin, a known methylpentaene macrolide antibiotic, was the main antifungal component of strain TS3RO as shown by TLC bioautography. This is the first report of endophytic whorl-forming *Streptomyces* from the medically important plant *Catharanthus roseus.*

4.2. Introduction

Over the past decade, studies on endophytic actinomycetes from medicinal plants have received increasing attention because plants with medicinal virtues have been considered as an important source of novel species and might host microorganisms that produce various compounds of pharmaceutical interest (Tan & Zou, 2001; Strobel *et al*., 2004). Investigations on medicinal plants-associated endophytic actinomycetes from different geographic locations worldwide have already shown promising results in this field. For examples, Zin *et al*. (2007) discovered in the Malay Peninsula three novel streptomycetes from plants with ethnobotanical uses. Li *et al*. (2008) isolated several endophytic actinomycetes from pharmaceutical plants in rainforest in Yunnan province, China, having antitumor and antimicrobial bioactivities. Similarly, El-Shatoury *et al*. (2009) reported from the medicinal plant, *Achillea fragrantissima* (Forssk) Sch. Bip. (Compositae), collected in Saint Katherine, South Sinai, Egypt, the isolation of twenty-five actinomycetes some of which being able to produce chitinase and siderophores.

Streptomyces spp. are aerobic, Gram-positive bacteria, with a high G + C content (69-73%) and cell wall chemotype I, inhabiting soil (Lechevalier & Lechevalier, 1970). They are also repeatedly isolated as endophytes (Araújo *et al*., 2000; Cao *et al*., 2004). Within this group of actinomycetes, the genus *Streptoverticillium* was proposed by Baldacci (1958) as a distinct group of whorl-forming *Streptomyces* species. However, the genus *Streptoverticillium* was later reunified to the genus *Streptomyces* and referred to in literature as whorl-forming *Streptomyces* (Hatano *et al*., 2003).

The Madagascar periwinkle *Catharanthus roseus* (L.) G. Don (Apocynaceae) is a medicinal plant producing indole alkaloids such as the

anticancer drugs vinblastine and vincristine, and antihypertensive compounds such as serpentine and ajmalicine (Dutta *et al,*. 2005). Besides, leaves of *C. roseus* have antifungal activity due at least to the presence of 5-hydroxy flavone (Roy & Chatterjee, 2010). Previous studies on microbial endophytes from *C. roseus* have focused mainly on fungi (Huang *et al*., 2008; Kharwar *et al*., 2008; Krishnamurthy *et al*., 2008). Zhang *et al*. (2000) and Tung *et al*. (2002) claimed that the fermentative broth of *Fusarium oxysporum*, a fungal endophyte associated with *C. roseus*, contained the same vincristine as that of the host plant. *C. roseus* was also used as an experimental host in greenhouse experiments to study the interaction of microbial endophytes and plant pathogens (Lacava *et al*., 2007; Musetti *et al*., 2007). However, very little information is available about the endophytic bacterial community of *C. roseus.* We were particularly interested in the occurrence of endophytic *Streptomyces* from this valuable medicinal plant because of the potential of these organisms to produce natural products with a wide range of bioactivities (Castillo *et al*., 2003; Shin *et al*., 2008; Taechowisan *et al*., 2007).

Here, we reported the isolation of a first verticillate endophytic *Streptomyces* sp. designated TS3RO from healthy stems of *C. roseus* showing antifungal activity against several fungal plant pathogens and human dermatophytic fungi. We also investigated its taxonomy and characterized its bioactive secondary metabolites.

4.3. Materials and Methods

4.3.1. Isolation of endophytes

Stems (approximately 3 mm diam.) of *C. roseus* were collected at random from plants grown at the botanical garden of the "*Institut Malgache de Recherches Appliquées*" Antananarivo, Madagascar and processed immediately after collection. Stems were thoroughly washed in running tap water and subsequently surface-sterilized by sequential immersion in 70% ethanol for 1 min followed by 3% calcium hypochlorite for 10 min. Stem samples were then rinsed three times with sterile distilled water before they were aseptically sliced and plated onto nutrient agar (Oxoid, Basingstoke, UK) supplemented with 15 µg mL^{-1} nalidixic acid to inhibit the growth of nonmycelial bacteria and 50 µg mL^{-1} nystatin to suppress fungal growth. Overall, 200 small discs of approximately 3 × 2 mm were made from forty 1 cm long fragments of surface-sterilized stems. Following incubation at 28°C in the dark, colonies that emerged from the stem discs were subcultured on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI, USA) to promote the growth of aerial mycelium and allow rapid isolation of *Streptomyces* species.

The effectiveness of the disinfection process was previously checked by carefully rolling the surface-sterilized stems onto nutrient agar. The test was validated whether no colonies was apparent after incubating the plates at 28°C for 3 weeks. In addition, vitality test described by Petrini (1984) was also used to check the effectiveness of the surface sterilization method. Aliquots of the isolates were dipped in the respective solution originally used for the surface sterilization of the stems and re-plated on the PDA under the same cultural conditions as above.

4.3.2. Morphological, cultural and physiological characterization

The International *Streptomyces* Project (ISP) media were used for morphological and cultural characterization of strain TS3RO and were prepared according to the methods of Shirling & Gottlieb (1966). After incubation at 28°C during 14 days, growth, aerial mycelium color, substrate mycelial pigmentation and production of soluble pigments were investigated on various ISP media including yeast extract/malt extract agar (ISP medium 2), oatmeal agar supplemented with ISP-trace salt solution (ISP medium 3), inorganic salts/starch agar (ISP medium 4), glycerol/asparagine agar (ISP medium 5), peptone/yeast extract/iron agar (ISP medium 6), tyrosine agar (ISP medium 7) and also on PDA and modified Bennett's agar (MBA; Jones, 1949). Light- and scanning electron microscopy (SEM) were used to study the aerial mycelium and spore-chain morphology of strain TS3RO from culture grown on MBA and ISP medium 3 for three weeks at 28°C. Melanoid pigment production was assessed after 4 days on ISP medium 6. Physiological tests including study of acid production from carbohydrates, hydrolysis of xanthine, starch and esculin and growth in the presence of 5% NaCl or at 10°C were carried out by using the media of Gordon *et al*. (1974). For comparative study of the phenotypic characteristics, data for *Streptomyces* species showing close relationship to strain TS3RO were retrieved from previously published data (Labeda *et al*., 1997).

4.3.3. 16S rRNA gene sequencing and phylogenetic analysis

The partial 16S rRNA gene sequence of strain TS3RO was performed at BCCM/LMG (LMG 26318). Briefly, total DNA was extracted according to the protocol of Niemann *et al*. (1997). The 16S rRNA gene was amplified

by PCR using the following primers 16F27 (5'-AGA GTT TGA TCC TGG CTC AG-3') and 16R1522 (5'-AAG GAG GTG ATC CAG CCG CA-3'). Sequencing reactions were performed using the BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an ABI Prism® 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Homology of the nucleotide sequence with known sequences of *Streptomyces* spp. from databanks was examined by the BLAST program.

The software package BioNumerics (AppliedMaths, Belgium) was used to analyze the nucleotide sequences after including the consensus sequence in an alignment of small ribosomal subunit sequences collected from the international nucleotide sequence library EMBL. This alignment was pairwise calculated using an open gap penalty of 100% and a unit gap penalty of 0%. A phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987).

4.3.4. Fermentation and extraction of metabolites

Fermentation was processed in each of twenty 2-liter Erlenmeyer flasks containing 500 mL of 3% oatmeal broth supplemented with 1 g L^{-1} of soya peptone. Each 500 mL culture broth was inoculated with ten 1 cm diam. discs from two week-old mycelium of strain TS3RO grown on oatmeal agar. Inoculated flasks were shaken on a rotary shaker at 150 rpm for 14 days at 28°C in the dark. After cultivation, the fermented whole broths were extracted three times with half volume of ethyl acetate by stirring for 2 h at room temperature and the mycelium was removed by centrifugation at 12000×*g* for 20 min. Organic phases were pooled, dried with anhydrous Na_2SO_4 and evaporated to dryness at reduced pressure with a rotary vapour to yield a crude extract of 3 g.

4.3.5. Screening for antifungal activities of the crude extract

The antifungal activity of the crude extract was assessed using the paper disk diffusion method described by Taechowisan *et al*. (2005) with some modifications. Briefly, each Petri plate containing 15 mL of PDA was inoculated in the centre with 5 mm mycelial disc cut from the periphery of 10-day-old cultures of each pathogenic fungus. Four 5 mm sterile paper discs per plates were loaded with 200 µg of crude extract dissolved in methanol (MeOH) and placed on the agar surface. Plates were incubated at 28°C in the dark and radial growths were measured when the control fungal colony margin touched the control disks filled only with MeOH. Results were expressed as the percentage of mycelial growth inhibition calculated as follows: [(RC-RT)/RC] ×100 with RC= colony radius in control plates and RT= colony radius in test plates. Three replicates were used for each treatment. Seven plant pathogenic fungi having a wide host range and worldwide distribution were used: *Pythium ultimum* MUCL 16164, *Sclerotinia sclerotiorum* MUCL 11553, *Rhizoctonia solani* MUCL 9417, *Colletotrichum gloeosporioides* MUCL 42003, *Cryphonectria parasitica* MUCL 7956, *Fusarium oxysporum* MUCL 7611 and *Pyrenophora tritici-repentis* MUCL 42329. In addition, two very common human dermatophytes were used as test organisms in the survey: *Epidermophyton floccosum* MUCL 39810 and *Trichophyton rubrum* MUCL 29301.

4.3.6. Direct bioautography on thin layer chromatography (TLC) plate for bioassay-guided isolation of active compounds of the TS3RO crude extracts

Direct TLC bioautography with *Cladosporium cucumerinum* MUCL 10091 was performed throughout the purification steps as bioassay-guided fractionation tool according to a modified method described by Homans & Fuchs (1970). Twenty microliters of the crude extracts (20 µg μL^{-1}) or the fractions (5 µg μL^{-1}) to be purified were applied to precoated silica gel TLC plates developed with the mixture ethyl acetate, methanol and water (100:13.5:10) and allowed to stand overnight under a stream of cool air for complete removal of solvents. The developed and dried TLC plates were sprayed with a spore suspension of *C. cucumerinum* in 2% malt agar and plates were incubated for 72 h in the dark in a moist atmosphere at 28°C. After the incubation period, the inhibition of fungal growth was assessed by visual examination. Active fractions appeared as clear inhibition zones against a dark background. Nystatin, a polyene antifungal drug to which *C. cucumerinum* is sensitive, was used as the positive control (1 µg).

4.3.7. Isolation of active compound from strain TS3RO and structure elucidation

Solvents were distilled before use and those for spectroscopic measurements were of high-pressure liquid chromatography (HPLC) grade. The crude extract (3 g) was initially fractionated by vacuum liquid chromatography on a silica gel 60 (Merck, Darmstadt, Germany) (0.2–0.5 mm) eluted successively with a mixture of dichloromethane (DCM) and MeOH (20:1 and 3:1). The active DCM-MeOH (3:1) extract was loaded at the top of an open column chromatography (50 × 1.5 cm) using silica gel 60

(0.063–0.2 mm) eluted with the mixture ethyl acetate, methanol, water (100:13.5:10). About 10 mL per tube were collected. Tubes containing active fractions were combined and subjected to Sephadex LH-20 (Sigma Aldrich, St. Louis, MO) (80 × 1 cm) eluted with (DCM-MeOH) (1:1). Active fractions were then purified by semi-preparative HPLC on a Prep Nova-Pak HR C18 column (6 µm, 30 × 300 mm) from Waters (Millipore corporation, Milford, MA). The elution was isocratic at 1 mL min^{-1}using acetonitrile-methanol-water (30:35:35) with 0.5% acetic acid as mobile phase. HPLC purifications were conducted on a Waters 600 Controller equipped with a Waters 481 Lambda Max LC spectrophotometer. An ultimate purification on Sephadex LH-20 with DCM-MEOH (1:1) yielded 4 mg of fine yellow needles referred here as compound A.

Compound A was then analyzed by liquid chromatography with mass spectrometry detection (LC-MS) and nuclear magnetic resonance (NMR) to determine its molecular weight and structure. Mass spectra in positive ion mode scanning from *m/z* 100 to *m/z* 1500 were acquired with a Thermo Scientific LTQ orbitrap XL mass spectrometer, equipped with an atmospheric-pressure chemical ionization (APCI) source, a Merck-Hitachi L6200 Intelligent Pump, a Rheodyne® 7125 manual injection valve with a 20 µL sample loop, a Kontron HPLC-322 UV detector, and the Xcalibur® 1.3 software. NMR data were measured in methanol, on a Bruker 500 NMR spectrometer with ^{1}H NMR spectra observed at 500 MHz and ^{13}C NMR spectra at 125 MHz in CD_3OD. IR spectra were recorded on a Perkin Elmer type FTIR 286 spectrometer with KBr.

4.4. Results and Discussion

4.4.1. Isolation and identification of strain TS3RO

No colony was observed on nutrient agar surface printed with the surface-sterilized stems after 3 weeks of incubation period. This suggested that all the epiphytes at the outer surface of the stems were suppressed and the surface-disinfection procedure was effective. In addition, isolates that emerged from the small stem discs did not survive the vitality test when they were re-subjected to surface sterilization, supporting their endophytic origin.

After 3 weeks of incubation, only three out of 200 small discs recorded colonies which were pale and hard on nutrient agar but switched to white-pinkish aerial mycelia after they were transferred onto PDA medium. The three colonies displayed the same morphological, cultural and cytological characteristics and could therefore be assigned to one morphospecies, designated here TS3RO. At first examination of the organism with a stereomicroscope, hyphae and spores were observed suspecting streptomycete-like organism. Thus, our isolation study indicated that *C. roseus* stems are seemingly rarely colonized by streptomycetes. Rare occurrence of endophytic actinomycetes in the aerial parts of *C. roseus* was also reported by Sharma *et al*. (2011). Only, seven endophytic actinomycetes were isolated from *C. roseus* twigs collected from different regions of India, suggesting a low level of colonization by this group of microorganisms in the aerial parts of *C. roseus*. This result is in agreement with those of other studies, which showed that *Streptomyces* and actinomycetes in general were less frequently recovered from the aerial parts of the plant than from the roots (Taechowisan *et al.* 2003; Verma *et al*., 2009). This trend could be explained by the fact that actinomycetes are common rhizosphere bacteria that could enter plants via roots where their occurrence are relatively high,

and then colonize the aerial parts of plants after overcoming the anatomic barrier of the internal plant tissues as well as the defense mechanisms of plants. On the other hand, a higher isolation rate was recorded by Kafur & Khan (2011) who isolated 38 isolates of endophytic actinomycetes from *C. roseus* leaves collected from different locations in Puducherry, India. Our study, however, could not be compared with that previous investigation (Kafur & Khan, 2011) since the endophytic microbiota and the frequency of a given endophytic species may be influenced by diverse factors including geographical and local site factors, diversity of the plant community, plant age and tissues, season of the collection, and the isolation procedure (Kowalski & Kehr, 1996).

The phenotypic properties were in accordance with the assignment of strain TS3RO to the genus *Streptomyces*. The cultural characteristics of the strain are shown in Table 7. Strain TS3RO developed well on most ISP media producing aerial mycelium and an extensively branched substrate mycelium. Colony on MBA and ISP medium 3 had a cottony consistency. Melanin pigments were produced from tyrosine (ISP medium 6). Microscopic observations using light microscopy at 40 X magnification showed that aerial mycelium consisted of long straight mycelial filaments bearing at more or less regular intervals verticils of branches displaying a typical "barbed wire" appearance (Fig. 13). No sclerotic granules, sporangium or zoospores were observed. SEM examination revealed that spores had a smooth surface and were produced from verticils. Such morphological characteristics are typical of strains previously assigned to whorl-forming *Streptomyces* species.

Table 7. Growth and cultural characteristics of strain TS3RO on various media.

Media	Growth	Aerial mycelium color	Substrate mycelium color	Soluble pigment
MBA	Abundant	Reddish	Red, pink	None
PDA	Abundant	Reddish, pink	Red-pink	None
ISP2	Abundant	Pink	Red-pink	Light brown
ISP3	Abundant	Light red	Pink	None
ISP4	Abundant	Light pink	Light pink	None
ISP5	Abundant	Light pink	Light pink	Light brown
ISP6	Moderate	None	Light brown	Light brown
ISP7	Abundant	Light pink	Light pink	None

The almost complete 16S ribosomal RNA gene sequence of strain TS3RO yielded 1476 nucleotides (nt) (GenBank accession number FR854235). Using nucleotide BLAST search, it was found to belong to the genus *Streptomyces* sp. Comparison of its almost full-length 16S rRNA gene sequence to those of *Streptomyces* species deposited in the international nucleotide sequence library EMBL showed that strain TS3RO shared high percentage 16S rRNA gene sequence nucleotide similarity values with other members of *Streptomyces* spp. The highest sequence similarities (> 98.9% for the complete sequences) were found with *S. cinnamoneus* subsp. *sparsus* JCM 10107 (99.5), *S. cinnamoneus* subsp. *lanosus* JCM 10106 (99.5), *S. hygroscopicus* subsp. *angustmyceticus* NRRL B-2347 (99.5), *S. thioluteus* LMG 20253 (99.4), *S. lilanicus* LMG 20059 (99.3), *S. subrutilus* NRRL B-12377 (99.2), *S. morookaensis* LMG 20074 (99.1), *S. luteosporeus* NRRL 2401 (99.1), *S. blastmyceticus* NRRL B-5480 (99.0), *S. varsoviensis* NRRL B-3589 (99.0), *S. mobaraensis* NRRL B-3729 (99.0), *S. griseocarneus* DSM 40004 (98.9), *S. ardus* DSM 40527 (98.9) and *S. eurocidicus* NRRL B-1676 (98.9). These values corresponded to 6–27 nt differences out of 1476 nt positions. Strain TS3RO might belong to one of these species. However, it

was reported that several validly described species that shared high percentage 16S rRNA gene sequence nucleotide similarity values within the ranges we found in this study could be separated to distinct species using a set of phenotypic properties (Kim *et al*. 1999, 2000).

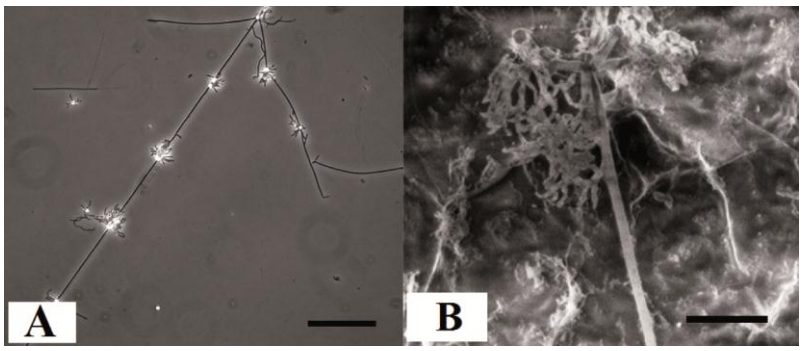


Fig. 13. Aerial mycelium of strain TS3RO. (A) Long, straight aerial mycelium bearing verticils at more or less regular intervals, light microscopy. Bar, 30 µm. (B) Verticil bearing umbels of spore chains, scanning electron microscopy. Bar, 5 µm.

Strain TS3RO was therefore compared phenotypically with other whorl-forming *Streptomyces* sharing high percentage of rDNA sequence similarities (> 99.0%). The diagnostic characteristics of strain TS3RO and its closely related strains are listed in Table 8. According to phenotypic properties and 16S rRNA gene sequence comparison, *S. cinnamoneus* subsp. *sparsus* ATCC 25185^T was the closest species to strain TS3RO. However, this latter can readily be differentiated from *S. cinnamoneus* subsp. *sparsus* by at least four differences in phenotypic traits. Strain TS3RO is able to grow at 10°C. Melanine is produced from tyrosine. Xanthine and starch are not degraded. In addition, the nucleotide sequence of strain TS3RO differed from the corresponding sequences of *S. cinnamoneus* subsp. *sparsus* by 6 nt differences at 1423 sites. Even though, both species gathered within the same clade in the phylogenetic tree the phylogenetic position of strain

TS3RO was rather far from *S. cinnamoneus* subsp. *sparsus* (Fig. 14). Based on the observed taxonomic properties strain TS3RO might then be assigned to at least a new subspecies within *S. cinnamoneus* groups. This latter already includes subspecies *cinnamoneus*, *albosporus*, *azacoluta*, *lanosus* and *sparsus* (Hatano *et al*., 2003).

Table 8. Diagnostic characteristics of TS3RO strain and its closest strains [a].

Taxa	Hydrolysis of			Acid production from:								Melanin from tyrosine	Growth in 5% NaCl	Growth at 10°C	Aerial mycelium color
	Xanthine	Starch	Esculin	Cellobiose	Fructose	Galactose	Mannitol	Salicin	Sucrose	Trehalose					
strain TS3RO	-	-	+	-	-	-	-	-	-	+	+	+	+	+	Pink red
S. cinnamoneus subsp. *sparsus* ATCC 25185^T	+	+	+	-	-	-	-	-	-	+	-	+	-		Reddish, cinnamon
S. cinnamoneus subsp. *lanosus* ATCC 25187^T	+	+	-	-	-	-	-	-	+	+	-	+	-		Cinnamon
S. thioluteus ATCC 12310^T	+	+	+	-	-	-	-	+	-	-	-	+	-		Beige
S. lilacinus NRRL B-1968^T	-	+	+	-	-	-	-	-	+	+	+	-	+		Cinnamon
S. morookaense NRRL B-12429^T	-	+	+	-	+	+	+	+	-	+	-	+	+		Gray
S. blastmyceticus NRRL B-5480^T	+	+	+	-	+	+	-	-	-	+	+	+	+		Bald
S. mobaraensis NRRL B-3729^T	+	-	+	+	-	-	-	-	+	+	+	+	-		Mouse gray

[a] Phenotypic data of other species were retrieved from Labeda *et al*. (1997)

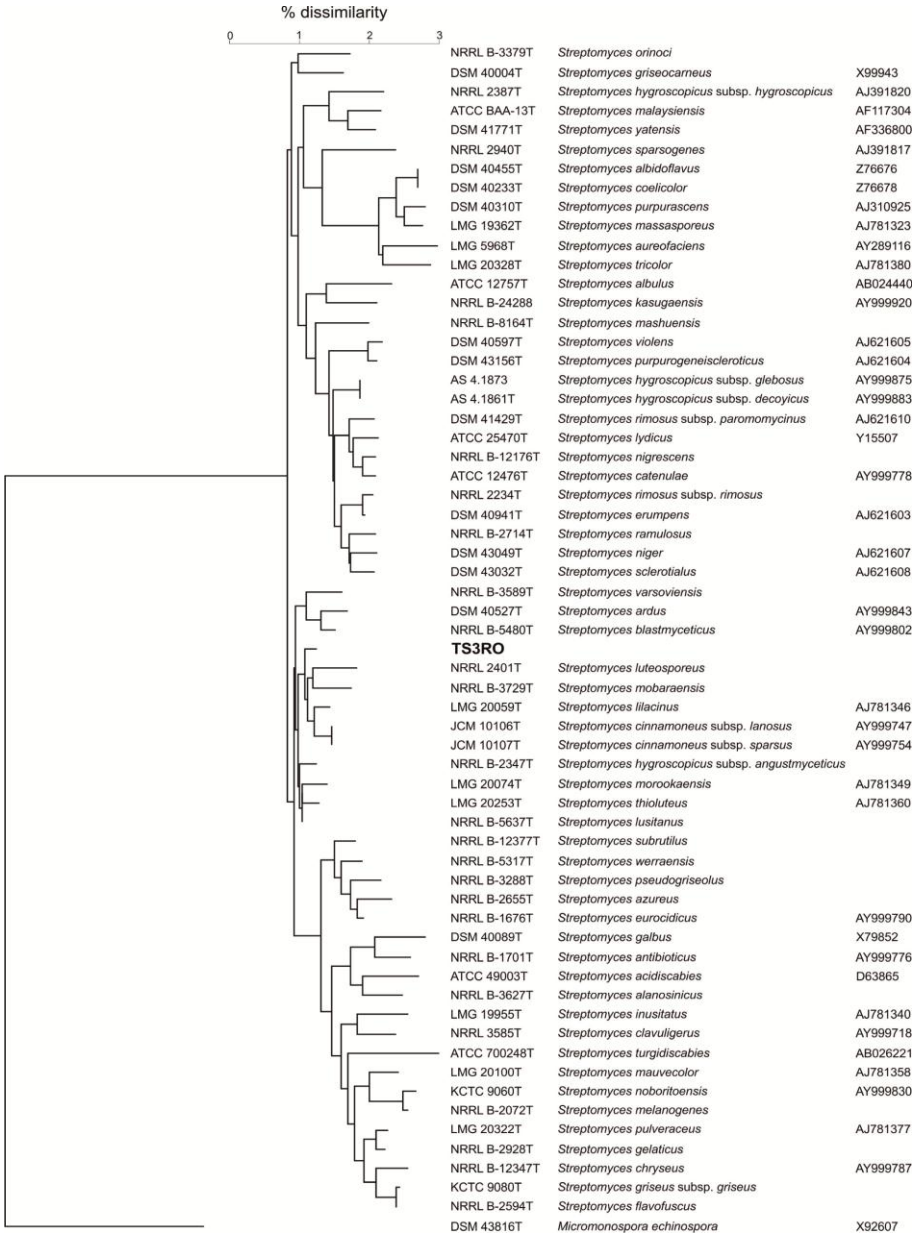


Fig. 14. Neighbour-joining tree based on 16S rRNA gene sequences showing relationships between strain TS3RO and related species of *Streptomyces*. *Micromonospora echinispora* DSM 43816^T was used as an outgroup.

4.4.2. Antifungal activity of the crude extract

Preliminary screening of the ethyl acetate crude extract from the fermentative broth of TS3RO isolate showed antifungal activity on a wide spectrum of test organisms, i.e. against agricultural important plant fungal pathogens as well as human fungal dermatophytes (Fig. 15). The crude extract was very active against *C. gloeosporioides*, the causative agent of anthracnose in a wide range of valuable crops and fruits (Chen *et al*., 2006; Freeman *et al*., 1998), with 53% growth inhibition (GI=53%). The radial mycelium growth of *C. gloeosporioides* was abruptly stopped far from the paper disks loaded with the crude antibiotic, suggesting that active compounds diffused within the agar (Fig. 15A).

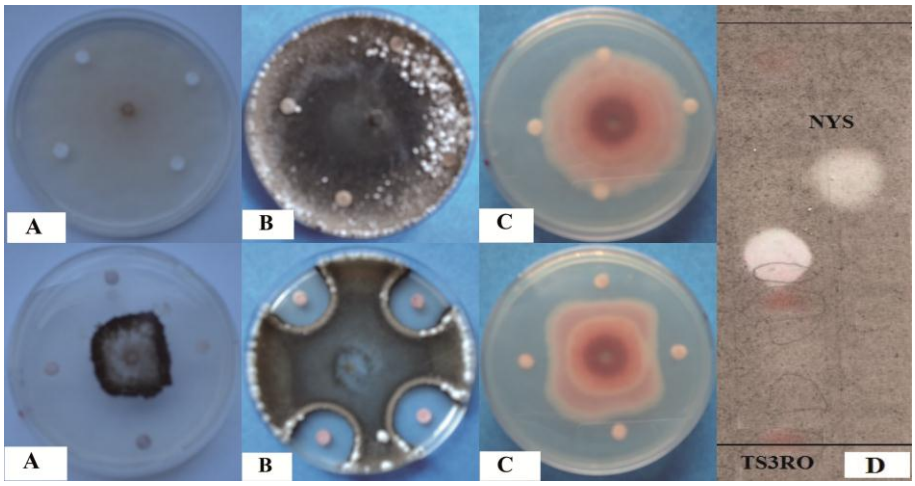


Fig. 15. Zones of growth inhibition induced by crude extract of *Streptomyces* sp. strain TS3RO. (A) *Colletotrichum gloeosporioides* on Petri plate. (B) *Sclerotinia sclerotiorum* on Petri plate. (C) *Fusarium oxysporum* on Petri plate. (D) *Cladosporium cucumerium* on TLC plate using direct bioautography with nystatin (NYS) as control positif. Control plates (above), paper disks were filled with MeOH. Test plates (below), paper disks were loaded with 200 µg of crude extract.

The zone of inhibition varied for the fungi. The inhibition of the radial growth of *F. oxysporum* (Fig. 15C) was the least pronounced (GI=25%), whereas clear zones of inhibition were observed around the paper disks loaded with the crude extract tested on *S. sclerotiorum* (GI=33.7%) (Fig. 15B), *R. solani* (GI=41%), *C. parasitica* (GI=33.3%) and *P. tritici-repentis* (GI=42.5%). TS3RO crude extract was also inhibitory to human dermatophytes, *Epidermophyton flocossum* (GI=58%) and *Trichophyton rubrum* (GI=62%). Interestingly, *Pythium ultimum*, a member of the oomycete was insensitive to the crude extract. Unlike fungi, oomycete cytoplasmic membranes lack ergosterol (Latijnhouwers *et al*., 2003), suggesting that TS3RO crude extract likely contained compounds that specifically bind to cell membrane sterol, primarily ergosterol, as polyene antibiotics do.

4.4.3. Purification and structural analysis of active compound

Direct TLC bioautography with *C. cucumerinum* was conducted for bioassay guided isolation of the compound(s) responsible for the observed antifungal activity of the crude extract. The technique permitted the localization of active constituent on the TLC plate and guided the isolation of pure compound with antifungal property throughout the purification steps (Hostettmann & Marston, 1994). Various natural products with antifungal property were obtained using this technique (Alves *et al*., 2003; Danelutte *et al*., 2003). A clear inhibition zone at Rf 0.45 was recorded when TS3RO crude extract or active fractions were subjected onto TLC bioautography using the mixture ethyl acetate, methanol and water (100:13.5:10) as mobile phase (Fig. 15D). At the end of the purification steps, 4 mg of fine yellow needles (named compound A) with antifungal activity was obtained from 3 g of crude extract.

The *m/z* spectrum of compound A showed dominant ions $[M+H]^+$ at 671.399, under positive ionisation conditions (Fig. 16A). Thus, the measured molecular weight was deduced to be 670.399, in good agreement with the calculated value of 670.828 of the molecular formula $C_{35}H_{58}O_{12}$. The infrared spectrum (KBr) (Fig. 16B) showed common feature of macrolide polyene antibiotic with absorption bands at 1/1717 cm, suggesting the presence of a carbonyl group of the lactone ring, at 1/3376 cm indicating several hydroxyl groups and at 1/850 cm a characteristic feature of methylpentaene (Whittfield *et al*., 1955). The UV spectrum of compound A in MeOH (Fig. 16C) was also in accordance with that of polyene compounds of the methylpentaene group with absorption maxima at 323, 339 and 358 nm (Berdy, 1980). A database searches showed that fungichromin, a methylpentaene antibiotic had a molecular mass of 670 Da. Furthermore, the ^{1}H NMR and the ^{13}C NMR data of compound A were consistent with those of fungichromin (Nogushi *et al*., 1988). We therefore concluded that the main antifungal compound of *Streptomyces* sp. strain TS3RO was fungichromin.

Polyene macrolide antibiotics are naturally occurring compounds that are commonly produced by actinomycetes mainly those belonging to the genus *Streptomyces*. Among the *S. cinnamoneus* group the subspecies *cinnamoneus* was known to produce fungichromin (Robison *et al*., 1971). This methylpentaene macrolide antibiotic was also produced by several *Streptomyces* species including *S. griseus* (Pandey *et al*., 1980), *S. cellulosae* (Nogushi *et al*., 1988) and *S. padanus* PMS 702 (Shih *et al*. 2003). It was demonstrated that fungichromin had inhibitory properties against *Rhizoctonia solani* (Shih *et al*., 2003) responsible for damping-off of seeds and seedlings in various crops. The antifungal activity of polyene macrolides was attributed to their interaction with cell membrane sterols resulting in the

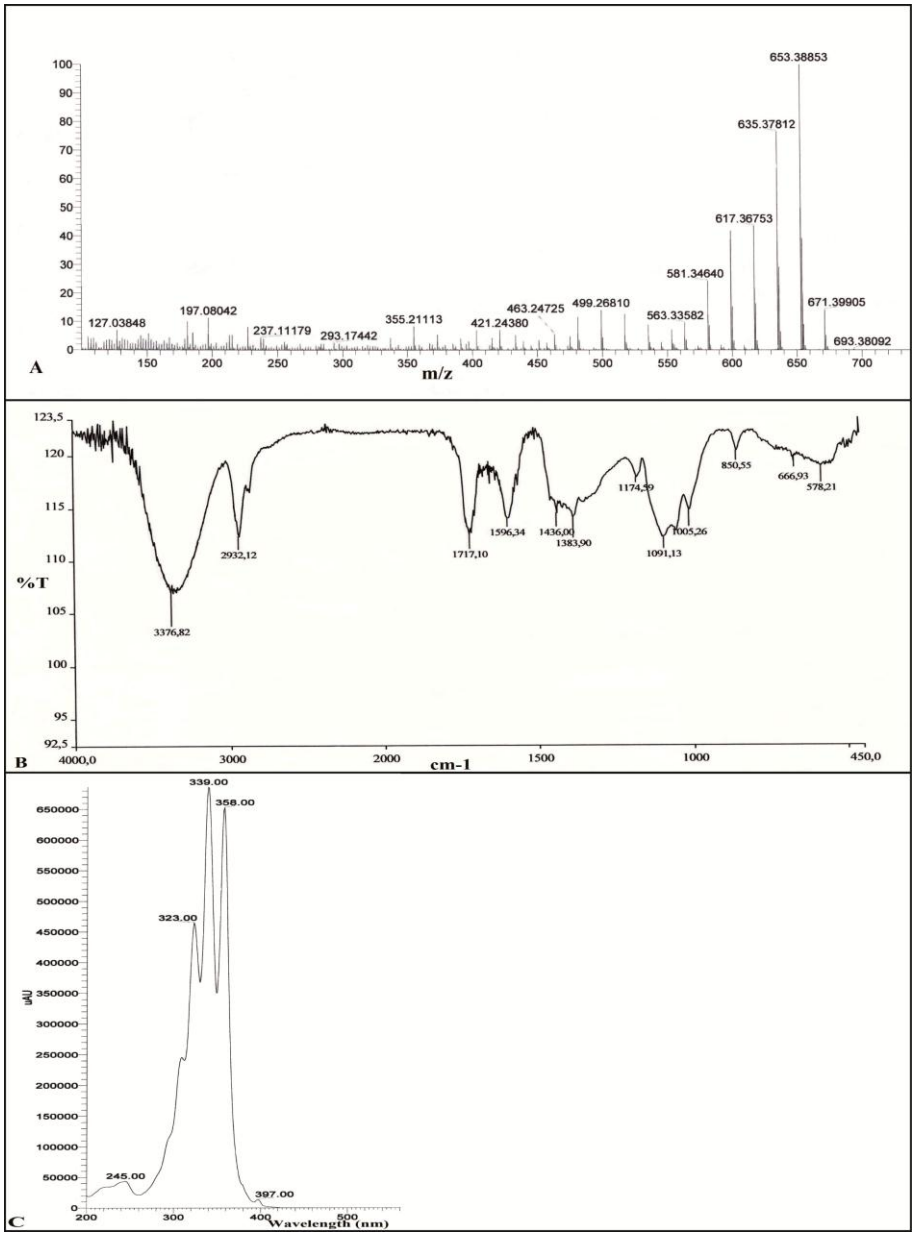


Fig. 16. Structural analysis of compound A. (A) mass spectrum in positive ionisation. (B) infrared spectrum on KBr. (C) UV-visible absorption.

formation of transmembrane pores, which lead to leakage of ions and small inorganic molecules from the cytoplasm and eventually to the cell damage or

death (De Kruijff *et al*., 1974; Bolard, 1986). In recent years, considerable efforts have been given to optimize cultural conditions in order to enhance fungichromin production (Huang *et al*., 2007; Wu *et al*., 2008) since some novel applications using fungichromin as treatment for skin yeast infections have been developed (Hedgpeth, 2005).

In conclusion, we demonstrated in this study that the whorl-forming *Streptomyces* sp. strain TS3RO is a new subspecies in the *S. cinnamoneus* group. More detailed investigations, notably the DNA-DNA relatedness between strain TS3RO and species in the *S. cinnamoneus* group would help in the future to better determine the exact taxonomic status of this newly isolated endophytic *Streptomyces* from *C. roseus* stem. Crude extract of *Streptomyces* sp. strain TS3RO and its major active compound, fungichromin, reduced the mycelial growth of most of the plant pathogenic fungi included in this survey. Further study is needed to verify the ability of *Streptomyces* sp. strain TS3RO in the biological control of plant diseases caused by these pathogens.

Acknowledgements

E.F.R. is a recipient of a fellowship from the ADRI (Administration de la Relation Internationale Belge). We gratefully acknowledge Dr S. Van Trappen (BCCM^{TM}/LMG) for helping in the identification of the strain and performing the phylogenetic tree. The authors gratefully thank the Belgian National Fund for Scientific Research (FNRS) (FRFC 2.4555.08), the Special Fund for Research (FSR) and the faculty of medicine of UCL.

GENERAL DISCUSSION

General discussion

The main results have been discussed extensively in the different chapters (papers) of this thesis. Therefore we took the option to discuss more deeply the methodology followed and some relevant results which have been influenced by the overall strategy adopted.

The main objective of this thesis was to investigate the microbial endophytes diversity from two Madagascan medicinal plants, *Centella asiatica* and *Catharanthus roseus* and to characterize their bioactive metabolites, especially their antifungal activities.

With respect to *C. asiatica*, leaves were collected in Mangoro region where Randriamampionona *et al*. (2007) observed the highest content in asiaticoside and total triterpenoid compared to *C. asiatica* samples from six different locations in Madagascar. It was supposed that the observed difference in triterpenoid content was attributed to genetic variability (Randriamampionona *et al*., 2007). However, the likely implication of endophytes could not be ruled out, because there are ample evidence that endophytes have the ability to enhance secondary metabolites production in plants (Yuan *et al*., 2007; Hao *et al*., 2010). This justified their isolation from the collected leaves for further investigation and possible subsequent valorization. For example, inoculation of the growth-promoting endophytic fungus *Piriformospora indica* to roots of *in vitro* plants of *C. asiatica* resulted in the rapid enhancement of root and shoot biomass of the host plant

and an increase in asiaticoside production in the leaves (Satheesan *et al*., 2012).

Studies on microbial endophytes have been booming since the discovery of taxol-producing endophytes from *Taxus* sp. (Stierle *et al*., 1993). During their long co-evolution, host plants and endophytes may have exchanged genetic materials and as a result, some endophytes, mostly endophytic fungi, were reported to have the ability to produce the same or similar bioactive compounds as those originated from their host plants such as paclitaxel, podophyllotoxin, camptothecine, vinblastine, hypericin, and diosgenin (Zhao *et al*., 2011). This finding is of great importance because it provides an alternative and promising approach to produce these valuable compounds from microbes rather than from plants. This may, in the future, help to reduce the over-collection of medicinal plants thanks to microbial engineering. As regards to *C. roseus*, for instance, it was reported that an endophytic fungus *Alternaria* sp. isolated from the phloem of this plant could produce vinblastine (Guo *et al*., 1998), while a *Fusarium oxysporum* of *C. roseus* (Zhang *et al*., 2000) and a mycelia sterilia 97CY3 endophyte from leaves of this plant (Yang *et al*., 2004) had the ability to produce vincristine. In addition to their ability to synthesize the same product originated from the host plants, endophytes have proved to be a rich source of bioactive compounds with various bioactivities (Tan & Zou, 2001; Strobel & Daisy, 2003; Joseph & Priya, 2011).

In the present study, we drew our attention on the antimicrobial activity of the secondary metabolites from microbial endophytes because the increasing levels of drug resistance by plant and human pathogens and the incidence of life-threatening fungal infections in immuno-compromised patients are becoming increasingly a community concern. Thus, there is an urgent need to discover novel antimicrobial compounds that is highly effective, possess

low toxicity, and will have a minor environmental impact (Strobel *et al*., 2004).

In this thesis, microbial endophytes were isolated from *C. asiatica* following surface disinfection of plant tissues and plating onto appropriate growth media (Schulz & Boyle, 2005). There are various microbiological isolation procedures (Bills, 1996), but all aim to eliminate epiphytic microorganisms from the surface of the plant tissues while preserving the endophytic microorganisms inside. In chapter I, II and IV, a three-step method (70-75% ethanol for 1 min, 3% calcium hypochlorite for 10 min, and 70-75% ethanol for 30 s) was found effective to sterilize the surface of *C. asiatica* leaves and *C. roseus* stems. No microbial growth was observed on malt extract agar (chapter I), tryptic soya agar (chapter II) and nutrient agar surface (chapter IV) after a 3 weeks incubation period either when aliquots of the final rinsing water were plated on the media or when the sterilized tissues were imprinted on the media surface (Schultz *et al*., 1998). Thus we concluded that all isolates recovered from this study were of endophytic-origin. The endophytic nature of the isolates (chapter IV) was further supported by performing the vitality test described by Petrini (1984) in which no microbial isolates survived when they were re-subjected to surface sterilization.

The disinfection method used allowed a quick isolation of culturable endophytes, but may have underestimated the whole endophytic community of the plant since only microorganisms that can grow under the cultural conditions used in the study were isolated (Hyde & Soytong, 2007). Disinfectants may also penetrate the plant tissues and cause killing effects on endophytes. In addition, rapid growing endophytes may conceal slow growing ones. According to Gamboa & Bayman (2001) the size of the plant fragments sampled had a strong impact on the number and the diversity of endophytic community. The smaller the plant fragments sampled, the higher

the number and the diversity of the isolated endophytes. In this study we took into account all these parameters in order to increase the probability to isolate the largest number of endophytes and to yield the highest value of microbial diversity. Calcium hypochlorite was preferred to sodium hypochlorite as disinfectant as calcium hypochlorite was reported to penetrate the tissue to a lesser extent than sodium hypochlorite (Boccon-Gibod, 1982). Leaves of *Centella asiatica* and stems of *Catharanthus roseus* were cut completely into very small segments of approximately 2 × 2 mm (chapter I) and into small discs of 3 × 2 mm (chapter IV), respectively and the emerging hyphae and colony were transferred immediately with the plant material to avoid that the growing mycelium masked the growth of the others. Eventually to capture the maximum diversity, morphologically near identical colonies were picked up and identified.

Direct identification of microbial endophytes within plant tissues using DNA-based techniques is an alluring alternative to characterize uncultivable endophytes and to raise the probability to acquire the near whole endophytic community of the plant (Reiter & Sessitsch, 2006; Tao *et al*., 2008). Briefly, these cultivation-independent techniques consist in direct extraction of the genomic DNA of the surface sterilized plant samples, then PCR amplification using bacterial or fungal-specific primers followed by separation methods such as random cloning, denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP) and finally, phylogenetic analysis of the generated sequences. Using this molecular approach, Duong *et al*. (2006) and Tao *et al*. (2008) reported the detection of more abundant fungal endophytes and higher diversities. Likewise, Sun *et al*. (2008) successfully studied endophytic bacterial diversity in rice roots using culture-independent approaches.

Molecular identification of the isolates found in this study has been completed using ribosomal DNA (rDNA) analysis, especially sequencing of the internal transcribed spacer (ITS) region for fungi (chapters I and III) and 16S rRNA gene for bacteria (chapters II and IV). The sequences obtained from the amplification of the genomic DNA of the isolates were compared to sequences available in public databases such as GenBank and EMBL and the identity of the strains was made based on similarity search results. As regards the identification of bacteria (chapters II and IV), there has been no agreement on a universal cut-off for bacterial species using 16S rRNA gene (Woo *et al*., 2009), so we used the threshold values proposed by Drancourt *et al*. (2000) who suggested that a sequence similarity higher than 97% was used as a cut-off for genus identification and by Janda & Abbot (2007) who recommended a minimum of > 99%, and ideally > 99.5% to be used for species identification. All the bacterial strains found in this study were well resolved to the genus level using these criteria. However, the 16S rRNA gene is not discriminative enough when two different bacterial species share similar 16S rRNA gene sequences. We observed the limitation of 16S rRNA gene sequences for instance, in the species diagnosis of *Streptomyces* sp. strain TS3RO (chapter IV), where it shared high percentage 16S rRNA nucleotide sequence similarity values (> 98.9% for the complete sequences) with 14 members of *Streptomyces* spp. In that case, additional phenotypic characters, biochemical tests and sequencing of additional gene loci were needed for definitive identification (Woo *et al*., 2009). Carrying out phenotypic characterization of the strain TS3RO as recommended by the International *Streptomyces* Project (Shirling & Gottlieb, 1966) we could resolve its identity to a new subspecies in *Streptomyces cinnamoneus.* However, the study of DNA-DNA relatedness would help ascertain the accurate taxonomic position of the strain TS3RO.

Sequencing of the ITS region has also been used widely for molecular systematics and identification of fungi from clinical and environmental samples (Henry *et al*., 2000; Iwen *et al*., 2002; O'Brien *et al*., 2005; Bellemain *et al*., 2010). In chapter I, strain NSS1, a sterile mycelium, was found to be the most frequently isolated fungal endophyte from *C. asiatica* collected in Mangoro (Madagascar). Due to the lack of morphological characters, sterile mycelia could not be identified. Using similarity search of the ITS region of strain NSS1 having 579 bp fragment (accession number AM403716) we resolved its belonging to the Xylariaceae. However, strain NSSI differed from *Xylaria longipes* (GenBank accession number AF163038) the closest related species proposed by the BLAST search by 46 bp (91% similarity). Guo *et al*. (2003) and Promputtha *et al*. (2007) have also successfully used the phylogenetic analysis of the ITS sequences in an attempt to identify sterile mycelia. However, caution should be taken when using only ITS sequences similarity for identification, since there have been numerous erroneous ITS sequences deposited in public databases (Nilsson *et al*., 2006).

On the other hand, ITS sequence is not accurate enough to differentiate complex species having similar ITS sequences such as in the genus *Colletotrichum* (Guerber *et al*., 2003; Du *et al*. 2005). In chapter III, we showed that *Colletotrichum gigasporum* could not be distinguished from *Glomerella septospora* which had the same ITS sequences. But separation to distinct species could be achieved using combined morphological characterization and phylogenetic analysis of the β-tubulin gene. In a polyphasic approach for studying *Colletotrichum* species, it was suggested to perform multi-gene phylogenetic analysis for a better resolution of the relationships among *Colletotrichum* species and for an accurate and reliable species diagnosis (Cai *et al*., 2009). In this study, we only sequenced the ITS region and the β-tubulin (tub2) gene, since in the phylogenetic tree of the

ITS sequences the taxonomic position of *C. gigasporum* was well resolved among the main group of straight conidia-producing *Colletotrichum* species and the phylogenetic tree of the β-tubulin gene enabled differentiating of *C. gigasporum* from *G. septospora*.

The mechanisms of sexual reproduction in *Colletotrichum* and the genetic basis of sexual compatibility in this genus remain unclear. Homothallic species were considered self-fertile, being able to produce fertile perithecia containing asci and ascospores by their own. For instance, *C. taiwanense* (Teleomorph, *Glomerella septospora*) was reported homothallic (Svanesan & Hsieh, 1993). In contrast, heterothallic species only produced the perfect stage (Teleomorph) from the outcross of self-sterile strains having opposite mating types. The two complementary mating types found in filamentous ascomycete fungi are termed MAT1-1 and MAT1-2 (Turgeon & Yoder, 2000). Recently, the teleomorph of some *Colletotrichum* species including *C. truncatum* (Armstrong-Cho & Banniza, 2006), *C. lindemuthianum* (Rodríguez-Guerra *et al*., 2005) and *C. acutatum* (Guerber & Correll, 2001) have been successfully produced using mating experiments. Other *Colletotrichum* species were reported to behave both ways being homothallic and heterothallic such as *C. graminicola* (Vaillancourt & Hanau, 1991) and in *C. acutatum* (Damm et al 2011).

In chapter III, we found that the sexual stage was only observed with *C. gigasporum* MUCL 44947 isolated from *C. asiatica*, but *C. gigasporum* MUCL 42034, 42044 and 42077 from *Stylosanthes guianensis* as well as *C. gigasporum* VEGA 508 from *Coffea arabica* failed to produce the teleomorph despite they were grown under the same cultural conditions. On PDA supplemented with 2 g L^{-1} *C. asiatica* powder and exposed constantly under near UV light and cool white fluorescent light isolate MUCL 44947 readily produced the sexual stage. Likewise, Svanesan & Hsieh (1993)

observed that the production of conidiomata and perithecia occurred more abundantly on the sterilized fragments of corn or rice sheaths than on agar.

The effect of illumination and culture media supplemented with host tissue on the sporulation of many fungi is well known (Leach, 1971; El-Fiki *et al*., 1999). Since 1928, Stevens observed the impact of ultra-violet rays on the induction of sexual stages in *Glomerella cingulata*. Low temperatures (16-20°C) and adequate light conditions (12 hours light per day) appeared to favour formation of perithecia in *Pestalotiopsis microspora* (Metz *et al*., 2000). In addition, Metz *et al*. (2000) demonstrated that the methylene chloride extract of yew (*Taxus cupsidata*) needles was able to induce the sexual stage of *P. microspora*, isolate NE-32. This indicated that chemical compounds in yew may be responsible for stimulation of perithercia growth in this fungus. However, it is not still clear how these cultural parameters such as light and temperature as well as plant-derived compounds triggered the MAT genes, which are thought to be necessary for the induction of the sexual cycle and production of sexual structures (Hiscock & Kües, 1999).

Several laboratory or greenhouse studies have demonstrated the beneficial effects of endophytic bacteria to host plants (Senghor *et al*., 2007; Kim *et al*., 2009; Fu *et al*., 2010). In chapter II, we demonstrated the role of several endophytic bacteria to enhance the fitness of *C. asiatica* under *in vitro* culture conditions. The inoculation assays showed that the plants challenged with *Bacillus subtilis* BCA31 and *Pseudomonas fluorescens* BCA08, three days before inoculation with the hemiobiotroph *Colletotrichum higginsianum* MUCL 44942, had a reduced anthracnose disease incidence and severity. The strong beneficial effects of *B. subtilis* BCA31 and *P. fluorescens* BCA08 was highlighted by the severity of the anthracnose symptoms noted on the control *C. asiatica* plants. In these plants, 100% of the leaves had anthracnose symptoms and 72% of the plants had a disease

severity score above 4, i.e., more than 50% of the leaf area was covered with lesions. The isolation study showed that leaves of the control plants contained a single bacterial flora of *Microbacterium* sp. Accordingly, the absence of antagonistic beneficial bacteria made those *in vitro* plants hypersensitive to the anthracnose disease. To the contrary, none of the tested *in vitro* plants had a disease score of 4 or 5 when *B. subtilis* BCA31 alone or in combination with *P. fluorescens* BCA08 was applied to leaves before inoculation with *C. higginsianum* MUCL 44942. The disease incidence rate was 32.2% and 36.3%, respectively.

Various mechanisms have been suggested to explain the role of these antagonistic microorganisms in the biocontrol of plants, including production of antibiotics, enzymes or toxins, competition with pathogens for nutrients and niches within the plant cells, hyperparasitism to reduce pathogen population and induction of plant defense mechanisms (Hallman *et al*., 1997; Kobayashi & Palumbo, 2000; Rajendran *et al*., 2006; Mahadtanapuk *et al*., 2007). In chapter II, we showed that the cell-free culture filtrates of *P. fluorescens* BCA08 and *B. subtilis* BCA31 were highly inhibitory to the growth of *C. higginsianum* MUCL 44942 even at the lowest concentration tested (i.e. at 20% of the culture filtrates incorporated to the malt extract agar) with a growth inhibition of 67% and 90% for BCA08 and BCA31 respectively. This suggested the production of diffusible antifungal compounds in the media. *B. subtilis* is well known to produce a wide variety of peptide antifungal antibiotics such as fengycin, iturins and surfactin (Touré *et al*., 2004; Romero *et al*., 2007) while fluorescent *Pseudomonas* species inhibited growth of numerous fungal plant pathogens due to their ability to produce a large array of bioactive metabolites including pyrrolnitrin (Hashimoto & Hattori, 1966), pyoluteorin (Howell & Stipanovic, 1980), siderophores (Loper, 1988), hydrogen cyanide (Voisard *et al*., 1989), phenazin 1-carboxylic acid (Thomashow *et al*., 1990), 2,4-

diacetyl phloroglucinol (Nowak-Thompson *et al*., 1994), pseudophomins A and B (Pedras *et al*., 2003) and aerugin (Lee *et al*., 2003).

Interestingly, Chen *et al*. (1994) suggested that the biocontrol effect of bacterial strains within plant tissues mainly resulted from enhanced host defense rather than from bacterial metabolites. This suggestion supported the findings of Wei *et al*. (1991) who first indicated that endophytic bacteria could elicit induced systemic resistance. It would be worth studying the impact of some endophytic bacteria found in this study on the defense mechanisms of *C. asiatica* against *Colletotrichum higginsianum*, notably on the salicilic acid- (SA) and ethylene/jasmonate (ET/JA)-dependent defense pathways. In fact, it is commonly admitted that the SA-dependent pathway is mainly involved against biotrophic pathogens, whereas ET/JA-dependent responses are essential against necrotrophic pathogens (Glazebrook, 2005). As *C. higginsianum* displayed both biotrophic and nectrotrophic lifestyles, it is likely that defense mechanisms related to these two pathways would be activated. Liu *et al*. (2007) demonstrated that a disturbance of genes linked to either the SA or ET pathway impaired resistance in *Arabidopsis thaliana* plants to *C. higginsianum*. It would also be interesting to determine the glycerol-3-phosphate (G3P) levels in *C. asiatica* grown in the field since high levels of G3P in plants were reportedly linked to basal resistance to *C. higginsianum* (Chanda *et al*., 2008).

Biological control of plant diseases using antagonistic endophytic bacteria is an interesting alternative to chemical control, given the cost of pesticides, the risks for the environment quality and human health (Perry *et al*., 2008) and the development of resistance to pesticides (Rosenberger & Meyer, 1981). For sustainable plant disease management, antagonistic *Bacillus* species and fluorescent pseudomonads have always been considered as excellent candidates for biocontrol (Bakker *et al*., 2007; Melnick *et al*., 2008, Melnick

et al., 2011) because of their ability to colonize rhizosphere, penetrate and inhabit the internal tissues of plants, occupying the same niches to that occupied by plant pathogens (Hallmann *et al*., 1997). After entering the plant through natural openings such as growing radicels or secondary roots or stomates in the aerial parts, endophytic bacteria may colonize plant tissues either locally or systematically (Hallman *et al*, 1997). Then, the effectiveness of endophytes as biological control agents depends on the population dynamics and pattern of host colonization, the ability to move within host tissues, and the ability to induce systemic resistance (Backman *et al*., 1997).

We adopted a different approach to investigate the endophytes of *C. roseus*. A targeted isolation of *Streptomyces* sp. was undertaken from this plant because very little information is available on the endophytic bacteria of this medicinal plant. The most recent studies reported mainly fungal endophytes (Zhang *et al*., 2000; Tung *et al*., 2002; Yang *et al*., 2004; Kharwar *et al*., 2008; Krishnamurthy *et al*., 2008). Moreover, the search for *Streptomyces* from diverse environmental sources remains a matter of attention given the enormous potential of this group of microorganisms to synthesize various bioactive compounds (Castillo *et al*., 2003; Shin *et al*., 2008; Taechowisan *et al*., 2007).

The result of the isolation study showed that *Streptomyces* rarely occurred in the stems of *C. roseus*. Only three out of 200 small discs recorded colonies which could be grouped into one morphospecies named TS3RO. This result was in agreement with that of Sharma *et al*. (2011). These authors were only able to isolate seven endophytic actinomycetes from *C. roseus* twigs collected from different regions of India. To the contrary, Kafur & Khan (2011) isolated 38 isolates of endophytic actinomycetes from *C. roseus* leaves collected from different locations in Puducherry, India. This discrepancy may be related to various environmental factors, host plants-

dependent factors (Arnold, 2007; Tao *et al*., 2008) and method-dependent process (Kowalski & Kehr, 1996) that impact the biodiversity of these microorganisms. Comparison with our study could not be made since we only explored one collecting site located at the botanical garden of the "*Institut Malgache de Recherches Appliquées*" Antananarivo, far from the natural habitat of *C. roseus*.

It was found that fungichromin, a pentaene macrolide antibiotic, was the main antifungal compound of *Streptomyces* sp. strain TS3RO. Other polyene macrolides were detected in the fermentative broth of strain TS3RO using high performance liquid chromatography-diode-array detector coupled to electrospray ionization mass spectrometric (HPLC-DAD-ESI-MS) detection in the positive ion mode (data not shown). These findings are in line with those of Bruheim *et al*. (2004) and Cai *et al*. (2007) who showed that *Streptomyces* species frequently produced more than one polyene macrolide antibiotic in the same fermentation broth. The HPLC allowed the separation of the components in the extract, which were then analyzed by the DAD displaying a UV-vis spectrum, before being injected into the MS-ESI to determine the molecular weight and the molecular formula. The UV-Vis spectra obtained from the HPCL-DAD allowed a preliminary identification of the components in the mixture using the characteristic absorption maxima of polyene macrolides, and proved to be a powerful analytical technology extremely valuable for dereplication of compounds. Polyene macrolide antibiotics have characteristic three-peaks UV spectra and the wavelengths of the peak maxima are function of the number of conjugated double bonds (Omara & Tanaka, 1984).

The inhibitory activity of the crude extract of strain TS3RO was at least attributed to fungichromin, which turned out to be the key chemical antifungal substance in the mixture as visualized by direct TLC

bioautography with *Cladosporium cucumerinum*, with a clear inhibition zone at Rf 0.45 using the mixture ethyl acetate, methanol and water (100:13.5:10) as mobile phase (chapter IV). The purification of a single polyene macrolide was and is still being a daunting task because this group of antibiotics is generally a mixture of several analogues or a complex. Bergy & Eble (1968) demonstrated that the pentaene antibiotic filipin was in fact a mixture of filipins I, II, III and IV and should be referred to as the filipin complex. Pandey *et al*. (1982) showed that fungichromin contained one major and several minor components. In this study we achieved the purification of fungichromin (4 mg) using a series of chromatographic techniques (vacuum liquid chromatography, open column chromatography, semi-preparative HPLC and Sephadex LH-20) and direct TLC bioautography with *C. cucumerinum* as a bioassay-guided isolation throughout the purification steps.

Polyene macrolide antibiotics are well known to possess prominent antifungal activity. Unfortunately, very few polyene macrolides, mainly those classified in triene (Rapamycin), tetraene (Nystatin) and heptaene (Amphotericin B), have found a current therapeutic use, due to the relatively high toxicities for mammalian cells and low water solubility (Zotchev, 2003; Nosanchuk, 2006). Recently, considerable efforts have been made to improve the production of fungichromin (Wu *et al*., 2008; Zang *et al*., 2011) since it has been used for the treatment of skin yeast infections (Hedgpeth, 2005) and vaginal infections (Balmer, 2009).

Overall, three major outcomes can be drawn from this study:
(1) There is a considerable potential of the Madagascan medicinal plants as a source of new and undescribed microbial species. This diversity should be explored!

(2) A number of beneficial endophytes isolated from these plants play a central role in the enhancement of their fitness (e.g. resistance to pathogens). They represent major means to control a number of diseases in plants as potential substitutes to chemical pesticides.

(3) Some of these endophytes produce bioactive compounds which are of high interest to the pharmaceutical and agricultural sector. Their isolation, testing and up-scaling needs to be considered.

GENERAL CONCLUSIONS AND PERSPECTIVES

General conclusions and Perspectives

The study of medicinal plants associated-microorganisms is still in its infancy in Madagascar. To date no information is available on endophytes of the Madagascan medicinal plants. However, the flora of Madagascar with its high level of endemicity and its rich diversity represent a hotspot to the discovery of new endophytes that may play diverse functions of interest to the pharmaceutical and agricultural sectors. This thesis has paved the way for many other forthcoming surveys on this pristine field. Here, we investigated the diversity of microbial endophytes from two medicinal plants from Madagascar, *Centella asiatica* and *Catharanthus roseus*, and characterized the antifungal secondary metabolites produced by some antagonistic strains hosted in these plants.

Three basic questions were addressed in the section "research objectives and outline of the thesis" and the following conclusions can be drawn from the present work:

1. Are medicinal plants from Madagascar potential sources for undescribed microbial species?

Our results have demonstrated that *Centella asiatica* and *Catharanthus roseus* are good niches for discovering undescribed microbial species. This is in good agreement with Bills' prediction (1996) that each vascular plant is host to at least two to four undescribed microbial species, specific to that plant species. As outlined in **chapter III**, a new species of

Colletotrichum, named, *Colletotrichum gigasporum* sp. nov., isolated as foliar endophyte from *C. asiatica* was described on the basis of morphological and molecular features. *C. gigasporum* and *G. septospora* are closely related species, since they gathered in the same clade in the phylogenetic analysis of the ITS sequences and both produced long ascospores up to 90 µm, separating them from typical *Glomerella* species. However, *C. gigasporum* and *G. septospora* could readily be separated into distinct species. *Glomerella septospora* mostly produced 3-septate ascospores whereas ascospores produced by the teleomorph of *C. gigasporum* are aseptate to medianly 1-septate. In addition, the phylogenetic analysis of the β-tubulin gene placed *C. gigasporum* and *G. septospora* in separate clades supported by high bootstrap value (83%).

C. gigasporum MUCL44947 readily produced the teleomorph when grown in a culture medium supplemented with sterilized leaf powder of *C. asiatica*. It would be interesting in the future to investigate the genetic basis of the sexual reproduction in *C. gigasporum.* Additional challenge should consider the impact of *C. asiatica* leaf extract on the regulation gene of the sexual stage.

Comparison of DNA sequences with those deposited in GenBank is a useful and rapid tool for the discovery of new or undescribed species from natural sources. Using BLAST searches, isolate BCA29 (**chapter II**), a bacterial endophyte from *C. asiatica,* can be assimilated to an undescribed species within the genus *Cohnella*, based on the proposition of Drancourt *et al*. (2000) who used as the cut-off for species and genus identification a sequence similarity over 99% and 97%, and according to the recommendation of Janda & Abbott (2007) who stated that a minimum of 99%, and ideally 99.5 % sequence similarity should be used for species identification. Isolate BCA29 demonstrated similarity with *Cohnella*

luojiensis HY-22R^T (accession number GQ214052), the closest species proposed by the BLAST search. But about 30 bp differences were noted between their 16S rRNA gene sequences, which accounted for 97% similarity. Further detailed investigations, notably phenotypic characterization, chemotaxonomic analyses and DNA–DNA hybridization are required to determine accurately to species level the taxonomic identity of the isolate BCA29 since we only sequenced 16S rRNA gene.

As demonstrated in **chapter IV**, the whorl-forming *Streptomyces* sp. strain TS3RO isolated from *C. roseus* stems is a new subspecies within the *Streptomyces cinnamoneus* group. Differentiation between *Streptomyces* sp. strain TS3RO and *S. cinnamoneus* subsp. *sparsus*, its closest related species, could be made by means of phenotypic properties, 16S rDNA sequence similarity and phylogenetic analyses. This is the first report of a verticillate *Streptomyces* from *C. roseus*.

Besides, the endophytic microbial community of *C. asiatica* displayed a high level of microbial diversity with a total of 475 fungal isolates (**chapter I**) and 31 bacterial isolates (**chapter II**), belonging to 45 different fungal taxa and 11 different bacterial genera. *C. asiatica* leaves used for the isolation study were collected in Mangoro (Madagascar), where the highest content in asiaticoside and total triterpenoid were recorded among the seven regions explored in Madagascar (Randriamampionona *et al*., 2007). Isolation study showed that the sterile mycelium, NSS1, and *Colletotrichum higginsianum* were the most isolated fungal endophytes with 19% and 13% isolation frequency (IF), respectively **(chapter I)**, whereas species of *Bacillus* (IF = 32%) and *Pseudomonas* (IF = 19%) were the most frequently isolated endophytic bacteria (**chapter II**). Isolation study should be extended to other areas of harvesting *C. asiatica* where different triterpenoid contents were recorded, in order to assess whether microbial endophytes could be involved

in the variability of the triterpenoid content observed. This assumption is supported by evidence that endophytes were reportedly able to convert betulinic acid (Bastos *et al*., 2007), a triterpenoid found in many plants, and in turn, triterpene can influence the colonization pattern of the endophytic microbial community (Mucciarelli *et al*., 2007).

2. Are Madagascan medicinal plants a good niche for the discovery of endophytes that may produce bioactive compounds of pharmaceutical or agricultural importance?

The crude ethyl acetate of the fermentation broth of *Streptomyces* sp. strain TS3RO isolated from *C. roseus* stems possesses good antifungal activity (**chapter IV**). It exhibited strong inhibitory activity against numerous plant and human pathogenic fungi. It turned out that fungichromin, a known methylpentaene macrolide antibiotic, is the main antifungal component of strain TS3RO. Structural elucidation of fungichromin was confirmed by means of LC/MS spectrometry, UV-visible spectra and NMR data.

Streptomyces sp. strain TS3RO is a potential polyene macrolide antibiotics-producing strain. Partial chemical characterizations of components in the semi-purified extract of TS3RO using LC-DAD coupled with a mass spectrometer (LC-DAD-MS) analysis revealed the presence of other polyene macrolide antibiotics. The searches from libraries and database indicated that some of these macrolide antibiotics are likely to be new compounds. Further study should therefore isolate and characterize these polyene macrolides and determine the optimal culture conditions for the production of the desired secondary metabolites.

3. Do endophytic microorganisms have the ability to protect their hosts against pathogens and enhance host fitness?

Under *in vitro* inoculation experiments using *Centella asiatica* plantlets, *Bacillus subtilis* BCA31 effectively controlled foliar anthracnose caused by *Colletotrichum higginsianum* with a control level of 66% (**chapter II**). Also, *Pseudomonas fluorescens* BCA08 significantly reduced the anthracnose disease severity caused by *C. higginsianum* MUCL 44942, but *P. fluorescens* BCA08 control level in the inoculation assays was less pronounced (about 19%) as compared to its antagonistic effects in the dual culture assays, with up to 82% growth inhibition of *C. higginsianum*.

Not only bacteria, but some fungal endophytes of *C. asiatica* would also take part in the inhibition of *C. higginsianum* in the plant. The most frequently isolated endophytic fungi from *C. asiatica* leaves, the sterile mycelium NSS1, and an unidentified ascomycete, designated ASCO1 significantly reduced by 35% and 31%, respectively, the mycelium growth of *C. higginsianum* (**chapter I**). These results suggested that the whole antagonistic endophytes, either bacteria or fungi, may be beneficial to *C. asiatica* for its protection against the pathogen *C. higginsianum*. Among the likely modes of actions of these antagonistic endophytes leading to the apparent inactivity of *C. higginsianum* in *C. asiatica* would be the production of extracellular compounds having antifungal properties on *C. higginsianum* associated with the enhancement of host fitness (**chapter II**). Since the endophytic community of *in vitro C. asiatica* plantlets was found to be composed of a single bacterial flora of *Microbacterium* sp., the impact of these antagonistic bacteria in enhancing host fitness can be evaluated in the inoculation assays. The absence of useful endophytic bacteria in the *in vitro* plants that served as positive controls made them highly susceptible to the anthracnose disease caused by *C. higginsianum*. However, *in vitro C.*

asiatica plantlets became more resistant when endophytic bacteria were applied on leaves three days before inoculation of the pathogen. Further study should therefore be conducted to investigate the nature of these allelochemicals toxic to *C. higginsianum* and assess the biocontrol potential of these beneficial endophytes under field conditions.

Within this thesis we provided information about the biodiversity of microbial endophytes from *C. asiatica*, the protective effect of some antagonistic endophytes on the host plant against the fungal pathogen *C. higginsianum* and the antifungal properties of endophytic *Streptomyces* from *C. roseus* collected in Madagascar.

Overall, a new fungal species, *Colletotrichum gigasporum*, a novel subspecies of *Streptomyces cinnamoneus* characterized morphologically and phylogenetically, and two undescribed species (*Cohnella* sp. BCA29 and *Xylaria* sp. NSS1) discovered by DNA analysis, were revealed. Furthermore, a good polyene macrolides producer, *Streptomyces* sp. strain TS3RO, was found, which would be worth investigating in more thorough study. Finally, a *B. subtilis* BCA31 for potential biocontrol of anthracnose under field conditions was unveiled. These findings offer additional insights on the high potential of endophytes from plants having medicinal virtues as a considerable source of new microbial species and bioactive compounds.

REFERENCES

Abang MM, Winter S, Green KR, Hoffmann P, Mignouna HD, Wolf GA (2002) Molecular identification of *Colletotrichum gloeosporioides* causing anthracnose of yam in Nigeria. Plant Pathology 51: 63–71.

Afkhami ME, Rudgers JA (2009) Endophyte-mediated resistance to herbivores depends on herbivore identity in the wild grass *Festuca subverticillata*. Environmental Entomology 38(4): 1086–1095.

Allen EA, Hoch HC, Steadman JR, Stavely RJ (1991) Influence of leaf surface features on spore deposition and epiphytic growth of phytopathogenic fungi. In: Andrews JH, Hirano SS (Eds.) Microbial ecology of leaves. Springer-Verlag, New York, pp. 87–110.

Alves TMA, Kloos H Zani CL (2003) Eleutherinone, a novel fungitoxic naphthoquinone from *Eleutherine bulbosa* (Iridaceae). Memórias do Instituto Oswaldo Cruz 98(5): 709–712.

Amarji B, Ajazuddin, Raghuwanshi D, Vyas SP, Kanaujia P (2007) Lipid nano spheres (LNSs) for enhanced oral bioavailability of amphotericin B: development and characterization. Journal of Biomedical Nanotechnology 3: 264–269.

Appa-Rao MVR, Srinivasan K, Rao K (1973) The effect of Mandukaparni (*Centella asiatica*) on the general mental ability (Medhya) of mentally retarded children. The Journal of research in Indian medicine 8: 9–16.

Ara N, Rashid M, Amran MS (2008) Comparison of hypotensive and hypolipidemic effects of *Catharanthus roseus* leaves extract with nifedipine on adrenaline induced hypertensive rats. Journal of Biological Sciences 8: 1082–1086.

Araújo JM, Silva AC, Azevedo JL (2000) Isolation of endophytic actinomycetes from roots and leaves of maize (*Zea mays* L.). Brazilian Archives of Biology and Technology 43: 447–451.

Arivudainambi US, Anand TD, Shanmugaiah V, Karunakaran C, Rajendran A (2011) Novel bioactive metabolites producing endophytic fungus *Colletotrichum gloeosporioides* against multidrug-resistant *Staphylococcus aureus*. FEMS immunology and medical microbiology 61(3): 340–345.

Armstrong-Cho CL, Banniza S (2006) *Glomerella truncata* sp. nov., the teleomorph of *Colletotrichum truncatum*. Mycological Research 110: 951–956.

Arnold AE, Maynard Z, Gilbert GS, Coley PD, Kursar TA (2000) Are tropical fungal endophytes hyperdiverse? Ecology Letters 3: 267–274.

Arnold AE, Maynard Z, Gilbert GS (2001) Fungal endophytes in dicotyledonous neotropical trees: patterns of abundance and diversity. Mycological Research 105: 1502–1507.

Arnold AE, Henk DA, Eells RL, Lutzoni FE, Vilgalys R (2007) Diversity and phylogenetic affinities of foliar fungal endophytes in loblolly pine inferred by culturing and environmental PCR. Mycologia 99: 185–206.

Backman PA, Wilson M, Murphy JF (1997) Bacteria for biological control of plant diseases. In: Rechcigl NA, Rechcigl JE (Eds.) Environmentally safe approaches to plant disease control. CRC/Lewis Press, Boca Raton, FL, pp. 95–109.

Bacon CW, Hinton DM (1996) Symptomless endophytic colonisation of maize by *Fusarium moniliforme.* Canadian Journal of Botany 75: 1195–1202.

Bailey JA, Jeger MJ (1992) *Colletotrichum*: Biology, Pathology and Control. Commonwealth Mycological Institute, Wallingford, p.388.

Bakker PAHM, Pieterse CMJ, van Loon LC (2007) Induced systemic resistance by fluorescent *Pseudomonas* spp. Phytopathology 97: 239–243.

Baldacci E (1958) Development in the classification of actinomycetes. Giornale di Microbiologia 6: 10–27.

Balmer JA (2009) Treatment of vaginal infections with intravaginal pentamycin in clinical practice.The Internet Journal of Gynecology and Obstetrics.Volume 11 Number 1.

Bastos DZ, Pimentel IC, de Jesus DA, de Oliveira BH (2007) Biotransformation of betulinic and betulonic acids by fungi. Phytochemisty 68: 834–839.

Belakhov VV, Shenin YL (2007) Synthesis, antifungal, and antiviral activity of hydrophosphoryl derivatives of mycoheptin. Pharmaceutical Chemistry Journal 41(6): 314–318.

Bellemain E, Carlsen T, Brochmann C, Coissac E, Taberlet P, Kauserud H (2010) ITS as an environmental DNA barcode for fungi: an in *silico* reveals potential PCR biases. BMC Microbiology 10: 189.

Berdy J (1980) CRC handbook of antibiotic compounds.CRC Press, Boca Raton, Florida.

Bergy ME, Eble TE (1968) The filipin complex. Biochemistry 7: 653–658.

Bills GF (1996) Isolation and analysis of endophytic fungal communities from woody plants. In: Redlin SC and Carris LM (Eds) Endophytic Fungi in Grasses and Woody Plants: Systematics, Ecology and Evolution. The American Phytopathological Society, Minnesota, pp. 31–65.

Boccon-Gibod J (1982) La technologie de la culture *in vitro*. In: Augé R, Beauchesne G, Boccon-Gibod J, Decourtye L, Digat B, Minier R, Morand JC, Oudin Y, Vidalie H (Eds) La culture *in vitro* et ses applications horticoles. Techniques et Documentation, Lavoisier, Paris, pp. 47–73.

Boddey RM, Urquiaga S, Reis V, Dobereiner J (1991) Biological nitrogen fixation associated with sugarcane. Plant and Soil 137: 111–117.

Boiteau P, Ratsimamanga AR (1956) L'asiaticoside extrait de *Centella asiatica* et ses emplois thérapeutiques dans la cicatrisation des plaies expérimentales et rebelles (lèpre, tuberculose cutanée et lupus). Thérapie 11(1): 125–149.

Bolard J (1986) How do the polyene macrolide antibiotics affect the cellular membrane properties? Biochimica et Biophysica Acta 86: 257–304.

Borris RP (1996) Natural products research: perspectives from a major pharmaceutical company. Journal of Ethnopharmacology 51: 29–38.

Brown KB, Hyde KD, Guest DI (1998) Preliminary studies on endophytic fungal communities of *Musa acuminata* species complex in Hong Kong and Australia. Fungal Diversity 1: 27–58.

Bruheim P, Borgos SEF, Tsan P, Sletta H, Ellingsen TE, Lancelin J-M, Zotchev SB (2004) Chemical diversity of polyene macrolides produced by *Streptomyces noursei* ATCC 11455 and recombinant strain ERD44 with genetically altered polyketide synthase NysC. Antimicrobial Agents Chemotherapy 48(11): 4120–4129.

Bussaban B, Lumyong S, Lumyong P, McKenzie EH, Hyde KD (2001) Endophytic fungi from *Amomum siamense.* Canadian Journal of Microbiology 47: 943–948.

Cai L, Hyde KD, Taylor PWJ, Weir BS, Waller J, Abang MM, Zhang JZ, Yang YL, Phoulivong S, Liu ZY, Prihastuti H, Shivas RG, McKenzie EHC, Johnston PR (2009) A polyphasic approach for studying *Colletotrichum*. Fungal Diversity 39: 183–204.

Cai P, Kong F, Fink P, Ruppen ME, Williamson RT, Keiko T (2007) Polyene antibiotics from *Streptomyces mediocidicus*. Journal of Natural Products 70 (2): 215–219.

Cannon PF, Simmons CM (2002) Diversity and host preference of leaf endophytic fungi in the Iwokrama forest reserve, Guyana. Mycologia 94: 210–220.

Cannon PF, Buddie AG, Bridge PD (2008) The typification of *Colletotrichum gloeosporioides*. Mycotaxon 104: 189–204.

Cao L, Qiu Z, You J, Tan H, Zhou S (2004) Isolation and characterization of endophytic *Streptomyces* strains from surface-sterilized tomato (*Lycopersicon esculentum*) roots. Letters in Applied Microbiology 39: 425–430.

Cao L, Qui Z, You J, Tan H, Zhou S (2005) Isolation and characterization of endophytic *Streptomycetes* antagonists of *Fusarium* wilt pathogen from surface sterilized banana roots. FEMS Microbiology Letters 247: 147–152.

Castillo U, Harper JK, Strobel GA, Sears J, Alesi K, Ford E, Lin J, Hunter M, Maranta M, Ge H, Yaver D, Jensen JB, Porter H, Robison R, Millar D, Hess WM, Condron M, Teplow D (2003) Kakadumycins, novel antibiotics from *Streptomyces* sp. NRRL 30566, an endophyte of *Grevillea pteridifolia*. FEMS Microbiology Letters 224: 183–190.

Castro MS, Fontes W (2005) Plant defense and antimicrobial peptides. Protein and Peptide Letters 12: 11–16.

Chanda B, Venugopal SC, Kulshrestha S, Navarre DA, Downie B, Vaillancourt L, Kachroo A, Kachroo P (2008) Glycerol-3-Phosphate levels are associated with basal resistance to the hemibiotrophic fungus *Colletotrichum higginsianum* in *Arabidopsis*. Plant Physiology 147: 2017–2029.

Chen C, Bauske EM, Muson G, Kloepper JW (1994) Biological control: Potential and population dynamics of endophytic bacteria in a cotton/*Fusarium* wilt system. In: Ryder MH, Stephens PM, Bowen GD (Eds) Improving plant productivity with rhizosphere bacteria Graphic Services, Adelaide, pp. 191–193.

Chen LS, Chu C, Liu CD, Chen RS, Tsay JG (2006) PCR-based detection and differentiation of anthracnose pathogens, *Colletotrichum gloeosporioides* and *Colletotrichum truncatum*, from vegetable soybean in Taiwan. Journal of Phytopathology 154: 654–662.

Cheng CL, Guo JS, Luk J, Koo MW (2004) The healing effects of *Centella extract* and asiaticoside on acetic acid induced gastric ulcers in rats. Life Science 74(18): 2237–2249.

Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, Thompson JD (2003) Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Research 31: 3497–3500.

Chung W-H, Kim J-J, Yamaoka Y, Uzunovic A, Masuya H, Breuil C (2006) *Ophiostoma breviusculum* sp. nov. (Ophiostomatales, Ascomycota) is a new species in the *Ophiostoma piacea* complex associated with bark beetles infesting larch in Japan. Mycologia 98(5): 801–814.

Clay K (1988) Fungal endophytes of grasses: a defense mutualism between plants and fungi. Ecology 69: 10–16.

Clay K (1989) Clavicipitaceous endophytes of grasses: their potential as biocontrol agents. Mycological Research 92: 1–12.

Costa R, Gomes NCM, Peixoto RS, Rumjanek N, Berg G, Mendonça-Hagler LC, Smalla K (2006) Diversity and antagonistic potential of *Pseudomonas* spp. associated to the rhizosphere of maize grown in a subtropical organic farm. Soil Biology and Biochemistry 38: 2434–2447.

Crouch JA, Clarke BB, White J, Hillman BI (2009) Systematic analysis of falcate-spored graminicolous *Colletotrichum* and a description of six new species from warm-season grasses. Mycologia 101: 717–732.

Damm U, Woudenberg JHC, Cannon PF, Crous PW (2009) *Colletotrichum* species with curved conidia from herbaceous hosts. Fungal Diversity 39: 45–87.

Damm U, Baroncelli R, Cai L, Kubo Y, O'Connell R, Weir B, Yoshino K, Cannon PF (2010) *Colletotrichum*: species, ecology and interactions. IMA Fungus: the global mycological journal 1(2): 161–165.

Damm U, Cannon PF, Woudenberg JHC, Johnston PR, Weir BS, Tan YP, Shivas RG, Crous PW (2012) The *Colletotrichum boninense* species complex. Studies in Mycology 73: 1–36.

Danelutte AP, Lago JH, Young MC, Kato MJ (2003) Antifungal flavanones and prenylated hydroquinones from *Piper crassinervium* Kunth. Phytochemistry 64(2): 555–559.

Darisipudi MN, Allam R, Rupanagudi KV, Anders H-J (2011) Polyene macrolide antifungal drugs trigger interleukin-1β secretion by activating the NLRP3 inflammasome. PLoS ONE 6(5):e19588.doi:10.1371.

De Kruijff B, Gerritsen WJ, Oerlemans A, Demel RA, van Deenen LL (1974) Polyene antibiotic-sterol interactions in membranes of *Acholeplasma laidlawii* cells and lecithin liposomes. I. Specificity of the membrane permeability changes induced by the polyene antibiotics. Biochimica et Biophysica Acta 339: 30–43.

De Pinho PG, Gonçalves RF, Valentão P, Pereira DM, Seabra RM, Andrade PB, Sottomayor M (2009) Volatile composition of *Catharanthus roseus* (L.) G. Don using solid-phase microextraction and gas chromatography/mass spectrometry. Journal of Pharmaceutical and Biomedical Analysis 49: 674–685.

Deckert RJ, Melville L, Peterson RL (2001) Structural features of a *Lophodermium* endophyte during the cryptic life-cycle in the foliage of *Pinus strobus*. Mycological Research 105: 991–997.

Dhanasekaran M, Holcomb LA, Hitt AR, Tharakan B, Porter JW, Young KA, Manyam BV (2009) *Centella asiatica* extract selectively decreases amyloid beta levels in hippocampus of Alzheimer's disease animal model. Phytotherapy Research 23(1): 14–19.

Dobelis IN (1986) Magic and Medicine of Plants. Pleasantville, NY. Reader's Digest Association.

Douville Y, Boland GJ (1992) A note on the antibiotic properties of *Bacillus subtilis* against *Colletotrichum trifolii*. Phytoprotection 73: 31–36.

Drancourt M, Bollet C, Carlioz A, Martelin R, Gayral JP, Raoult D (2000) 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. Journal of Clinical Microbiology 38: 3623–3630.

Du M, Schardl CL, Nuckles EM, Vaillancourt LJ (2005) Using mating-type gene sequences for improved phylogenetic resolution of *Colletotrichum* species complexes. Mycologia 97: 641–658.

Duke JA (1985) Handbook of Medicinal Herbs. Boca Raton, FL. CRC Press.

Duong LM, Jeewon R, Lumyong S, Hyde KD (2006) DGGE coupled with ribosomal DNA gene phylogenies reveal uncharacterized fungal phylotypes. Fungal Diversity 23: 121–138.

Dutta A, Batra J, Pandey-Rai S, Singh D, Kumar S, Sen J (2005) Expression of terpenoid indole alkaloid biosynthetic pathway genes corresponds to accumulation of related alkaloids in *Catharanthus roseus* (L.) G. Don. Planta 220: 376–383.

Ecker JA, Massire C, Hall TA *et al*. (2006) Identification of *Acinetobacter* species and genotyping of *Acinetobacter baumannii* by multilocus PCR and mass spectrometry. Journal of Clinical Microbiology 44: 2921–2932.

El-Fiki AII, El-Habbaa GMD, Eid KE, Ismail IA (1999) Induction of better growth and sporulation of *Uromyces fabae* (pers.) de Bary grown in axenic culture. Annals of Agricultural Science, Moshtohor 37 (2): 1187–1200.

El-Shatoury S, El-Kraly O, El-Kazzaz W, Dewedar A (2009) Antimicrobial activities of actinomycetes inhabiting *Achillea fragrantissima* (Family: Compositae). Egyptian Journal of Natural Toxins 6(2): 1–15.

Espinosa-García FJ, Rollinger JL, Langenheim JH (1996) Coastal redwood leaf endophytes: their occurrence, interactions and response to host volatile terpenoids. In: Redlin SC, Carris LM (Eds.) Endophytic Fungi in Grasses and Woody Plants. APS Press, San Diego, California, pp. 101–120.

European Food Safety Authority (2009) Scientific opinion on the use of natamycin (E 235) as a food additive. EFSA Journal 7(12): 1412.

Faeth SH, Hammon KE (1997) Fungal endophytes in oak trees: long-term patterns of abundance and associations with leafminers. Ecology 78: 810–819.

Ferreres F, Pereira DM, Valentão P, Andrade PB, Seabra RM, Sottomayor M (2008) New phenolic compounds and antioxidant potential of *Catharanthus roseus*. Journal of Agricultural and Food Chemistry 56: 9967–9974.

Filippini R, Caniato R, Piovan A, Cappelletti EM (2003) Production of anthocyanins by *Catharanthus roseus*. Fitoterapia 74: 62–67.

Fischhof PK, Moslinger-Gehmayr R, Herrmann WM, Friedmann A, Russmann DL (1996) Therapeutic efficacy of vincamine in dementia. Neuropsycobiology 34(1): 29–35.

Fisher PJ, Anson AE, Petrini O (1986) Fungal endophytes in *Ulex europaeus* and *Ulex galli*. Transactions of the British Mycological Society 86: 153–193.

Fisher PJ, Petrini O, Lappin-Scott M (1992) The distribution of some fungal and bacterial endophytes in maize (*Zea mays* L.). New phytologist 122: 299–305.

Fjærvik E, Zotchev SB (2005) Biosynthesis of the polyene macrolide antibiotic nystatin in *Streptomyces noursei*. Applied Microbiology and Biotechnolgy 67: 436–443.

Freeman EM (1904) The seed fungus of *Lolium temulentum* L., the darnel. Philosophical Transactions of the Royal Society of London, Series B 196: 1–27.

Freeman S, Rodriguez RJ (1993) Genetic conversion of a fungal plant pathogen to a nonpathogenic, endophytic mutualist. Science 260: 75–78.

Freeman S, Katan T (1997) Identification of *Colletotrichum* species responsible for anthracnose and root necrosis of strawberry in Israel. Phytopathology 87: 516–521

Freeman S, Katan T, Shabi E (1998) Characterization of *Colletotrichum* species responsible for anthracnose diseases of various fruits. Plant Disease 82: 596–605.

Fröhlich J, Hyde KD, Petrini O (2000) Endophytic fungi associated with palms. Mycological Research 104: 1202–1212.

Fu G, Huang SL, Ye YF, Wu YG, Cen ZL, Lin SH (2010) Characterization of a bacterial biocontrol strain B106 and its efficacy in controlling banana leaf spot and post-harvest anthracnose diseases. Biological Control 55: 1–10.

Fuhrmann J (1994) Isolation of microorganisms producing antibiotics. In: Weaver RW, Angle S, Bottomley P, Bezdicek D, Smith S, Tabatabai A & Wollum A (Eds.) Methods of Soil Analysis, Part 2. Microbiological and Biochemical properties-SSSA book series, no.5, Madison, WI, pp. 379–405.

Gama-Lopez S, Munaut F, Vander Stappen J, Scheldeman X, Van Damme V (2007) Diversity studies in the interaction between the anthracnose fungus *Colletotrichum gloeosporioides* and its host plant *Stylosanthes* spp. in Mexico. Technical report of Bioversity International, Rome, Italy. ISBN: 978-92-9043-769-7. 82 p.

Gamboa MA, Bayman P (2001) Communities of endophytic fungi in leaves of a tropical timber tree (*Guarea guidonia*: Meliaceae). Biotropica 33: 352–360.

Ganachari MS, Babu SVV, Katare SS (2004) Neuropharmacology of an extract derived from *Centella asiatica*. Pharmaceutical Biology 42(3): 246–252.

Gao F-K, Dai C-C, Liu X-Z (2010) Mechanisms of fungal endophytes in plant protection against pathogens. African Journal of Microbiology Research 4: 1346–1351.

Guerber JC, Correll JC (2001) Characterization of *Glomerella acutatum*, the teleomorph of *Colletotrichum acutatum*. Mycologia 93: 216–229.

Glazebrook J (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annual Review of Phytopathology 43: 205–227.

Glass NL, Donaldson GC (1995) Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Applied and Environmental Microbiology 61(4): 1323–1330.

Glenner GG, Wong CW (1984) Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. Biochemical and Biophysical Research Communications 120: 885–890.

Gordon RE, Barnett DA, Handerhan JE, Pang CHN (1974) *Nocardia coeliaca*, *Nocardia autotrophica*, and the nocardin strain. International Journal of Systematic Bacteriology 24: 54–63.

Griffith GS, Boddy L (1990) Fungal communities in attached angiosperm twigs. I. Decay community development in ash, beech and oak. New phytologist 116: 407–415.

Guerber JC, Liu B, Correll JC, Johnston PR (2003) Characterization of diversity in *Colletotrichum acutatum* sensu lato by sequence analysis of two gene introns, mtDNA and intron RFLPs, and mating compatibility. Mycologia 95: 872–895.

Guo B, Li H, Zhang L (1998) Isolation of the fungus producing vinblastine. Journal of Yunnan University (Natural Science Edition) 20: 214–215.

Guo LD, Huang GR, Wang *et al*. (2003) Molecular identification of white morphotype strains of endophytic fungi from *Pinus tabulaeformis*. Mycological Research 107: 680–688.

Hallmann J, Quadt-Hallmann A, Mahaffee WF, Kloepper JW (1997) Bacterial endophytes in agricultural crops. Canadian Journal of Microbiology 43: 895–914.

Hamilton-Miller JMT (1973) Chemistry and biology of the polyene macrolide antibiotics. Bacteriological Reviews 37: 166–196.

Hanlin RT (1990) Illustrated genera of Ascomycetes. APS Press, Minnesota. 263 p.

Hao G, Du X, Zhao F, Ji H (2010) Fungal endophytes-induced abscisic acid is required for flavonoid accumulation in suspension cells of *Ginkgo biloba*. Biotechnology Letters 32: 305–314.

Harrison DE, Strong R, Sharp ZD *et al*. (2009) Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. Nature 460: 392–395.

Hartman GL, Manandhar JB, Sinclair JB (1986) Incidence of *Colletotrichum* spp. on soybeans and weeds in Illinois and pathogenicity of *Colletotrichum truncatum*. Plant Disease 70: 780–782.

Hassan MN, Afghan S, Hafeez FY (2010) Suppression of red rot caused by *Colletotrichum falcatum* on sugarcane plants using plant growth-promoting rhizobacteria. International Organization for Biological Control 55: 531–542.

Hashimoto M, Hattori K (1966) Isopyrrolnitrin: a metabolite from *Pseudomonas.* Bulletin of the Chemical Society of Japan 39: 410.

Hata K, Atari R, Sone K (2002) Isolation of endophytic fungi from leaves of *Pasania edulis* and their within-leaf distributions. Mycoscience 43: 369–373.

Hatano K, Nishii T, Kasai H (2003) Taxonomic re-evaluation of whorl-forming *Streptomyces* (formerly *Streptoverticillium*) species by using phenotypes, DNA-DNA hybridization and sequences of *gyrB*, and proposal of *Streptomyces luteireticuli* (*ex* Katoh and Arai 1957) corrig., sp. nov., nom. rev. International Journal of Systematic and Evolutionary Microbiology 53: 1519–1529.

Hazen EL, Brown R (1950) Two antifungal agents produced by a soil actinomycete. Science 112: 423.

Hedgpeth DL (2005) ‘‘Adhesive Treatment for Skin Yeast Infections,’’ U.S. Patent, 6942875.

Henry T, Iwen PC, Hinrichs SH (2000) Identification of *Aspergillus* species using internal transcribed spacer regions 1 and 2. Journal of Clinical Microbiology 38: 1510–1515.

Higgins BB (1917) A *Colletotrichum* leafspot of turnips. Journal of Agricultural Research 10: 157–161.

Hiscock S, Kües U (1999) Cellular and molecular mechanisms of sexual incompatibility in plants and fungi. International Review of Cytology 193: 165–295.

Homans AL, Fuchs A (1970) Direct bioautography on thin-layer chromatograms as a method for detecting fungitoxic substances. Journal of Chromatography 51: 327–329.

Hostettmann K, Marston A (1994) Search for new antifungal compounds from higher plants. Pure and Applied Chemistry 66: 2231–2234.

Howell CR, Stipanovic RD (1980) Suppression of *Pythium ultimum*-induced damping-off of cotton seedlings by *Pseudomonas fluorescens* and its antibiotic, pyoluteorin. Phytopathology 70: 712–715.

Huang JW, Shih HD, Huang HC, Chung WC (2007) Effects of nutrients on production of fungichromin by *Streptomyces padanus* PMS-702 and efficacy of control of *Phytophthora infestans*. Canadian Journal of Plant Pathology 29(3): 261–267.

Huang WY, Cai YZ, Xing J, Corke H, Sun M (2007) A potential antioxydant resource: Endophytic fungi from medicinal plants. Economic Botany 61: 14–30.

Huang WY, Cai YZ, Hyde KD, Corke H, Sun M (2008) Biodiversity of endophytic fungi associated with 29 traditional Chinese medicinal plants. Fungal Diversity 33: 61–75.

Hudson JB, Lee MK, Rasoanaivo P (2000) Antiviral activities in plants endemic to Madagascar. Pharmaceutical Biology 38(1): 36–39.

Hyde KD, Soytong K (2007) Understanding microfungal diversity – a critique. Cryptogamie Mycologie 28: 1–9.

Hyde KD, Cai L, Cannon PF, Crouch JA, Crous PW, Damm U, Goodwin PH, Chen H, Johnston PR, Jones EBG, Liu ZY, McKenzie EHC, Moriwaki J, Noireung P, Pennycook SR, Pfenning LH, Prihastuti H, Sato T, Shivas RG, Tan YP, Taylor PWJ, Weir BS, Yang YL, Zhang JZ (2009) *Colletotrichum* – names in current use. Fungal Diversity: 147–182.

Ibáñez JP, Monteverde ML, Goldberg J, Diaz MA, Turconi A (2005) Sirolimus in pediatric renal transplantation. Transplantation Proceedings 37: 682–684.

Inamdar PK, Yeole RD, Ghogare AB, de Souza NJ (1996) Determination of biologically active constituents in *Centella asiatica*. Journal of Chromatography A 742: 127–130.

Iwen PC, Hinrichs SH, Rupp ME (2002) Utilization of the internal transcribed spacer regions as molecular targets to detect and identify human fungal pathogens. Medical Mycology 40: 87–109.

Janda JM, Abbott SL (2007) 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. Journal of Clinical Microbiology 45(9): 2761–2764.

Jiang ZY, Zhang XM, Zhou J (2005) New triterpenoid glycosides from *Centella asiatica*. Helvetica Chimica Acta 88(2): 297–303.

Jones KL (1949) Fresh isolates of actinomycetes in which the presence of sporogeneous aerial mycelia is a fluctuating characteristic. Journal of Bacteriology 57: 141–145.

Jordan MA, Himes RH, Wilson L (1985) Comparison of the effects of vinblastine, vincristine, vindesine, and vinepidine on microtubule dynamics and cell proliferation *in vitro*. Cancer Research 45: 2741–2747.

Joseph B, Priya RM (2011) Bioactive compounds from endophytes and their potential in pharmaceutical effect: a review. American Journal of Biochemistry and Molecular Biology 1: 291–309.

Jumpponen A, Mattson K, Trappe JM (1998) Mycorrhizal functioning of *Phialocephala fortinii* with *Pinus contorta* on glacier forefront soil: interactions with soil nitrogen and organic matter. Mycorrhiza 7: 261–265.

Kafur A, Khan AB (2011) Isolation of endophytic actinomycetes from *Catharanthus roseus* (L.) G. Don leaves and their antimicrobial activity. Iranian Journal of Biotechnology 9(4): 302–306.

Kangatharalingam N, Pierce ML, Essenberg M (2003) A technique for precise inoculation of the internal phyllosphere of cotton with *Xanthomonas campestris* pv. *malvacearum.* Phytopathology 93: 1204–1208.

Kartnig T (1988) Clinical application of *Centella asiatica* (L.) Urb. In: Craker LE and Simon JE (Eds.) Herbs, Spices and Medicinal Plants. Oxyx Press, Phoenix, pp. 145–173.

Kayser O, Olbrich C, Yardley V, Kiderlen AF, Croft SL (2003) Formulation of amphotericin B as nanosuspension for oral administration. International Journal of Pharmaceutics 254: 73–75.

Khan Z, Doty SL (2009) Characterization of bacterial endophytes of sweet potato plants. Plant and Soil 322: 197–207.

Kharwar RN, Verma VC, Strobel G, Ezra D (2008) The endophytic fungal complex of *Catharanthus roseus* (L.) G. Don. Current Science 95(2): 228–233.

Kim B, Sahin N, Minnikin DE, Zakrzewska-Czerwinska J, Modarski M, Goodfellow M (1999) Classification of thermophilic *Streptomyces* including the description of *Streptomyces thermoalcalitolerans* sp. nov. International Journal of Systematic Bacteriology 49: 7–17.

Kim B, Al-Tai AM, Kim SB, Somasundaram P, Goodfellow M (2000) *Streptomyces thermocoprophilus* sp. nov., a cellulase-free endo-xylanase-producing streptomycete. International Journal of Systematic Bacteriology 50: 505–509.

Kim GH, Lim MT, Hur J-S, Yum K-J, Koh YJ (2009) Biological control of tea anthracnose using an antagonistic bacterium of *Bacillus subtilis* isolated from tea leaves. Plant Pathology Journal 25: 99–102.

Kimura Y, Sumiyoshi M, Samukawa K, Satake N, Sakanaka M (2008) Facilitating action of asiaticoside at low doses on burn wound repair and its mechanism. European Journal of Pharmacology 584: 415–423.

Kleemann J, Rincon-Rivera LJ, Takahara H, Neumann U, van Themaat EVL, *et al.* (2012) Sequential delivery of host-induced virulence effectors by appressoria and intracellular hyphae of the phytopathogen *Colletotrichum higginsianum*. PLoS Pathogens 8(4): e1002643. doi:10.1371/journal.ppat.1002643.

Kloepper JW, Ryu C-M (2006) Bacterial endophytes as elicitors of induced systemic resistance. In: Schulz B, Boyle C, Sieber TN (Eds.) Microbial Root Endophytes. Soil Biology Vol. 9. Springer-Verlag, Berlin Heidelberg, pp. 33–52.

Ko KS, Suh JY, Kwon KT, Jung S-I, Park K-H, Kang CI, Chung DR, Peck KR, Song J-H (2007) High rates of resistance to colistin and polymyxin B in subgroups of *Acinetobacter baumannii* isolates from Korea. Journal of Antimicrobial Chemotherapy 60: 1163–1167.

Kobayashi DY, Palumbo JD (2000) Bacterial endophytes and their effects on plants and uses in agriculture. In: Bacon CW, White JF (Eds.) Microbial endophytes. Dekker, New York, pp. 199–233.

Kowalski T, Kehr RD (1996) Fungal endophytes of living branch bases in several European tree species In: Redlin SC, Carris LM (Eds.) Endophytic Fungi in Grasses and Woody Plants: Systematics, Ecology and Evolution. The American Phytopathological Society, St. Paul, Minnesota, pp. 67–86.

Krishnamurthy RG, Senut MC, Zemke D, Min J, Frenkel MB, Greenberg EJ, Yu SW, Ahn N, Goudreau J, Kassab M, Panickar KS, Majid A (2009) Asiatic acid, a pentacyclic triterpene from *Centella asiatica*, is neuroprotective in a mouse model of focal cerebral ischemia. Journal of Neuroscience Research 87(11): 2541–2550.

Krishnamurthy Y, Naik SB, Jayaram S (2008) Fungal communities in herbaceous medicinal plants from the Malnad region, Southern India. Microbes and Environments 23(1): 24–28.

Kuklinsky-Sobral J, Araújo WL, Mendes R, Geraldi IO, Pizzirani-Kleiner AA, Azevedo JL (2004) Isolation and characterization of soybean associated bacteria and their potential for plant growth promotion. Environmental Microbiology 6(12): 1244–1251.

Kuldau G, Bacon C (2008) Clavicipitaceous endophytes: their ability to enhance resistance of grasses to multiple stresses. Biological Control 46: 57–71.

Kumar MHV, Gupta YK (2003) Effect of *Centella asiatica* on cognition and oxidative stress in an intracerebroventricular streptozotocin model of Alzheimer's disease in rats. Clinical and Experimental Pharmacology & Physiology 30: 336–342.

Kumaresan V, Suryanarayanan TS (2001) Occurrence and distribution of endophytic fungi in mangrove community. Mycological Research 105: 1388–1391.

Labeda DP, Lechevalier MP, Testa RT (1997) *Streptomyces stramineus* sp. nov., a new species of the verticillate streptomycetes. International Journal of Systematic Bacteriology 47(3): 747–753.

Lacava PT, Araújo WL, Marcon J, Maccheroni Jr W, Azevedo JL (2004) Interaction between endophytic bacteria from citrus plants and the phytopathogenic bacteria *Xylella fastidiosa*, causal agent of citrus-variegated chlorosis. Letters in Applied Microbiology 39: 55–59.

Lacava PT, Li W, Araújo WL, Azevedo JL, Hartung JS (2007) The endophyte *Curtobacterium flaccumfaciens* reduces symptoms caused by *Xylella fastidiosa* in *Catharanthus roseus*. Journal of Microbiology 45(5): 388–393.

Latijnhouwers M, de Wit PJGM, Govers F (2003) Oomycetes and fungi: similar weaponry to attack plants. Trends in Microbiology 11: 462–469.

Lechevalier MP, Lechevalier H (1970) Chemical composition as a criterion in the classification of aerobic actinomycetes. International Journal of Systematic Bacteriology 20: 435–443.

Leach CM (1971) A guide to the effects of visible lights and ultraviolet light on fungi. In: Booth C (Ed) Methods in microbiology, Vol. 4. Academic Press, London, pp. 609–664.

Lee JY, Moon SS, Hwang BK (2003) Isolation and antifungal and antioomycete activities of aerugine produced by *Pseudomonas fluorescens* strain MM-B16. Applied and Environmental Microbiology 69: 2023–2031.

Lee SB, Milgroom MG, Taylor JW (1988) A rapid, high yield mini prep method for isolation of total genomic DNA from fungi. Fungal Genetics Newsletter 35: 23–24.

Leuchtmann A (1992) Systematics, distribution and host specificity of grass endophytes. Natural toxins 1: 150–162.

Li C-T, Li Y, Wang Q-J, Sung C-K (2008) Taxol production by *Fusarium arthrosporioides* isolated from yew, *Taxus cuspidata*. Journal of Medical Biochemistry 27: 454–458.

Li HZ, Wan JY, Zhang L, Zhou QX, Luo FL, Zhang Z (2007) Inhibitory action of asiaticoside on collagen-induced arthritis in mice. Acta pharmacologica Sinica 42: 698–703.

Li J, Zhao GZ, Chen HH, Wang HB, Qin S, Zhu WY, Xu LH, Jiang CL, Li WJ (2008) Antitumor and antimicrobial activities of endophytic streptomycetes from pharmaceutical plants in rainforest. Letters in Applied Microbiology 47: 574–580.

Li JY, Harper JK, Grant DM *et al.* (2001) Ambuic acid, a highly functionalized cyclohexenone with antifungal activity from *Pestalotiopsis* spp. and *Monochaetia* sp. Phytochemistry 56: 463–468.

Lin HF, Chen TH, Liu SD (2011) The antifungal mechanism of *Bacillus subtilis* against *Pestalotiopsis eugeniae* and its development for commercial applications against wax apple infection. African Journal of Microbiology Research 5: 1723–1728.

Liu G, Kennedy R, Greenshields DL, Peng G, Forseille L, Selvaraj G, Wei Y (2007) Detached and attached *Arabidopsis* leaf assays reveal distinctive defense responses against hemibiotrophic *Colletotrichum* spp. Molecular Plant Microbe Interaction 20: 1308–1319.

Liu M, Dai Y, Li Y, Luo Y, Huang F, Gong Z, Meng Q (2008a) Madecassoside isolated from *Centella asiatica* herbs facilitates burn wound healing in mice. Planta Medica 74(8): 809–815.

Liu M, Dai Y, Yao X, Li Y, Luo Y, Xia Y, Gong Z (2008b) Anti-rheumatoid arthritic effect of madecassoside on type II collagen-induced arthritis in mice. International Immunopharmacology 8: 1561–1566.

Liu X, Xie X, Duan J (2007) *Colletotrichum yunnanense* sp. nov., a new endophytic species from *Buxus* sp. Mycotaxon 100: 137–144.

Loper JE (1988) Role of fluorescent siderophore production in biological control of *Pythium ultimum* by a *Pseudomonas fluorescens* strain. Phytopathology 78: 166–172.

Lu G, Cannon PF, Reid A, Simmons CM (2004) Diversity and molecular relationships of endophytic *Colletotrichum* isolates from the Iwokrama Forest Reserve, Guyana. Mycological Research 108: 53–63.

Lu H, Zou WX, Meng JC *et al*. (2000) New bioactive metabolites produced by *Colletotrichum* sp., an endophytic fungus in *Artemisia annua*. Plant Science 151: 67–73.

Magnani GS, Didonet CM, Cruz LM, Picheth CF, Pedrosa FO, Souza EM (2010) Diversity of endophytic bacteria in Brazilian sugarcane. Genetics and Molecular Research 9(1): 250–258.

Mahadtanapuk S, Sanguansermsri M, Cutler RW, Sardsud V, Anuntalabhochai S (2007) Control of anthracnose caused by *Colletotrichum musae* on *Curcuma alismatifolia* Gagnep. using antagonistic *Bacillus* spp. American Journal of Agricultural and Biological Sciences 2 (2): 54–61.

Malewicz B, Momsen M, Jenkin HM, Borowski E (1984) Potentiation of antiviral activity of acyclovir by polyene macrolide antibiotics. Antimicrobial Agents Chemotherapy 25(6): 772–774.

Malinowski DP, Belesky DP (2000) Adaptations of endophyte-infected cool season grasses to environmental stresses: mechanisms of drought and mineral stress tolerance. Crop Science 40: 923–940.

Mano H, Morisaki H (2008) Endophytic bacteria in the rice plant. Microbes and Environments 23(2): 109–117.

Markiewicz M, Kalicinski P, Teisseyre J, Ismail H, Kaminski A, Teisseyre M (2003) Rapamycin in children after liver transplantation. Transplantation Proceedings 35: 2284–2286.

Martín M, García-Figueres F (1999) *Colletotrichum acutatum* and *Colletotrichum gloeosporioides* cause anthracnose on olives. European Journal of Plant Pathology 105: 733–741.

Matsuda H, Morikawa T, Ueda H, Yoshikawa M (2001) Medicinal foodstuffs. XXVI. Inhibitors of aldose reductase and new triterpene and its oligoglycoside, centellaspogenol A and centellasponin A from *Centella asiatica* (*Gotu Kola*). Heterocycles 55(8): 1499–1504.

Matsuoka M, Umezawa H (1960) Unamycin, an antifungal substance produced by *Streptomyces fungicidicus*. The Journal of Antibiotics 13A: 114–120.

McInroy JA, Kloepper JW (1995) Survey of indigenous bacterial endophytes from cotton and sweet corn. Plant and Soil 173: 337–342.

Mejía LC, Rojas EI, Maynard Z, Van Bael SA, Arnold E, Hebbar P, Samuels GJ, Robbins N, Herre EA (2008) Endophytic fungi as biocontrol agents of *Theobroma cacao* pathogens. Biological Control 46: 4–14.

Melnick RL, Zidack NK, Bailey BA, Maximova SN, Guiltinan M, Backman PA (2008) Bacterial endophytes: *Bacillus* spp. from annual crops as potential biological control agents of black pod rot of cacao. Biological Control 46: 46–56.

Melnick RL, Suárez C, Bailey BA, Backman PA (2011) Isolation of endophytic endospore-forming bacteria from *Theobroma cacao* as potential biological control agents of cacao diseases. Biological Control 57: 236–245.

Metz AM, Haddad A, Worapong J, Long DM, Ford EJ, Hess WM, Strobel GA (2000) Induction of the sexual stage of *Pestalotiopsis microspora*, a taxol-producing fungus. Microbiology 146: 2079–2089.

Moriwaki J, Tsukiboshi T (2009) *Colletotrichum echinochloae*, a new species on Japanese barnyard millet (*Echinochloa utilis*). Mycoscience 50: 273–280.

Mucciarelli M, Camusso W, Maffei M, Panicco P, Bicchi C (2007) Volatile terpenoids of endophyte-free and infected peppermint (*Mentha piperita* L.): chemical partitioning of a symbiosis. Microbial Ecology 54: 685–696.

Mulks MH, Nair MG, Putnam AR (1990) *In vitro* antibacterial activity of faeriefungin, a new broad-spectrum polyene macrolide antibiotic. Antimicrobial Agents and Chemotherapy 34(9): 1762–1765.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15: 473–497.

Musetti R, Polizzotto R, Grisan S, Martini M, Borselli S, Carraro L, Osler R (2007) Effects induced by fungal endophytes in *Catharanthus roseus* tissues infected by Phytoplasmas. Bulletin of Insectology 60(2): 293–294.

Mustafa NR, Verpoorte R (2007) Phenolic compounds in *Catharanthus roseus*. Phytochemistry Reviews 6: 243–258.

Muthukumar A, Bhaskaran R, Sanjeevkumar K (2010) Efficacy of endophytic *Pseudomonas fluorescens* (Trevisan) migula against chilli damping-off. Journal of Biopesticides 3(1 Special Issue): 105–109.

Myers N, Mittermeier RA, Mittermeier CG, Da Fonseca GAB, Kent J (2000) Biodiversity hotspots for conservation priorities. Nature 403: 853–858.

Nair MG, Putnam AR, Mishra SK, Mulks MH, Taft WH, Keller JE, Miller JR, Zhu P-P, Meinhart JD, Lynn DG (1989) Faeriefungin: a new broad-spectrum antibiotic from *Streptomyces grise*us var. *autotrophicus*. Journal of Natural Products 52: 797–809.

Narusaka Y, Narusaka M, Park P *et al*. (2004) RCH1, a locus in *Arabidopsis* that confers resistance to the hemibiotrophic fungal pathogen *Colletotrichum higginsianum*. Molecular Plant-Microbe Interaction 17(7): 749–762.

Nguyen PTH, Pettersson OV, Olsson P, Liljeroth E (2010) Identification of *Colletotrichum* species associated with anthracnose disease of coffee in Vietnam. European Journal of Plant Pathology 127: 73–87.

Niemann S, Puehler A, Tichy H-V, Simon R, Selbitschka W (1997) Evaluation of the resolving power of three different DNA fingerprinting methods to discriminate among isolates of a natural *Rhizobium meliloti* population. Journal of Applied Microbiology 82: 477–484.

Nilsson RH, Ryberg M, Kristiansson E, Abarenkov K, Larsson K, Kõljalg U (2006) Taxonomic reliability of DNA sequences in public sequence databases: a fungal perspective. PLoS ONE 1: e59.

Noble RL (1990) The discovery of the vinca alkaloids– chemotherapeutic agents against cancer. Biochemistry and Cell Biology 68: 1344–1351.

Nogushi H, Harrison PH, Arai K, Nakashima TT, Trimble LA, Vederas JC (1988) Biosynthesis and full NMR assignment of fungichromin, a polyene antibiotic from *Streptomyces cellulosae*. Journal of the American Chemical Society 110(9): 2938–2945.

Nosanchuk JD (2006) Current status and future of antifungal therapy for systemic mycoses. Recent Patents on Anti-Infective Drug Discovery 1: 75–84.

Nowak-Thompson B, Gould SJ, Kraus J, Loper JE (1994) Production of 2,4-diacetyl phloroglucinol by the biocontrol agent *Pseudomonas fluorescens* Pf-5. Canadian Journal of Microbiology 40: 1064–1066.

O'Brien H, Parrent J, KJackson J, Moncalvo J, Vilgalys R (2005) Fungal community analysis by large-scale sequencing of environmental samples. Applied and Environmental Microbiology 71(9): 5544–5550.

Oyedeji OA, Afolayan AJ (2005) Chemical composition and antibacterial activity of the essential oil of *Centella asiatica* growing in South Africa. Pharmaceutical Biology 43(3): 249–252.

Pandey RC, Kalita CC, Aszalos AA, Geoghegan R Jr, Garretson AL, Cook JC Jr, Rinehart KL Jr (1980) Field desorption mass spectrometry in structural studies of polyene macrolide antibiotics: Isolation and early identification of a pentaene macrolide antibiotic. Biomedical Mass Spectrometry 7(3): 93–98.

Pandey RC, Guenther EC, Aszalos AA, Brajtburg J (1982) Physicochemical and biological comparison of polyene macrolide antibiotics fungichromin, lagosin and cogomycin. The Journal of Antibiotics (Tokyo) 35(8): 988–996.

Pandey RC (1992) (Xechem, Inc.) US5159002.

Péchard G, Antona M, Aubert S, Babin D (2005) Ressources phytogénétiques, contrats et application de la Convention biodiversité à Madagascar : une approche prospective. Bois et Forêts des Tropiques 284(2): 45–58.

Pedras MS, Ismail N, Quail JW, Boyetchko SM (2003) Structure, chemistry, and biological activity of pseudophomins A and B, new cyclic lipodepsipeptides isolated from the biocontrol bacterium *Pseudomonas fluorescens*. Phytochemistry 62: 1105–1144.

Perry MJ (2008) Effects of environmental and occupational pesticide exposure on human sperm: a systematic review. Human Reproduction Update 14 (3): 233–242.

Petrini O (1984) Endophytic fungi in British Ericaceae: a preliminary study. Transactions of the British Mycological Society 83: 510–512.

Petrini O (1986) Taxonomy of endophytic fungi of aerial plant tissues. In: Fokkeman NJ, Van den Heuvel J (Eds) Microbiology of the Phyllosphere. Cambridge University Press, Cambridge, pp. 175–187.

Petrini O (1991) Fungal endophytes of tree leaves. In: Andrews JH, Hirano SS (Eds.) Microbial Ecology of Leaves. Springer Verlag, New York, pp. 179–187.

Petrini O, Sieber TH, Toti L, Viret O (1992) Ecology, metabolite production and substrate utilization in endophytic fungi. Natural Toxins 1: 185–196.

Petrini O, Stone J, Carroll FE (1982) Endophytic fungi in evergreen shrubs in western Oregon: a preliminary study. Canadian Journal of Botany 60: 789–796.

Photita W, Lumyong S, Lumyong P, Hyde KD (2001) Endophytic fungi of wild banana (*Musa acuminata*) at Doi Suthep Pui National Park, in Thailand. Mycological Research 105: 1508–1513.

Photita W, Lumyong S, Lumyong P, McKenzie EHC, Hyde KD (2004) Are some endophytes of *Musa acuminata* latent pathogens? Fungal Diversity 16: 131–140.

Photita W, Taylor PWJ, Ford R, Hyde KD, Lumyong S (2005) Morphological and molecular characterization of *Colletotrichum* species from herbaceous plants in Thailand. Fungal Diversity18: 117–133.

Phoulivong S (2011) *Colletotrichum*, naming, control, resistance, biocontrol of weeds and current challenges. Current Research in Environmental & Applied Mycology 1(1): 53–73.

Pietrosiuk A, Furmanowa M, Lata B (2007) *Catharanthus roseus*: micropropagation and *in vitro* techniques. Phytochemistry Reviews 6: 459–473.

Pimentel IC, Glienke-Blanco C, Gabardo J, Stuart RM, Azevedo JL (2006) Identification and colonization of endophytic fungi from soybean (*Glycine max* (L.) Merril) under different environmental conditions. Brazilian Archives of Biology and Technology 49: 705–711.

Pointel JP, Boccalon H, Cloarec M *et al*. (1987) Titrated extract of *Centella asiatica* (TECA) in the treatment of venous insufficiency of the lower limbs. Angiology 38 (1 Pt 1): 46–50.

Porter JK, Bacon CW and Robbins JD (1979) Lysergic acid amide derivatives from *Balansia epichloe* and *Balansia claviceps* (Clavicipitaceae). Journal of Natural Product 42: 309–314.

Prihastuti H, Cai L, Chen H, McKenzie EHC, Hyde KD (2009) Characterization of *Colletotrichum* species associated with coffee berries in Chiang Mai, Thailand. Fungal Diversity 39: 89–109.

Procópio REL, Araújo WL, Maccheroni Jr.W, Azevedo JL (2009) Characterization of an endophytic bacterial community associated with *Eucalyptus* spp. Genetics and Molecular Research 8: 1408–1422.

Promputtha I, Lumyong S, Dhanasekaran V, McKenzie EHC, Hyde KD, Jeewon RA (2007) Phylogenetic evaluation of whether endophytes become saprotrophs at host senescence. Microbial Ecology 53: 579–590.

Pruesse E, Quast C, Knittel K, Fuchs B, Ludwig W, Peplies J, Glöckner FO (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Research 35: 7188–7196.

Przybył K, Żłobińska-Podejma M (2000) Effects of some bacteria (*Pseudomonas* spp. and *Erwinia herbicola*) on *in vitro* growth of *Piptoporus betulinus*. Forest Pathology 30: 321–328.

Puja G, Shenoy BD, Hyde KD, Bhat DJ (2006) *Echinosphaeria macrospora* sp. nov., teleomorph of *Vermiculariopsiella endophytica* sp. nov. Cryptogamie Mycologie 27(1): 11–20.

Raaijmakers JM, Weller DM (2001) Exploiting genotypic diversity of 2, 4-diacetylphloroglucinol-producing *Pseudomonas* spp.: characterization of superior root-colonizing *P. fluorescens* strain Q8r1-96. Applied and Environmental Microbiology 67: 2545–2554.

Rafat A, Philip K, Muniandy S (2012) A novel source of bioactive compounds: endophytic bacteria isolated from *Centella asiatica*. Journal of Pure and Applied Microbiology 6: 11–20.

Rai R, Dash PK, Prasanna BM, Singh A (2007) Endophytic bacterial flora in the stem tissue of a tropical maize (*Zea mays* L.) genotype: isolation, identification and enumeration. World Journal of Microbiology and Biotechnology 23(6): 853–858.

Rajendran L, Saravanakumar D, Raguchander T, Samiyappan R (2006) Endophytic bacterial induction of defence enzyme against bacterial blight of cotton. Phytopathologia Mediterranea 45: 203–214.

Rakotoniriana EF, Munaut F, Decock C, Randriamampionona D, Andriambololoniaina M, Rakotomalala T, Rakotonirina EJ, Rabemanantsoa C, Cheuk K, Ratsimamanga SU, Mahillon J, El-Jaziri M, Quetin-Leclercq, Corbisier AM (2008) Endophytic fungi from leaves of *Centella asiatica*: occurrence and potential interactions within leaves. Antonie van Leeuwenhoek 93: 27–36.

Rakotoniriana EF, Chataigné G, Raoelison G, Rabemanantsoa C, Munaut F, El Jaziri M, Urveg-Ratsimamanga S, Marchand-Brynaert J, Corbisier A-M, Declerck S, Quetin-Leclercq J (2012) Characterization of an endophytic whorl-forming *Streptomyces* from *Catharanthus roseus* stems producing polyene macrolide antibiotic. Canadian Journal of Microbiology 58(5): 617–627.

Ramani S, Jayabaskaran C (2008) Enhanced catharanthine and vindoline production in suspension cultures of *Catharanthus roseus* by ultraviolet-B light. Journal of Molecular Signaling 3: 9.

Randriamampionona D, Diallo B, Rakotoniriana EF, Rabemanantsoa C, Cheuk K, Corbisier AM, Mahillon J, Ratsimamanga S, El Jaziri M (2007) Comparative analysis of active constituents in *Centella asiatica* samples from Madagascar: application for *ex situ* conservation and clonal propagation. Fitoterapia 78: 482–489.

Rasoanaivo P, Ramanitrahasimbola D, Rafatro H, Rakotondramanana D, Robijaona B, Rakotozafy A, Ratsimamanga-Urveg S, Labaied M, Grellier P, Allorge L *et al*. (2004) Screening extracts of Madagascan plants in search of antiplasmodial compounds. Phytotherapy Research 18(9): 742–747.

Redman RS, Dunigan DD, Rodriguez RJ (2001) Fungal symbiosis from mutualism to parasitism: who controls the outcome, host or invader? New Phytologist 151: 705–716.

Reiter B, Sessitsch A (2006) Bacterial endophytes of the wildflower *Crocus albiflorus* analyzed by characterization of isolates and by a cultivation-independent approach. Canadian Journal of Microbiology 52(2): 140–149.

Richardson TI, Rychnovsky SD (1997) Total synthesis of filipin III. Journal of the American Chemical Society 119: 12360–12361.

Risovic V, Rosland M, Sivak O, Wasan KM, Bartlett K (2007) Assessing the antifungal activity of a new oral lipid-based amphotericin B formulation following administration to rats infected with *Aspergillus fumigatus*. Drug Development and Industrial Pharmacy 33: 703–707.

Robinson JG (2004) An island of evolutionary exuberance. Science 304 (5667): 53.

Robison RS, Aszalos A, Kraemer N, Giannini SM (1971) Production of fungichromin by *Streptoverticillium cinnamomeum* subsp. *cinnamomeum* NRRL B-1285. Journal of Antibiotics 24(4): 273.

Rodrigues KF (1994) The foliar fungal endophytes of the Amazonian palm *Euterpe oleracea*. Mycologia 86: 376–385.

Rodrigues KF, Drandarov K, Heerklotz J *et al*. (2001) Guignardic acid, a novel type of secondary metabolite produced by the endophytic fungi *Guignardia* sp.: isolation, structure elucidation and asymmetric synthesis. Helvetica Chimica Acta 84: 3766–3772.

Rodrigues KF, Petrini O (1997) Biodiversity of endophytic fungi in tropical regions. In: Hyde KD (Ed.) Biodiversity of tropical microfungi. Hong Kong University Press, Hong Kong, pp. 57–69.

Rodríguez-Guerra RJ, Ramírez-Rueda M-T, Cabral-Enciso M, García-Serrano M, Lira-Maldonado Z, Guevara-González RG, González-Chavira M, Simpson J (2005) Heterothallic mating observed between Mexican isolates of *Glomerella lindemuthiana*. Mycologia 97: 793–803.

Rojas EI, Rehner SA, Samuels GJ, Van Bael SA, Herre EA, Cannon P, Chen R, Pang J, Wang R, Zhang Y, Peng YQ, Sha T (2010) *Colletotrichum gloeosporioides* s.l. associated with *Theobroma cacao* and other plants in Panama: multilocus phylogenies distinguish host-associated pathogens from asymptomatic endophytes. Mycologia 102(6): 1318–1338.

Romero D, de Vicente A, Olmos JL, Dávila JC, Pérez-García A (2007) Effect of lipopeptides of antagonistic strains of *Bacillus subtilis* on the morphology and ultrastructure of the cucurbit fungal pathogen *Podosphaera fusca*. Journal of Applied Microbiology 103: 969–976.

Rosenberger DA, Meyer CW (1981) Post harvest fungicides for apples: development of resistance to benomyl, vinclozolin and iprodione. Plant Disease 65: 1010–1013.

Rouillard GF, Robin JR, Ratsimamanga AR, Ratsimamanga S, Rasoanaivo P (1997) Etude comparative de *Centella asiatica* d'origine malgache et d'origine indienne. Acta Botanica Gallica 144: 489–493.

Roy S, Chatterjee P (2010) A non-toxic antifungal compound from the leaves of *Catharanthus roseus* characterized as 5-hydroxy flavone by UV spectroscopic analysis and evaluation of its antifungal property by agar-cup method. Industrial Crops and Products 32: 375–380.

Saccardo PA (1899) Sylloge fungorum 14: 1017.

Saccardo PA, Saccardo D (1906) Sylloge fungorum 18: 469.

Saccardo PA, Trotter A (1913) Sylloge fungorum 22: 1205.

Saikkonen K, Faeth SH, Helander M, Sullivan TJ (1998) Fungal endophytes: a continuum of interactions with host plants. Annual Review of Ecology and Systematics 29: 319–343.

Saito A, Minamisawa K (2006) Evaluation of the nitrogen-fixing ability of the endophytic *Clostridia* based on acetylene reduction and reverse transcription-PCR targeting the *nifH* transcript and ribosomal RNA. Microbes and Environments 21(1): 23–35.

Saitou H, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4: 406–425.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Sarode PD, Rane MR, Chaudhari BL, Chincholkar SB (2009) Siderophoregenic *Acinetobacter calcoaceticus* isolated from wheat rhizosphere with strong PGPR activity. Malaysian Journal of Microbiology 5(1): 6–12.

Satheesan J, Narayanan AK, Sakunthala M (2012) Induction of root colonization by *Piriformospora indica* leads to enhanced asiaticoside production in *Centella asiatica*. Mycorrhiza 22: 195–202.

Schaffner CP, Plescia OJ, Pontani, D. Sun D, Thornton A, Pandey RC, Sarin PS (1986) Anti-viral activity of amphotericin B methyl ester: inhibition of HTLV-III replication in cell culture. Biochemical Pharmacology 35: 4110–4113.

Schaneberg BT, Mikell JR, Bedir E *et al.* (2003) An improved HPLC method for quantitative determination of six triterpenes in *Centella asiatica* extracts and commercial products. Pharmazie 58: 381–384.

Schulz B, Guske S, Dammann U, Boyle C (1998) Endophyte-host interactions II. Defining symbiosis of the endophyte-host interaction. Symbiosis 25: 213–227.

Schulz B, Rommert A-K, Dammann U, Aust H-J, Strack D (1999) The endophyte-host interaction: a balanced antagonism. Mycological Research 103: 1275–1283.

Schulz B, Boyle C, Draeger S *et al*. (2002) Endophytic fungi: a source of biologically active secondary metabolites. Mycological Research 106: 996–1004.

Schulz B, Boyle C (2005) The endophytic continuum. Mycological Research 109: 661–686.

Schulz B, Boyle C (2006) What are endophytes? In: Schulz B, Boyle C, Sieber TN (Eds.) Microbial Root Endophytes. Soil Biology, Volume 9. Springer- Verlag Berlin Heidelberg, pp. 1–13.

Seam P, Janik JE, Longo DL, Devita VT Jr (2009) Role of chemotherapy in Hodgkin's lymphoma. The Cancer Journal 15(2): 150–154.

Senghor AL, Liang W-J, Ho W-C (2007) Integrated control of *Colletotrichum gloeosporioides* on mango fruit in Taiwan by the combination of *Bacillus subtilis* and fruit bagging. Biocontrol Science and Technology 17: 865–870.

Sergeeva V, Spooner-Hart R, Nair NG (2008) First report of *Colletotrichum acutatum* and *C. gloeosporioides* causing leaf spots of olives (*Olea europaea*) in Australia. Australasian Plant Disease Notes 3: 143–144.

Sharma R, Mithilesh , Jain DA, Kumar U, Kumar TS, Raghvendra , Yadav A, Mishra A (2011) Isolation and screening of endophytic actinomycetes producing antibacterial compound from different parts of *Catharanthus rosea*. International Journal of Pharma World Research, Vol 2, Issue 1.

Shen L, Jiao RH, Ye YH, Wang XT, Xu C, Song YC, Zhu HL, Tan RX (2006) Absolute configuration of new cytotoxic and other bioactive tricothecene macrolides. Chemistry-A European Journal 12: 5596–5602.

Shih HD, Liu YC, Hsu FL, Mulabagal V, Dodda R, Huang JW (2003) Fungichromin: a substance from *Streptomyces padanus* with inhibitory effects on *Rhizoctonia solani*. Journal of Agricultural and Food Chemistry 51(1): 95–99.

Shin HJ, Kim TS, Lee HS, Park JY, Choi IK, Kwon HJ (2008) Streptopyrrolidine, an angiogenesis inhibitor from a marine-derived *Streptomyces* sp. KORDI-3973. Phytochemistry 69: 2363–2366.

Shiomi HF, Silva HSA, Melo IS, Nunes FV, Bettiol W (2006) Bioprospecting endophytic bacteria for biological control of coffee leaf rust. Scientia Agricola 63(1): 32–39.

Shipton WA, Brown JF (1962) A whole-leaf clearing and staining technique to demonstrate host-pathogen relationships of wheat stem rust. Phytopathology 52: 1313.

Shirling EB, Gottlieb D (1966) Methods for characterization of *Streptomyces* species. International Journal of Systematic Bacteriology 16: 313–340.

Sinclair JB, Cerkauskas RF (1996) Latent infection vs. endophytic colonization by fungi. In: Redlin SC, Carris LM (Eds.) Endophytic Fungi in Grasses and Woody Plants: Systematics, Ecology and Evolution. The American Phytopathological Society, St. Paul, Minnesota, pp. 3–29.

Sinz CJ, Rychnovsky SD (2002) Total synthesis of the polyene macrolide dermostatin A. Tetrahedron 58: 6561–6576.

Sivanesan A, Hsieh WH (1993) A new ascomycete, *Glomerella septospora* sp. nov. and its coelomycete anamorph, *Colletotrichum taiwanense* sp. nov. from Taiwan. Mycological Research 97(12): 1523–1529.

Spilman P, Podlutskaya N, Hart MJ, Debnath J, Gorostiza O, Bredesen D, Richardson A, Strong R, Galvan V (2010) Inhibition of mTOR by rapamycin abolishes cognitive deficits and reduces amyloid-β levels in a mouse model of Alzheimer's disease. PLoS ONE 5(4): e9979. doi:10.1371/journal.pone.0009979.

Srivastava R, Shalini (2008) Antifungal activity of *Pseudomonas fluorescens* against different plant pathogenic fungi. Electronic Journal of Environmental, Agricultural and Food Chemistry 7*:* 2789–2796.

Stanley SJ (1992) Observations on the seasonal occurrence of marine endophytic and parasitic fungi. Canadian Journal of Botany 70: 2089–2096.

Stevens FL (1928) The sexual stage of fungi induced by ultra-violet rays. Science 67: 514-515.

Stierle A, Strobel G, Stierle D. (1993) Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew. Science 260: 214–216.

Stoltzfus JR, So R, Malarvithi PP, Ladha JK, de Bruijn FJ (1997) Isolation of endophytic bacteria from rice and assessment of their potential for supplying rice with biologically fixed nitrogen. Plant and Soil 194: 25–36.

Stone JK (1987) Initiation and development of latent infections by *Rhabdocline parkeri* on Douglas-fir. Canadian Journal of Botany 65: 2614–2621.

Stone JK, Bacon CW, White JF (2000) An overview of endophytic microbes: endophytism defined. In: Bacon CW, White JF (Eds.) Microbial Endophytes. Marcel Dekker, New York, pp. 3–29.

Strobel GA, Yang X, Sears J, Kramer R, Sidhu R, Hess WM (1996) Taxol from *Pestalotiopsis microspora*, an endophytic fungus of *Taxus wallachiana*. Microbiology 142: 435–440.

Strobel GA, Daisy B (2003) Bioprospecting for microbial endophytes and their natural products. Microbiology and Molecular Biology Reviews 67: 491–502.

Strobel GA, Daisy B, Castillo U, Harper J (2004) Natural products from endophytic microorganisms. Journal of Natural Products 67: 257–268.

Struyk AP, Hoette I, Drost G, Waisvisz JM, van Eek T, Hoogerheide JC (1958) Pimaricin, a new antifungal antibiotic. Antibiotics Annual 5: 878–884.

Sun L, Qiu F, Zhang X, Dai X, Dong X, Song W (2008) Endophytic bacterial diversity in rice (Oryza sativa L.) roots estimated by 16S rDNA sequence analysis. Microbial Ecology 55: 415–424.

Sutton BC (1980) The Coelomycetes. Fungi imperfecti with pycnidia, acervuli and stromata. Surrey, England: Commonwealth Mycological Institute, 696 p.

Sutton BC (1992) The genus *Glomerella* and its anamorph *Colletotrichum*. In: Bailey JA, Jeger MJ (Eds.) *Colletotrichum*: Biology, Pathology and Control. Wallingford, UK: CAB International, pp. 1–26.

Swatzell LJ, Powell MJ, Kiss JZ (1996) The Relationship of endophytic fungi to the gametophyte of the fern *Schizaea pusilla.* International Journal of Plant Sciences 157: 53–62.

Swofford DL (2000) PAUP*: phylogenetic analysis using parsimony (*and other methods). Version 4.0b10. Sunderland, Massachusetts: Sinauer Associates.

Taechowisan T, Peberdy JF, Lumyong S (2003) Isolation of endophytic actinomycetes from selected plants and their antifungal activity. World Journal of Microbiology and Biotechnology 19: 381–385.

Taechowisan T, Lu C, Shen Y, Lumyong S (2005) Secondary metabolites from endophytic *Streptomyces aureofaciens* CMUAc130 and their antifungal activity. Microbiology 151: 1691–1695.

Taechowisan T, Lu C, Shen Y, Lumyong S (2007) Anti-tumor activity of 4-arylcoumarins from endophytic *Streptomyces aureofaciens* CMUAc130. Journal of Cancer Research and Therapeutics 3: 86–91.

Tan RX, Zou WX (2001) Endophytes: a rich source of functional metabolites. Natural Product Reports 18: 448–459.

Tanaka A, Tapper BA, Popay A, Parker EJ, Scott B (2005) A symbiosis expressed non-ribosomal peptide synthetase from a mutualistic fungal endophyte of perennial ryegrass confers protection to the symbiotum from insect herbivory. Molecular Microbiology 57: 1036–1050.

Tang XL, Yang XY, Jung HJ, Kim SY, Jung SY, Choi DY, Park WC, Park H (2009) Asiatic acid induces colon cancer cell growth inhibition and apoptosis through mitochondrial death cascade. Biological and Pharmaceutical Bulletin 32(8): 1399–1405.

Tansuwan S, Pornpakakul S, Roengsumran S, Petsom A, Muangsin N, Sihanonta P, Chaichit N (2007) Antimalarial benzoquinones from an endophytic fungus, *Xylaria* sp. Journal of natural Products 70: 1620–1623.

Tao G, Liu ZY, Hyde KD, **Liu XZ, Yu ZN (2008)** Whole rDNA analysis reveals novel and endophytic fungi in *Bletilla ochracea* (Orchidaceae). Fungal Diversity 33: 101–122.

Than PP, Shivas RG, Jeewon R, Pongsupasamit S, Marney TS, Taylor PWJ, Hyde KD (2008) Epitypification and phylogeny of *Colletotrichum acutatum* J.H. Simmonds. Fungal Diversity 28: 97–108.

Thirumalachar MJ, Menon SK (1962) Dermostatin, a new antifungal agent. Hindustan Antibiotics Bulletin 4: 106–108.

Thomashow LS, Weller DM, Bonsall R, Pierson LS (1990) Production of the antibiotic phenazine 1-carboxylic acid by fluorescent pseudomonas species in the rhizosphere of wheat. Applied and Environmental Microbiology 56: 908–912.

Toki K, Saito N, Irie Y, Tatsuzawa F, Shigihara A, Honda T (2008) 7-O-Methylated anthocyanidin glycosides from *Catharanthus roseus*. Phytochemistry 69: 1215–1219.

Tor-Agbidye J, Blythe LL, Craig AM (2001) Correlation of endophyte toxins (ergovaline and lolitrem B) with clinical disease: fescue foot and perennial ryegrass staggers. Veterinary and Human Toxicology 43(3): 140–146.

Toti L, Chapela IH, Petrini O (1992) Differential attachment by conidia of the endophyte, *Discula umbrinella* (Berk. & Br.) Morelet, to host and non-host surfaces. New Phytologist 121: 469–475.

Touré Y, Ongena M, Jacques P, Guiro A, Thonart P (2004) Role of lipopeptides produced by *Bacillus subtilis* GA1 in the reduction of grey mould disease caused by *Botrytis cinerea* on apple. Journal of Applied Microbiology 96: 1151–1160.

Tung CY, Yang DB, Gou M (2002) A preliminary study on the condition of the culture and isolate of endophytic fungus producing vincristine. Journal of Chuxiong Normal University 6: 39–41.

Turgeon B, Yoder O (2000) Proposed nomenclature for mating type genes of filamentous Ascomycetes. Fungal Genetics and Biology 31: 1–5.

Ulrich K, Ulrich A, Ewald D (2008) Diversity of endophytic bacterial communities in poplar grown under field conditions. FEMS Microbiology Ecology 63: 169–180.

Umezawa S, Tanaka Y, Ooka M, Shiotsu S (1958) A new antifungal antibiotic, pentamycin. The Journal of Antibiotics 11A: 26–29.

Vaillancourt LJ, Hanau RM (1991) A method for genetic analysis of *Glomerella graminicola* from maize. Phytopathology 81: 530–534.

van der Heijden R, Jacobs DI, Snoeijer W, Hallard D,Verpoorte R (2004) The *Catharanthus* alkaloids: pharmacognosy and biotechnology. Current Medicinal Chemistry 11: 607–628.

Vandeputte J, Wachtel JL, Stiller ET (1955) Amphotericins A and B, antifungal antibiotics produced by a streptomycete. II. The isolation and properties of the crystalline amphotericins. Antibiotics Annual 3: 587–591.

Vanier MT (2010) Niemann-Pick disease type C. Orphanet Journal of Rare Diseases 5: 16.

Vázquez-Flota F, Hernández-Domínguez E, de Lourdes Miranda-Ham M, Monforte-González M (2009) A differential response to chemical elicitors in *Catharanthus roseus in vitro* cultures. Biotechnology Letters 31: 591–595.

Vega FE, Pava-Ripoll M, Posada F, Buyer JS (2005) Endophytic bacteria of *Coffea arabica* L. Journal of Basic Microbiology 45: 371–380.

Veiga M, Traba MP, Fabregas J (1983) Tetrafungin, a new polyene macrolide antibiotic. II. Taxonomy of the producing organism and comparison with nystatin by means of high performance liquid chromatography. The Journal of Antibiotics 36: 776–783.

Verma VC, Gond SK, Kumar A, Mishra A, Kharwar RN, Gange AC (2009) Endophytic actinomycetes from *Azadirachta indica* A. Juss: isolation, diversity, and antimicrobial activity. Microbial Ecology 57(4): 749–756.

Vézina C, Kudelski A, Sehgal SN (1975) Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle. The Journal of Antibiotics 28: 721–726.

Voisard C, Keel C, Haas D, Dèfago G (1989) Cyanide production by *Pseudomonas fluorescens* helps suppress black root rot of tobacco under gnotobiotic conditions. Journal of the European Molecular Biology Organization 8: 351–358.

von Arx JA (1957) Die Arten der Gattung *Colletotrichum* Cda. Journal of Phytopathology 29: 413–468.

Wanderlei-Silva D, Neto ER, Hanlin R (2003) Molecular systematics of the Phyllachorales (Ascomycota, Fungi) based on 18S ribosomal DNA sequences. Brazilian Archives of Biology and Technology 46(3): 315–322.

Wani MC, Taylor HL, Wall ME, Coggon P, McPhail AT (1971) Plant antitumor agents VI: the isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. Journal of the American Chemical Society 93: 2325–2327.

Weber D, Sterner O, Anke T, Gorzalczancy S, Martino V, Acevedo C (2004) Phomol, a new anti-inflammatory metabolite from an endophyte of the medicinal plant *Erythrina crista-galli*. Journal of Antibiotics 57: 559–563.

Wei G, Kloepper JW, Tuzun S (1991) Induction of systemic resistance of cucumber to *Colletotrichum orbic*ulare by select strains of plant growth-promoting rhizobacteria. Phytopathology 81: 1508–1512.

Weir BS, Johnston PR (2010) Characterisation and neotypification of *Gloeosporium kaki* Hori as *Colletotrichum horii* nom. nov. Mycotaxon 111: 209–219.

West CP (1994) Physiology and drought tolerance of endophyte-infected grasses. In: Bacon CW, White JF Jr (Eds.) Biotechnology of Endophytic Fungi of Grasses. CRC press, Boca Raton, pp. 87–99.

Whalley AJS, Edwards RL (1995) Secondary metabolites and systemic arrangement within the Xylariaceae. Canadian Journal of Botany 73: 802–810.

White JF Jr, Cole GT (1985) Endophyte-host associations in forage grasses. III. *In vitro* inhibition of fungi by *Acremonium coenophialum*. Mycologia 77: 487–489.

White TJ, Bruns TD, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ & White TJ (Eds.) PCR Protocols: A Guide to Methods and Applications. Academic Press, Inc., New York, pp. 315–322.

Whittfield G, Brock TD, Ammann A, Gottlieb D, Carter HE (1955) Filipin, an antifungal antibiotic: isolation and properties. Journal of the American Chemistry and Society 77: 4799–4801.

Wikee S, Cai L, Pairin N, McKenzie EHC, Su Y-Y, Chukeatirote E, Thi HN, Bahkali AH, Moslem MA, Abdelsalam K, Hyde KD (2011) *Colletotrichum* species from Jasmine (*Jasminum sambac*). Fungal Diversity 46: 171–182.

Williams RB, Norris A, Miller JS, Razafitsalama LJ, Andriantsiferana R, Rasamison VE, Kingston DG (2006) Two new cytotoxic naphthoquinones from *Mendoncia cowanii* from the rainforest of Madagascar. Planta Medica 72: 564–566.

Wilson D (1993) Fungal endophytes: out of sight but should not be out of mind. Oikos 68: 379–384.

Wilson D, Carroll GC (1994) Infection studies of *Discula quercina*, an endophyte of *Quercus garryana*. Mycologia 86: 635–647.

Wilson D (2000) Ecology of woody plant endophytes. In: Bacon CW, White JF (Eds.) Microbial Endophytes. New York & Basel: Marcel Dekker, pp. 389–420.

Wiyakrutta S, Sriubolmas N, Panphut W, Thongon N, Danwiserkanjana K, Ruangrungsi N, Meevootisom V (2004) Endophytic fungi with antimicrobial, anti-cancer, anti-malarial activities isolated from Thai medicinal plants. World Journal of Microbiology and Biotechnology 20: 265–272.

Wong MK, Hyde KD (2001) Diversity of fungi on six grasses species of Gramineae and one species of Cyperaceae in Hong Kong. Mycological Research 105: 1485–1491.

Woo PC, Teng JL, Wu JK, Leung FP, Tse H, Fung AM, Lau SK, Yuen KY (2009) Guidelines for interpretation of 16S rRNA gene sequence-based results for identification of medically important aerobic Gram-positive bacteria. Journal of Medical Microbiology 58: 1030–1036.

Wu JY, Huang JW, Shih HD, Lin WC, Liu YC (2008) Optimization of cultivation conditions for fungichromin production from *Streptomyces padanus* PMS-702. Journal of the Chinese Institute of Chemical Engineers 39: 67–73.

Yang X, Zhang L, Guo B, Guo S (2004) Preliminary study of a vincristine-producing endophytic fungus isolated from leaves of *Catharanthus roseus*. Zhongcaoyao 35(1): 79–81 (Abstract).

Yang Y, Liu ZY, Cai L, Hyde KD, Yu ZN, Mckenzie EHC (2009) *Colletotrichum* anthracnose of *Amaryllidaceae*. Fungal Diversity 39: 123–146.

Yang Y, Cai L, Yu Z, Liu Z, Hyde KD (2011) *Colletotrichum* species on *Orchidaceae* in southwest China. Cryptogamie Mycologie 32: 229–253.

Yoshida M, Fuchigami M, Nagao T, Okabe H, Matsunaga K, Takata J, Karube Y, Tsuchihashi R, Kinjo J, Mihashi K, Fujioka T (2005) Antiproliferative constituents from Umbelliferae Plants VII. Active triterpenes and rosmarinic acid from *Centella asiatica*. Biological and Pharmaceutical Bulletin 28(1): 173–175.

Yuan Z-L, Dai C-C, Chen L-Q (2007) Regulation and accumulation of secondary metabolites in plant-fungus symbiotic system. African Journal of Biotechnology 6 (11): 1266–1271.

Zainol MK, Abd-Hamid A,Yusof S, Muse R (2003) Antioxidative activity and total phenolic compounds of leaf, root and petiole of four accessions of *Centella asiatica* (L.) Urban. Food Chemistry 81(4): 575–581.

Zang C-Z, Chang Y-N, Chen H-B, Wu J-Y, Chen C-I, Huang J-W, Shih H-D, Liu Y-C (2011) Deciphering the roles of fatty acids and oils in fungichromin enhancement from *Streptomyces padanus*. Journal of the Taiwan Institute of Chemical Engineers 42: 413–418.

Zhang HW, Song YC, Tan RX (2006) Biology and chemistry of endophytes. Natural Product Reports 23(5): 753–771.

Zhang L, Guo B, Li H, Zeng S, Shao H, Gu S, Wei R (2000) Preliminary study on the isolation of endophytic fungus of *Catharanthus roseus* and its fermentation to produce products of therapeutic value. Chinese Traditional and Herbal Drugs 31: 805–807.

Zhang P, Zhou P, Yu L (2009) An endophytic taxol-producing fungus from *Taxus media*, *Cladosporium cladosporioides* MD2. Current Microbiology 59: 227–232.

Zhang Y, Arpin CC, Cullen AJ, Mitton-Fry MJ, Sammakia T (2011) Total synthesis of dermostatin A. The Journal of Organic Chemistry 76(19): 7641–7653.

Zhao J, ShanT, Mou Y, Zhou L (2011) Plant-derived bioactive compounds produced by endophytic fungi. Mini Reviews in Medicinal Chemistry 11(2): 159–168.

Zheng CJ, Qin LP (2007) Chemical components of *Centella asiatica* and their bioactivities. Zhong Xi Yi Jie He Xue Bao 5: 348–351.

Zhou D, Hyde KD (2001) Host-specificity, host-exclusivity and host-recurrence in saprobic fungi. Mycological Research 105: 1449–1457.

Zimmermann A (1901) Über einige an tropischen Kulturpflanzen beobachtete Pilze. I. Centbl. Bakteriol. Parasitenk.7: 139–147.

Zin NM, Sarmin NIM, Ghadin N, Basri DF, Sidik NM, Hess WM, Strobel GA (2007) Bioactive endophytic streptomycetes from the Malay Peninsula. FEMS Microbiology Letters 274(1): 83–88.

Zotchev SB (2003) Polyene macrolide antibiotics and their applications in human therapy. Current Medicinal Chemistry 10(3): 211–223.

OVERVIEW OF THE SCIENTIFIC ACHIEVEMENTS

I. Scientific publications

Rakotoniriana EF, Chataigné G, Raoelison G, Rabemanantsoa C, Munaut F, El Jaziri M, Urveg-Ratsimamanga S, Marchand-Brynaert J, Corbisier AM, Declerck S, Quetin-Leclercq J. (2012) Characterization of an endophytic whorl-forming *Streptomyces* from *Catharanthus roseus* stems producing polyene macrolide antibiotic. **Canadian Journal of Microbiology** 58(5): 617–627.

Rakotoniriana EF, Munaut F, Decock C, Randriamampionona D, Andriambololoniaina M, Rakotomalala T, Rakotonirina EJ, Rabemanantsoa C, Cheuk K, Urveg-Ratsimamanga S, Mahillon J, El-Jaziri M, Quetin-Leclercq J, Corbisier AM (2008) Endophytic fungi from leaves of *Centella asiatica*: occurrence and potential interactions within leaves. **Antonie van Leeuwenhoek** 93: 27–36.

Rakotoniriana EF, Rafamantanana M, Randriamampionona D, Rabemanantsoa C, Urveg-Ratsimamanga S, El Jaziri M, Munaut F, Corbisier AM, Quetin-Leclercq J, Declerck S. Study *in vitro* of the impact of endophytic bacteria isolated from *Centella asiatica* on the disease incidence caused by the hemibiotrophic fungus *Colletotrichum higginsianum*. **Antonie van Leeuwenhoek**, submitted.

Rakotoniriana EF, Scauflaire J, Rabemanantsoa C, Urveg-Ratsimamanga S, Corbisier AM, Quetin-Leclercq J, Declerck S, Munaut F. *Colletotrichum gigasporum* sp. nov., a new species of *Colletotrichum* producing long straight conidia. **Mycological Progress**, accepted for publication.

II. Other publications

A. Published papers

Rakotoniriana EF, Rajaonarison JF, Raoelison EG, Rajaonarivelo JP, Manga N, Solofoniaina M, Rakotonirina B, Randriamampionona D, Rabemanantsoa C, Cheuk K, Urveg-Ratsimamanga S, Quetin-Leclercq J (2010) Antimicrobial activity of 23 endemic plants in Madagascar. **Tropical Journal of Pharmaceutical Research** 9(2): 165–171.

Randriamampionona D, Rafamantanana M, Rabemananstoa C, **Rakotoniriana EF**, Cheuk K, Corbisier AM, Mahillon J, Ratsimamanga S, El Jarizi M (2008) *Ex Situ* conservation and clonal propagation of the Malagasy *Syzygium cuminii* (L.), an antidiabetic plant. **Belgium Journal of Botany** 141(1): 14–20.

Randriamampionona D, Diallo B, **Rakotoniriana EF**, Rabemanantsoa C, Cheuk K, Corbisier AM, Mahillon J, Ratsimamanga S, El Jaziri M (2007) Comparative analysis of active constituents in *Centella asiatica* samples from Madagascar: Application for *ex situ* conservation and clonal propagation. **Fitoterapia** 78: 482–489.

Rangasamy O, Raoelison G, **Rakotoniriana EF**, Cheuk K, Urverg-Ratsimamanga S, Quetin-Leclercq J, Gurib-Fakim A, Subratty AH (2007) Screening for anti-infective properties of several medicinal plants of the Mauritians flora. **Journal of Ethnopharmacology** 109: 331–337.

Rakotoniriana EF (2004) Approche chimio-microbiologique de l'activité des huiles essentielles produites à l'Institut Malgache de Recherches Appliquées. **Mémoire pour l'obtention du Diplôme**

d'études de Formations Spécialisées (DEFS) en Biologie Médicale, Madagascar.

Rakotoniriana EF (2000) Etude rétrospective des suspicions de thrombopénies induites par l'héparine pendant l'année 1999 à l'hôpital Haut-Lévêque. **Mémoire pour l'obtention de l'Attestation de Formation Spécialisée (AFS) en Biologie Médicale**, Bordeaux, France.

Rakotoniriana EF (1996) La maladie de Lobstein. A propos de six cas observés sur quatre générations. **Thèse pour l'obtention du diplôme de doctorat en médecine**, Madagascar.

B. Conference

Oral presentation

Rakotoniriana EF. Biodiversity and antimicrobial properties of endophytes from medicinal plants: cases of *Centella asiatica* and *Catharanthus roseus*. MBLA, Université catholique de Louvain, March 2008, Belgium.

Rakotoniriana EF. Les microorganismes endophytes: rôle dans les interactions plante-microorganismes pour la production de métabolites bioactifs contenus dans la plante. Colloque international "Biodiversité et biotechnologie à Madagascar", IMRA, 2006, Madagascar.

Rakotoniriana EF. Endophytisme: source potentielle de nouveaux taxa. Cas des champignons endophytes des écorces de *Cedrelopsis grevei*, une plante endémique malgache. Conférence Internationale 11ème NAPRECA, Panorama, Août 2005, Madagascar.

Posters

Rakotoniriana EF, Andriambololoniaina M, Rajaonarivelo AJ, Randriamampionona D, Raoelison G, Rabemanantsoa C, Cheuk K, Urveg-Ratsimamanga S, El-Jaziri M, Mahillon J, Quetin-Leclercq J, Corbisier AM. Screening for antifungal properties from endophytic fungi. Colloque international "Contrôle et Standardisation des produits d'origine végétale", IMRA, July 2007, Madagascar.

Rakotoniriana EF, Randriamampionona D, Ralijerson B, Ratiarivelo V, Raoelison G, Rabemanantsoa C, Cheuk K, Quetin-Leclercq J, S, El-Jaziri M, Corbisier AM, Van Brandt P, Urveg-Ratsimamanga Approach to the protection, valorization and quality control of malagasy medicinal plants. Symposium international "Bioresources towards drug discovery and development", IUPAC Satellite, University of Mauritius, February 2004, Mauritius Island.

III. Scientific trainings

Gestion des ressources microbiennes. Chaire Francqui, Mars 2008, Gembloux, Belgium.

Management du laboratoire. NUSESA, Octobre 2005, Madagascar.

Initiation à la mycologie: Isolement, identification et conservation des microorganismes endophytes. MUCL, 2001-2004, Louvain-la-Neuve, Belgium.

RECENT TITLES IN THE COLLECTION

2009

N°148, Pokrzywa Wojcieh, Targeting of the yeast Sna3p and Sna4p to the endosomal pathway depends on their interaction with ubiquitin ligase Rsp5p (Promoteur: Morsomme P. / février 2009)

N°149, de Lespinay Alexis, Study of seed priming mechanisms of three plant species used in revegetation of industrial sites (Promoteurs : Lutts S., Declerck S. / mai 2009)

N°150, Hoton Florence, Insights into the Ecology and Genetic Diversity of Cereulide-producing Bacillus cereus strains (Promoteur : Mahillon J. / mai 2009)

N°151, Fornelos Martins Nadine, GIL01 and relatives, a new generation of tectiviruses infecting the *Bacillus cereus* group (Promoteur : Mahillon J. / mai 2009)

N°152, Marghadi Saïd, Intégration des méthodes d'aide à la décision dans l'aménagement multifonctionnel des forêts au Maroc (Promoteurs : Devillez F., Farcy Ch. / juillet 2009)

N°153, Harahagazwe Dieudonné, Heat tolerance assessment of the potato crop: evaluation of CIP clones in the lowlands of Burundi (Promoteurs: Ledent J-F., Rusuku G. / août 2009)

N°154, Godet Marie, Survey of filamentous fungi and development of molecular tools for their rapid and accurate identification, in advanced life support systems like the International Space Station (Promoteurs: Declerck S., Munaut F. / septembre 2009)

N°155, Verbelen Claire, Nanoscale probing of the mycobacterial cell wall (Promoteur: Dufrêne Y. / octobre 2009)

N°156, Gaidashova Svetlana, Effect of plant parasitic nematodes and arbuscular mycorrhizal fungi on banana (*Musa* spp.) in the East African highland cropping systems (Promoteurs: Declerck S., De Waele D. / octobre 2009)

N°157, Gyuricza Veronika, Influence of arbuscular mycorrhizal fungi on the uptake and accumulation of radiocesium by plants and on its redistribution between plants (Promoteur: Declerck S. / novembre 2009)

N°158, Stenuit Benoît, Biological Degradation of 2,4,6-Trinitrotoluene (TNT): Bioprospecting for TNT-Denitrating Bacteria and Deciphering of Multiple TNT Denitration Pathways (Promoteur : Agathos S. / octobre 2009)

N°159, De Muynck Benoit, Expression of a functional antibody in *Nicotiana tabacum* plants and culture cells: analysis of proteolytic products and identification of putative extracellular peptidases involved (Promoteurs: Boutry M., Navarre C. / décembre 2009)

N°160, Mattern Samuel, Mapping and source identification of groundwater pollution by nitrate: Theory and application to the Brusselian sand groundwater body (Promoteur: Bogaert P. / décembre 2009)

N°161, Mehrvar Mohsen, Harahagazwe, Dieudonné, Diversity of soil-borne sugar beet viruses in Iran (Promoteur: Bragard C. / novembre 2009)

2010

N°162, Radoux Julien, Updating lan cover maps by GIS-driven analysis of very high resolution satellite images (Promoteur: Defourny P. / 13 janvier 2010)

N°163, Cochonneau Daphné, Characterization of yeast polymerase gamma mutations associated with human disorders (Promoteur: Foury F. / 24 mars 2010)

N°164, Obsomer Valérie Daphné, Multiscale environmental analysis and prediction for insects vector of disease : Application to malaria vectors in Southest Asia (Promoteur: Defourny P. / 7 mai 2010)

N°165, Lebrun Anne-Sophie, Towards functional and structural characterization of maize ZmSIP proteins belonging to a divergent aquaporin subfamily (Promoteur: Chaumont F. / 8 mars 2010)

N°166, Kalonda Mbulu Gabriel, Analyse de la transmission des prix internationaux des produits agricoles sur les marchés des pays ACP (Promoteur: Henry de Frahan B. / 15 mars 2010)

N°167, Fiamohe Rose, Facteur determinant les échanges de produits vivriers au Bénin : Rôle des institutions de marché (Promoteur : Henry de Frahan B. / 20 avril 2010)

N°168, Hochstenbach Jean-François, The yeast proteolipid Pmp3p is a new component of the plasma membrane microdomains associated with the eisosomes (Promoteur : Morsomme P. / 4 mai 2010)

N°169, Laloy Eric, Measuring and modeling the impact of intercrop management on plotscale runoff and erosion in a continuous maize cropping system (Promoteur : Bielders C. / 31 mai 2010)

N°170, Debecker Damien, MoO3-based heterogeneous catalysts for the metathesis of propene (Promoteur : Gaigneaux E. / 28 mai 2010)

N°171, Cornelis Jean-Thomas, Impact of tree species on silicon cycling in temperate soil-tree systems (Promoteur : Delvaux B. / 25 mai 2010)

N°172, Bontemps Sophie, Towards an automated satellite-based alarm system for global forest monitoring (Promoteur : Defourny P. / 17 juin 2010)