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Important pests and diseases of plantation grown *Pinus* and *Eucalyptus* in Colombia and their control

By

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DECLARATION

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

Carlos Alberto Rodas Pelaez.

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Preface

Colombia covers an area of approximately 114 million hectares (ha) of which the potential forestly land has been estimated at 60.7 million ha, about 53% of the total area. Only 54 million ha are considered as natural forest, leaving approximately 29 million ha to be used for livestock and agriculture. In order to provide an alternative source of timber, Colombian groups have planted approximately 327 000 ha to different species of *Pinus, Eucalyptus* and native species. This clearly represents only a small proportion of the total area that might be used for forestry, which is set to grow in the future.

In general, trees established in plantations have been grown as monocultures that allow for substantial productivity per management unit. But this is also a homogeneous genetic resource that is highly susceptible to damage caused by insect pests and diseases. It is therefore, surprising that relatively little work has been conducted on pests and pathogens negatively affecting plantation foresty in Colombia. In this regard, the studies presented in this thesis present the first comprehensive treatment of the topic for the country. It is consequently hoped that these will form a basis for the future management and sustainability of forestry in Colombia.

In the **first Chapter** of this thesis, I provide a historical background and the current pest and disease situation for the forestry sector in Colombia. This includes an overview of the main pest and diseases affecting commercial non-native trees, especially species of *Pinus* and *Eucalyptus*. A wide range of sites occurring in Colombia were considered and the document also includes the impact of different climatic conditions on the incidence and management of the various pests and diseases treated.

Some of the most important defoliators in Colombia reside in the order Phasmatodea and one of these, *Litosermyle ocanae,* was treated in studies presented in **chapter two**. The overall aim was to contribute basic knowledge of *L. ocanae* including an understanding of the biology, egg population dynamics, and potential biological control assessments based on early detection of the insect.

Chapter three includes the discovery of one of the members of the Geometridae that causes serious damage due to defoliation of *Pinus* and *Eucalyptus* plantations. This pest, known as *Chrysomima semiluteraria*, has been known in Colombia for many years and this study included a comprehensive evaluation of its biology and field monitoring contributing to an Integrated Pest Management strategy for it. The insect was studied under field and laboratory conditions and a special emphasis was placed on its biological control using the egg parasitoid *Telenomus alsophilae*.

Pinus plantations in Colombia have been affected by numerous pests, including recently, the adelgid *Pineus* sp. To establish management strategies to assist commercial forestry operations, the life cycle of this insect and the susceptibility of different *Pinus* species were determined in **Chapter four**. In addition, the possible role of a *Ceraeochrysa* sp. as a biological control agent was investigated.

In **chapter five,** *Fusarium circinatum* is recorded for the first time on *Pinus* spp. in Colombia. The discovery of this fungus, known to cause the disease Pitch Canker has had an important impact on forestry, especially with regard to management strategies aimed at reducing its impact. Studies in this chapter included those to evaluate the susceptibility of families and provenances of *Pinus* spp.*,* which are currently used in forestry planting programs in Colombia.

The first emergence of the foliage pathogen *Dothistroma septosporum* as an important constraint to pine forestry in Colombia is treated in **chapter six**. This fungus is a serious pathogen of many species of *Pinus* around the world. It was, however, not recognised as a serious threat to forestry in Colombia until it emerged as a serious source of damage to *Pinus tecunumanii*. In this chapter, the aim was firstly to confirm the identity of the pathogen based on DNA sequence data. Subsequently, the host range and distribution of the pathogen was established in different Colombian forestry areas. The impact of the disease and the susceptibility of different provenances of *P. tecunumanii* was also assessed.

In **chapter seven,** the main objective was to described the susceptibility of *E. grandis* clones to a new species of *Ceratocystis* and to identify clones resistant to it. The fungus was described as *Ceratocystis neglecta* and management strategies are suggested for it.

This thesis includes two Appendices treating new reports of insect pests and an important *Eucalyptus* pathogen. In Appendix 1, I report on the importance of three different pest insects. Appendix 2 includes a description of a new disease, as well as the potential risks that this disease poses for the Colombian forestry sector.

Chapter 1

Plantation Forestry in Colombia with special reference to Pest and Diseases of *Eucalyptus* and *Pinus*

1. Introduction

Latin America and the Caribbean region have a forest area cover of approximately 49%, which represents 22% of the total woodland cover in the world. In the region, Brazil has the largest forested area with 13% cover and Peru, Colombia, Bolivia, Venezuela and Brazil collectively comprise the 84% of the total forestry area. The commercial plantations in Latin America and the Caribbean include approximately 14.9 million hectares (ha), which represents only 6% of the total area planted in the world. However, in the last decade Brazil, Chile, Argentina, Uruguay and Peru have reported an increase of the planted area at an annual rate of 3.2% (FAO 2011a).

Colombia has a surface area of 114 million ha, of which natural forests make up 60.7 million (53%) (PROEXPORT 2010). Approximately 327 000 ha are used for plantation forestry (MADR 2010). Of this area, *Pinus* species represent one of the most commonly grown non-native trees with 75 000 ha planted. This is followed by *Eucalyptus* species covering 46 000 ha, and other native species occupy 206 000 ha (Anonymous 2011).

In Colombia, protection of the natural forestry resource has strongly influenced plantation forestry. Thus, in 2006, approximately 2 million m^3 of timber, pulp and other forest products were produced in the territory. The requirement for wood production in 2015 has been estimated to be about 7.2 million m^3 and this will be from sustainable forestry plantations (MADR 2006).

In order to develop a sustainable industry, Colombian forestry companies have focused on utilizing natural resources to satisfy the demand for wood products. Thus, in recent years, there has been an increase of approximately 100 000 ha of new plantations established. As an important consequence, the Colombian government has re-structured the forestry sector, which has included a long-term vision for wood management and appropriate measures

for sustainable industries using reforestation to reduce negative impacts on the environment (MADR 2006).

The varied geography in Colombia offers many agro-ecological advantages for planting *Pinus* and *Eucalyptus.* This is due to the wide range of altitudes, weather conditions and soil quality. These provide both genera with optimal and varied conditions that allow rapid growth, adaptability and a high potential to increase yields.

The variable climate in different regions of Colombia has resulted in a wide range of tree pathogens and insects affecting forestry resources. The very different climatic zones are conducive to different pathogens and pests where they are also adapted to the different tree genotypes being propagated (Woods et al. 2005). In the case of diseases, globalization has contributed to the spread of pests and pathogens with dispersal barriers regularly being overcome (Watt et al. 2009, Wingfield et al. 2010, 2011a, 2011b, 2013). Likewise, the genetic uniformity of plantations increases the risks of severe damage occurring.

In their native environments, most pests and pathogens of *Pinus* and *Eucalyptus* do not cause substantial damage. However, when exotic tree species are cultivated in new environments, some insects and pathogens can cause very serious damage. A good example is found in the case of *Cryphonectria* canker of *Eucalyptus,* which has been shown to be caused by a suite of pathogens that have adapted to infect non-native trees (Hodges et al. 1986, Davison and Coates 1991, Wingfield 2003, Gryzenhout et al. 2009). Another classic case is found for the rust fungus *Puccinia psidii*, a native on Myrtaceae in South America that has adapted to infect *Eucalyptus* spp. and other members of the Myrtaceae (Coutinho et al. 1998, Glen et al. 2007). Similarly, in Colombia *Chrysoporthe* species and *Chrysoporthella hodgesiana* have been found on native members of Melastomataceae and these fungi have been shown to also affect non-native *Eucalyptus* spp*.* in plantations (Wingfield et al. 2001, Rodas et al. 2005a, Gryzenhout et al. 2006).

In Colombia, plantations of *Pinus* spp. have been severely damaged by insects in orders including the Lepidoptera: Geometridae; Phasmatodea: Heteronemiidae and Pseudophasmatidae; and Hymenoptera: Formicidae. Here, wide ranges of climate and altitude plus a large diversity of hosts and the adaptability of insects, have contributed to a wide distribution of these pests across the country. This has also resulted in a large number of areas where severe outbreaks have occurred and these have resulted in tremendous financial losses.

Unlike the case with insects, commercial plantation forestry in Colombia has not been seriously affected by outbreaks of pathogens until recently. In the 1980's, the important plant pathogen *Diplodia pinea* was reported affecting *Pinus patula* plantations in the Eastern region, causing substantial damage to plantations. This was in the altitude range between 1500-2000 masl, which is not suitable for this species. Subsequently, *P. patula* has had to be eliminated as a planting stock for these areas (Hoyos 1987, Rodas and Osorio 2008a).

Pinus spp. in Colombia are considered to be seriously threatened by new diseases and insect pests. For example, a serious threat lies in pitch canker caused by *Fusarium circinatum* that has appeared recently in the country affecting *P. patula* (Steenkamp et al. 2012). Likewise, *Dothistroma septosporum* causing red band needle blight of pine is currently damaging *P. tecunumanii, P. kesiya* and *P. oocarpa.* In the nurseries, a recently discovered and described fungus, *Calonetria brassicae,* has caused serious damage to *Pinus* spp. (Lombard et al. 2009).

Eucalyptus spp. in Colombian plantations have been severely damaged by pests and pathogens. For example leaf-cutting ants (Hymenoptera: Formicidae), sucking insects (Hemiptera: Miridae), wood borers (Lepidoptera: Hepialidae) and defoliator insects, mainly in the Geometridae have caused serious problems in some areas. These groups of insects are all native and have adapted to the wide environmental conditions in which *Eucalyptus* spp.

have been planted. Diseases of *Eucalyptus* have been caused by pathogens such as *Neofusicoccum ribis* that damages plantations where trees are stressed (Rodas et al. 2009). Likewise, basal canker caused by *Chrysoporthe cubensis* has resulted in substantial damage in low altitude areas having high humidity and temperature. In this case, the damage has been especially severe on *E. grandis* compared to *E. urograndis,* which is more tolerant to infection (Rodas 2003). Diseases caused by *Cylindrocladium* species have been reported in young plantations and also in nurseries (Crous and Wingfield 1994, Lombard et al. 2010). In Colombia, *Cylindrocladium spathulatum* was reported as an important foliar pathogen including young and mature plantations of *E. grandis* and *E. "urograndis"* (Rodas et al. 2005b)*.*

A new disease in *Eucalyptus* plantations has recently appeared in Colombia (2011) and the pathogen is the rust fungus *P. psidii*. This pathogen was reported several years ago causing a common disease in non-native trees such as *Syzygium jambos* and *Psidium guajava* (Glen et al. 2007, M. J. Wingfield and C.A. Rodas unpublished data), but it has now apparently adapted to infect some *Eucalyptus* clones and seed sources.

The National Institute of Natural Renewal Resources and Environment (INDERENA) in Colombia had the responsibility for forest protection between 1968 and 1993 and it developed a Sanitary Program for Forestry Plantations. Since 1994, forest protection in the country has been managed by private forestry companies. The aim of this review is to present a summary of knowledge relating to forestry in Colombia and to provide an overview of the major pests and pathogens affecting this industry. The focus is on commercial forestry, which is based on non-native trees, especially species of *Pinus* and *Eucalyptus*.

2.0   Plantation Forestry in Colombia

The commercial forest plantation industry in Colombia comprises approximately 327 000 (MADR 2010) ha and this is made up of 23% *Pinus*

species, 14% *Eucalyptus* species and 63% native species. Generally, forestry is poorly developed if one considers that Colombia has nearly 17 million ha of available land for reforestation projects (MADR 2006). Commercial reforestation begun in Colombia in the 1940's with various *Cupressus* species, including *C. lusitanica* being planted. In addition, *Eucalyptus* species such as *E. globulus, E. viminalis,* and *E. citriodora* and some *Pinus* species such as *P. radiata, P. ponderosa* and *P. rigida* were established (UNAL 1955).

By the 1960's, *P. patula* and *Eucalyptus* species were mainly used for commercial plantation purposes. As forestry areas expanded, various native insects began to adapt to feed on these introduced species. It has consequently become evident, similar to the situation in other countries of the world, that the damage by pests and pathogens and consequently the costs of exotic plantation forestry will rise (Wingfield 2003, Slippers et al. 2005, Wingfield et al. 2010, 2011a, 2011b, 2013).

The plantation forest area in Colombia has increased rapidly in recent years to accommodate increased demand for wood products. The most important products obtained from these plantations are solid wood (round logs and saw timber) and pulp, and this accounts for about 0.3% of worldwide production. Most wood produced in Colombia is used locally. However, this situation is changing as new trade with countries such as those in the MERCOSUR (Brazil, Argentina, Paraguay and Uruguay) as well as the United States, El Salvador, Guatemala, Honduras, Canada and some other European emerging countries grows (MADR 2006). While trade agreements and globalization are important trends, they also open up opportunities for the introduction of new pests and pathogens (Wingfield et al. 2008, Liebhold 2012, Wingfield et al. 2013). These represent a serious threat to forestry in Colombia.

In order to increase productivity and yields, the application of biotechnology and appropriate control of pests and diseases is needed. Because *Pinus* and *Eucalyptus* plantation forestry is a long term business, these trees will

continually be exposed to pathogens and insects that may appear at any time. Thus, these agents have become a national concern because they threaten the economic viability and long term sustainability of the forestry industry as well as reforestation programs. For this reason, ICA (Instituto Colombiano Agropecuario) is developing the National Forest Protection Programme for the country.

Pinus patula and *Eucalyptus* spp. are the most important components of commercial plantation forestry in Colombia. *Pinus patula* was introduced into Colombia from Mexico in the 1960's for reforestation. The physiological characteristics of this species were well suited to the varying conditions of climate and soil in the country and the total area planted to this species is now approximately 40 000 ha (Anonymous 2011).

Eucalyptus species were introduced into Colombia from Australia in 1868. Initially, *E. globulus* was used for ornamental purposes but later commercial plantations of this species were established in the Central and Southern region in order to produce solid wood (Noguera 1982). Currently, *Eucalyptus* species cover an area of approximately 46 000 ha in Colombia. The most important planted species is *E. grandis* (Wright and Osorio 1996); due to its superior capacity to adapt to a multiplicity of sites; ease of vegetative propagation, rapid growth and the high quality of its wood and pulp.

3.0   Insect Pests and Diseases in Plantations

3.1   Insect Pests on *Pinus* species

As mentioned above, there are many insect pests that have caused damage to *Pinus* spp. in Colombia and a complete list is presented in Table 1. The following section treats only the most important examples and in this case, it is also based on a relatively limited available knowledge.

3.1.1   Lepidoptera: Geometridae

Several members of the Geometridae are important forest pests (Triplehorn et al. 2005). The first outbreaks of defoliator insects in Colombia were recorded in 1953 and the insect *Oxydia trychiata* was responsible for extensive damage to *C. lusitanica* plantations (Gallego 1959). Since the 1960's, there have been other reports of Geometridae including the native *Glena bisulca, O. trychiata* (Vélez 1972)*, Cargolia arana* (Wiesner and Madrigal 1983)*,* and *Chrysomima semilutearia* (Rodas 1994)*,* among other species that have damaged plantations of *P. patula, C. lusitanica, P. maximinoi, P. tecunumanii,* and *E. grandis.*

The larvae of the Geometridae are responsible for the outbreaks and thus the economic losses that go with them. Favourable environmental conditions usually result in the recovery of these plantations; for example rainfall can result in the restoration of foliage. The main factor affecting the incidence of defoliators in forest plantations in Colombia is the lack of vegetation within plantations that provides habitats for natural enemies of these native pests. As a result, plantations older than six years are most susceptible to infestation. Management of Geometridae is focused on early detection as well as Integrated Pest Management (IPM) with an emphasis on biological control. All of these activities are interrelated and focus on reducing the pest populations.

Integrated pest management for Geometridae in Colombia includes evaluation of the populations based on light trap data. Cultural control includes thinning and pruning of trees to increase their vigor. Biological control using parasitoids, predators, and microbes represents a key component of the management strategy. Here, food substrates such as honey, sugar, and molasses are provided to increase predator and parasitoid populations. In Colombia, bacteria and fungi such as *Bacillus thuringiensis, Beauveria bassiana, Metarhizium anisopliae* and a *Cordyceps* sp. (Bustillo and Drooz 1977, Bustillo 1978, Rodas 1996, Madrigal 2003) are also used to control

populations of the most important Geometridae including *G. bisulca, O. trychiata, C. arana* and *C. semilutearia* (Rodas 1997)*.* These insect pests are treated individually below.

3.1.1.1 *Glena bisulca* **(Lepidoptera: Geometridae)**

Glena bisulca is one of the most frequently occurring and most harmful species in Colombian *P. patula* plantations (Bustillo 1976a, Ladrach 1992). This native insect also affects other forestry plantation species such as different species of *Pinus* and *Eucalyptus.* Larvae feed on the foliage as they pass through five larval stages. The pupae are found concentrated between the litter at the bottom of trees. Adults orientatc themselves parallel to the bark where females lay individual eggs in the crevices, which facilitate easy detection (Drooz and Bustillo 1972, Bustillo 1979) (Figure 1). As is true of many forest pests, their populations rise when there is inappropriate management of the plantations or when there are environmental conditions such as prolonged summers that adversely affect populations of natural enemies.

Biological control is best applied after early detection. The most useful insects for biological control of *G. bisulca* are parasitoids of larvae such as *Cratichneumon* sp. (Hymenoptera: Ichneumonidae), *Elachertus* sp. (Hymenoptera: Eulophidae), *Rogas* sp. (Hymenoptera: Braconidae), and *Siphoniomyia melaena* (Diptera: Tachinidae), which is the most important larval parasitoid for *G. bisulca.* Predators of larvae residing in the Vespidae (Hymenotera) such as *Parachartergus* sp., *Polybia* sp., *Polistes erythrocephalus* and the Pentatomidae (Hemiptera), *Podisus* sp., can also be important. Likewise, birds belonging to the families: Tyrannidae, Trogonidae, Momotidae, Parulidae, Turdidae (Madrigal 2003) and microorganisms such as *B. thuringiensis, Beauveria bassiana* and *Cordyceps* sp. can reduce populations of the pest (Rodas 1996, Madrigal 2003).

3.1.1.2 *Oxydia trychiata* **(Lepidoptera: Geometridae)**

This insect is widely distributed in most South American countries (FAO 2007). In Colombia, it has continuously affected forest trees since 1953, especially *P. patula*, which is the most susceptible host (Gallego 1959, Bustillo 1976b). *Oxydia trychiata* is one of the most devastating insect pests on *P. patula* with high levels of tree mortality being common. However, this insect is also found causing damage to *Eucalyptus* spp., coffee as well as native tree species. Adult moths lay masses eggs under *Pinus* foliage and associated vegetation. The pupae can be found on the ground around infested trees and adult insects commonly mimic dried leaves (Madrigal 2003) (Figure 2).

Silvicultural management is generally poor in plantations where defoliators are problematic. The weather also has a substantial influence on the presence and extent of damage, with infestations being most severe in the dry season. Various biological control options are available to reduce losses due to *O. trychiata*. These include applications of *B. thuringiensis,* which is most frequently used. The parasitoid *Cotesia* sp. (Hymenoptera: Braconidae) and a *Xanthoepalpus* sp. (Diptera: Tachinidae) are also important larval parasitoids (Madrigal 2003). In Colombia, *Telenomus alsophilae* Viereck (Hymenoptera: Scelionidae) is propagated in large numbers on the eggs of *C. semilutearia.* This small wasp was introduced from Virginia (USA) in 1975 for *O. trychiata* control (Bustillo 1976b, Bustillo and Drooz 1977, Drooz et al. 1977). Since its introduction, the egg-parasitoid *T. alsophilae* has been continuously used as the best option for biological control.

3.1.1.3 *Cargolia arana* **(Lepidoptera: Geometridae)**

Cargolia arana is known only in South American countries including Colombia, Peru, Bolivia and Argentina (Covell 1964). In Colombia, it is one of

the most frequently encountered defoliator insects in forestry plantations (Wiesner and Madrigal 1983, Madrigal et al. 1985). *Cargolia arana* outbreaks were originally common in *C. lusitanica, P. patula* and *E. grandis* plantations. However, since 2004, newly established tree species have been seriously infested including *P. maximinoi* and *P. tecunumanii.* This pest causes the same type of damage as other Geometridae*,* effectively cutting needles or parts of tree leaves. Adult moths lay masses eggs on bark of tree stems and branches and the pupae can also be found on the branches and tree trunks (Figure 3).

   Cargolia arana can be found solitary or in groups with other insects such as *G. bisulca, O. trychiata, C. semilutearia, O. trychiata, Sabulodes glaucularia* (Madrigal et al. 1985, FAO 2007). As is true for other Geometridae, outbreaks generally occur where there has been a breach in management strategies, where trees are densely planted or where thinning or pruning have been neglected. Natural enemies of *C. arana* include the egg parasites *T. alsophilae* Viereck (Hymenoptera: Scelionidae), and *Trichogramma* spp. (Hymenoptera: Trichogrammatidae), larval parasites such as *Cratichneumon* sp. and *Pimpla* sp. (Hymenoptera: Ichneumonidae) and predators such as *Podisus* sp. (Hemiptera: Pentatomidae) and the *Polistes* sp. (Hymenoptera: Vespidae) as well as some birds (Madrigal 2003).

3.1.1.4   *Chrysomima semilutearia* (Lepidoptera: Geometridae)

This insect pest was first detected in Colombia in 1991 causing severe damage to *P. patula* plantations (Rodas 1994). *Pinus* spp. have the capacity to refoliate after damage caused by *Chysomima semilutearia.* In contrast, *C. lusitanica* cannot recover from an attack caused by any of the five instars of the larvae. The insect is also commonly encountered in *Eucalyptus* plantations and in natural forests. The adults lay egg masses on the stems and branches and the pupae are found in the same location (Figure 4).

The egg parasitoid *T. alsophilae* introduced to control *O. trychiata* rapidly became an effective biological control agent for *C. semilutearia* resulting in excellent control. Therefore, this small wasp has been mass-reared for release and biological control (Bustillo 1976a). Additionally, in Colombian *Pinus* and *Eucalyptus* plantations, the predator *Podisus* sp. (Hemiptera: Pentatomidae) and important microorganisms such as *Bacillus thuringiensis,* and *B. bassiana* are used as alternative control measures (Rodas 1994). As is clear, most of the strategies used to control *Oxydia trychiata* are also used to control *C. semilutearia,* although some methodological adaptations are required (Rodas 1997).

3.1.2 Phasmatodea: Heteronemiidae, Pseudophasmatidae and Bacillidae

Members of the Order Phasmatodea do not have enlarged hind femora and are not capable of jumping. They are commonly referred to as "walking stick" insects and are slow-moving, herbivorous and nocturnal. When their numbers are great, they can cause very serious damage to trees (Madrigal 2003, Triplehorn et al. 2005). In Colombia, members of all families are forest defoliators.

The first substantial populations of stick insects were detected in Colombian plantations in 1975, but they were not considered to be a serious threat at the time (Madrigal 2003). However, in the early 1980's, their populations exploded and they emerged as a serious threat to forestry (CAR unpublished data). Since 1986, there have been sporadic outbreaks in *P. patula* plantations. The first of these was in the municipality of Pensilvania (Caldas, Colombia), where *Planudes cortex* and *Libethroidea inusitata* appeared as the responsible pests (Madrigal and Abril 1994, Madrigal 1997).

Phasmatodea have a wide range of hosts providing them with different food sources and greater areas of colonization. When an outbreak occurs, the infestation can be associated with more than one species of this order. After

emerging, phasmids cut and feed on needles of *P. patula* and thus nymph and adult's stages are both responsible of the damage. Eggs are dropped into the soil and nymphs emerge and climb trees to feed on the needles. These insects display both asexual and sexual reproduction (Bedford 1978).

Outbreaks of Phasmatodea in Colombia are always associated with *Pinus* spp. in plantations over eigth years old. These are typically also in plantations where ideal silvicultural practices have been neglected. Management of Phasmatodea includes physical control where sticky bands are placed on tree trunks to trap insects and controlled burning is used to reduce populations. Optimal silvicultural conditions such as thinning and pruning also reduce population levels.

Biological control is the most important action used to reduce populations of Phasmatodea in Colombia. Here, native plants are allowed to develop alongside plantations to encourage a build-up of populations of parasitoids. The parasitoid *Adelphe* sp. (Hymenoptera: Chrysididae) identified by Dr. Carlos Sarmiento of the Universidad Nacional de Colombia (CES unpublished data) is one of the most effective egg parasites. Thus, the phasmids are very well managed in the field with the parasitoid populations being increased by providing food substrates such as molasses, sugar and honey for biological control. In addition, the insect pathogen *B. bassiana* has been used to reduce populations of Phasmatodea (CAR unpublished data).

The most representative species associated with forest plantations in Colombia were identified by Dr. David Nickle of The National Museum of Natural History of the Smithsonian Institution during the period 1990-1993 for Smurfit Kappa Cartón de Colombia (SKCC). These included the following: Phasmatodea: Heteronemiidae: *L. inusitata* Hebard; *Litosermyle* sp. near *ocanae* Hebard (Figure 5); *Libethra strigiventrus* Hebard; *Libethra* sp.; *L. spinicollis* Hebard; *Ceroys quadrispinosus* Hebard (Figure 6); *Heteronemia striatus*; Phasmatodea: Pseudophasmatidae: *P. cortex* Hebard (Figure 7); Phasmatodea: Bacillidae: *Acanthoclina* sp.; *Acanthoclina* sp. near *hystrix*

(Nickle personal communication). More recently, 74 new species residing in four new genera of stick insects have been described in Colombia (Conle et al. 2011).

3.1.3 Hymenoptera: Formicidae

The Leaf-cutting ants are considered as one of the five most severely limiting plagues in forestry plantations of South America, especially in Brazil (Forti and Castellani 1997, Camargo et al. 2006). The economic repercussions of damage by ants depends heavily on the age of trees and the environmental conditions that contribute to the development of secondary pest or disease agents that often kill trees (Mendes Filho 1981). The Formicidae are eusocial insects that include at least three castes: queens, males, and workers, therefore, the colony is defined by the division of labor between physical castes (size and morphological differences) (Weber 1972, Verza et al. 2007). Amongst the workers, polymorphism is high and related to the form of labor that is performed (Triplehorn et al. 2005, Camargo et al. 2006). In this regard, they make use of a symbiotic fungus that is cultivated on leaf tissue and which is the main food source for their larvae (Zanetti et al. 2003, Camargo et al. 2006). The fungus *Leucoagaricus gongylophorus* thus forms a mutualistic relationship with both the genera *Atta* and *Acromyrmex,* providing nutrition, and energy for a healthy development of the colony (Weber 1972, Quinlan and Cherret 1979, Silva et al. 2003, Camargo et al. 2008).

In Colombia, the genera *Atta* and *Acromyrmex* are the responsible for leaf-cutting in agricultural crops as well as in *Eucalyptus* and *Pinus* plantations. Four species of *Atta* are most important and these include *A. cephalotes*, *A. columbica*, *A. laevigata* and *A. sexdens* (Mackay and Mackay 1986).

Integrated pest management for leaf-cutting ants in Colombia includes a combination of chemical, cultural, mechanical, biological, and microbial control strategies. Such management includes early detection of the mating flight to prevent new nest formation. Biological control with natural enemies is

also important and includes the use of parasitoids such as *Eibesfeldtphora attae* (Diptera: Phoridae) (Uribe 2012), predators such as *Vescia angrensis* (Hemiptera: Reduviidae), other ants such as *Nomamyrmex esenbeckii* and *N. hartigi* (Hymenoptera: Formicidae). In addition, *Canthon virens* (Coleoptera: Scarabaeidae) and *Taeniolobus sulcipes* (Coleoptera: Carabidae) are useful. Birds such as *Tyrannus melancholicus* (Tyrannidae)*, Pitangus sulphuratus* (Tyrannidae)*,* among others, also represent means to reduce population levels (Madrigal 2003). Fungi such as *M. anisopliae, Trichoderma harzianum* and *B. bassiana* are also being studied as possible control measures for ants (Ortiz 1998, Ortiz and Guzmán 2007). Yet, chemical control remains the most effective and widely used approach to kill ants. This is generally achieved using toxic pellets (Sulfluramid, Fipronil) (Forti et al. 2007), as well as fumigation with chlorpyrifos.

3.1.3.1 *Atta cephalotes* (Hymenoptera: Formicidae)

In Colombia*, A. cephalotes* is the most important species affecting agricultural crops and forestry plantations (Figure 8.1 and 8.2). Effective silviculture and maintaining a healthy condition of the plantations (including age of trees) are essential in order to reduce the damage caused by *A. cephalotes* (Mendes Filho 1981). *Pinus patula* and some species of *Eucalyptus* are able to recover from three repeated defoliations without their productivity being dramatically reduced. However, partial or total defoliation of plants can result on tree death in some cases (Camargo et al. 2006). One difference that can be found between damage caused by *Atta* spp. and *Acromyrmex* spp., is the altitude at which trees are damaged. *Atta cephalotes* is damaging only up to a maximum altitude of 2000 masl (Weber 1972).

In the laboratory, *A. cephalotes* is controlled using some strains of *M. anisopliae* and *T. harzianum.* Pellets including the fungus are made with a mixture of orange juice, corn and wheat (Verza et al. 2006). This biological control option works by the fungi infesting the fungal symbiont of the ants and thus competing with them for food.

3.1.4 Minor insect pests in *Pinus* plantations

There are various insects other than those mentioned above that are less important but considered to be potentially relevant pests in terms of Colombian reforestation. In order of importance, a *Pineus* sp. (Hemiptera: Adelgidae) (Figure 9) first detected in 2008 is found on *P. kesiya, P. maximinoi* and *P. tecunumanii* causing a severe defoliation, slowing growth and affecting productivity. The defoliator *Platycoelia nigrosternalis* (Coleoptera: Scarabaeidae) found in 2008 can damage *P. patula* in plantations (Figure 10), the defoliator *Mesoscia eriophora* (Lepidoptera: Megalopygidae) found in 1992 (Figure 11), and others like *Oiketicus kirbyi* (Lepidoptera: Psychidae) (Figure 12), *Dirphia somniculosa* (Lepidoptera: Saturniidae) damage *P. patula, P. maximinoi* and *P. tecunumanii* (Figure 13). Control of these more minor pests is achieved through broad IPM with an emphasis on biological control.

3.2   Insect Pests on *Eucalyptus* species

In Colombia, *Eucalyptus* plantations have been mainly infested by defoliators such as the Geometridae including *Oxydia vesulia* (Figure 14)*, Bassania schreiteri* (Figure 15)*, Sabulodes caberata* (Figure 16)*,* commonly found at low elevations (900-1500 masl), and that can result in severe defoliation in 4-6 year-old trees. Insects of the order Coleoptera represented by Chrysomelidae, Curculonidae, and Scarabaeidae cause foliar damage and injuries to the roots. The defoliator *Lichnoptera gulo* (Lepidoptera: Noctuidae) causes extensive damage in mature plantations (Figure 17). *Selenothrips rubrocinctus* (Thysanoptera: Thripidae) are sucking insects that can damage the foliage of trees. *Glycaspis brimblecombei* (Hemiptera: Psyllidae) has been recorded sporadically since 2005 and its importance has increased dramatically in recent years (Figure 18). Other minor insects such as *Aepytus* sp. (Lepidoptera: Hepialidae) is an important wood borer in young plantations. *Monalonion velezangeli* and *Horciasisca signatus* (Hemiptera: Miridae) are

among the most important sucking insects in young plantations. Early detection and adequate control measurements including biological control contributes to retaining a natural equilibrium between insect population and *Eucalyptus* plantation.

3.2.1 *Monalonion velezangeli* (Hemiptera: Miridae)

In 2009, *M. velezangeli* was documented for the first time in Colombia affecting *Eucalyptus* in plantations. This insect had previously been known only due to damage on other trees such as avocado (Carvalho and Costa 1988, Londoño and Vargas 2010a), coffee (Ramírez et al. 2007), guava and cocoa (Giraldo et al. 2009), among others (Londoño and Vargas 2010b). However, in more recent years, nymphs and adults have been shown to contribute to serious damage on *Eucalyptus* species, especially in young plantations (Figure 19).

The damage to *Eucalyptus* trees is relatively minor but these trees become seriously damaged due to secondary infections by the fungus *N. ribis* (Rodas et al. 2009). This is very similar to the damage to *Eucalyptus* in Indonesia, where *Helopeltis* spp. (Hemiptera: Miridae) damage stems and this is followed by secondary infections by species of Botryosphaeriaceae (MJW personal communication).

The main damage caused by *M. velezangeli* is through sap sucking on very young buds and leaves. This results in die-back and the loss of apical dominance of trees. After one hour of sap-sucking, dark lesions appear and these rapidly lead to more wide spread necrotic lesions, presumably due to the effects of a toxic saliva. Chemical management of this insect is not the best option in forest plantations, and for this reason, the main strategy is focused on screening of *Eucalyptus* spp. clones for tolerance. Management of weeds to reduce the high moisture levels that favour the pest is also helpful.

3.2.2 Minor insects on *Eucalyptus*

3.2.2.1   *Aepytus* sp. **(Lepidoptera: Hepialidae)**

The wood borer, *Aepytus* sp. is considered as a secondary pest on *Eucalyptus* in plantations (Madrigal 2003). Nevertheless, it could become a major pest when there is poor weed management. Visible symptoms of the damage include galleries constructed on the surface of the xylem. The entrance holes of the pest are covered with a silky material. The galleries of this insect lead to a weakening of the stems that become dry and are easily broken (Figure 20). Management of this insect, as with other minor pests, is preventive and removing weeds from the plantations can be very effective. For control, *B. bassiana* can be effective; however, it is difficult to apply this biological control fungus.

3.2.2.2   *Horciasisca signatus* **(Hemiptera: Miridae)**

Horciasisca signatus is an important pest in nurseries as well as in young plantations, in which nymphs and adults cause damage on leaves and buds, producing yellowish injury (Figure 21). This is due to the presence of toxic saliva and the symptoms are thus similar to those caused by *M. velezangeli*. Damage due to *H. signatus* can result in a need to replant affected areas. Overall, management is preventive and weed control is crucially important**.**

3.3 Diseases of *Pinus* **species**

Relatively little is known regarding the diseases of *Pinus* spp. in Colombia (Table 2). Until very recently, and as noted previously in this review, the only pathogen known to cause damage in pine plantations is *D. pinea*. Other pathogens have emerged more recently and while very little is known about them, these are treated briefly below.

3.3.1 *Fusarium circinatum* **(Pitch Canker)**

Fusarium circinatum is the causal agent of the serious pine disease known as Pitch Canker (Wingfield et al. 2008). The pathogen can cause very serious damage in nurseries, but is best-known for the cankers that it causes on mature trees (Wingfield et al. 2008, Peréz-Sierra et al. 2007).

Fusarium circinatum was first discovered in Colombia in 2006. The most common symptoms associated with infection by the pathogen in Colombia include foliar wilt, shoot, twig die-back and roots with small resin-soaked necrotic lesions, and resinous cankers on trunks and branches (Figure 22). *Pinus patula* is the most susceptible species followed by *P. tecunumanii* (High Elevation) in Colombia. *Pinus maximinoi,* and *P. tecunumanii* (Low Elevation) have shown tolerance to infection and they will thus become increasingly important for commercial forestry in the future (Steenkamp et al. 2012).

Fusarium circinatum is important due to its ability to infect seedlings in nurseries and to result in poor establishment in young plantations. In some countries such as South Africa and Chile, this pathogen has been reported as a major constraint to nursery propagation and in the establishment of new plantations (Viljoen et al. 1992, Wingfield et al. 2002, Wingfield et al. 2008, Mitchell et al. 2011). Efforts to find an effective control measure for Pitch Canker have been focused on the screening for resistant *Pinus* species and nursery sanitation.

3.3.2 *Dothistroma septosporum* **(Red band needle blight)**

Dothistroma septosporum causes a disease known as Red Band Needle Blight. It is a widely distributed pathogen that has been spread to many parts of the world where pines are grown as non-natives in plantations (Gibson 1972, Barnes et al. 2004, Barnes et al. 2008). In Colombia, the only Pine Needle Blight disease recorded (Gibson 1979, 1980) is caused by *Scirrhia acicola* (Dearn.) Siggers. Only recently in 2008, *D. septosporum* was reported

as an important foliar disease in the country, causing very severe damage and yield loss in plantations of *P. tecunumanii, P. oocarpa* and *P. kesiya* (M. J. Wingfield and C. A. Rodas unpublished data). The damage is clearly related to the age of the trees that are most severely affected when they are 2-4 years old. Trees such as these can die due to severe infection (Brown et al. 2003).

Moisture and high levels of precipitation provide favorable conditions in which spores germinate and penetrate through the stomata on the needle surfaces (Brown et al. 2003). The disease in Colombia appears to occur throughout the year due to there being no distinct seasons, which is also very different to the situation in for example New Zealand or Chile. The first symptoms are yellow bands, which then become dark-red to brown, with acervuli at the centers of the lesions. The infection progresses upwards in the crowns from bottom to top and from the inside of the crowns, outwards (Figure 23). For control, screening for tolerant species and silvicultural practices to reduce humidity levels are import.

3.3.3 *Diplodia pinea* (Diplodia shoot blight)

Shoot blight caused by *D. pinea* was the first disease to be recorded in *Pinus* plantations in Colombia. The first report of this disease was made in Cauca Valley in 1984, on *P. patula* in a 7-year-old plantation (Hoyos 1987). However, more recently and since 2004, 4-year-old pines have also been affected in many areas in the country (Rodas and Osorio 2008a). At first, Diplodia shoot blight was confused with Boron deficiency in Colombia. Generally the disease is most serious where *P. patula* has been planted in environments that are not conducive to growth (Hoyos 1987). These areas were typically represented by low altitudes between 1500 to 2000 masl and in plantations older than 7-years of age. However, since 2004 infections by *D. pinea* have been observed affecting younger plantations (4 years of age), as well as at altitudes between 1500 to 2500 masl. This situation has resulted in a reduced feasibility to continue planting *P. patula* in the country.

Pinus patula plantations are wide-spread in Colombia, and the species has been planted at many different altitudes and under stressful conditions. This has resulted in serious outbreaks of shoot blight caused by *D. pinea* (Rodas and Osorio 2008a). Initial symptoms include die-back, cankers, deformed twigs and buds, blighted needles with presence of black fruiting bodies (pycnidia) filled with spores to initiate new infections (Hoyos 1987, Rodas and Osorio 2008a) (Figure 24). It has been observed that needles fall and are continuously replaced, only if the environmental conditions are favorable for growth. Where trees are stressed, they do not recover and typically die (Hoyos 1987). To reduce the impact of the disease, silvicultural practices are required to reduce stress but most importantly, appropriate sites need to be chosen for plantation establishment.

3.3.4 *Calonectria* species (death of *Pinus* cuttings in nurseries)

A new disease has been reported in nurseries in Colombia that was first recognized in 2007 and is caused by the fungal pathogens *C. brassicae* and *C. brachiatica*. These fungi infect *P. tecunumanii,* and *P. maximinoi* hedge plants and young rooted cuttings in the nursery. Symptoms of infection include wilting, collar and root rot, and dieback, discolouration of the vascular tissue with abundant resin formation (Lombard et al. 2009) (Figure 25). Management of this disease is focused on screening of *Pinus* for tolerance to infection, but in the nursery, sanitation is of paramount importance to remove the inoculum.

3.4   Diseases on *Eucalyptus* species

In Colombia, very little work had been conducted on the diseases of *Eucalyptus* until the late 1990's. This coincided with the first extensive planting of these trees in plantations. Some of the most important diseases of *Eucalyptus* in Colombian plantations are due to pathogens such as *C. cubensis* that causes a serious canker disease (Wingfield et al. 2001, Rodas et al. 2005b) and *N. ribis*, affecting shoots, branches and stems and resulting

in losses to *Eucalyptus* spp. in plantations (Rodas et al. 2009). Species of *Calonectria* but particularly the asexual form *Cylindrocladium spathulatum* is an important causal agent of defoliation and low wood productivity (Rodas et al. 2005b). Likewise, the rust fungus *P. psidii* has recently appeared for the first time on *Eucalyptus* in the country (MJW and CAR unpublished data). These diseases are treated briefly in the following section.

3.4.1 *Chrysoporthe cubensis* (Basal stem canker)

Chrysoporthe cubensis, first recorded as *Cryphonectria cubensis,* is one of the most important pathogens in *E. grandis* plantations and is restricted to low altitude areas of Colombia, where average temperatures are over 25°C and relative humidity (HR) is over 75%. The disease is known to cause a basal canker of *Eucalyptus* and in some cases can result in tree death (Sharma et al. 1985, Wingfield et al. 2001). Infection in Colombia due to *C. cubensis* appears in plantations over 6 months of age; common symptoms are sudden die-back of isolated trees or trees in small patches with basal cankers that gradually increase in size on the stems (Rodas 2003) (Figure 26). In other countries similar symptoms have been observed on *Eucalyptus* in plantations, for example in South Africa (Wingfield et al. 1989, Gryzenhout et al. 2004) and Brazil (Hodges et al. 1976, Seixas et al. 2004) but the species of *Chrysoporthe* involved are not all the same. The most effective management option is to plant resistant clones and hybrids of *Eucalyptus* species and also to promote silvicultural practices that reduce the production of wounds, which are sites for infection (Van Der Merwe et al. 2001, Rodas 2003, Alfenas et al. 2004).

3.4.2 *Neofusicoccum ribis* (Shoot blight and stem canker)

Species of the Botryosphaeriaceae have a cosmopolitan distribution in the tropics and sub-tropics and are commonly associated with *Eucalyptus* diseases. The most common symptoms include stem and branch cankers, die-back, bleeding necrosis, coppice failure and seed capsule abortion (Webb

1983, Barnard et al. 1987, Smith et al. 1994, Smith et al. 2001, Alfenas et al. 2004). *Eucalyptus* spp. are infected through wounds, open stomata and lenticels and these fungi are opportunistic wound and stress related pathogens, which can exist for long periods of time as endophytes in asymptomatic tissue (Luttrell 1950, Wene and Schoeneweiss 1980, Schoeneweiss 1981, Slippers and Wingfield 2007, Slippers et al. 2009).

Botryosphaeria canker has been recognized as an important constraint to the productivity of *E. grandis* plantations in Colombia. Since the 1990's, disease caused by *N. ribis* also known as *Botryosphaeria ribis* in its sexual state (Rodas et al. 2009) has been recorded in zones with high moisture, altitude and frequent rainfall. *Eucalyptus grandis* plantations commonly affected by this disease range in age from 6 to 36 months, with the most susceptible trees being those between 18 to 26 months old. Usual symptoms include small necrotic lesions at the points of insertion of twigs on the shoots and these develop to form large irregular cankers causing die-back of shoots. In addition, cankers are located on branches and main stems, giving rise to an abundant production of kino (Figure 27), which degrades the wood quality and produces weak stems that can break during wind or harvesting (Rodas 2003, Rodas et al. 2009).

Control of canker caused by *N. ribis* includes an appropriate selection of *E. grandis* tolerant clones and hybrids. In addition, silvicultural practices that contribute to vigor are important. Furthermore, this disease is associated with stress including insect infestation (see section on *Monalonion velezangeli* above) and it is important to reduce stress wherever possible.

3.4.3 *Calonectria spathulatum* (Shoot and leaf blight)

Calonectria species and their *Cylindrocladium* anamorphs represent important pathogens associated with diverse plant hosts in tropical and subtropical regions of the world (Crous et al. 1994, Crous 2002, Rodas et al. 2005b). These fungi are associated with a wide variety of symptoms including

damping off, root rot, crown canker, leaf spot, seedlings and shoot blight, needle blight, wilt, fruit rot, tuber rot, cutting rot, die-back and stem lesions (Schoch 1999, Crous 2002, Old et al. 2003, Alfenas et al. 2004, Lombard et al. 2010, Chen et al. 2011). In Colombia, the main symptoms of infection by the most important species on *Eucalyptus*, *C. spathulatum* in commercial *E. grandis* plantations include leaf necrosis on the mature leaves on lower branches of young trees. Defoliation develops from the base of trees upwards (Figure 28), therefore, in severe cases tree death can also occur (Rodas 2003, Rodas et al. 2005b). The expression of the disease is usually associated with high humidity and frequent rainfall, which are conditions favoring infection caused by this group of fungi. Infections typically first appear when trees are 12-months-old, and where canopies close, leading to an increase in humidity and leaf wetness.

3.4.4 *Puccinia psidii* (Eucalyptus rust)

The disease caused by *P. psidii* is known as Eucalyptus rust, although this name is misleading given that the pathogen is native in South and Central America where *Eucalyptus* is exotic. This disease is widely distributed in countries of Central and South America, including Argentina, Brazil, Venezuela, Uruguay, Paraguay, Ecuador, Colombia and until recently in Australia (Coutinho et al. 1998, Old et al. 2003, Glen et al. 2007; Peréz et al. 2011, Carnegie et al. 2012, Roux et al 2013).

The main disease symptoms include egg-yellow uredinia that develop on both surfaces of young leaves and shoots leading to leaf deformation and shoot death (Old et al. 2003, Alfenas et al. 2004). In addition, *P. psiidi* can produce defoliation, die-back and stunted growth (FAO 2011b). Young *Eucalyptus* trees are the most susceptible and they remain highly susceptible until about 2-years of age and where humidity in plantations reduces (Old et al. 2003, Alfenas et al. 2004, Pérez et al. 2011).

In Colombia, Eucalyptus rust was not known until 2011 although the pathogen was well-known on other Myrtaceae like *S. jambos* and *P. guajava* (Figure 29). All control measures are now focused on screening clones and hybrids for disease tolerance, which is a strategy commonly applied in Brazil (Alfenas et al. 2004, Glen et al. 2007).

3.4.5 *Ceratocystis* **spp.**

In the 1990´s, wilt and die-back of young *Eucalyptus* trees was recorded in the Republic of Congo (Roux et al. 2000) and Brazil (Laia et al. 1999, Roux et al. 2000). The disease was later found in Uruguay where trees had been recently pruned (Barnes et al. 2003) and the pathogen was identified as *C. fimbriata sensu lato.* This pathogen has also been found in South Africa, although this was in the absence of disease symptoms (Roux et al. 2004). The pathogen on *Eucalyptus* has recently been described as *C. eucalypticola* (van Wyk et al. 2012). In Colombia, die-back and wilt of *Eucalyptus* has been associated with another *Ceratocystis* species that was described as *C. neglecta* (Rodas et al. 2008b). The pathogen enters wounds on the stems of trees and symptoms can include die-back, internal stem discoloration and a rapid death of affected trees (Figure 30). Management is focused on the identification of tolerant *Eucalyptus* species and clones.

4.0   Conclusions

Very little was known regarding pests and pathogens of *Pinus* spp. and *Eucalyptus* spp. in Colombia until relatively recently. But these agents of disease and tree death have gradually increased in importance and they represent a serious and growing threat to plantation productivity in the future. This, alongside the fact that plantation forestry is set to increase in magnitude, suggests strongly that investment in this field will be essential to future sustainability.

In the case of insect pests, the Geometridae are the main group of defoliators that cause economic losses in plantations of *Pinus* and *Eucalyptus* species. Amongst the most important of these pests are *G. bisulca*, *O. trychiata, C. semilutearia* and *C. arana,* but damage in recent years has been reduced below economic thresholds due to very effective Integrated Pest Management with an emphasis on biological control. Walking stick insect (Phasmatodea) populations are being effectively managed with the egg parasitoid *Adelphe* sp. and *B. bassiana* as biological control agents. Leaf cutting ants (*Atta* spp.) remain a serious threat to plantations and this is especially true in the case of the latter group of pests.

Diseases such as those caused by the pathogens *D. pinea, D. septosporum, F. circinatum* and *Calonectria* spp. on *Pinus* spp. and *N. ribis, C. cubensis, C. spathulatum,* and *P. psidii* on *Eucalyptus* spp. have recently been recognized as economically important. Damage due to these diseases is largely being sought through the selection of disease tolerant planting stock. But silvicultural techniques, ensuring that conditions favourable to infection do not prevail in plantations are also considered important and are being pursued.

One of the most important strategies to reduce losses in plantations due to pests and diseases is high quality monitoring and risk assessment. This will also ensure early detection of new pest and disease problems that seriously threaten plantation forestry in Colombia. These approaches form an integral part of the Integrated Pest and Disease Management program that is being established in Colombia.

Research to gain knowledge of pest and diseases present in plantations as well as to understand the biology of the causal agents is essential if losses are to be reduced in the future. These will strongly inform the development of management strategies and the research needs to be an ongoing priority for forestry companies as well as for the Government in Colombia. The aim of the research presented in the chapters of this thesis form part of an effort to

improve the knowledge of the most important pests and diseases in *Eucalyptus* and *Pinus* plantations in Colombia.

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Table 1: Important insect pests on forestry plantation species in Colombia

Order	Family	Species	Year of detection	Damage	Life cycle (days)	Stage*	Host	Report by	Figure
Lepidoptera	Geometridae	*Glena bisulca* Rindge	1968	Defoliation	90-120	L	Pp, Pt, Pm, Cl, Eg	Bustillo 1970, Drooz and Bustillo 1972, Vélez 1972	Figure 1
		Oxydia trychiata (Guenée)	1953	Defoliation	120	L	Pp, Pm, Cl, Eg	Gallego 1959	Figure 2
		Cargolia arana Dognin	1979	Defoliation	74-95	L	Pp, Cl, Pt, Pm	Wiesner and Madrigal 1983	Figure 3
		Chrysomima semilutearia (Felder & Rogenhofer)	1990	Defoliation	94	L	Pp, Cl, Pt, Pm, Eg	Rodas 1994[1]	Figure 4
		Oxydia vesulia Cramer	1997	Defoliation	--------	L	Eg, Eu, Es	Madrigal 2003	Figure 14
		Sabulodes caberata (Guenée)	1997	Defoliation	--------	L	Eg, Eu, Es	Madrigal 2003	Figure 16
		Bassania schreiteri Schaus	1979	Defoliation	98	L	*Pinus* spp., Eg	Wiesner and Madrigal 1983	Figure 15
	Hepialidae	*Aepytus* sp. Herrich-Schäffer	1992	Borer	--------	L	*Eucalyptus* spp.	Madrigal 2003	Figure 20
	Megalopygidae	*Mesoscia eriophora* Sepp.	1992	Defoliation	--------	L	*Pinus* spp.	Madrigal 2003	Figure 11
	Psychidae	*Oiketicus kirbyi* (L&G)	1975	Defoliation	1 year	L	*Pinus* spp., Cl, Eg	Díaz and Ordoñez 1975, Newman 1980	Figure 12
	Saturniidae	*Dirphia somniculosa* (Cramer)	1980	Defoliation	1 year	L	*Pinus* spp., Tl	Wiesner and Madrigal 1983	Figure 13
	Noctuidae	*Lichnoptera gulo* H.S.	1971	Defoliation	103	L	Pp, Eg, Cl	Bustillo 1975	Figure 17
Hemiptera	Psyllidae	*Glycaspis brimblecombei* Moore	2005	Defoliation	---------	N, A	Et, Ec, Eg	LYR personal communication, CAR unpublished data	Figure 18
	Adelgidae	*Pineus* sp. Shimer	2008	Sucking	68	L, A	*Eucalyptus* spp.	CAR unpublished data	Figure 9
	Miridae	*Monalonion velezangeli* Carvalho & Costa	2009	Sucking	61	N, A	Eg	CAR unpublished data	Figure 19
		Horciasisca signatus Distant.	1995	Sucking	--------	N, A	Eg, Eu, Es	Madrigal 2003	Figure 21
Hymenoptera	Formicidae	*Atta cephalotes* (L.)	1958	Defoliation	177	A	*Pinus* spp., Cl, *Eucalyptus* spp.	Mackay and Mackay 1986	Figure 8.1 and 8.2
Coleoptera	Scarabaeidae	*Platycoelia nigrosternalis* Ohaus	2008	Defoliation	--------	A	Pp	CAR unpublished data	Figure 10
Phasmida	Heteronemiidae	*Litosermyle* sp. nr. *ocanae* Hebard	1988	Defoliation	199	N, A.	Pp	Rodas1990[2], Madrigal 2003	Figure 5
		Ceroys quadrispinosus Redt.	1997	Defoliation	-------	N, A	Pp	Rodas 1990[2], Madrigal 1997	Figure 6
	Pseudophasmatidae	*Planudes cortex* Hebard	1987	Defoliation	192	N, A.	Pp	Rodas1990[2], Madrigal 2003	Figure 7

(*) Stage of the insect in which the damage is produced.
L: Larvae; **N**: Nymph; **A**: Adult; **Eg**: *E. grandis*; **Eu**: *E. urograndis*; **Ec**: *E. camaldulensis;* **Et**: *E.tereticornis;* **Es**: *E. saligna;* **Pp**: *P. patula;* **Pk**: *P. kesiya;* **Po**: *P. oocarpa;* **Pt**: *P. tecunumanii;*
Pe: *P. elliottii***; Pm**: *P. maximinoi*; **Cl**: *Cupressus lusitanica;* **Tl**: *Tibouchina lepidota.*
[1]Identification made by Dr. Douglass Ferguson, Taxonomic Services Unit of the Systematic Entomology Laboratory, PSI, USDA.
[2]Identification was made through C.A. Rodas in 1990, by Dr. David Nickle, Research entomologist, Systematic Entomology Laboratory, PSI, USDA.

Table 2: Important diseases on *Pinus* and *Eucalyptus* in Colombia

Family	Pathogen	Year of detection	Age of trees	Host	Reported by	Figure
Nectriaceae	*Fusarium circinatum*	2005	From Nursery	Pp, Pm, Pt	Steenkamp et al. 2012	Figure 22
	Calonectria spp.	2007	Nursery	Pm, Pt	Lombard et al. 2009	Figure 25
	Cylindrocladium spathulatum	1995	From 1 year	*Eucalyptus* spp.	Rodas et al. 2005b	Figure 28
Mycosphaerellaceae	*Dothistroma septosporum*	2008	From 3 months old	Pt, Pk, Po, Pm	MJW and CAR unpublished data	Figure 23
Botryosphaeriaceae	*Diplodia pinea*	1984 -2004	From 4 years old	Pp, Pt	Hoyos 1987, Rodas et al. 2008a	Figure 24
	Neofusicoccum ribis	1991	From 6 months old	Eg, Eu	Rodas et al. 2009	Figure 27
Cryphonectriaceae	*Chrysoporthe cubensis*	1995 - 2003	From 6 months old	Eg, Eu	Wingfield et al. 2001, Rodas 2003	Figure 26
Pucciniaceae	*Puccinia psidii*	2011	From Nursery until 2 years old	Eg, Eu	MJW and CAR unpublished data	Figure 29
Ceratocystidaceae	*Ceratocystis neglecta*	2008	From 6 months old	Eg, Eu	Rodas et al. 2008b	Figure 30

Pp: *P. patula*; **Pk**: *P. kesiya*; **Po**: *P. oocarpa*; **Pt**: *Pinus tecunumanii*; **Eg**: *E. grandis*; **Eu**: *E. urograndis.*

Figure 1: The moth *Glena bisulca* Rindge (Lepidoptera: Geometridae) and some of its natural biological control agents. A. Eggs. B. Larvae. C. Pupae. D. Adult in a typical position on stem. E. The parasitoid *Siphoniomyia melaena* (Diptera: Tachinidae). F. The parasitoid *Rogas* sp. (Hymenoptera: Brachonidae). G. A species of *Siphoniomyia* sp. that parasitizes larvae.
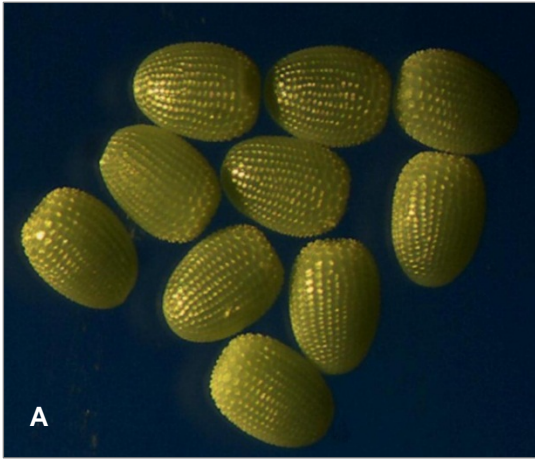
Figure 2: The moth *Oxydia trychiata* (Guenee) (Lepidoptera: Geometridae) showing its different biological stages and some of its natural enemies encountered in Colombia*.* A. Eggs. B. Larvae. C. Pupae. D. Adult. E. Larvae infected with *Bacillus thuringiensis.* F. A bird predator *Momotus momota.* G. A larvae parasitoid *Euplectrus* sp. (Hymenoptera: Eulophidae). H. A Pupae infected by a *Cordyceps* sp. (Hypocreales: Clavicipitaceae) I. *Podisus* sp. (Hemiptera: Pentatomidae) feeding on larvae.

Figure 3: Life stages of the moth *Cargolia arana* (Lepidoptera: Geometridae) and some natural biological control agents encountered in Colombia. A. Eggs on bark of *Pinus patula*. B. Larvae. C. Pupae. D. Adult female. E. The predator *Podisus* sp. (Hemiptera: Pentatomidae). F. The larval and pupal parasitoid *Chratichneumon* sp. (Hymenoptera: Ichneumonidae). G. The egg parasitoid *Telenomus alsophilae* (Hymenoptera: Scelionidae).

Figure 4: The moth *Chrysomima semilutearia* (Lepidoptera: Geometridae) and some of its commonly encountered biological control agents in Colombia. A. Eggs. B. Larvae. C. Pupae. D. Adult female. E. The egg parasitoid *Telenomus alsophilae* (Hymenoptera: Scelionidae). F. The predator *Podisus* sp. (Hemiptera: Pentatomide). G. Larvae infected with the fungal pathogen *Beauveria bassiana* (Hypocreales: Cordycipitaceae).

Figure 5: Life stages of the stick insect *Litosermyle ocanae* (Phasmatodea: Heteronemiidae) and natural biological control agents. A. Eggs. B,C. Nymphs. D. Adult female. E. Adult male. F. A reduviid predator (Hemiptera: Reduviidae). G. An insect infected with the pathogenic fungus *B. bassiana.* H. A female parasitoid wasp *Adelphe* sp. (Hymenoptera: Chrysidadae) emerging from egg. I. *Adelphe* sp. wasp parasitizing eggs. J. Adult male *Adelphe* sp.
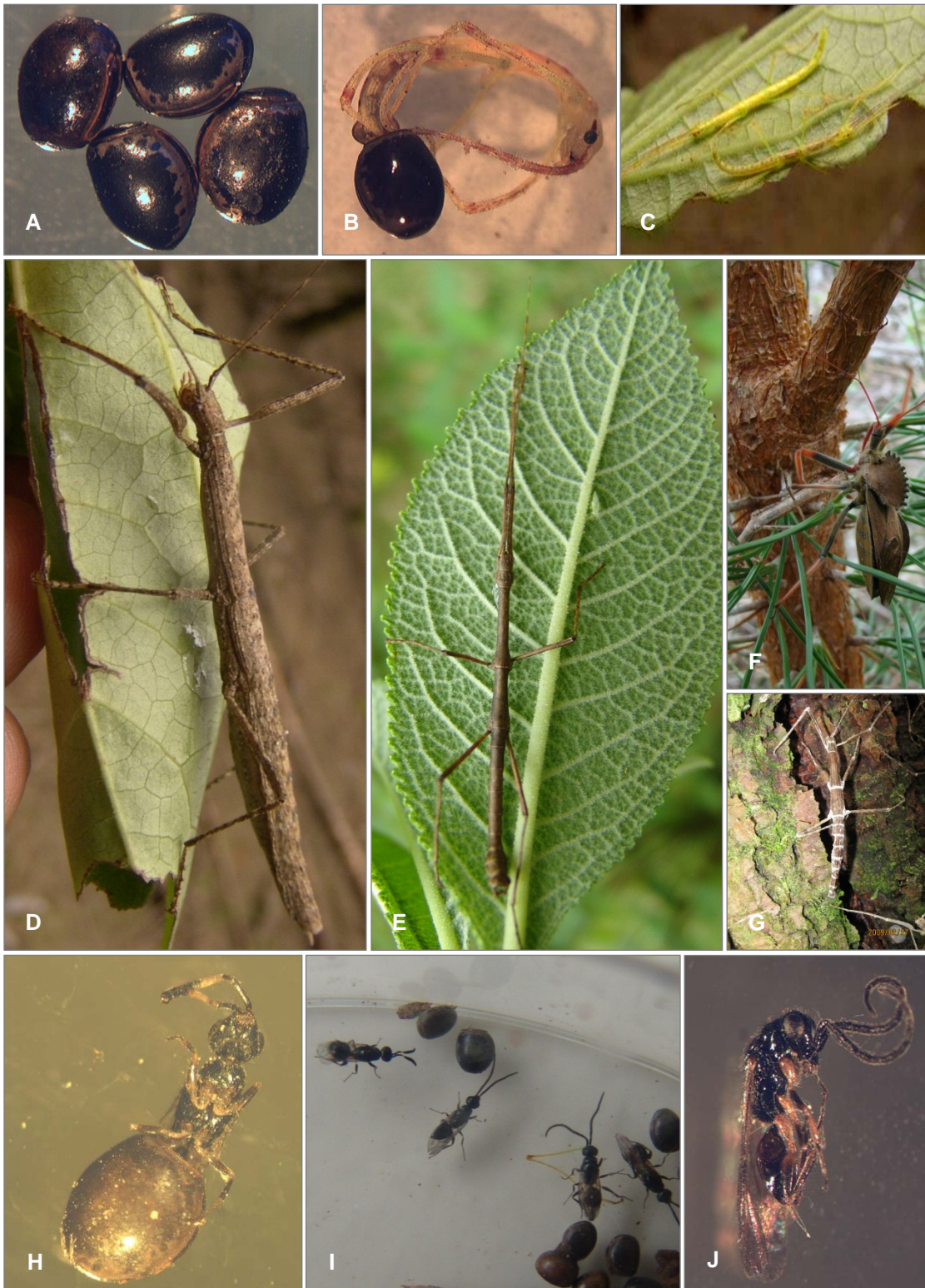
Figure 6: Life stages of the stick insect *Ceroys quadrispinosus* (Phasmatodea: Heteronemiidae)*.* A,B. Eggs. C. Last nymphal instar. D. Adult female feeding *P. patula* needles. E. Adult male on the bark of stem of *P. patula.*

Figure 7: Life stages of the stick insect *Planudes cortex* (Phasmatodea: Pseudophasmatidae)*.* A. Eggs. B. Nymph. C. Adult male showing wings. D. Apterous adult female.
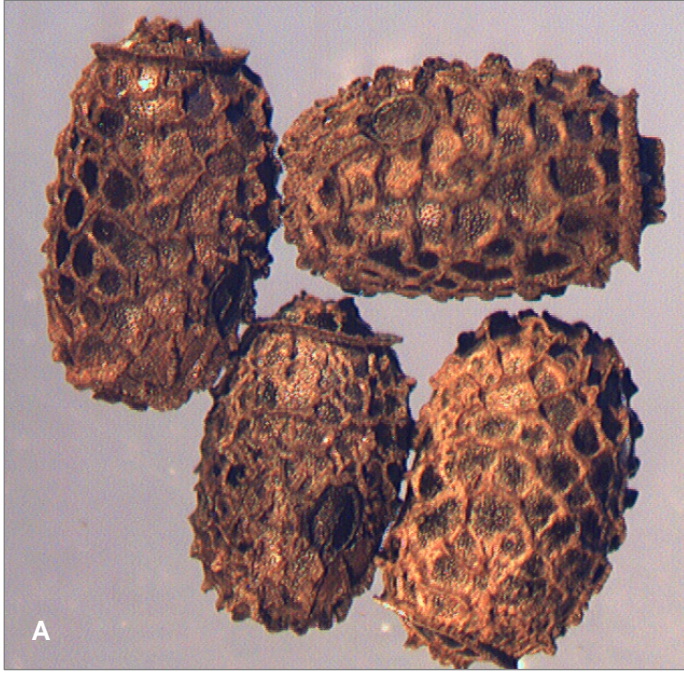
Figure 8.1: Life stages of the leaf-cutting and *Atta cephalotes* (Hymenoptera: Formicidae) and some naturally occurring biological control agents. A. Eggs. B. Larvae. C. Pupae. D. Gardener caste. E. Cutter caste. F. Foragers and explorer caste. G. Soldier caste. H. Gardener ant feeding larvae.

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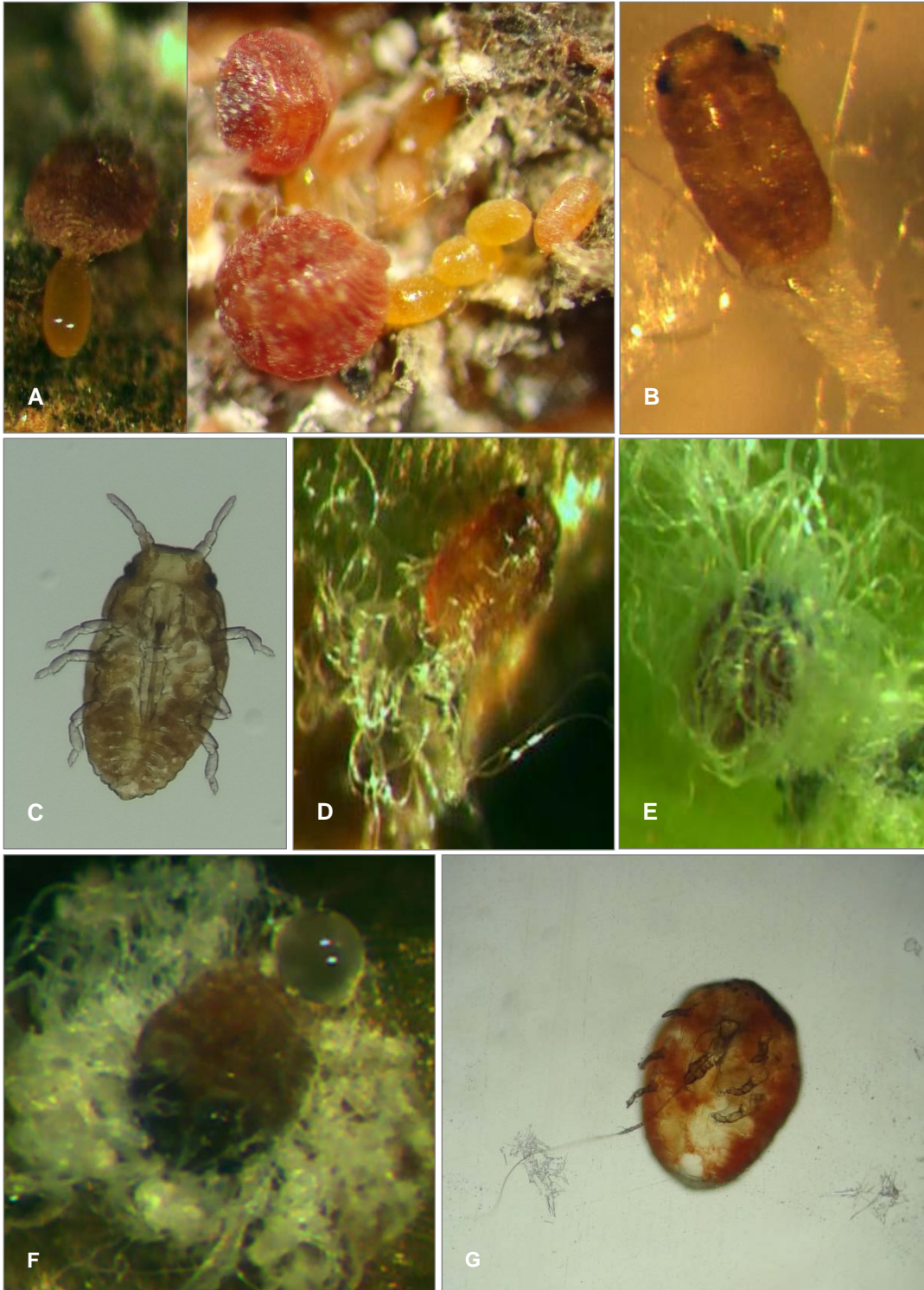
Figure 10: Life stages of the June beetle *Platycoelia nigrosternalis* (Coleoptera: Scarabaeidae) and natural biological control. A. Adult female laying eggs. B. Emerging adult. C. Adults feeding on *P. patula* needles. D. Male and female adults. E. Larvae infected with *Metarhizium* sp. (Hypocreales: Clavicipitaceae).

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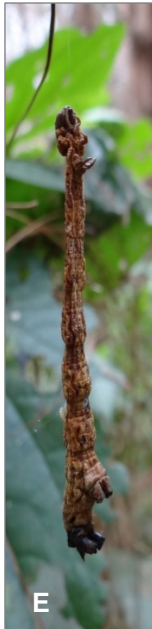
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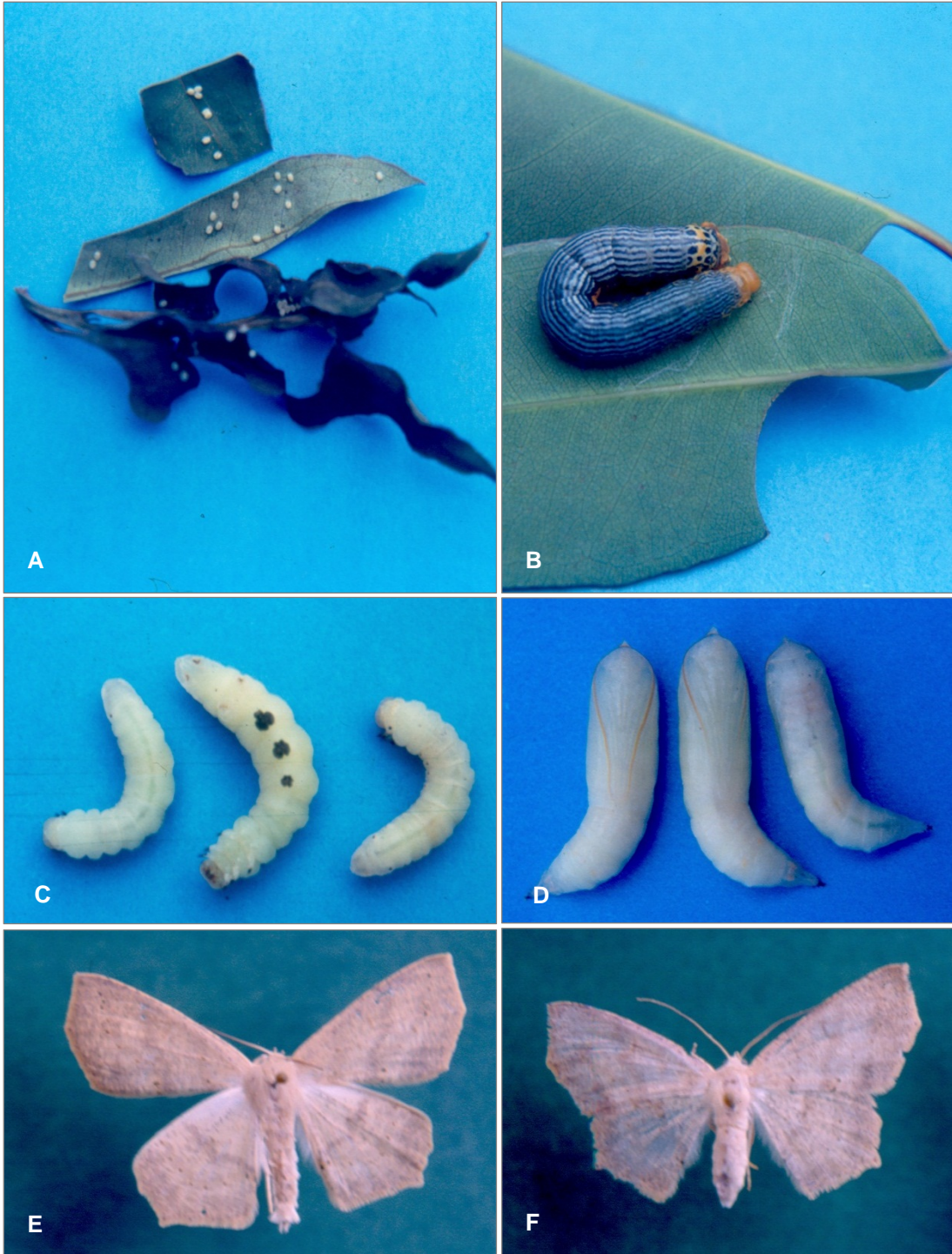
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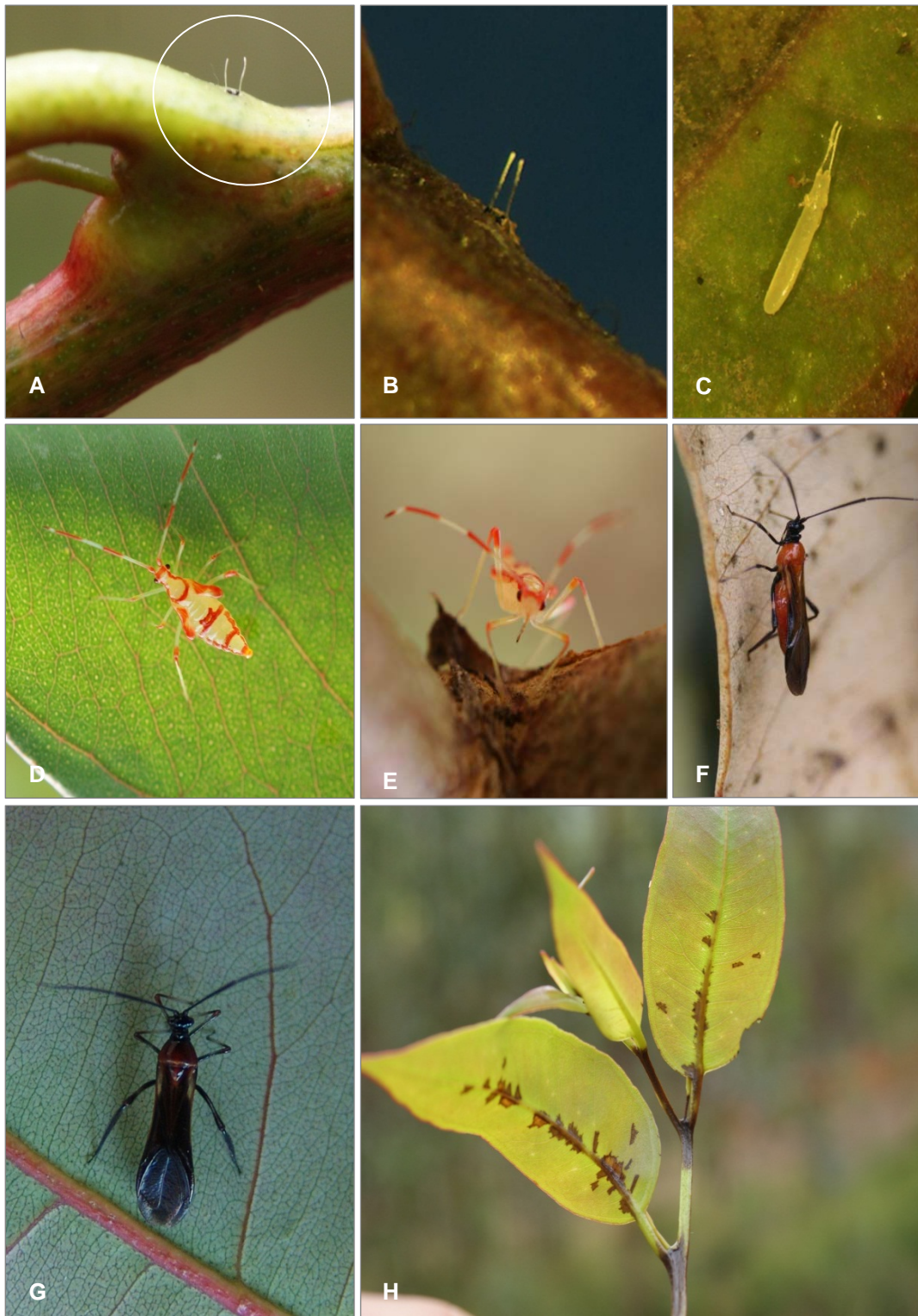
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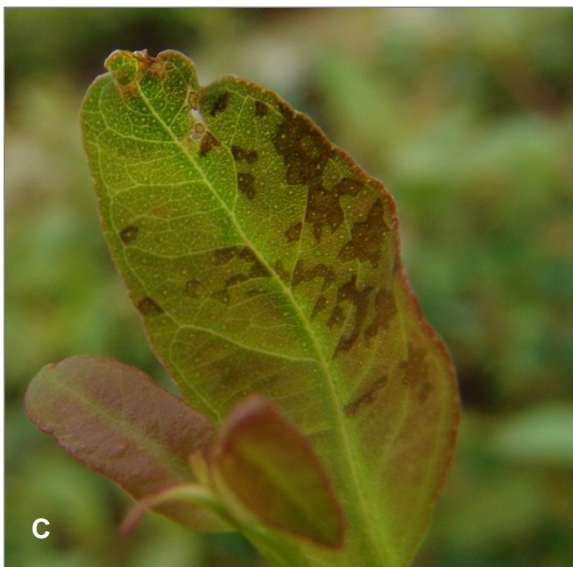
Figure 22: Symptoms associated with the infection of *Pinus* spp. with the canker pathogen *Fusarium circinatum*. A. Stem discoloration with drops of resin seeping from diseased tissue. B,C. Die-back and dead seedlings. D. Constricted root collar with copious resin exudation from infected tissues.

Figure 23: Symptoms associated with the infection by the needle pathogen *Dothistroma septosporum.* A. Initial lesions on needles showing typical yellow to red band. B. Lesions spreading from middle to the tips of needles. C. Acervulae of the pathogen formed on the needles. D. Mass of needles showing death of the tips. E. Affected branches showing apparently healthy current year needles and severely affected needle from the previous growing season. F. Infected trees showing development of the disease from the bottom towards the tops.

Figure 24: Symptoms associated with infection by the fungal pathogen *Diplodia pinea*. A. Lesions at the bases of the needles showing fruiting bodies (pycnidia). B. Small cankers on the young branches and growing tips. C. Dead tips resulting in branch deformation. D. Affected *P. patula* trees in a plantation with most severe damage in moist areas.

Figure 25: Damage to nursery seedlings by the fungus *Calonectria brassiacae.* A. Girdled stem of *Pinus* spp. B. Exposed root collar with discolouration and resin exudation. C,D. Exposed pine seedling root collars showing girdling and discolouration of the cambium.

Figure 26: Symptoms of infection by fungal canker pathogen *Chrysoporthe cubensis.* A. Canker at the base of an *E. grandis* stem. B. Cankers at the base and higher up on stems (insert showing cross section through a canker). C. Fungal fruiting bodies (pycnidia and perithecia) on the surface of the bark covering cankers.

Figure 27: Infection of *E. grandis* by the opportunistic pathogen *Neofusicoccum ribis.* A. Die-back shoots. B,C. Cankers on the branches and main stem. D. Extensive kino production and concomitant weakening of the stems.

Figure 28: Symptoms of infection on *E. grandis* clones by the leaf pathogen *Cylindrocladium spathulatum.* A,B. Leaf necrosis on mature leaves. C,D. Infection typically first appears on lower branches of trees causing with defoliation developing upwards into the crowns of trees.

Figure 29: Symptoms and signs associated with the rust pathogen *Puccinia psidii* on *Eucalyptus* and other Myrtaceae. A-C, Lesions on the leaves and fruit of *S. jambos,* respectively. D,E. Lesions on the leaves and fruits of *P. guajava*, respectively. F,G. Lesions and necrosis on the young growing tips and leaves of *E. citriodora.* H,I. Infected *E. grandis* leaves and shoots.
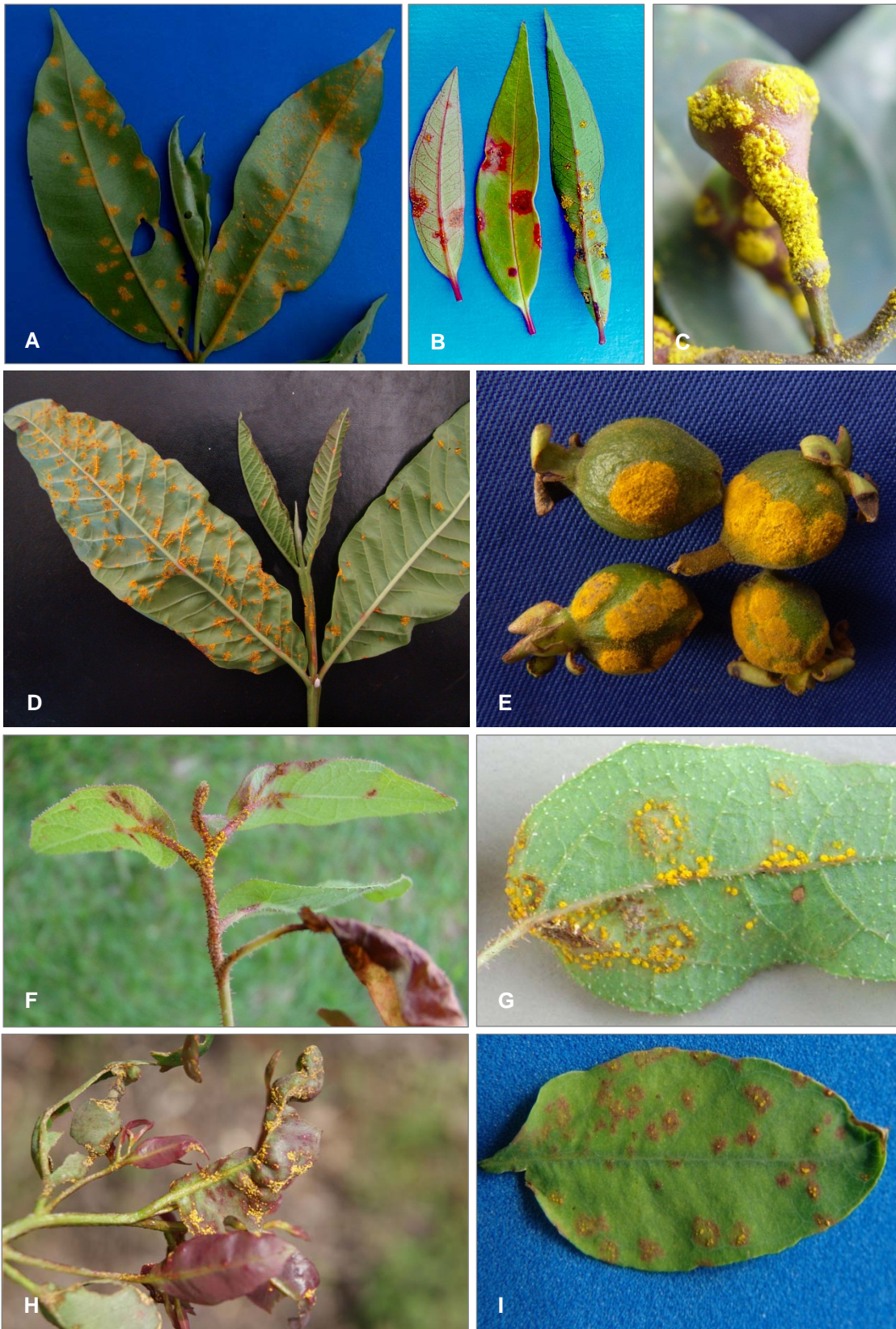
Figure 30: Infection of *Eucalyptus grandis* stems by the fungus *Ceratocystis neglecta* after inoculation*.* A,B. External and internal lesion after one month respectively. C. Lesions after four months illustrating a high level of susceptibility.

Chapter 2

Biology and control of *Litosermyle ocanae* Hebard **in Colombian** *Pinus patula* **plantations**

Abstract

Plantations of *Pinus* spp. in Colombia are severely damaged by various Phasmid (Phasmatodea: Heteronemiidae) insects. Of these, *Litosermyle ocanae* is one of the most serious defoliators. Since 1988, several outbreaks have been recorded in *Pinus patula* plantations including those in the Caldas, Antioquia, Quindío, and Cauca departments, resulting in substantial economic loss. The aim of this study was to identify the most important Phasmatid species found in forestry plantations, to describe its life cycle and to consider strategies to manage losses. To achieve this goal, the research was carried out in both the field and in laboratory studies. An evaluation was made of 28 egg traps randomly placed in a 19-year-old plantation of *P. patula* in the Cauca department. The results showed a total life cycle for males of 225 days and for females 235 days; a complete four instar nymph cycle of 121 days for males and 116 days for females. The sex ratio was 1:1.7 female to male and female individuals laid an average of 112 eggs. Natural parasitism was caused by an undescribed wasp belonging to the genus *Adelphe* (Hymenoptera: Chrysididae), that gave rise to 68.7% parasitism of 7200 eggs. Different food sources were tested to increase the wasp population for control purposes, in which honey and sugar contributed to the longest life cycles. The results of this study provide fundamental data that will be useful in the biological control of *L. ocanae* in *Pinus* plantations in Colombia.

Introduction

From approximately 1950, commercial forestry plantations in Colombia have been negatively affected by a large group of defoliating insects. The most important of these insects reside in the Lepidoptera (Geometridae) (Gallego 1959, Vélez 1972, Bustillo 1976, Rodas 1994), Hymenoptera (Formicidae) (Mackay and Mackay 1986), and Phasmatodea (Heteronemiidae) (Madrigal 1997). Since 1975, there have been heavy levels of infestation, particularly by phasmids that have caused extensive damage to plantations of *Pinus patula* in

different geographic regions of the country (Madrigal and Abril 1994, Madrigal 1997, 2003).

Phasmatodea, commonly referred to as phasmids, phasmatids, stick-insects or walking stick insects are well known defoliators, including in forests (Gullan and Cranston 2005, Conle et al. 2011). The Phasmatodea are well-known as a predominantly tropical order of more than 3000 species that lack a phylogenetically based classification (Otte and Brock 2005, Conle et al. 2011). The Phasmatodea exhibit varied body shapes including cylindrical and stick-like, flattened or leaf-like (Gullan and Cranston 2005) and have mandibulate mouthparts (Key 1991, Gullan and Cranston 2005). Members of the Phasmatodea do not have enlarged hind femora and are thus not capable of jumping (Triplehorn and Johnson 2005), slow-moving, phytophagous and mostly mimic various plant parts such as stems, sticks, and leaves (Costa Lima 1938, Key 1970, Bedford 1978, Gullan and Cranston 2005). Additionally, phasmids have a nocturnal habit (Brock 1999) and display thanatosis, autotomy, diapause in eggs, and both sexual and asexual reproduction (Bedford 1978). When they are in very large numbers, they can cause serious damage to trees in Colombia (Madrigal 2003, Triplehorn and Johnson 2005), principally *P. patula* that is widely established in plantations.

The first outbreaks of phasmids in forestry plantations were recorded in the early 1980's. Between 1986 and 1993, occasional outbreaks caused alarm in the forestry sector. The stick insects involved included a complex of species, including *Ceroys quadrispinosus, Planudes cortex, Heteronemia striatus, Libethroidea inusitata* and *Libethra* sp. (Madrigal 1997, 2003). In 1990, an outbreak caused by *H. striatus* in Riosucio on the Cebada farm (Caldas department), prompted studies to evaluate different biological control options (C.A Rodas, unpublished data). During subsequent years and beginning in 1991, severe outbreaks were documented in *P. patula* plantations in El Tambo and Sotara forest regions (Cauca department) and these continue today.

Between 1990 and 1993, Dr. David Nickle of the National Museum of Natural History of the Smithsonian Institution identified various species of Phasmatodea from Colombia. These included the following: Heteronemiidae: *Libethroidea inusitata*, *Litosermyle* sp. near *ocanae* Hebard, *Libethra strigiventrus* Hebard, *Libethra* sp., *Libethra spinicollis* Hebard, *C. quadrispinosus* Hebard, *Heteronemia striatus;* Phasmatodea: Pseudophasmatidae: *Planudes cortex* Hebard; Phasmatodea: Bacillidae: *Acanthoclina* sp., *Acanthoclina* sp.nr. *hystrix* (Nickle, personal communication). An additional 74 new species and four new genera of phasmids in Colombia were described by Conle et al. (2011), giving a total of 182 described species. Conle et al (2011) estimated that at least 300 species are present in Colombia. Despite the great diversity, information on this group remains incomplete and confusing, largely due to the extensive polymorphism amongst phasmid species and the lack of keys to identify them (Ramírez 2009).

Little is known regarding the biology of the phasmids in Colombian plantation environments, although there have been a number of unpublished studies on the topic. The aim of the present study was, therefore, to determine the life cycle of *Litosermyle ocanae*, one of the most important defoliators in *P. patula* plantations between 1993 to 2012. An additional aim was to assess the population levels of the insect as well as to identify a potential biological control agent and to consider its possible effect on the populations of *L. ocanae*.

Materials and Methods

Study sites

The Sombreros farm (Salinas, Cauca department) was used to assess the population of *L. ocanae* and to collect eggs for the life cycle study. This site is located at 3°51'45''N - 76°29'49''W in plantations of 19-year-old *P. patula*. Climatic conditions included an annual average temperature of 16°C, precipitation of 2175 mm, and the site was 2632 masl. The life cycle studies were performed during 2007 and 2008 at the entomology facilities of Smurfit

Kappa Cartón de Colombia (SKCC) located at 3°51'21''N - 76°30'28''W where weather conditions included an annual average temperature of 20°C, precipitation of 1192 mm, and the laboratories are situated at 1500 masl in Restrepo, Valle del Cauca department (Figure 1).

Population of *L. ocanae*

Insect populations were measured by randomly placing 28 egg traps having a 1 x 1 m surface area and including a 10 x 10 cm mesh center to allow water to pass through. The egg traps were erected 70 cm above soil level (Figure 2A) and eggs were collected from each egg trap every five days between January 2007 and December 2012. Eggs in 28 traps were counted and packed separately in Petri dishes marked with date, trap number, number of eggs, and transported to the laboratory. The mean monthly number of eggs was determined.

Life cycle of *L. ocanae*

To obtain a laboratory population of stick insects, insects emerging from field collected eggs were kept in glass bottles closed with mesh at the top, containing 500 g of sterile saw dust and maintained at 76% relative humidity between 23 - 24°C. Newly laid eggs from the laboratory reared stick insects were removed daily and used for the study. The eggs were placed in separate petri dishes according to the date of oviposition and the duration of eclosion determined. Emerged first instar nymphs were counted and transferred into 5 L glass bottles containing mature pine foliage as a food source. Each nymph was marked on the thorax with water paint to determine the change to the second and followed nymph instar. Nymphs were examined daily and newly moulted nymphs were transferred to separate containers, marked with the date of transfer. This process was continued until the adult stage, and the duration of the nymphal instars determined.

At the adult stage, male and female insects were counted to determine the sex ratio, and placed in separate containers where adult longevity of the different sexes was determined. For the female insects, the pre and post-oviposition periods and number of eggs per female was determined. Only data from insects that completed their life cycle was used to determine the duration of the different life stages. Measurements of the different life stages, not necessarily from the same insects included in the data for the duration study, were taken during the course of the study.

Egg eclosion was further examined under three different environmental conditions to investigate the influence of environment. In the first trial, eggs were collected in the field and then transferred to the laboratory for eclosion; in a second trial, eggs were collected and monitored under field conditions and in a third trial, eggs were collected and monitored from females bred under laboratory conditions.

Evaluation of tree mortality

In order to measure the impact of *L. ocanae* as a defoliator, a study was conducted between 2008 and 2010 on the Sombreros farm in three different areas. These study areas had no previous history of defoliation by the insect but there was a good possibility of their being affected by *L. ocanae*. In each of the study areas, 12 randomly selected groups of 25 trees were chosen to include trees that were dominant, co-dominant and suppressed. In all, there were 300 trees per area available for evaluation. Tree mortality was assessed annually.

Natural Parasitism

Eggs collected from the litter in the field were used to determine whether naturally occurring egg parasitoids were present (Figure 2B). Parasitoid wasps emerging from eggs were enumerated from all eggs collected during the four-year study period. A single parasitoid identified (Sarmiento, Universidad Nacional de Colombia, personal communication) as a species of *Adelphe*

(Hymenoptera: Chrysididae) was found. The sex ratio for the parasitoid was determined from the emerging insects. Additionally, the parasitism percentage was calculated from a total of 7200 eggs.

An appropriate food source for adult *Adelphe* sp. was sought in order to maintain populations of the parasitoid for further investigations. Three different food sources were tested including (i) a water solution containing 30% honey; (ii) a 30% sugar cane solution; and (iii) a water solution containing 30% molasses. Each solution was provided to 20 adult individuals of the *Adelphe* sp. per treatment.

Statistical analyses

Data were analysed using a descriptive analysis in SAS (Statistical Analysis System, 2009). Natural parasitism was analysed using Duncan's Multiple Range Test based on a grouping mean classification.

Results

Population of *L. ocanae*

The egg population fluctuation evaluated between November 2007 and December of 2012 showed the same trends in which the greatest numbers of eggs were produced in November (Figure 3A). This was consistent with the period of highest defoliation (pers. observation) (Figure 3B). The egg production decreased gradually from November to May and from May to August the eggs production remained at a low level, suggesting a high presence of nymphs. During the evaluation period, most nymphs became adults by August. In 2009, it was found that the greatest number of eggs was 25 338, followed by 2011 with 22 470 eggs, 20 567 eggs produced in 2008, and a similar egg production in 2012 with 20 414 eggs collected. During November and December 2007, 9208 eggs were collected (Figure 3B). Data from July to December 2010 was not collected due to traps being destroyed.

Life cycle of *L. ocanae*

Development of *L. ocanae* from eggs to adult death required an average of 225.8 days (200 - 242; *n* = 23) for adult males, and 235.5 days (187 - 257; *n* = 55) for adult females. Egg incubation under laboratory conditions required an average of 65.8 (65 - 68) days for males and 69.8 (56 - 94) days for females. The nymphal stage was 121.6 (103 - 135) days for the males and 116.7 (99 - 136) days for females. The adult stage was an average of 38.3 (16 - 53) days for males and 49.5 (26 - 70) days in the case of females (Table 1).

Results of comparisons between three different environmental conditions for egg incubation were as follows; eggs collected in the field and incubated in the laboratory conditions required an average of 98.9 days (55 - 123; *n* = 167) and eggs collected and incubated in field required an average incubation of 96.6 days (77 - 169; *n* = 537). Eggs that were laid by females bred under laboratory conditions required an average of 66.7 (56 - 94; *n* = 155) days of incubation (Table 2). The *L. ocanae* eggs were measured and presented an average of 2.5 mm (2.1 - 2.8 mm; *n* = 100) long and 2.0 mm (1.7 - 2.5 mm; *n* = 100) wide (Figure 2C).

Litosermyle ocanae had a total of four instars. Nymphs and adults had small anatomical differences differing in color in each instar, first being light yellow and gradually becoming greenish-brown. The duration of each nymphal instar was as follows: for male nymph I, 33.8 (25 - 38) days, nymph II, 30.5 (25 - 37) days, nymph III, 26.1 (22 - 31) days and for nymph IV, 31.1 (18 - 36) days (*n* =23). For females, the nymphal stage I was 29.8 (19 -38) days, nymphal stage II was 28.0 (17 - 37) days, nymphal stage III was 26.7 (19 - 46) days and for nymphal stage IV was 32.1 (18 - 38) days (*n* = 55) (Table 1).

Adults and the fourth instar nymphs differed in size and the nymphs also lacked wings. Emerged nymphs of the first instar had a nymph to egg size ratio of 6:1, giving the impression that they had been rolled inside the eggs (Figure 2D). The range of body length for the first instar nymphs was 12.0 (10.1 - 13.2)

mm (*n* = 20). Nymphal stage II had a body length of 15.3 (10.3 - 19.2) mm (*n* = 21). Third instar nymphs were 27.4 (23.6 - 31.1) mm (*n* = 17) and the fourth instar nymphs were 32.2 (29.8 - 36.9) mm (*n* = 21) long. The average body length for adult males was 33.3 (29.8 - 36.9) mm (*n* = 11) (Figure 2E) while the female body length was an average of 31.9 (30.7 - 32.7) mm (*n* = 8) (Figure 2F). Body length measurements of the different *L. ocanae* stages are described in Table 3.

The adult females displayed a pre-oviposition period of 7.8 days; oviposited for an average of 37.5 days, had an average post-oviposition period of 4.2 days (*n* = 55), and produced an average of 111.7 eggs per female (*n* = 55) (Table 4). The sex ratio for 600 adults was 1:1.7 (female: male).

Evaluation of tree mortality

The evaluation of impact of defoliation on the three categories (dominant, co-dominant and suppressed) of trees in three evaluated areas with 300 trees in each area showed variable results for trees under different levels of stress. In each of the three areas assessed, the highest mortality levels were observed in dominant trees with 1% for 2008, 43% for 2009 and 44% in 2010. This was followed by co-dominant group having mortalities of 8% in (2008), 9% (2009) and 14% for (2010). In the case of the suppressed trees, the average percentage mortality was 10% (2008), 10% (2009) and 11% in (2010), respectively.

Natural Parasitism

From a total of 7200 evaluated eggs, 2087 had been parasitized by *Adelphe* sp., of which 787 were male and 841 were female wasps, giving a sex ratio of 1:1.1 male to female. There were 459 unemerged wasps and 952 emerged nymphs. The remaining unhatched eggs (4161) were considered unviable. Based on this, the percentage parasitism (of viable eggs) was 68.7 % (Table 5).

In the evaluation of food sources to perpetuate the life-cycle of *Adelphe* sp., the water solution with molasses was least effective and allowed survival of wasps for an average of 4.2 days. For the water/honey solution and the water/sugar solution, wasps were able to survive for a mean of 20.8 and 20 days respectively (Table 6). The parasitoid was not found to breed under laboratory conditions and details of its life-cycle must still be determined.

Recorded control agents in this study included the egg parasitoid wasp *Adelphe* sp. (Figure 4A and B) and in addition, some adults were naturally infected by the entomopathogenic fungus *Beauveria bassiana* in field (Figure 4C). Likewise, *Podisus* sp. (Hemiptera: Pentatomidae) (Figure 4D) and flies identified as *Anisia* sp. (Diptera: Tachinidae) (Figure 4E) were found to occasionally act as predators and parasitoids of *L. ocanae* adults and nymphs respectively. Furthermore, some adults of unidentified reduviids (Hemiptera: Reduviidae) (Figure 4F and G) were occasionally observed as predators of *L. ocanae* nymphs.

Discussion

In this study we investigated the life cycle of *L. ocanae*. In particular, we determined the duration and size of the different life stages, pre- and post-oviposition period, potential female fecundity and sex ratio. In addition, we examined the extent of tree mortality caused by *L. ocanae* and investigated the presence of natural enemies. This study represents the first study on the life cycle of *L. ocanae* in Colombia or elsewhere in the world.

Results of this study showed that *L. ocanae* has a life cycle which is different to other members of the Heteronemiidae such as *Libethroidea inusitata*. In the latter insect, adult males lived an average of 217.4 days and adult females lived an average of 198.9 days and it has five nymphal instars (Madrigal and Abril 1994). *Litosermyle ocanae* had similar male and female lifespans (225.8 and 236.6 days, respectively) but only with four nymphal instars. Future studies

should consider the life histories of the many other Heteronemiidae in Colombia as these might also show interesting and different patterns of development.

In the Phasmatodea, the average number of eggs per female during its life cycle is between 60 - 700 eggs/female (Key 1970). In Colombia, *L. inusitata* displayed a biological potential of 220 eggs/female (Madrigal and Abril 1994), whereas in this study *L. ocanae* had an average of 111.7 eggs/female. Such knowledge of the behaviour and population dynamics of each of the life stages of *L. ocanae* emerging from this study, provides some basis on which to devise effective management strategies such as for instance the optimal times for biological or chemical applications.

Stick insects exhibit localized infestations in plantations, mostly due to their reduced mobility. However, observations from this study showed that severe defoliation tends not to extend for long periods of time. This is apparently due to competition with other insects and the impact of natural biological control agents. This is a common characteristic of many native insect pests that damage plantation forestry in Colombia (Gallego 1959, Vélez 1972, Bustillo 1976, Rodas 1994). In this study and over a period of 24 years, *L. ocanae* had between two and three generations before the outbreaks disappeared. In the absence of control measures, this trend will likely continue in the future.

Areas affected by *L. ocanae* have increased in Colombia due to the large range of food sources for this insect and its relatives. Nymphs and adults are responsible for cutting and feeding on needles of *P. patula* plantations over eight years old. This is also a period where silvicultural practices are not effectively applied, due to the economic costs for the long periods needed to propagate these trees.

The discovery of a relatively abundant parasitoid, *Adelphe* sp. holds some promise for the management of *L. ocanae* in Colombia. *Adelphe* spp., are commonly referred to as cuckoo wasps and are well-known stick insect egg parasitoids (Krombein 1956, Annonymous 1985, Kimsey 1986). These

parasitoids are adapted to feed on the fluid contents of the eggs after oviposition (Krombein 1986). The best food source for *Adelphe* sp. used under laboratory conditions was the water/honey solution that contributed to the longest period of survival with an average of 20.8 days. Food sources for insects such as *Adelphe* sp., are of vital importance in biological control programmes (Debach 1968). In Colombia, an important issue for biological control of forest insect pests like *Glena bisulca*, *Oxydia trychiata* (Lepidoptera: Geometridae) and other defoliatiors, is to feed their respective biological control agents, *Siphoniomya melaena* and *Xanthoepalpus* sp. (Diptera: Tachiniidae), with different sources of carbohydrates and protein. In this study, the best evaluated source of nutrition for *Adelphe* sp. were the honey/water and sugar/water solutions, which contributed to longer survival and the efficiency of parasitism resulting in a 70% of reduction of the *L. ocanae* population.

This study provided information on the life cycle and importance of *L. ocanae* as a pest of *Pinus* species in Colombia. Furthermore, we identified an egg parasitoid of *L. ocanae* which shows promise to use as a native biological control agent. These results are important in the development of a management strategy for this insect in Colombia. Continued monitoring is required to assess the spread and impact of this insect. In addition, further work on the rearing and effectiveness of the *Adelphe* sp. is needed, as well as research on other management tactics including host resistance.

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Table 1: Life stages of *Litosermyle ocanae* male (*n* = 23) and female (*n* = 55) in days.

Biological stage		Male				Female			
		Min.	Max.	Mean	SD	Min.	Max.	Mean	SD
Egg		65	68	65.8	1.4	56	94	69.8	10.7
Nymph	I	25	38	33.8	4.4	19	38	29.8	6.8
	II	25	37	30.5	4.3	17	37	28.0	6.8
	III	22	31	26.1	2.3	19	46	26.7	4.2
	IV	18	36	31.1	4	18	38	32.1	4.4
Total Nymph		103	135	121.6	9.7	99	136	116.7	12.4
Adults		16	53	38.3	11.2	26	70	49.5	9
Total		200	242	225.8	9.8	187	257	235.5	14.2

Table 2: Comparison between three different environments for egg eclosion in days

Environment	Min.	Max.	Mean	SD	*n*
Field – Laboratory	55	123	98.9	16.6	167
Field	77	169	96.6	11.2	537
Laboratory	56	94	66.7	8.8	155

Table 3: Size (mm) of the different states of *L. ocanae.* For the eggs, length and width and for and nymphs and adults, only body length was measured.

Biological state		Min.	Max.	Mean	SD	CV	*n*
Egg	Wide	1.7	2.5	2.0	0.1	5.5	100
	Length	2.1	2.8	2.5	0.1	5.6	100
Nymph	I	10.1	13.2	12.0	1.1	9.3	20
	II	10.3	19.2	15.3	2.4	15.6	21
	III	23.6	31.1	27.4	2.3	8.3	17
	IV	29.8	36.9	32.2	1.8	5.6	21
Male Adult		29.8	36.9	33.3	2.3	6.8	11
Female Adult		30.7	32.7	31.9	1.1	3.5	8

Table 4: Longevity of adult female *L. ocanae* (n =55), in days, and eggs per female.

Female adult stage	Min.	Max.	Mean	SD
Pre-oviposition	2	17	7.8	3.5
Oviposition	18	51	37.5	9
Post-oviposition	1	11	4.2	3.3
Total adult	21	79	49.5	3.23
Eggs/female	16	193	111.7	38.6

Table 5: Details of *Adelphe* sp. (Hym.: Chrysididae) from eight different *Litosermyle ocanae* egg collections.

	Eggs number	Male	Female	Sex ratio	Wasps unemerged	Total wasps	Total Nymphs	% Parasitism
	1000	76	64	1:0.8	109	249	18	93.3
	1000	104	102	1:1	135	341	211	61.8
	1000	82	72	1:0.9	86	240	32	88.2
	1000	57	66	1:1.2	83	206	128	61.7
	1300	190	239	1:1.3	0	429	179	70.6
	450	60	72	1:1.2	21	153	97	61.2
	450	24	136	1:5.7	25	185	108	63.1
	1000	194	90	1:0.5	0	284	179	61.3
Total	7200	787	841	1:1.1	459	2087	952	68.7

Table 6: Comparison between three different food sources for wasps (*Adelphe* sp.), in days. Duncan groups A (Honey and Sugar) and B (Molasses) presents statistical differences among them.

Carbohydrate sources	Min.	Max.	Mean	SD	*n*	Duncan group[1]
Honey	17	26	20.8	2.4	20	A
Sugar	14	25	20	2.6	20	A
Molasses	1	7	4.2	1.6	20	B

[1]Duncan grouping is based on the mean analyses using SAS, 2009

Figure 1: Localities where research was conducted in Colombia; Sombreros farm in Cauca department and the Entomology laboratory in Restrepo (Valle del Cauca department).
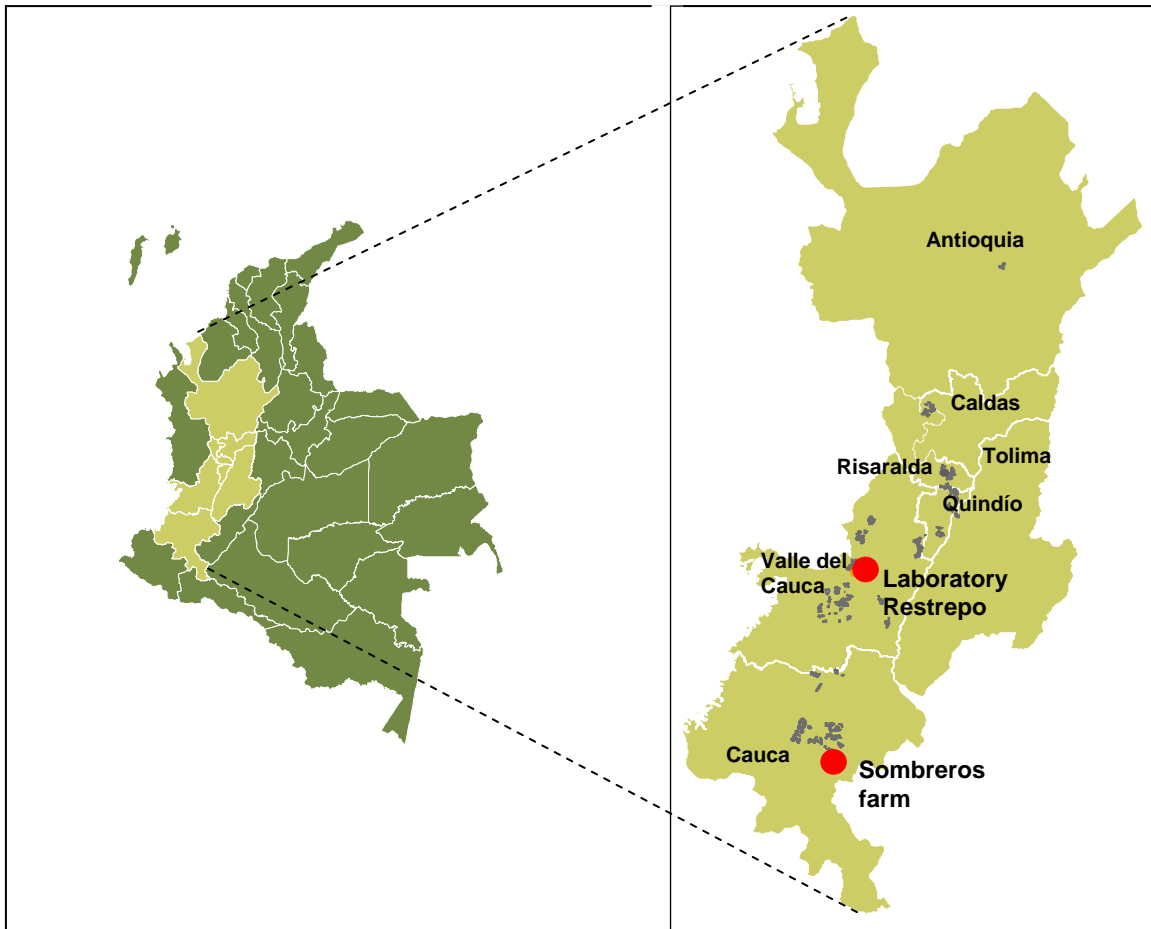
Figure 2: *Litosermyle ocanae* (Phasmatodea Heteronemiidae). A. Egg traps. B. Eggs on litter. Measures of: C. Eggs. D. Nymph I with an egg size comparison. E. Adult male. F. Adult female.

L :2.1–2.8 (2.4) x W: 1.7- 2.5 (2.0) mm

L: 10.1 -13.2 (11.9) mm

L: 29.8 -26.9 (33.3) mm

L: 30.7 – 32.7 (31.9) mm
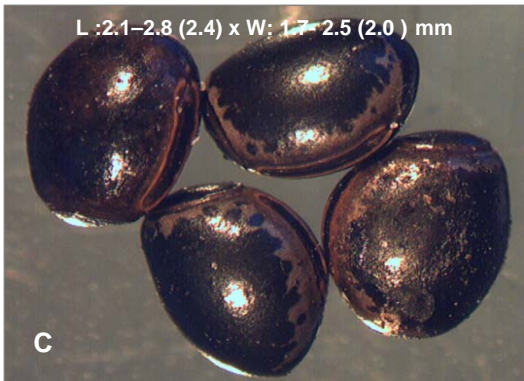
Figure 3: A. Egg population fluctuation in *Litosermyle ocanae* collected between 2007 and 2012. B. Affected area in which egg traps were installed.
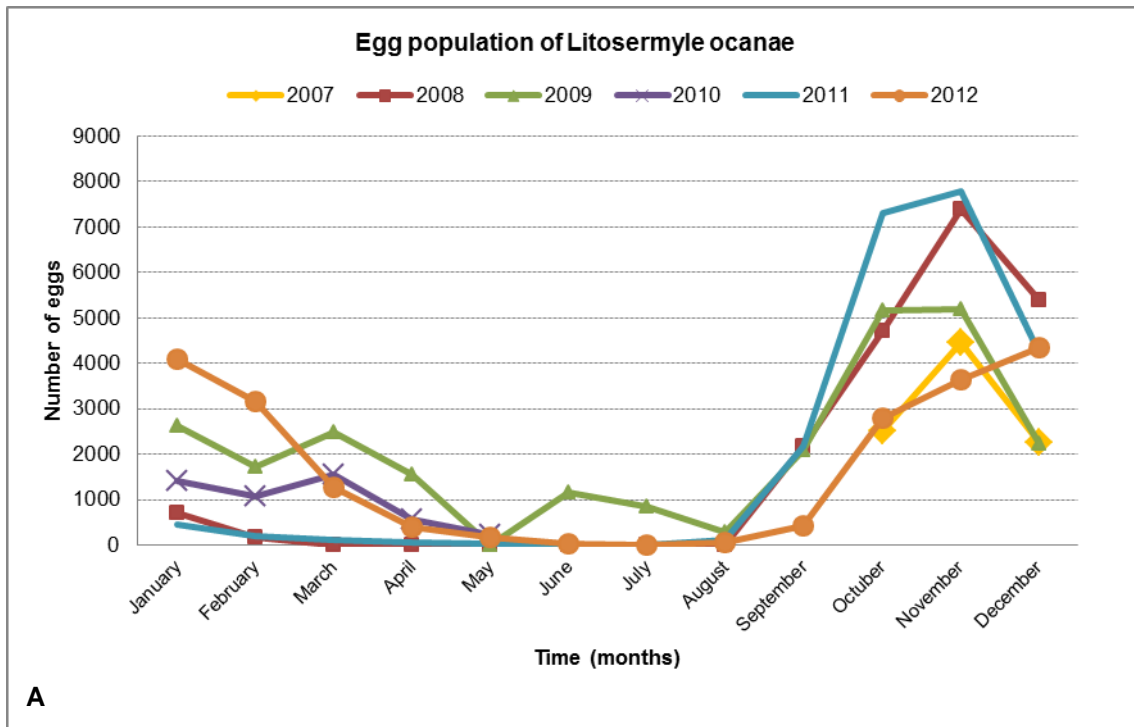
Figure 4: Parasitoids, predators and pathogens of *Litosermyle ocanae* (Phasmatodea: Heteronemiidae). A. *Adelphe* sp. (Male). B. *Adelphe* sp. (Female). C. Adult *L. ocanae* infected with the pathogenic fungus *Beauveria bassiana*. D. *Podisus* sp. (Hemiptera: Pentatomidae) predating an adult *L. ocanae*. E. Pupae of *Anisia* sp. (Diptera: Tachinidae) alongside to an adult *L. ocanae*. F,G. Unidentified reduviid predators (Hemiptera: Reduviidae), predating on adults and nymphs of *L. ocanae.*

Chapter 3

Biology and biological control of *Chrysomima*

***semiluteraria* in Colombian *Pinus* plantations**

Abstract

Geometrid insects are amongst the most important insect pests of commercial forestry in Colombia. In 1991, *Chrysomima semilutearia* (Lepidoptera: Geometridae) was detected as a new defoliator causing damage on *Pinus patula, Cupressus lusitanica* and *Eucalyptus grandis.* Very little is known regarding this insect and this study sought to understand its life-cycle and opportunities for biological control using the egg-parasitoid *Telenomus alsophilae.* The life cycle of *C. semilutearia* was studied on *P. patula* and the efficiency of *T. alsophilae* was considered using *Oxydia trychiata* and *C. semilutearia* as rearing hosts. *Chrysomima semilutearia* had a total life cycle of 97.6 days, comprising a seven day egg stage followed by a 50.2 day larval stage, a 25.4 day pupal and a 15.1 day adult stage. *Telenomus alsophilae* had a life cycle of 28.81 days when reared on *C. semilutearia* (sex ratio 2.68:1, female: male), while on *O. trychiata* the life cycle was 27.17 days (sex ratio 2.36:1, female: male). The percentage emergence of *T. alsophilae* was 87.87% on *O. trychiata,* and the lowest percentage emergence was 73.74% on *C. semilutearia. Telenomus alsophilae* parasitized a maximum of 230 (average 126.1) eggs per female on *C. semilutearia* and 190 (average 98.1) eggs on *O. trychiata.* Overall, *C. semilutearia* was the most efficient rearing host for *T. alsophilae,* which can be mass reared for biological control of the pest.

Introduction

Since the 1950's, Colombian Forestry plantations had been negatively affected by a large group of defoliating insects. These included Lepidoptera (Geometridae) (Gallego 1959, Vélez 1972, Bustillo 1976, Rodas 1994, Rodas and Madrigal 1996), Hymenoptera (Formicidae) (Mackay and Mackay 1986), and Phasmatodea (Heteronemiidae) (Madrigal 1997). These insects result in serious damage to various species of *Pinus* and especially *P. patula*, which is widely grown in Colombia.

Several members of the Geometridae are considered as important forest pests worldwide (Triplehorn and Johnson 2005). Likewise, in Colombia, this family of defoliators represents one of the most important agents of damage to commercially propagated *Pinus* spp. In 1953, *Oxydia trychiata* was recorded as the first geometrid to damage Colombian plantations, causing a severe infestation in *Cupressus lusitanica* plantations (Gallego 1959). Since 1956, other geometrids such as *Glena bisulca* (Vélez 1972)*, Cargolia arana* (Wiesner and Madrigal 1983, Madrigal et al. 1985), *Melanolophia commotaria* (Madrigal 1982, Wiesner and Madrigal 1983), and *Chrysomima semilutearia* (Rodas 1994) have emerged as relevant defoliators, particularly in *Pinus* plantations of seven years and older. These insect species also commonly co-occur in a plantation, which exacerbates the resulting damage.

In 1991, *Chrysomima semilutearia* was detected as a new defoliator causing damage on *Pinus patula, Cupressus lusitanica* and *Eucalyptus grandis* (Rodas 1994, Rodas and Madrigal 1996)*,* with *P. patula*, the most severely affected species. The identification of *C. semilutearia* was made by Dr. Douglass Ferguson of the Systematic Entomology Laboratory of the Agricultural Department of United States in 1992 (Ferguson personal communication).

Management strategies using biological control provide an important alternative to reduce the impact of insect pests in plantations. Biological control using mass-reared and released insect parasitoids, represent a highly effective component of an Integrated Pest Management strategy for plantation forestry. For the biological control of Lepidopteran defoliators *Telonomus* spp. (Scelionidae: Hymenoptera) are commonly used (Krombein et al. 1979, Orr 1988, Hébert et al. 2001). *Telenomus alsophilae* (Hymenoptera: Scelionidae) has been used in USA as an effective biological control agent of *Alsophila pometaria* and *Abbotana clemataria* (Lepidoptera: Geometridae), with *A. clemataria* used as the main rearing host (Drooz et al. 1977). In Colombia, *T. alsophilae* was introduced in 1975 for the biological control of the geometrid defoliator *O. trychiata* (Drooz 1975, Drooz et al. 1977).

In Colombia and worldwide, very little is known of *C. semilutearia* as a forest pest. The purpose of this study was to determine the life cycle and biology of this geometrid defoliator on *P. patula* in Colombia. In addition, the efficiency of the introduced egg parasitoid *T. alsophilae* was evaluated in two different hosts; *O. trychiata* and *C. semilutearia.* The life cycle of *T. alsophilae* including, emergence percentage, sex ratio, number of parasite eggs per female and longevity were compared on the two host geometrids.

Materials and Methods

Life cycle

Pupae of *C. semilutearia* were collected in La Colonia farm located in Popayán (Cauca) in Colombia at 2°34′50′′N - 76°38′18′′W [T°: 19°C, 1769 masl, RH: 70%, precipitation: 2260 mm/year], and then transferred to the entomology laboratory at 3°51'21''N - 76°30'28''W [T°: 20°C, 1450 masl, RH: 75%, precipitation: 856 mm]. The study was conducted between January and June 2012. Life cycle studies began with pupae of known age stored in sterile sawdust in plastic containers (20 x 20 cm) covered with mesh. Newly emerged adults were placed in wooden cages (1 x 1 x 1 m), with paper strips hanging from the ceilings that served as sites for oviposition. Adult moths were fed with a solution of honey (30%) and eggs were collected daily and placed in glass bottles with dates of oviposition recorded. Incubation, pre-oviposition, oviposition and post-oviposition periods, percentage of emergence, number of eggs, retained eggs, and reproduction capacity (total oviposited eggs + retained eggs for each female) per female were then determined. Newly emerged larvae were placed individually in glass bottles (500 ml) containing mature *P. patula* needles (replaced weekly) as a food source and duration of the different larvae instars was calculated. The size of the different life stages was measured and the morphology noted.

Life cycle and parasitism of *T. alsophilae*

Oxydia trychiata and *C. semilutearia* were used as hosts for *T. alsophilae*. To determine the life cycle of the parasitoids, 1724 eggs of *O. trychiata* and 1301 eggs of *C. semilutearia* were exposed to *T. alsophilae* for 24 hours at 25°C and relative humidity (HR) of 76%. The eggs used were approximately 12 hours old. After the exposure to the parasitoids, eggs were transferred to a development room (21°C, 75% RH) for adult emergence. Adult females were taken and divided in two additional rooms; one for parasitism studies (23°C, 75% RH) and one to conserve parasitoids (14°C, 75% RH) where longevity could be determined under two different environments for *T. alsophilae* infested *C. semilutearia* eggs. Emerged adults were counted and wasp sex ratio, emergence percentage, and adult longevity were counted.

To compare parasitism capacity between *C. semilutearia* and *O. trychiata*, 20 female and 20 male wasps of *T. alsophilae* that emerged from eggs of each of these hosts were collected. Each pair (female: male) were placed individually in glass bottles (3.8 cm high and 2.5 cm diameter). Every 24 hours, 10 eggs of *C. semilutearia* were introduced in each bottle containing a pair that emerged from *C. semilutearia* eggs and 10 eggs of *O. trychiata* were introduced in each bottle containing a pair that emerged from *O. trychiata*. This was continued until the females died. The eggs were removed and the number of emerging *T. alsophilae* recorded to determine parasitism levels.

Results

Life cycle

The total life cycle of *C. semilutearia* took an average of 97.6 (48 -162) days. Eggs hatched after an average of 7 days, the larvae fed for an average of 50.2 days, having six instars; the pupal stage was an average of 25.4 days and the adult stage an average of 15.1 days (Table 1). All of the eggs collected (*n* = 114) hatched in 7 days. The average egg length was 0.62 (0.51 - 0.71) mm with an average width of 0.67 (0.36 - 0.79) mm (Table 2). Eggs were irregular laid in

masses on the trunks and twigs of trees and were cylindrical except for the micropylar area that was flattened with a crown area and slightly convex operculum (Figure 1A). The eggs were olive green when newly laid, but after 24 hours they became pale yellow. By the second day the eggs were reddish-brown and became dark red between the 5th and 6th day, and dark grey just prior to eclosion.

Chrysomima semilutearia had a total of six larval instars with a total larval development of 50.2 (22 – 85) days. Newly hatched larvae had an average length of 2.59 (1.60 - 3.40) mm with an average cephalic capsule width of 0.33 (0.10 - 0.45) mm, and were black with a longitudinal stripe in the pleural area. Fully developed larvae in the sixth instar had an average length of 39.39 (29.50 - 50.10) mm with a cephalic capsule width of 3.20 (2.20 - 3.80) mm and varied in colour from greenish-brownish to brownish-red. Post second instar larvae had: 1) a horn-like structure on the anterodorsal pronotum above the head; 2) a small dorsal prominence on the second abdominal segment; 3) a small ventral prominence on the third abdominal segment and; 4) conically shaped dorsal prominence on the eighth abdominal segment (Figure 1B). *Chrysomima semilutearia* larvae had a nocturnal feeding habit and during the day maintained a rigid position to mimic the needles and small branches.

Chrysomima semilutearia larvae searched for small holes on the tree bark trunk and branches for pupation. The pupal colour varied from light brown to dark brown, and it was possible to differentiate between sexes based on size. The pupal stage lasted between 16 and 40 (25.4) days and male pupae were an average of 15.86 (14.30 - 21.00) mm long and an average of 2.29 (2.00 - 2.50) mm wide, while female pupae were an average of 22.71 (19.40 - 30.60) mm long and an average of 6.20 (5.20 - 6.80) mm wide (Figure 1C).

The adults of *C. semilutearia* had marked sexual dimorphism, nocturnal habits were phototropic and remained on the bark of trees during the day (Figure 1D). The male adults had a lifespan of 16.8 (3 - 30) days; were an average of 15.40 (13.00 – 17.00) mm long and had a wingspan between 30.00

and 41.00 mm (average 34.83 mm) (Figure 1E). The female adults lived for an average of 14.2 (3 - 26) days, were an average length of 19.25 (17.00 – 23.00) mm, and had an average wingspan of 50.42 (40.00 – 57.00) mm (Figure 1F).

The female adults displayed a pre-oviposition period of 3.9 (1 – 11) days, an average oviposition period of 7.2 (1 – 20) days and an average post-oviposition period of 3.0 (1 – 13) days (Table 3). The total number of eggs laid per adult female was an average of 389.0 (10 – 1044). Masses of eggs varied between 263 – 1006 (n = 18). The average number of eggs retained per female was 242.7 (5 – 671; n = 27) from which 43 of 70 insects did not retain eggs. Reproduction capacity per female was an average of 483.8 (10 – 1211) eggs.

Life cycle and parasitism of *T. alsophilae*

Results obtained from the two different life cycles using *O. trychiata* and *C. semilutearia* as rearing hosts for *T. alsophilae* showed that the life cycle of *T. alsophilae* was only slightly shorter using *O. trychiata* as a host, with an average of 27.17 (23 – 30) days. *Telenomus alsophilae* reared on *C. semilutearia* had a lifespan of 28.81 (25 – 31) days (Table 4). The host insect thus appeared to have no influence in the life cycle of *Telenomus*, indicating that both are reliable for rearing under laboratory conditions.

To consider the effectiveness of *Telenomus* as an egg parasitoid, the percentage of emergence on different hosts was evaluated. The highest emergence percentage of *T. alsophilae* in *O. trychiata* was 87.87%, and 73.74% on *C. semilutearia* (Table 5). The sex ratio for *T. alsophilae* was 2.68: 1 when raised on *C. semilutearia* and 2.36: 1 when raised on *O. trychiata* (Table 6).

The female longevity of *T. alsophilae* in *C. semilutearia* was determined in two different environments (parasitism and conservation rooms). In both cases the longevity was higher for females than males. Significant differences were found between the two environments, with an average lifespan of 25.8 days in

the parasitism room (23°C, 75% RH), and 75.1 days in the cooler conservation room (14°C, 75% RH) (Table 7).

Parasitism capacity

Results of parasitism capacity of *T. alsophilae* under laboratory conditions using *C. semilutearia* and *O. trychiata* showed no substantial differences for the two hosts. Each female *T. alsophilae* wasp had an average parasitism of 98.1 (56 – 190) on *O. trychiata* eggs and 126.1 (73 – 230) on *C. semilutearia* eggs (Table 8).

The following equation was calculated to measure the host efficiency for *T. alsophilae* using evaluated parameters in this study.

$$He = \left(\frac{1}{t} \times 100\right)\left(\frac{\% E}{100}\right)\left(\frac{\% fe}{100}\right)\left(\frac{PC}{100}\right)$$

He = Host efficiency
t = Life cycle duration
E = Emergence percentage
fe = Emerged female percentage
PC = Parasitism capacity

$$He\ (C.semilutearia) = 3.47 \times 0.734 \times 0.728 \times 1.261 = 2.348$$

$$He\ (O.trychiata) = 3.68 \times 0.879 \times 0.698 \times 0.981 = 2.210$$

Considering that the maximum value for the host efficiency equation is 5 for a life cycle of 20 days under optimal conditions, results showed that the most efficient host for *T. alsophilae* was *C. semilutearia* with a *He* of 2.348, being higher than *O. trychiata* with 2.210.

Discussion

This study represents a complementary investigation to preliminary data undertaken in Rodas (1994), and Rodas and Madrigal (1996), in which the first approximation to the life cycle and biological control of *C. semilutearia* in Colombia was provided. In the present study, new biological parameters were considered to understand important assessments to develop strategies for biological control. The egg parasitoid, *T. alsophilae* presented a high potential for the biological control of *C. semilutearia,* as well as a new alternative rearing host*.*

The duration of the life cycle was determined in previous studies (Rodas 1994, Rodas and Madrigal 1996). Different conditions such as climate, host condition, and biological aspects of the insect pest may alter the population dynamics (Karuppaiah and Sujayanad 2012). In Colombia, *C. semilutearia* has approximately 3.7 generations per year, and its presence throughout the year is related with the wide range of altitudes and weather conditions. The number of generations per year in turn influences the release of biological control agents such as *Telenomus.*

In biological control programs, host preference is an important parameter (Goulart et al. 2011). In Colombia, the introduction of *T. alsophilae* for *O. trychiata* control and the massive rearing on this host was considered an efficient strategy (Drooz 1975, Drooz et al. 1977). However, during the 20 years of investigation of *C. semilutearia,* it was determined that *C. semilutearia* was more efficient as a mass-rearing host than *O. trychiata. C. semilutearia* showed great adaptability and easy handling under laboratory conditions, and resulted in a high female to male ratio of *T. alsophilae,* namely 2.68:1 (Figure 2A and B).

In Colombia, *T. alsophilae* has been an efficient biological control agent for a number of geometrid defoliators including *C. semilutearia, O. olivata, O. geminata, O. platypterata, O. trychiata, C. arana, C. pruna, Bassania schreiteri* and *Neuromelia ablinearia.* The successive rearing on an alternative host could

affect the host preference of the natural enemy, and this could possibly alter control efficiency (Corbet 1985). However, in this study the selected hosts were target forest pests, thus, the preference and efficiency would not be altered.

Integrated pest management includes the establishment of natural enemies within plantations and silvicultural practices to reduce the population levels. The biological control in the forestry company Smurfit Kappa Carton de Colombia (SKCC) is being used since 1988 with a massive rearing program for *T. alsophilae* (Figure 2C). Currently, this insect is considered as one of the most important biological control agents for Lepidoptera in Colombia. This is consistent with the reports that species of the genus *Telenomus* are among the most efficient egg parasitoids of forest defoliating Lepidoptera (Orr 1988).

In the Integrated Pest Management context in Colombia, early detection is key to the management of Geometrids. For example, early detection of *C. semilutearia* allows the early release of *T. alsophilae*. If high populations of *C. semilutearia* are present, light traps are used as a complementary measurement for attracting population densities of Geometrids, after which *T. alsophilae* are released at 500 000 – 600 000 wasps / ha divided between five to six light traps (Figure 2D-F). If low populations of *C. semilutearia* are present, or if undetected, inoculative releases of *T. alsophilae* are done at 20 000 – 30 000 wasps / ha. This is provided that the presence of alternative hosts in the plantation has been confirmed.

Geometrids are one of the most important pests in Colombia that causes serious damages to *P. patula* plantations. This study has provided a better understanding of the life cycle of *C. semilutearia*, and demonstrated the efficiency of the biological control agent as part of the management strategy. Continuous monitoring of damage caused by *C. semilutearia* on *P. patula* and alternative hosts such as *Pinus* spp., *E. grandis* and *C. lusitanica,* and rearing programs of natural enemies of *C. semilutearia,* are required for the management of this pest.

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Table 1: Life cycle duration of *C. semilutearia* in days.

Biological stage		Min.	Max.	Mean	SD	CV	*n*
Egg		7	7	7	0	0	114
Larvae	I	4	15	12.0	1.9	16.1	94
	II	3	17	7.1	2.3	32.6	91
	III	3	12	7.2	2.0	27.9	87
	IV	4	15	8.1	2.4	29.5	60
	V	4	13	8.3	2.6	31.0	35
	VI	4	13	7.5	3.3	44.4	17
Total Larvae		22	85	50.2	14.6	181.4	384
Pupae		16	40	25.4	4.4	17.4	78
Adult		3	30	15.1	6.3	41.7	105
Total life cycle		48	162	97.6	25.3	240.5	681

Table 2: Body length and width measurements (mm) of eggs, larvae, pupae and adults of *C. semilutearia* in Colombia

	Egg		Larvae				Pupae				Adult			
			I		VI		Female		Male		Female		Male	
	L	W	L	CC	L	CC	L	W	L	W	L	Wi	L	Wi
Min.	0.51	0.36	1.60	0.10	29.50	2.20	19.40	5.20	14.30	2.00	17.00	40.00	13.00	30.00
Max.	0.71	0.79	3.40	0.45	50.10	3.80	30.60	6.80	21.00	2.50	23.00	57.00	17.00	41.00
Mean	0.62	0.67	2.59	0.33	39.39	3.20	22.71	6.20	15.86	2.29	19.25	50.42	15.40	34.83
SD	0.04	0.06	0.52	0.08	5.09	0.18	2.01	0.32	1.12	0.14	1.67	3.30	0.77	2.14
CV	6.98	9.02	20.17	23.32	12.92	5.79	8.86	5.22	7.09	6.05	8.68	6.55	5.01	6.14
n	82	82	42	42	100	100	42	42	46	46	82	82	65	65

L Length

W Width

CC Cephalic Capsule Width

Wi Wingspan

Table 3: Longevity of adult female (days) and number of eggs per female of *C. semilutearia*

Female adult stage	Min.	Max.	Mean	SD	CV	*N*
Pre-oviposition	1	11	3.9	2.9	74.8	70
Oviposition	1	20	7.2	5.2	72.1	70
Post-oviposition	1	13	3.0	3.1	103.3	70
Total adult	3	26	14.2	5.7	40.5	70
Retain eggs/female	5	671	242.7	188.9	77.8	27
Total eggs/female	10	1044	389.0	262.6	67.5	69
Reproduction capacity	10	1211	483.8	256.7	53.1	70
Male adult stage						
Total adult	3	30	16.8	7	41.6	35

Table 4: Life cycle duration of *T. alsophilae* in two different rearing hosts, in days

Rearing host	Mean	Min	Max	SD	Conf. Int.	CV	*N*
O. trychiata	27.17	23	30	1.69	27.16 -27.18	6.24	1724
C. semilutearia	28.81	25	31	1.128	28.79 -28.87	3.91	1301

Table 5: Emergence percentage of *T. alsophilae* on *O. trychiata* and *C. semilutearia*

Rearing host	Emergency (%)	SD	CV	*N*
O. *trychiata*	87.87	23.145	26.34	83
C. semilutearia	73.74	25.27	34.27	139

Table 6: Female percentage obtained by rearing *T. alsophilae* on *O. trychiata* and *C. semilutearia*

Rearing host	Female (%)	Sex Ratio (female: male)	SD	CV	*N*
O. *trychiata*	69.81	2.36:1	30.54	43.75	83
C. semilutearia	72.87	2.68:1	23.03	31.60	140

Table 7: Longevity of *T. alsophilae* adults reared on *C. semilutearia*, under conditions in two different environments, such as parasitism room and conservation room in days (75% RH in both rooms)

Longevity	Parasitism Room (23°C)		Conservation Room (14°C)	
	Female	**Male**	**Female**	**Male**
Mean	25.8	23.0	75.1	67.1
SD	6.058	3.132	5.110	8.15
n	64	43	8	8
CV (%)	23.45%	13.61%	6.80%	12.14%
	Mean F+M: 24.65 days (Min. 21, Max. 36)		Mean F+M: 71 days (Min. 60, Max. 82)	

Table 8: Parasitism capacity of *T. alsophilae* in the rearing hosts *O. trychiata* and *C. semilutearia*

Eggs / female	Rearing Host	
	C. semilutearia	*O. trychiata*
Mean	126.1	98.1
SD	60.64	10.97
Min.	73	56
Max.	230	190
***n* (females)**	20	20
CV (%)	48.08%	11.18%

Figure 1: Life stages of the moth *Chrysomima semilutearia* (Lepidoptera: Geometridae). A. Eggs. B. Larvae. C. Pupae. D. Adult female and male copulating. E. Adult male. F. Adult female.
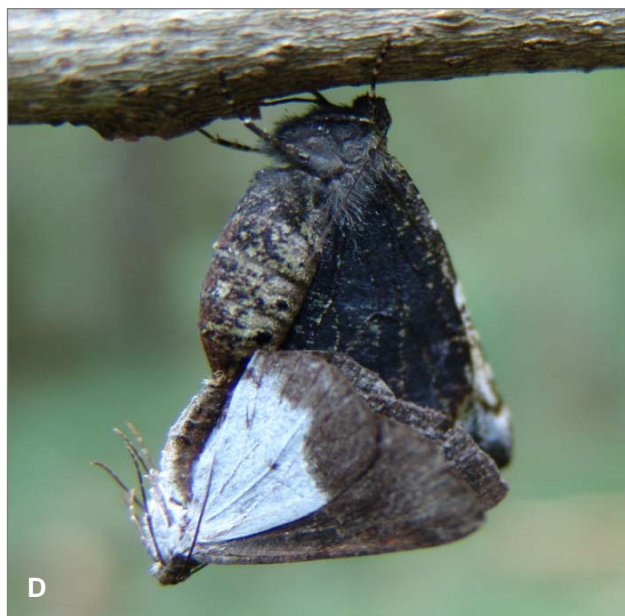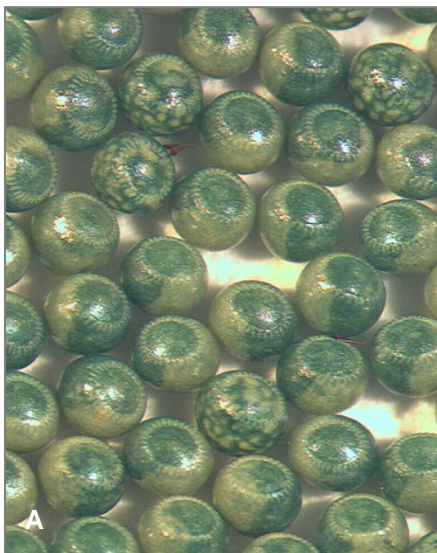
Figure 2: Physical and biological control agent of *Chrysomima semilutearia* (Lepidoptera: Geometridae). A,B. The egg parasitoid *Telenomus alsophilae* (Hymenoptera: Scelionidae) female and male, respectively. C. Rearing process of *T. alsophilae* in laboratory. D. Light traps used for concentration of adults moths and eggs. E. Inoculation and inundative methods for *T. alsophilae* releasing. F. *T. alsophilae* wasp parasites *C. semilutearia* eggs in field.

Chapter 4

Biology, damage and management of *Pineus* sp.
(Hemiptera: Adelgidae) in Colombian Pine plantations

Abstract

Adelgids (Hemiptera) in the genus *Pineus* have been reported as introduced insect pests causing serious losses to *Pinus* plantations worldwide. In 2008, a *Pineus* sp. was recorded for the first time in Colombia, with infestations recorded on *Pinus kesiya, P. tecunumanii, P. maximinoi* and *P. oocarpa*. The lack of information of this insect in Colombia necessitated an understanding of its life cycle and the development of potential management strategies. To achieve this goal, studies were carried out in the field and laboratory in Valle del Cauca department, Colombia. The results showed an anholocyclic life cycle comprised of four instars with a complete duration between 49 and 97 days. Feeding of the *Pineus* sp. was preferentially in the middle and upper parts of trees. *Pinus kesiya* and *P. maximinoi* had the highest levels of susceptibility in field as well as in greenhouse trials. A survey of naturally infested trees showed *P. tecunumanii* to be moderately susceptible while *Pinus patula* and *P. oocarpa* had low levels of susceptibility in the greenhouse trial but were not susceptible in the field. Investigations considering the predatory effect of *Ceraeochrysa* sp. (Neuroptera: Chrysopidae), a commonly occurring biological control agent already present in *Pinus* plantations in Colombia, showed a high predation rate of up to 140 *Pineus* consumed per day by a single *Ceraeochrysa* individual. Other predators of the *Pineus* sp. were recorded but were not sufficiently common to warrant detailed study.

Introduction

The Hemiptera (Superfamily: Aphidoidea) includes more than 170 species of pine sap-feeders distributed in the Adelgidae, Phylloxeridae, and Aphididae (Blackman and Eastop 1994). The Adelgidae is a family known to feed exclusively on conifers (Scholtz and Holm 1985, Triplehorn and Johnson 2005), and with a complex multiple-generation life cycle. Here, individuals can either be holocyclic (includes sexual reproduction) or anholocyclic (parthenogenetic reproduction) (Havill and Foottit 2007a, Havill et al. 2007b, Foottit et al. 2009). Some adelgids display a host-alternating behaviour (Dimond 1974), in which

Picea is the primary host associated with sexual reproduction, and other genera such as *Abies, Larix, Pseudotsuga* or *Pinus* are secondary hosts associated with parthenogenetic reproduction (Havill and Foottit 2007a, Havill et al. 2007b, Sano and Ozaki 2012).

Species in the adelgid genus *Pineus,* commonly referred to as wooly aphids, have emerged as some of the most serious pest in forests. Since 1918, reports of *Pineus* infestations affecting both primary and secondary hosts throughout Europe were documented (Pierce 1918). *Pineus* spp. have subsequently spread throughout Africa, North America, South America, Australasia and Asia (Blackman and Eastop 1994, FAO 2007, Lazzari and Cardoso 2011) and species such as *P. pini* and *P. boerneri* have been reported as serious pests of *Pinus*, including *P. mugo* and *P. sylvestris* in Europe (Blackman and Eastop 1994, Soria et al. 1996), *P. patula* and *P. elliotti* in Tanzania (Petro and Madoffe 2011), and *P. kesiya* in Malawi (Chilima and Leather 2001). Symptoms of infestation include defoliation and in extreme cases, dieback and tree death. There is uncertainty regarding the taxonomy of various *Pineus* species, but life cycle characteristics can aid in their identification, such as is the case for *P. orientalis* (holocyclic) and *P. pini* (anholocyclic) (Foottit et al. 2009).

A *Pineus* sp. was recorded in Colombia for the first time in 2008. Infestations have been observed on *Pinus kesiya, P. tecunumanii, P. maximinoi* and *P. oocarpa* between the ages of 2.1 and 3.4 years (CAR unpublished data), in the departments of Valle del Cauca, Cauca, Caldas, Quindío and Risaralda (Figure 1). Characteristic symptoms of infestation were observed when populations of the pest were high. These symptoms included yellowing of needles (Figure 2A), early senescence, tip die-back, growth reduction, shoot death, and in extreme cases tree death. In addition, the presence of white cottony tufts (Figure 2B-E) and reduced productivity of stands of *Pinus* spp. was observed.

The purpose of this study was to investigate the life cycle of the *Pineus* sp. in *Pinus* plantations in Colombia. A further goal was to consider the susceptibility of different *Pinus* species planted in the country and to assess the impact of

infestation. The possibility of using a *Ceraeochrysa* sp., an already established predator of adelgids in Colombia for biological control, was also considered.

Materials and Methods

Identification

A preliminary identification of the *Pineus* sp. found in Colombia was made using the taxonomic patterns documented by Blackman and Eastop (1994).

Life cycle

The life cycle of the *Pineus* sp. was investigated under controlled laboratory conditions. Insects were collected in the Aguaclara Plantation farm (La Cumbre, Valle del Cauca) at 3°41´31´´N - 76°32´48´´ W [T°: 20.3°C, 1489 masl, precipitation: 1489 mm/year] in a three year-old *P. kesiya* compartment. The life cycle studies were performed between September and December 2010 at the Smurfit Kappa Cartón de Colombia (SKCC) laboratory, located in Restrepo (Valle del Cauca) in Colombia at 3°51´45´´N - 76°29´49´´W [T°: 22°C, 1450 masl, RH: 75%, precipitation: 1405 mm/year] using 100 seven-month-old *P. kesiya* trees.

Eggs (1000) were taken from field-collected *Pineus* sp. and incubated in Petri dishes on wet sterile filter paper, separated in groups of 10 eggs per dish. Emerged nymphs were transferred to the trees, with 10 nymphs per tree. For each tree, a 20 ml syringe barrel was placed mid-way over the main branch to prevent escape of the adelgids (Figure 3A-D). Observations were made to assess aspects of the life cycle, including duration of the different life stages, percentage emergence from eggs, percentage nymph survival, percentage mortality, and the number of eggs per female. The length and width of each life stage individual was also measured.

Evaluation of the impact on *Pinus* plantations

To measure the impact caused by *Pineus* sp., evaluations were made monthly between November 2010 and September 2011 on 2-year-old *P. kesiya* trees in plantations at two different sites, namely La Ponderosa Farm [3°51´17´´N – 76°29´39´´W, T°: 20.4°C, 1473 masl], and San Quin Farm [3°34´40´´N – 76°41´13´´W, T°: 21.1°C, 1524 masl]. At each site, 4 plots with 10 trees per plot were randomly selected. The percentage infestation per plot was calculated based on the total number of trees infested.

To calculate the population level of *Pineus* sp., a tree was randomly selected within each plot and divided in three sections representing the bottom, middle and top. Each section was divided into three parts, namely two lateral branches and the main stem. A circular sample (disc) of bark of 2 cm^2 was cut from the middle of each of these tree parts. These samples were taken to the SKCC Laboratory (Restrepo, Valle del Cauca). The number of eggs, nymphs and adults were counted per disc and the total calculated per farm and per section over the 11-month period.

Susceptibility of *Pinus* species

To assess the susceptibility of *Pinus* species to infestation by the *Pineus* sp., a survey was conducted using naturally infested trees as well as artificially infested trees in the greenhouse.

To assess natural infestation, five *Pinus* species were planted 1 x 1 m apart within a plantation of two-year-old *P. kesiya* at La Ponderosa Farm, in random blocks of 20 trees per test species. The test species included *P. oocarpa, P. patula, P. kesiya, P. maximinoi, P. tecunumanii* High Elevation (HE). The trees were planted in May 2011 and monitored monthly until September of 2011. In order to examine infestation where trees had not been planted in close proximity to an infested area, 6-month-old trees of the same species were planted in plastic trays (29 trees per species), and placed at a site in the

Rancho Grande farm. Monthly evaluations of these trees were made from September 2010 to July 2011. In both cases, the number of infested trees was counted to determine the percentage infestation.

For the greenhouse trial, the same five *Pinus* species (20 trees per species) were used. The trees were nine-months-old and were planted in plastic trays. Individuals of the *Pineus* sp. were collected from Rancho Grande farm [3°51′43′′N –76°30′48′′W, T°: 21°C, 1200 masl] on 3.8 years old *P. tecunumanii*. Twenty individuals of nymphal stage I were placed on each tree. Individuals of Nymph I were identified using a Nikon stereoscope SMZ800 C-DS. Monthly evaluations were made between September 2010 and January 2011. The number of infested trees was enumerated to determine the percentage infestation.

Predation capacity of *Ceraeochrysa* **sp. and other predators present**

Observations in the field showed predation of *Pineus* sp. by a *Ceraeochrysa* sp. (Neuroptera: Chrysopidae). This predator is commonly found in *Pinus* plantations in Colombia. The presence of other less common predators was also noted and these insects were identified.

Individuals of the *Ceraeochrysa* sp. were reared in the laboratory to assess their predation capacity on the *Pineus* sp. Sixteen larvae of one-day-old *Ceraeochrysa* sp. were individually separated and placed in Petri dishes. In each dish, 10 eggs, 10 nymphs and 10 adults of *Pineus* sp. were provided initially for the newly emerged *Ceraeochrysa* sp. Thereafter, the number of *Pineus* sp. individuals provided was increased according to the predation capacity of the growing *Ceraeochrysa* sp. The total numbers of consumed insects were counted every day and the cumulative predatory capacity for the different larvae instars of *Ceraeochrysa* sp. was determined.

Results

Identification

Observations made under laboratory conditions based on taxonomic patterns of the cephaloprothoracic shield aspect and wax pores (Blackman and Estop 1994) suggested that the *Pineus* sp. found in Colombia is *Pineus boerneri.*

Life cycle

During the examination of the life cycle of the *Pineus* sp. in Colombia, the presence of males was not observed. This suggests a parthenogenetic reproductive strategy. Additionally, infestations of *Pineus* sp. were observed only on *Pinus* species. From observations in the laboratory, the duration and dimensions of the different life stages were calculated (Table 1 and 2). The complete life cycle ranged from 49 to 97 days, with an average of 65.6 days. The average egg length was 0.32 mm (0.30 – 0.35 mm) with an average width of 0.17 mm (0.16 – 0.18 mm). The egg eclosion time was on average 6.5 days (5 - 8 days), and the percentage emergence was 90.5% from a total of 200 eggs. Eggs were a light yellow color when newly laid, becoming red before hatching (Figure 4A).

Four instars of *Pineus* sp. were observed. The total duration of the nymph stage was on average 24.2 days (22 – 29 days). Nymph stages of the *Pineus* sp. produced white woolly tufts (Figure 4B-F). The nymphs had small anatomical differences from the adults, having a variety of colors such as light yellow to reddish-brown. Nymph I started to form white cottony tufts on the bark, had the longest duration (mean = 10.9 days, range = 10 – 12 days), and an average body length and width of 0.34 mm (0.30 - 0.38 mm) and 0.21 mm (0.19 – 0.22 mm), respectively. Nymph II had an average duration of 5.2 days (5 – 6 days), and an average body length and width of 0.39 mm (0.37 – 0.41 mm) and 0.28 mm (0.24 – 0.30 mm), respectively. Nymph III had a duration of 4.1 days (4 – 6 days), and an average body length and width of 0.42 mm (0.40 – 0.46

mm) and 0.35 mm (0.31 – 0.39 mm), respectively. Nymph IV had a duration of 4 days (3 – 5 days), and an average body length and width of 0.46 mm (0.40 – 0.48 mm) and 0.40 mm (0.38 - 0.41 mm), respectively. Nymph survival rate for instars I, II, III and IV was 93.9% (n=148), 90.7% (n=139), 90.5% (n=126) and 76.3% (n=114), respectively. The adult had a duration of 34.9 days (22 – 60 days), and an average body length and width of 0.53 (0.46 - 0.58 mm) and 0.44 mm (0.42 – 0.47 mm), respectively (Figure 4G).

Examination of the duration of the adult life stage presented a pre-oviposition period of 1 day, an average oviposition period of 33 days (20 – 55 days) and an average post-oviposition period of 2.2 days (1 – 4 days) (Table 3). The average number of eggs oviposited per female was 65.6 (40 – 125 eggs/female). The number of eggs decreased across the duration of the adult state, with an average of 2.6 eggs / day at 2nd day to an average of 0.2 eggs / day at 58th day (Figure 5).

Impact on *Pinus* plantations

Infestation levels of the *Pineus* sp. at La Ponderosa Farm were 85% at the time of the first observation (November 2010) and 92.5% when the last observation was made (September 2011) (Table 4A). Infestation levels at San Quin were similar, with 87.5% infestation at the time of first observation and 95% when the last observation was made (Table 4B). The population of *Pineus* sp. was highest in September 2011 (mean = 38.6) at La Ponderosa, and highest in March (mean = 37.7) at San Quin (Table 4A and B). The population levels over the 11-month study period were higher at La Ponderosa Farm (total = 2418 eggs, 1848 nymphs and adults) than at San Quin Farm (total = 1623 eggs, 1316 nymphs and adults) distributed in the different tree sections (Table 5A and B). *Pineus* sp. showed a preference for laying eggs in the upper and middle tree sections at La Ponderosa Farm (mean = 36.5 and 36.4, respectively) and in the upper and middle branch 1 at San Quin Farm (mean = 24.6 and 19.8, respectively) (Table 5A). A greater number of nymphs and adults was

registered in the higher tree sections at both farms over a period of eleven months (mean = 49.0 for La Ponderosa, mean = 18.1 for San Quin) (Table 5B).

Susceptibility of different *Pinus* spp.

The most susceptible species in all trials were *P. kesiya* and *P. maximinoi* (Table 6). Infestation percentages (number of trees infested) varied between the different assessments. Where natural infestation was assessed, mean infestation on *P. kesiya* was 88.3% at Rancho Grande and 23.4% at La Ponderosa farm, and mean infestation on *P. maximinoi* was 67.4% at Rancho Grande and 11.2% at La Ponderosa. In the greenhouse trial, mean infestation was higher on *P. kesiya* (93.8%) than on *P. maximinoi* (80.2%). *Pinus oocarpa* and *P. patula* had mean infestation levels in the greenhouse trial of 15.0% and 28%, respectively, but were not naturally infested at either of the two field sites (Table 6). *Pinus tecunumanii* HE showed a mean infestation of 23% in the greenhouse trial. Natural infestation of this species was observed at Rancho Grande, with 8.9% infestation, but not at La Ponderosa, most likely because infestation levels were generally lower at that site.

Predation capacity of *Ceraeochrysa* sp.

The *Ceraeochrysa* sp. was the most commonly encountered biological control agent found on *Pineus* sp. in Colombian plantations (Figure 6A). The *Ceraeochrysa* sp. emerged from the eggs after an average of 6 days (*n*=110) (Figure 6B and C). The duration of the instars was 7.2, 7.7 and 10.2 days for Larvae I (*n*=109) (Figure 6D and E), Larvae II (*n*=71) and Larvae III (*n*=19) instar (Figure 6F), respectively. The average predation capacity of *Pineus* sp. (eggs, nymphs and adults combined) per 24 hours by the different *Ceraeochrysa* sp. instars was 15 (Larvae I), 33 (Larvae II) and 98 (Larvae III) individuals. The highest predation capacity per day was obtained by an individual of Larvae III, with 45 eggs, 47 nymphs and 50 adults, with a total of 142 *Pineus* consumed per day. The average predation of *Pineus* sp. over the 33 days exposed to the 16 *Ceraeochrysa* sp. instars was 2437 (817 eggs, 813

nymphs, 807 adults) (Figure 7). The *Ceraeochrysa* sp. showed no obvious discrimination of food source in terms of the various *Pineus* sp. instars (Figure 6G). The *Ceraeochrysa* pupae were also found (Figure 6H and I).

Other predators observed in this study were *Harmonia axyridis* (Coleoptera: Coccinellidae) (Figure 8A-E), *Chrysoperla* spp. (Neuroptera: Chrysopidae) (Figure 8F-G) and *Cryptolaemus* sp. (Coleoptera: Coccinellidae) (Figure 8H).

Discussion

This study represents the first investigation of the life cycle, impact and host susceptibility of a *Pineus* sp., tentatively identified as *P. boerneri* in Colombia. The study was also the first report of the predation capacity of an existing natural enemy of *Pineus* sp. in Colombia. Results showed the presence of an anholocyclic lifespan of *Pineus* sp. in Colombia as reported in other countries (Blackman and Eastop 1994, Chilima and Leather 2001). Clear patterns were observed with regards to the section of the trees infested as well as host susceptibility. The predator, *Ceraeochrysa* sp. represented encouraging potential for the biological control of *Pineus* sp. The overall findings presented will inform management decisions for this new pest and potentially offset losses to plantation forestry in Colombia.

Adelgids can become obligate and anholocyclic when there is limited migration and the absence of an alternate host (Havill and Foottit 2007a). These conditions were found in Colombia, where all *Pineus* sp. individuals were apterous and none were found on other nearby vegetation. This suggests that *Pineus* sp. in Colombia is anholocyclic. Reported anholocyclic *Pineus* species include *P. strobi* (Blackman and Eastop 1994, Sano and Ozaki 2012) in *P. strobus* in North America (Blackman and Eastop 1994), *P. boerneri* on *P. radiata* in California (Blackman and Eastop 1994) and *P. pini* on *P. sylvestris* in Europe (Blackman and Eastop 1994, Soria et al. 1996).

The duration of the life cycle has not been determined for an anholocyclic *Pineus* sp., due to the complexity of the insect (Foottit et al. 2009). According to Sano and Ozaki (2012), the life cycle could be completed within a year with a reduced number of generations. The generation time is closely related with climate, host condition and insect size, and can include between two and six generations per year (Arthur and Hain 1984, Havill and Foottit 2007a, Lazzari and Cardoso 2011). In Colombia, the parthenogenetic generation time for *Pineus* sp. was approximately 3.7 per year, the varied geography offers a wide range of altitudes and weather conditions that would allow the *Pineus* sp. to be present throughout the year. The data gathered on the number of generations per year will be useful in devising control strategies such as those concerning the release of biological control agents.

Morphological measurements of parthenogenetic females of *Pineus* sp. have been reported with a body length between 0.5 - 0.8 mm, and eggs with an average length of 0.3 mm and width of 0.2 mm (Cibrián-Tovar et al. 1995). This is a similar range to the adult females in the present study. According to Lazzari and Cardoso (2011), differences in morphology are influenced by climatic conditions, where winter morphs are smaller. The effect of climatic conditions on the size of *Pineus* sp. in Colombia should be determined in future studies.

The number of affected trees was not related with the population of insects in the areas studied. For example, La Ponderosa had an infestation of 92.5% and a mean population of 38.6, while San Quin had an infestation of 95%, but a mean population of only 14.7. In addition, higher population fluctuations over time were recorded at San Quin, which could be associated with the presence of different generations. This is in contrast to La Ponderosa where the population gradually increased until the last month of evaluation. Nutritional quality as well as protection offered by branches is known to impact strongly on colonization sites for *Pineus* spp. such as *P. boerneri* and *P. coloradensis* (McClure 1991). At both farms studied, younger tissues in the upper parts of trees harboured the greatest number of eggs, nymphs and adults. The *Pineus*

sp. was also present throughout the year despite varying climatic conditions between the seasons.

In Colombia, the most susceptible *Pinus* hosts were *P. kesiya* and *P. maximinoi.* Although all five of the *Pinus* species tested showed susceptibility to *Pineus* sp. when infestation was induced, *P. oocarpa* and *P. patula* showed no natural infestation, and *P. tecunumanii* HE showed low natural infestation at one site, with no infestation at another site. A wide diversity of *Pinus* hosts has been documented including *P. maximinoi, P. cembroides, P. hartwegii* for *Pineus* sp. (Cibrián-Tovar et al. 1995), *P. oocarpa, P. patula* (Blackman and Eastop 1994, Oliveira et al. 2008)*,* and *P. kesiya* (Chilima and Leather 2001, Oliveira et al. 2008) for *Pineus boerneri*, and *P. sylvestris* and *P. mugo* for *P. pini* (Blackman and Eastop 1994, Soria et al. 1996). This study is the first to report on the susceptibility of *P. maximinoi* and *P. tecunumanii* to infestation by *Pineus* sp. in Colombia.

Management strategies for forest insect pests can include the conservation, mass production and release of naturally occurring predators, as a form of biological control (Núñez 1988). The Neuroptera includes some of the most promising biological agents such as species of Chrysopidae, which are commonly known as known as Green Lacewings (Tauber and Tauber 2009). For example, the genus *Chrysopa*, commonly found in tropical and subtropical regions of America (Vargas et al. 1987), have been widely used as biocontrol agents (Klingen et al. 1996, Santa-Cecília et al. 1997, Cadena et al. 2007). In this study, the green lacewing *Ceraeochrysa* sp. showed encouraging potential as a biological control agent for *Pineus* sp. due to its high predation capacity on all life stages of the insect.

The *Pineus* sp. considered in this study is a recent pest invasion in Colombia that has the potential to cause serious losses to *Pinus* plantations. This study has provided a better understanding of its life cycle in Colombia, and it has demonstrated the potential to use host resistance and biological control to reduce its impact. Continuous monitoring of *Pineus* sp. in Colombia and rearing

of natural enemies will be required for the effective management of this pest. Morphological characteristics of *Pineus* sp. found in Colombia were similar to those of *Pineus boerneri,* indicating the possible presence this species in *Pinus* plantations in Colombia. This is the species also known to occur in nearby Brazil (Oliveira et al. 2008) and in other countries such as South Africa (Blackman and Eastop 1994, Lazzari and Cardoso 2011) where *Pinus* spp. are propagated intensively as non-natives in plantations. However, to ensure accuracy, the identification of the *Pineus* sp. in Colombian plantations should be confirmed using more detailed morphological as well as molecular studies.

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Table 1: Life cycle of *Pineus* sp. (Hemiptera: Adelgidae) on *Pinus kesiya* under controlled laboratory conditions (data recorded in days).

Biological stage		Min.	Max.	Mean	SD	CV	*n*
Egg		5	8	6.5	0.6	9.4	100
Nymph	I	10	12	10.9	0.3	2.9	148
	II	5	6	5.2	0.4	8.2	139
	III	4	6	4.1	0.3	7	126
	IV	3	5	4	0.3	7.4	114
Total Nymph		22	29	24.2	1.3	34.8	527
Adult		22	60	34.9	11	31.7	87
Total life cycle		49	97	65.6	12.9	75.9	714

Table 2: Body length and width measurements (mm) of eggs, nymphs and adult females of *Pineus* sp. (Hemiptera: Adelgidae) found in Colombia.

	Egg		Nymph								Adult	
			I		II		III		IV			
	L	**W**	**L**	**W**	**L**	**W**	**L**	**W**	**L**	**W**	**L**	**W**
Min.	0.30	0.16	0.30	0.19	0.37	0.24	0.40	0.31	0.40	0.38	0.46	0.42
Max.	0.35	0.18	0.38	0.22	0.41	0.30	0.46	0.39	0.48	0.41	0.58	0.47
Mean	0.32	0.17	0.34	0.21	0.39	0.28	0.42	0.35	0.46	0.40	0.53	0.44
SD	0.02	0.01	0.03	0.01	0.01	0.02	0.02	0.02	0.02	0.01	0.04	0.02
CV	4.81	4.31	7.48	5.27	3.21	7.21	4.19	6.82	5.16	2.73	6.91	3.94
n	10	10	10	10	10	10	10	10	10	10	10	10

L: Length
W: Width

Table 3: Longevity of adult female (days) and number of eggs per female of *Pineus* sp. (Hemiptera: Adelgidae).

Female adult stage	Min.	Max.	Mean	SD	CV	*n*
Pre-oviposition	1	1	1	0	0	87
Oviposition	20	55	33	11.2	33.9	87
Post-oviposition	1	4	2.2	0.5	24.8	87
Total adult cycle	22	60	34.9	11	31.7	87
Eggs/female	40	125	65.6	18.1	27.6	87

Table 4: Mean population levels (eggs, nymphs and adults combined) and average percentage of infestation (%) of *Pineus* sp. (Hemiptera: Adelgidae) at two sites over an eleven month period. A. La Ponderosa farm. B. San Quin farm

(A) La Ponderosa

Evaluation	Population	Min.	Max.	Mean	SD	CV	Infestation (%)
November	237	0	37	13.2	9.3	70.5	85
December	314	0	40	17.4	11.5	66.1	87.5
January	496	16	45	27.6	8.2	29.9	92.5
February	326	2	39	18.1	10.4	57.3	92.5
March	264	2	39	14.7	8.7	59.2	92.5
April	283	2	38	15.7	8.1	51.2	92.5
May	304	2	43	16.9	9.1	54	92.5
June	296	3	38	16.4	8.2	49.9	92.5
July	496	14	55	27.6	11.6	42	92.5
August	555	11	107	30.8	22.7	73.8	92.5
September	695	10	68	38.6	14.8	38.2	92.5
n	4266	0	107	21.6	4.4	20.2	

(B) San Quin

Evaluation	Population	Min.	Max.	Mean	SD	CV	Infestation (%)
November	83	0	31	4.6	8.1	174.5	87.5
December	330	0	42	18.3	14.5	78.8	90.0
January	341	2	40	18.9	9.9	52.2	95
February	140	0	18	7.8	5.4	69.7	95
March	678	10	72	37.7	20	53	95
April	140	1	17	7.8	4.7	60.6	95
May	155	1	18	8.6	5.4	62.5	95
June	370	9	44	20.6	9.3	45	95
July	204	1	26	11.3	6.9	60.8	95
August	233	1	43	12.9	9.9	76.3	95
September	265	4	38	14.7	8.9	60.3	95
n	2939	0	72	14.8	4.5	30.15	

Table 5: Mean population level of *Pineus* sp. (Hemiptera: Adelgidae) for the different tree parts sampled, at two sites and over an eleven month period. A. Eggs. B. Nymphs and adults

(A)

Eggs	La Ponderosa						San Quin					
	Total	Min.	Max.	Mean	SD	CV	Total	Min.	Max.	Mean	SD	CV
Lower section	135	0	44	12.3	14.2	115.9	133	0	52	12.1	15.6	128.8
Middle section	400	20	107	36.4	25.2	69.3	167	0	44	15.2	12.6	82.7
Higher section	401	15	55	36.5	12.3	33.8	271	1	68	24.6	19.2	77.8
Lower branch 1	292	15	51	26.6	11.1	41.9	204	2	45	18.6	16.7	89.9
Middle branch 1	312	15	68	28.4	17	60	218	0	72	19.8	20.9	105.2
Higher branch 1	214	0	50	19.5	15.9	81.7	179	0	41	16.3	14.2	87.5
Lower branch 2	207	13	39	18.8	8.8	46.8	84	0	22	7.6	9.2	119.8
Middle branch 2	193	2	50	17.6	17.1	97.5	156	0	38	14.2	12.2	86.2
Higher branch 2	264	11	41	24	7.5	31.3	211	1	55	19.2	15.9	82.8
TOTAL	**2418**	**0**	**107**	**24.4**	**16.6**		**1623**	**0**	**72**	**16.4**	**15.6**	

(B)

Nymph- Adult	La Ponderosa						San Quin					
	Total	Min.	Max.	Mean	SD	CV	Total	Min.	Max.	Mean	SD	CV
Lower section	81	0	20	7.4	6	80.9	181	0	67	16.5	17.9	109
Middle section	223	13	43	20.3	8.8	43.4	151	0	27	13.7	8.4	61.1
Higher section	294	18	52	49.0	77.8	158.8	199	8	40	18.1	9.1	50.3
Lower branch 1	221	8	48	20.1	10.9	54.3	61	0	14	5.6	4.2	75.8
Middle branch 1	273	14	49	24.8	9.3	37.5	169	5	42	15.4	9.8	63.9
Higher branch 1	169	6	40	15.4	8.9	57.8	117	2	28	10.6	7.1	66.8
Lower branch 2	172	7	26	15.6	6	38.2	88	1	16	8	5	62.3
Middle branch 2	227	12	48	20.6	11.1	53.8	185	3	31	16.8	9.1	53.8
Higher branch 2	188	7	29	17.1	7.1	41.5	165	5	47	15	12.3	81.9
TOTAL	**1848**	**0**	**52**	**18.7**	**10.1**		**1316**	**0**	**67**	**13.3**	**10.4**	

Table 6: Percentage infestation of *Pineus* sp. (Hemiptera: Adelgidae) on different *Pinus* species, in three different trials: Natural Infestation at Rancho Grande trial between September 2010 to July 2011, and La Ponderosa farm trial between May and September of 2011. Thus, Induced Infestation on the Green House trial between September 2010 and January of 2011

Year - Month	2010				2011									Mean	SD	CV	*n*
	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sept.				
Species							**Natural Infestation - Rancho Grande Trial**									
P. kesiya	79	86	86	90	90	90	90	90	90	90	90	-	-	88.3	3.5	3.9	29
P. maximinoi	59	66	66	72	72	72	72	69	69	62	62	-	-	67.4	4.7	7	29
P. oocarpa	0	0	0	0	0	0	0	0	0	0	0	-	-	0	0	0	29
P. patula	0	0	0	0	0	0	0	0	0	0	0	-	-	0	0	0	29
P. tecunumanii HE	7	7	10	10	10	10	10	10	10	7	7	-	-	8.9	1.5	17	29
						Natural Infestation - La Ponderosa farm Trial											
P. kesiya	-	-	-	-	-	-	-	-	0	5	5	25	82	23.4	34.1	145.9	20
P. maximinoi	-	-	-	-	-	-	-	-	0	0	5	18	33	11.2	14.2	127.1	20
P. oocarpa	-	-	-	-	-	-	-	-	0	0	0	0	0	0	0	0	20
P. patula	-	-	-	-	-	-	-	-	0	0	0	0	0	0	0	0	20
P. tecunumanii HE	-	-	-	-	-	-	-	-	0	0	0	0	0	0	0	0	20
						Induced Infestation - Green House Trial											
P. kesiya	74	95	100	100	100	-	-	-	-	-	-	-	-	93.8	11.3	12	20
P. maximinoi	56	75	90	90	90	-	-	-	-	-	-	-	-	80.2	15	18.7	20
P. oocarpa	0	0	20	25	30	-	-	-	-	-	-	-	-	15	14.1	94.3	20
P. patula	25	25	30	30	30	-	-	-	-	-	-	-	-	28	2.7	9.8	20
P. tecunumanii HE	0	20	25	35	35	-	-	-	-	-	-	-	-	23	14.4	62.6	20

Figure 1: Map of Colombia with the current distribution of the *Pineus* sp. (Hemiptera: Adelgidae). Red areas indicates where the *Pineus* sp. has been reported and green areas indicates forestry areas.
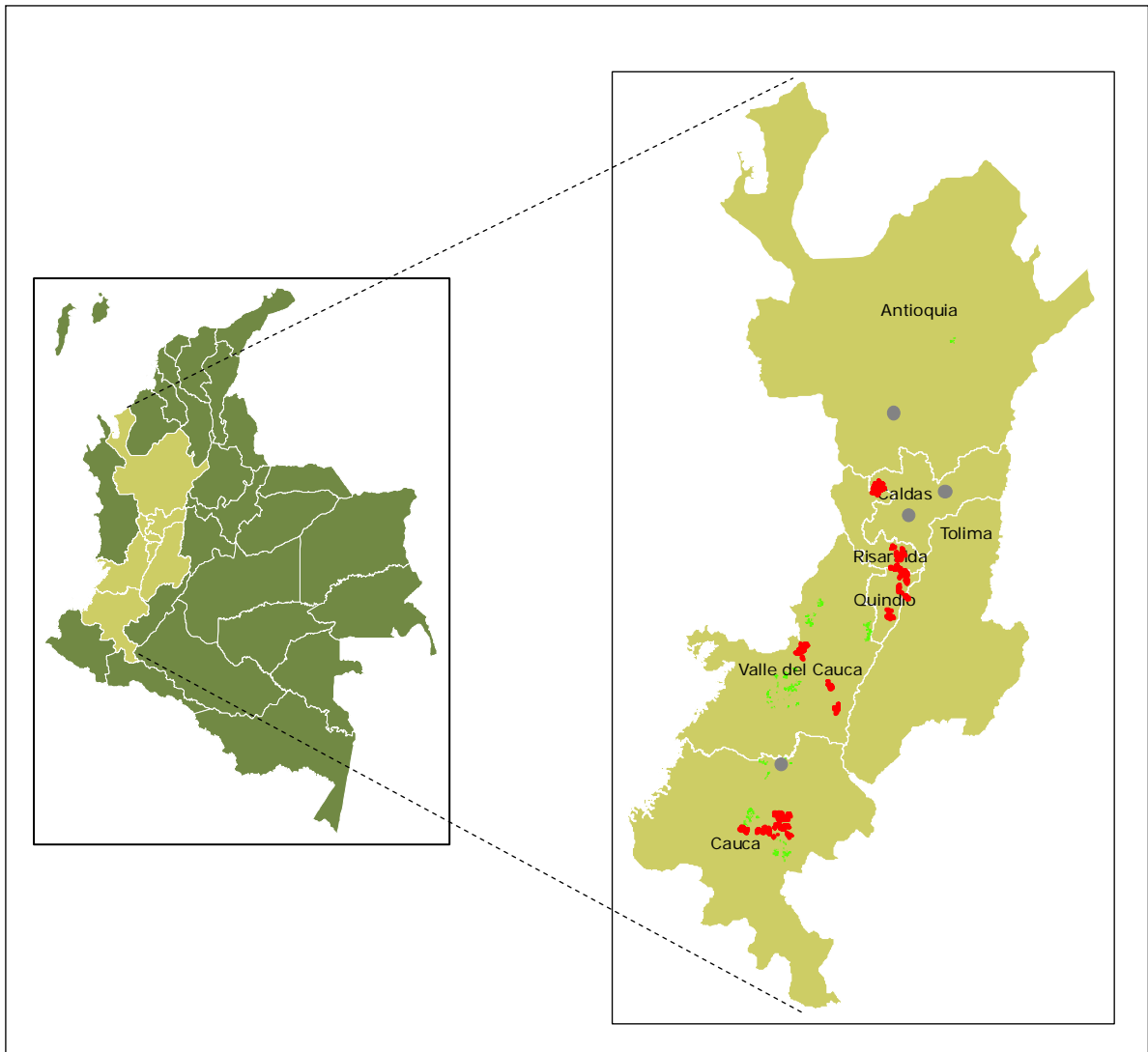
Figure 2: Infestation by *Pineus* sp. (Hemiptera: Adelgidae) in Colombia. A. Infestation in three years-old *Pinus kesiya*. B. Low infection level on bark. C,D. Moderate infection level on bark. E. High levels of white cottony tufts observed in the bark.

Figure 3: Method used for the evaluation of the woolly aphid *Pineus* sp. (Hemiptera: Adelgidae) life cycle in greenhouse. A. Syringe 20 ml and rubber disc. B,C. Installed syringe barrel and rubber disc in trees for the evaluation of the biological cycle of the insect. D. Syringe barrels and trees into greenhouse with contained insects.
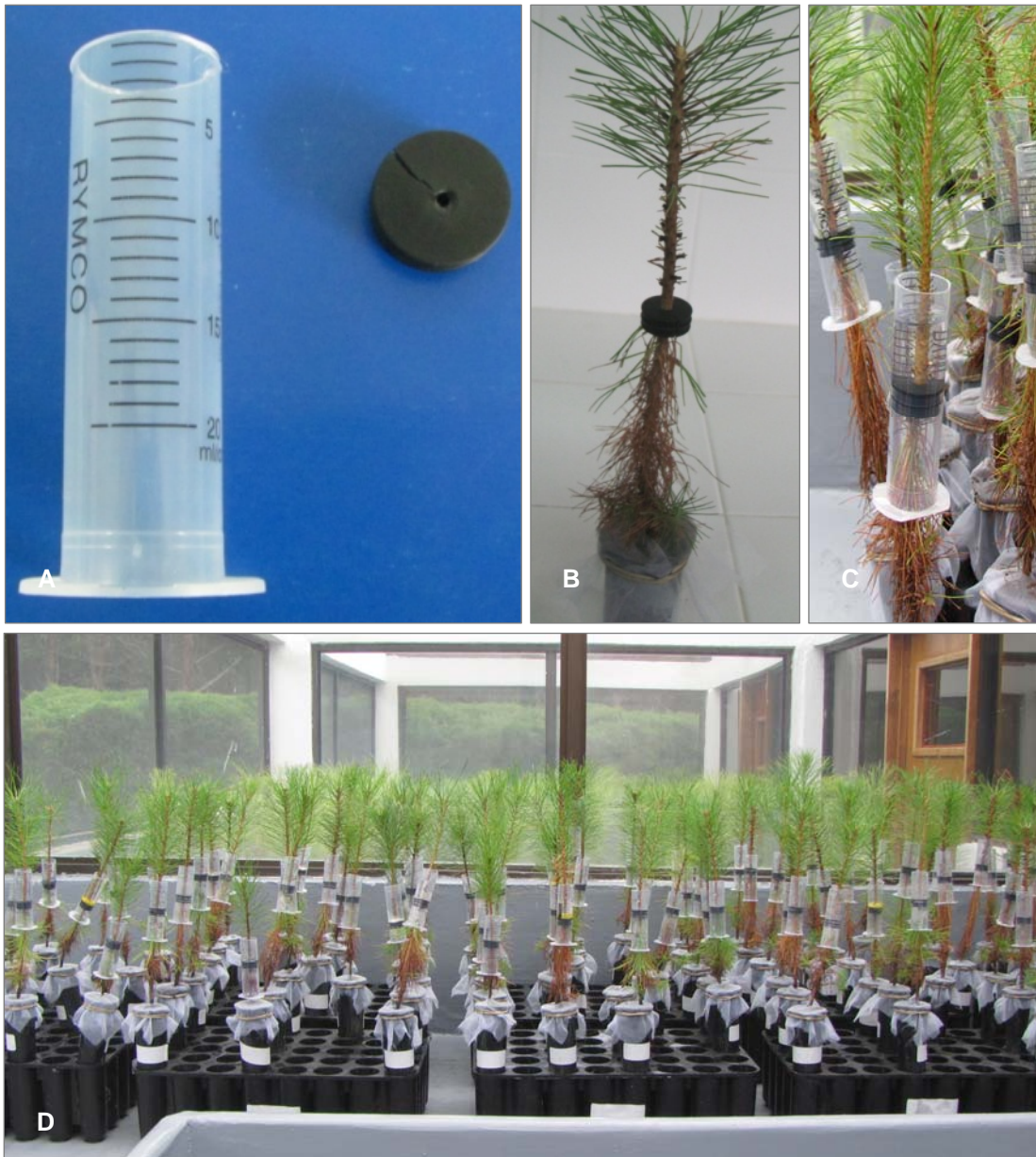
Figure 4: Life stages of the *Pineus* sp. (Hemiptera: Adelgidae). A. Adult laying eggs. B. Nymphal instar I emerging from an egg. C. Nymphal instar I showing long proboscis. D-F. Nymphal instar II, III and IV, respectively. G. Adult and eggs in white woolly tufts.
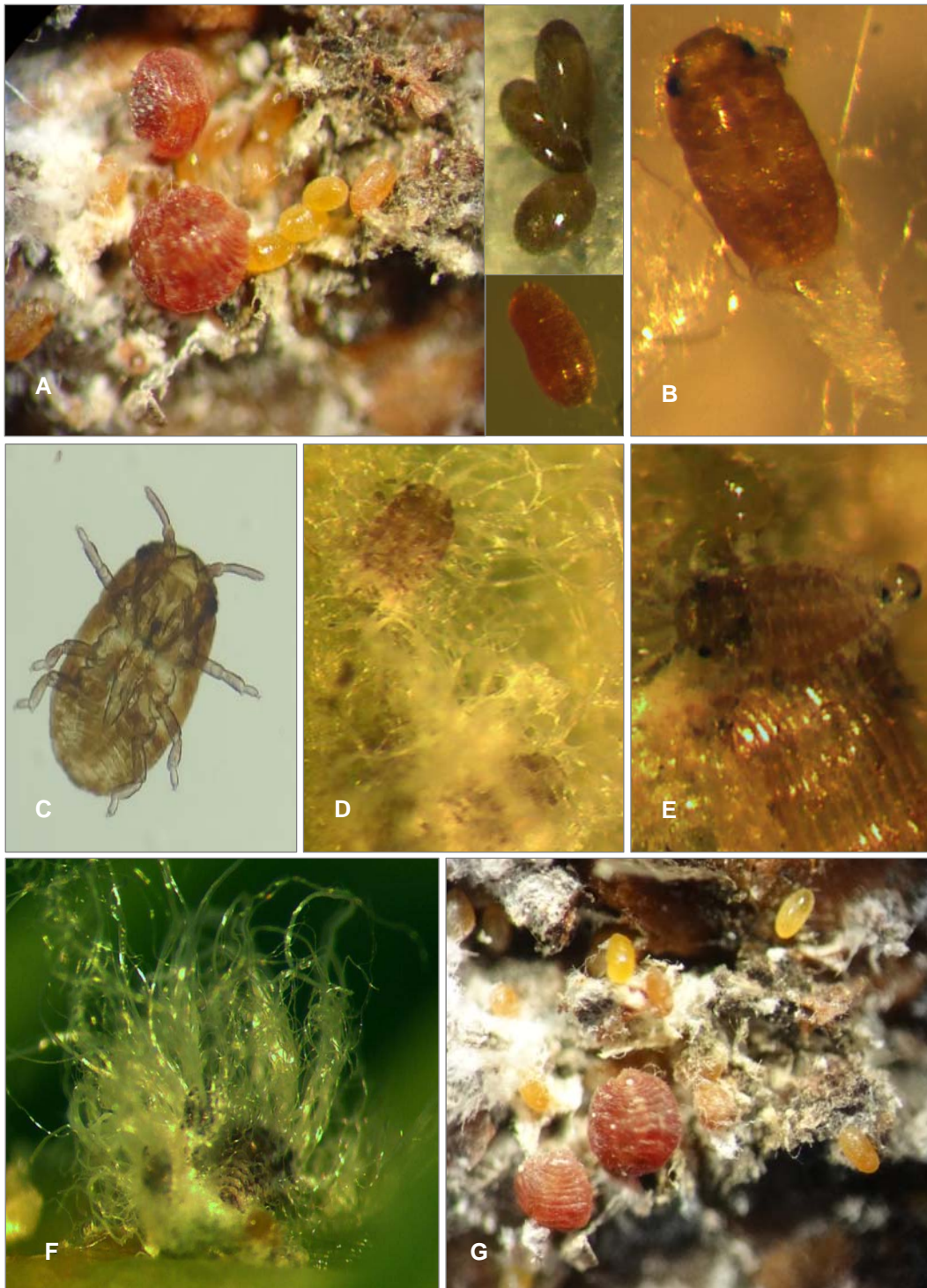
Figure 5: Rate of oviposition of *Pineus* sp. (Hemiptera: Adelgidae) per day

Rate of oviposition of *Pineus* sp. per day
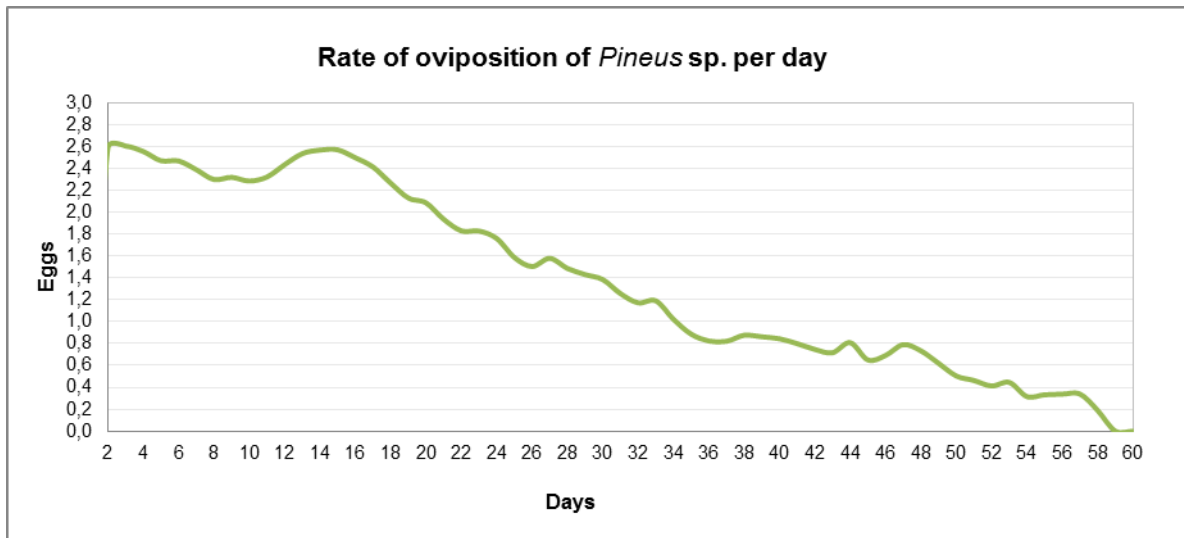
Figure 6: Important biological control agent of the *Pineus* sp. (Hemiptera: Adelgidae). A. *Ceraeochrysa* sp. (Neuroptera: Chrysopidae) Adult. B,C. Eggs of *Ceraeochrysa* sp. D,E. Larval instar I emerging from the egg covered with white wax. F. Larval instar III with white wax covering. G. Last larval instar of *Ceraeochrysa* sp. H,I. Pupae and open pupae from *Ceraeochrysa* sp., respectively
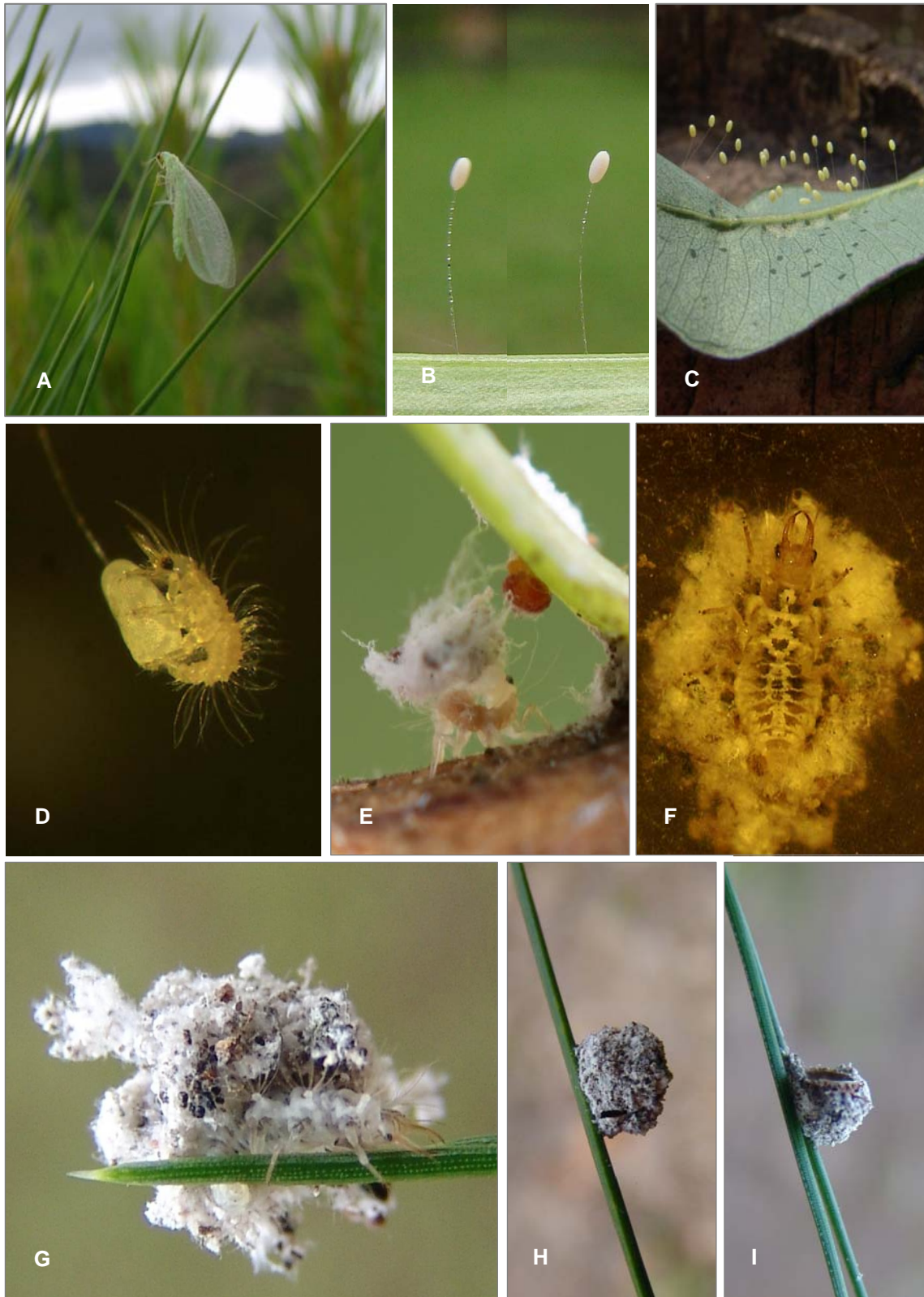
Figure 7: Average *Pineus* sp. (Hemiptera: Adelgidae) predated by 16 *Ceraeochrysa* sp. (Neuroptera: Chrysopidae) per day

Total *Pineus* sp. predated by *Ceraeochrysa* sp. each day
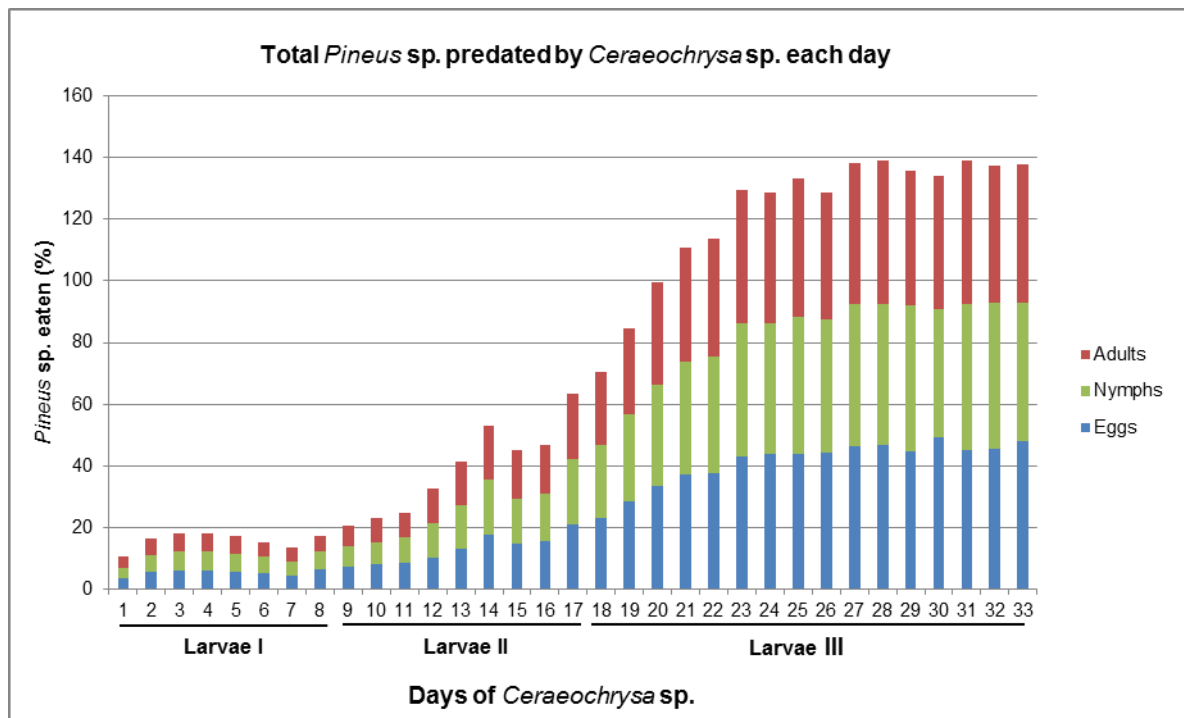
Figure 8: Naturally occurring biological control agents of the *Pineus* sp. in Colombia. A. Adults of *Harmonia axyridis* (Coleoptera: Coccinellidae). B. Eggs of *Harmonia axyridis.* C-E. Larvae feeding on *Pineus* sp. F,G. Adults and larvae of *Chrysoperla* sp. (Neuroptera: Chrysopidae) H. *Cryptolaemus* sp. (Coleoptera: Coccinellidae) feeding on *Pineus* sp.

Chapter 5

Fusarium circinatum **and Pitch canker of** *Pinus* **in Colombia**

Abstract

Pitch canker, caused by the ascomycete fungus *Fusarium circinatum*, infects a wide range of *Pinus* species. The pathogen has a global distribution and limits plantation productivity wherever susceptible *Pinus* species are commercially cultivated. During 2005-2007, symptoms typical of those associated with *F. circinatum* were observed in Colombia on nursery seedlings of *P. maximinoi*, *P. tecunumanii* and *P. patula*, as well as established *P. patula* and *P. kesiya* trees in plantations. Symptoms on seedlings included collar and root disease while shoot dieback and resinous stem cankers were found on trees in plantations. The aim of this study was to isolate and identify the causal agent of these symptoms and to evaluate the relative tolerance of various families of *Pinus* species commonly grown in Colombia. By making use of morphology and DNA-based methods, as well as pathogenicity tests on *P. patula* seedlings, it was possible to show that the symptoms observed in the nursery and field were caused by *F. circinatum*. Furthermore, the results of pathogenicity tests with two virulent isolates of the pathogen indicated that *P. tecunumanii* from low-elevation sources and *P. maximinoi* are significantly more tolerant to infection by *F. circinatum* than *P. tecunumanii* from high-elevation sources and *P. patula*. These results show that there is substantial opportunity to avoid losses due to infection by *F. circinatum* through deployment of resistant planting stock.

Introduction

Pitch canker of pine is caused by the ascomycete fungus *Fusarium circinatum* (teleomorph = *Gibberella circinata*) (Nirenberg and O'Donnell 1998, Wingfield et al. 2008). Usually, the first symptoms of the disease are wilting and discolouration of needles, followed by dieback due to the development of resinous cankers at the sites of infection (reviewed by Wingfield et al. 2008). Because *F. circinatum* is capable of infecting vegetative and reproductive structures of susceptible hosts of all ages, the pathogen can affect roots, shoots, stems, flowers, cones, seed and seedlings. In the case of seedlings, the

pathogen causes root disease and girdling of root collars, which can result in substantial losses during propagation.

Fusarium circinatum has a global distribution and is capable of infecting a wide range of *Pinus* species, as well as *Pseudotsuga menziesii* (Wingfield et al. 2008). In its suggested center of origin (*i.e.*, Mexico and neighboring Central America) (Wikler and Gordon 2000), the pathogen is known to be associated with native species such as *P. douglasiana* and *P. durangensis*, as well as planted *P. radiata* and *P. halepensis* (Santos and Tovar 1991, Guerra-Santos 1999). In the south-eastern United States, *P. elliottii* and *P. taeda* have been affected most severely (Hepting and Roth 1946), and in California pitch canker has devastated natural and planted stands of *P. radiata* (Gordon et al. 2001). The disease also occurs on native pine species in Haiti (Hepting and Roth 1953), Japan (Kobayashi and Muramoto 1989), Korea (Woo et al. 2010) and Italy (Carlucci et al. 2007). In Spain, South Africa, and Korea pitch canker occurs in planted stands of important non-native forestry species (Lee et al. 2000, Landeras et al. 2005, Coutinho et al. 2007, Woo et al. 2010, Iturritxa et al. 2011). In Spain, South Africa, Chile, Portugal and Uruguay, *F. circinatum* further also limits seedling production in commercial forestry nurseries (Wingfield et al. 2002a, 2002b, Landeras et al. 2005, Alonso and Bettucci 2009, Bragança et al. 2009).

In Colombia, symptoms resembling those caused by *F. circinatum* were observed on four *Pinus* species important for commercial forestry in that country (Figure 1A-F). In November 2005, seedlings of *P. patula* displaying symptoms such as wilt, shoot dieback and roots with small resin-soaked necrotic lesions were observed in a nursery located in Valle del Cauca. In this nursery, similar symptoms also were observed subsequently on seedlings of *P. tecunumanii* and *P. maximinoi*. In March 2006, symptoms typical of pitch canker (e.g., shoot and twig dieback, and the presence of resinous cankers on trunks and branches) were observed on 11-year old *P. patula* trees near Santa Rosa (Risaralda). During 2006 and 2007, shoot dieback was also observed on established *P. patula* and *P. kesiya* trees in plantations in Valle del Cauca and Antioquia, respectively.

The association of *F. circinatum* with *Pinus* species is almost always correlated with significant economic losses (Dwinell et al. 1985). This could be due to reduction in tree growth volume (Bethune and Hepting 1963, Arvanitis et al. 1984) and in establishment of seedlings in the field (Crous 2005, Mitchell et al. 2011). An increase in the mortality of nursery seedlings (Wingfield et al. 1999) and trees in natural stands or plantations (Blakeslee and Oak 1979) can also cause dramatic losses. Therefore, if the pathogen occurs on commercially important *Pinus* species in Colombia, similar losses could occur. Approximately 35% of the total forestry plantation area in Colombia is planted to *Pinus* species (IDEAM 2009) and the export of timber and related products contributes considerably to the country's gross domestic product (Mendell et al. 2006). Understanding the potential involvement of the pitch canker pathogen in contributing to the symptoms observed on the four non-native *Pinus* species is thus important; not only from a disease management point of view, but also in considering the long-term consequences of pitch canker in Colombia.

The aims of this study were firstly to isolate and identify the fungal species associated with the diseased *P. kesiya*, *P. maximinoi*, *P. tecunumanii* and *P. patula* during 2005-2007. For this purpose we employed methodologies based on fungal morphology and DNA sequence data. A second goal was to determine the pathogenicity of the isolated fungi on commercially important families of *P. patula, P. maximinoi* and *P. tecunumanii*.

Materials and Methods

Fungal isolates

The *Fusarium* isolates used in this study were obtained from diseased plants in four different geographic locations in Colombia (Table 1). Climatically, these locations range from being cool temperate with average day temperatures of 17-22ºC and annual precipitation of 1125- 2688 mm. Diseased tissues of three to four-month old *P. patula*, *P. tecunumanii* and *P. maximinoi* seedlings were collected from a nursery near Restrepo in Valle del Cauca. Samples were collected from 11-year old *P. patula* trees near Santa Rosa de Cabal (Risaralda) and from six-month old *P. patula* trees near Santa Rosa de

Osos (Antioquia). Diseased tissue samples were also obtained from 18-month old *P. kesiya* plants in the Cumbre area (Valle del Cauca).

For the isolation of fungi, diseased tissue samples were surface-sterilized for 1 minute in a commercial bleach solution containing 1.5% sodium hypochlorite and then rinsed well with sterile distilled water. Small pieces of tissue, cut from the edges of lesions, were placed directly onto medium containing Potato Dextrose Agar (PDA, 20 g l^{-1} PDA [Merck, Germany], 5 g l^{-1} agar [Merck]), and incubated for seven days at 22°C. Fungi resembling those in the genus *Fusarium* were transferred to Petri plates containing PDA. After another round of incubation at 22°C for seven days, the fungi on these plates were used to prepare pure cultures by transferring single germinating conidia to fresh PDA medium. All cultures were deposited in and may be obtained from the culture collection of the Tree Protection Cooperative Programme, Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa.

Identification of fungi

All isolates were grown on synthetic low nutrient agar (Nirenberg 1976) and carnation leaf agar (Fisher et al. 1982) for identifications using morphology. Following incubation for about ten days at 25°C under near ultraviolet light, the fungi were examined microscopically. We specifically considered the diagnostic characters proposed by Nirenberg and O'Donnell (1998), Britz et al. (2002) and Leslie and Summerell (2006).

For identifications based on DNA sequence information, total DNA was extracted from seven-day old PDA cultures (Steenkamp et al. 1999). These DNA extracts were used as templates in PCRs with primers EF1 and EF2 (O'Donnell et al. 1998b) and primers T1 and T2 (O'Donnell and Cigelnik 1997) to amplify diagnostic portions of the genes encoding translation elongation factor 1-alpha (TEF) and β-tubulin, respectively. These primers were also used for sequencing the respective fragments. All PCRs and sequencing reactions were performed as described previously (Kvas et al. 2008) by making use of a GeneAmp® PCR system 9700 (Applied Biosystems, Foster City, California) and a 3730 DNA Analyzer (Applied Biosystems).

Raw sequence files were analysed with Chromas Lite 2.0 (Technelysium, Australia) and BioEdit version 7.0.5.2 (Hall 1999). Sequences were compared to those in GenBank® (Benson et al. 2011) using nucleotide BLAST searches. The TEF sequences were also compared to those in the *Fusarium* Identification Database (Geiser et al. 2004, http://isolate.fusariumdb.org/index.php). Multiple alignments and phylogenies based on Bayesian inference and maximum likelihood were constructed using previously described procedures (Kvas et al. 2008) and, respectively, MAFFT v6 (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/) (Katoh et al. 2002), MrBayes v3.1.2 (Ronquist and Heuelsenbeck, 2003) and PHYML v2.2.4 (Guindon and Gascuel 2003).

Pathogenicity tests

All pathogenicity tests with *F. circinatum* were performed on nine- or eight-month old *Pinus* seedlings in a greenhouse at Restrepo in Colombia. The plants were grown in plastic containers and maintained at approximately 22°C under natural light at Rancho Grande Nursery in Restrepo. Before inoculation, the trees were allowed to acclimatize in the greenhouse for four weeks. Conidial suspensions (50 000 conidia ml^{-1} sterile water) were prepared from eight-day old fungal cultures grown on PDA. These suspensions were used as inoculum, where 100 µl of the suspension were placed onto wounds left after removal of terminal buds. For the control treatments, inoculations were performed with 100 µl of sterile water. To prevent desiccation of the inoculum, inoculated plants were covered with plastic bags for 12 hours, after which the bags were removed.

Results of pathogenicity tests were evaluated 16 weeks after inoculation by measuring the length of the internal lesions that developed. These lesions were exposed by scraping away the bark at the point of inoculation with a sterile scalpel. To confirm that the lesions were caused by the isolates used for inoculation, re-isolations were made from three randomly selected plants per isolate and identifications were made as described above. Statistical analyses were performed with the SAS/STAT software (SAS Institute 2009). Analysis of Variance (ANOVA) was used to determine significant differences among

treatments and their interactions, while the Sidak method was used for comparing treatment differences (SAS Institute 2009).

Two separate sets of pathogenicity tests were performed. The first set of tests was conducted with seven isolates of *F. circinatum* that originated from four *Pinus* species in Colombia (Table 1). These were used in pathogenicity tests on nine-month old *P. patula* plants, where twenty trees were inoculated with each isolate, as well as with sterile water for the control inoculations. The entire trial was repeated three times, giving a total of 480 trees inoculated. The second set of tests was conducted with two isolates (CMW 21140, CMW 21144) of *F. circinatum* that were selected based on their high level of pathogenicity in the first trial. The two isolates were used to inoculate eight-month old seedlings of five different *Pinus* families, which were selected to represent the main species planted in the region. The five families included *P. tecunumanii* (PTECCATA) originating from a high elevation source (Cauca, Colombia) and *P. tecunumanii* (PTECSUIZ) originating from a low elevation source (Valle del Cauca), as well as two families of *P. maximinoi* (PM1564CA and PM1517CA) and a family of *P. patula* (PPATPNEG). For this set of tests, ten plants were inoculated with each isolate and sterile water was again used for the control inoculations. The entire trial was repeated three times, giving a total of 450 trees inoculated.

Results

Identification of Fungi

Isolates resembling members of the genus *Fusarium* were obtained from diseased trees and seedlings of *P. patula, P. maximinoi, P. tecunumanii* and *P. kesiya* collected at four different locations in Colombia (Table 1). Microscopic examination revealed that they generally produced sterile coiled hyphae, lunate macroconidia, branched conidiophores and polyphiallides with two to five conidiogenous openings. These traits are characteristic of the pitch canker pathogen, *F. circinatum* (Nirenberg and O'Donnell 1998, Britz et al. 2002, Leslie and Summerell 2006).

The TEF sequences for the seven isolates from Colombia were identical and comparison to those in the *Fusarium* Identification Database and GenBank®, showed that they were highly similar to known isolates of the pitch canker fungus (see Table 1 for GenBank® assession numbers). For example, their sequences contained only eight nucleotides that were different from that of isolate NRRL 25331, the ex-holotype of *F. circinatum* (Nirenberg and O'Donnell 1998), and one nucleotide different from those of the *F. circinatum* mating-type tester strains MRC 7488 and MRC 6123 (Britz et al. 1999). With regards to their β-tubulin sequences, the Colombian isolates were identical to one another and to the three isolates mentioned above (see Table 1 for GenBank® assession numbers). Phylogenetic analysis of the data further showed that the isolates from Colombia form part of a well-supported group that also include isolates of *F. circinatum* from South Africa, Japan and USA (Figure 2). These results thus confirmed that the fungi isolated from the diseased *P. patula, P. maximinoi, P. tecunumanii* and *P. kesiya* plants in Colombia represent the pitch canker pathogen, *F. circinatum*.

Pathogenicity tests

Five of the *F. circinatum* isolates (CMW 21140, CMW 21144, CMW 25510, CMW 25518 and CMW 25520) tested for pathogenicity on nine-month old *P. patula* plants produced lesions that were significantly larger ($P \leq 0.0001$) than those observed for the control treatments (Table 2). The longest lesions were produced by isolates CMW 21144, CMW 25520 and CMW 21140, which were all obtained from *P. patula.* The mean lesion lengths caused by isolates CMW 25509 (isolated from *P. maximinoi*) and CMW 25519 (isolated from *P. patula*) were not significantly different from those of the control treatment. However, *F. circinatum* was successfully re-isolated from all of the examined inoculated plants and not from the control plants. This confirmed Koch's postulates showing that the observed symptoms were caused by the pitch canker pathogen.

Isolates CMW 21144 and CMW 21140 were used to evaluate the resistance to *F. circinatum* of five pine families. All inoculations with the two isolates produced lesions that were significantly ($P \leq 0.0001$) larger than the control treatments. The results of the two-way ANOVA indicated that the response

variable, lesion length, is strongly dependent on the genotype of the fungus and the plant, as well as the interaction between these two factors (Table 3). Although these data indicate that typical genotype-by-genotype interactions underlie resistance of *Pinus* to the pitch canker fungus (Lambrechts et al. 2006, Gordon and Leveau 2010), both of the *F. circinatum* isolates produced significantly larger lesions on *P. patula* and *P. tecunumanii* (PTECCATA) than on the other *P. tecunumanii* (PTECCSUIZ) family and the two *P. maximinoi* families (Table 4).

Discussion

In this study, we show conclusively that the disease symptoms observed in Colombia on the roots of *P. patula*, *P. tecunumanii* and *P. maximinoi* seedlings, as well as on the branches and trunks of *P. kesiya* and *P. patula* in established plantations were caused by the pitch canker fungus *F. circinatum*. Together with this first report from Colombia, pitch canker and its causal fungus, *F. circinatum* is now known from three South American countries. In 2002, *F. circinatum*-associated root disease was detected in both containerized and open rooted clonal hedges of *P. radiata* in a commercial nursery in Chile (Wingfield et al. 2002b). More recently in 2009, the pathogen was reported from seedlings of *P. taeda* in Uruguay (Alonso and Bettucci 2009). However, in contrast to the situation in Colombia, the symptoms of *F. circinatum* appears to be limited to pine seedling nurseries in Chile and Uruguay, as pitch canker symptoms have not yet been observed in plantation trees in these countries.

Apart from the nursery seedlings affected by *F. circinatum*, symptoms were also observed on 18-month old *P. kesiya* and six-month old *P. patula* plants. In South Africa and Chile, mortality of, respectively, *P. patula* and *P. radiata* plants in these age classes has also been observed to be due to infection by *F. circinatum* (Mitchell et al. 2004, Crous 2005, Wingfield et al. 2008). In *P. patula*, mortality usually starts three to six months post-planting and can persist for up to two years (Crous 2005). The sources of such infections are believed to originate from infected but asymptomatic plants (Storer et al. 1998) in the nursery. Although it is not known how and why the pathogen switches from a latent phase

to actively infecting the host plant, the stresses associated with out-planting and post-planting establishment in the field are probably important (Wingfield et al. 2008). Therefore, some of the disease symptoms observed in Colombia could reflect a similar problem with seedling establishment after planting in the field, which is associated with significant economic losses in South Africa (Mitchell et al. 2011). Extensive surveys are required to fully quantify the extent to which *F. circinatum*-associated post-planting establishment problems in pine plantations might impact Colombian forestry.

The fact that pitch canker occurred on 11-year old *P. patula* trees in one area in Risaralda, suggests the pitch canker pathogen in Colombia is not only a problem in seedlings and their establishment in the field, but also of older established plantation trees. A similar situation occurs in South Africa where the pitch canker pathogen was first discovered on *P. patula* seedlings in a single nursery in 1990, after which it spread to almost all commercial forestry nurseries (Wingfield et al. 2008). It was only recently, in 2005, that an outbreak of the disease occurred on five- and nine-year old *P. radiata* (Coutinho et al. 2007). Although it remains unclear why pitch canker in established plantations only emerged 15 years after the pathogen was first recorded in South Africa, the involvement of the non-native deodar weevil (*Pissodes nemorensis*) have been suggested (Coutinho et al. 2007). It is possible that the disease in Risaralda on *P. patula* trees could also be attributable to the infection of wounds created by pine-feeding insect species that have been introduced to that region. Future studies should thus seek to determine how infection courts are being created in plantation trees in this region. Such information, together with knowledge regarding ecological and environmental factors that might facilitate infection (Wingfield et al. 2008) would be invaluable for preventing or managing this and other field outbreaks of pitch canker in Colombia.

The results of the pathogenicity tests on nine-month old *P. patula* seedlings suggested a significant level of genetic diversity within the Colombian population of the pathogen, although only seven isolates of *F. circinatum* were examined in this study. The isolates varied widely in terms of the length of lesions that they induced on the seedlings (Table 2). An analysis of more extensive collections of

isolates and the application of markers such as microsatellites or vegetative compatibility groups will provide a much clearer view of the population biology and origin of *F. circinatum* in Colombia. Such studies have shown, for example, that *F. circinatum* in California was introduced from the southeastern United States (Wikler and Gordon 2000) and that pitch canker in northern Spain is probably due to one or a few introductions (Iturritxa et al. 2011). A detailed understanding of the population biology of *F. circinatum* in Colombia should, therefore, reveal whether the pathogen originated from contaminated seed that was imported from Mexico or Central America, as have been shown for the pathogen in South Africa (Wikler and Gordon 2000, Britz et al. 2001), or from some other source.

Of the five pine species that were evaluated in this study, *P. tecunumanii* originating from high-elevation sources and *P. patula* were most susceptible to the pitch canker fungus. In contrast, very small lesions were induced by the pathogen on the two *P. maximinoi* families and the *P. tecunumanii* family originating from low-elevation sources (Table 4). These findings are in agreement with what has been demonstrated previously (Hodge and Dvorak 2000, 2007). The apparent resistance of species such as *P. maximinoi*, *P. tecunumanii* and *P. oocarpa* to pitch canker has boosted their popularity in tropical and subtropical regions. Furthermore, the results of a recent study that modeled the potential impact of climate change on *P. patula* and *P. tecunumanii,* suggest that provenances of *P. tecunumanii* from low-elevation sources in Central America are predicted to be most productive in the future (Leibing et al. 2009). The latter species, together with *P. maximinoi* are expected to become increasingly important for commercial forestry in Colombia.

The results of previous pitch canker resistance screenings and climate change predictions should be interpreted with care. Although the results of greenhouse trials are generally predictive of host resistance under field conditions (Gordon et al. 1998a, 1998b), the possibility that different virulence and pathogenicity factors might be operational in the greenhouse and in the field (Matheson et al. 2006) is rarely considered. Also, in most cases, the relative resistance or tolerance of a *Pinus* species or family to *F. circinatum* is based on tests with a limited number

of isolates. However, one genotype of the pathogen will not necessarily express similar levels of pathogenicity in different *Pinus* species or genotypes, which is also evident from our results (Table 3) and those reported previously (Hodge and Dvorak 2007). This because virulence is dependent on genetic factors determined by both the host and the pathogen (e.g., Thompson and Burdon 1992, Lambrechts et al. 2006, Gordon and Leveau 2010). Furthermore, the climate change models suggested for *P. patula* and *P. tecunumanii* (Leibing et al. 2009) did not take into account the pitch canker fungus and its inherent genetic diversity or its ability to change with time. Like its plant host, *F. circinatum* will likely adapt to climate change, but predictions regarding such adaptations are confounded by a general lack of understanding of the pathogen's ecology and biology. Therefore, as is the case wherever *Pinus* species are commercially cultivated, the continued and sustainable use of pine in Colombia will be dependent on a multi-faceted and integrated approach that considers not only the host and its associated diversity and adaptability, but also the biology of the pathogen and environmental factors involved in the disease.

Acknowledgements

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Table 1: Host, geographic origin and sequence accession numbers for the isolates of *F. circinatum* used in this study

Isolate [a]	*Pinus* host	Area in Colombia	GPS coordinates, rainfall, and average temperature [b]	Sequence accession numbers [c]
CMW 21140	*P. patula*	Restrepo (Valle del Cauca)	1450 (76°29'49" W 3°51'45" N) 1067, 20	JN642099, JN642106
CMW 21144	*P. patula*	Restrepo (Valle del Cauca)	1450 (76°29'49" W 3°51'45" N) 1067, 20	JN642100, JN642107
CMW 25509	*P. maximinoi*	Restrepo (Valle del Cauca)	1450 (76°29'49" W 3°51'45" N) 1067, 20	JN642101, JN642108
CMW 25510	*P. tecunumanii*	Restrepo (Valle del Cauca)	1450 (76°29'49" W 3°51'45" N) 1067, 20	JN642102, JN642109
CMW 25518	*P. kesiya*	Cumbre (Valle del Cauca)	1525 (76°31'46"W, 3°42'10"N) 1108, 21	JN642103, JN642110
CMW 25519	*P. patula*	Santa Rosa de Cabal (Risaralda)	1971 (75°36'21"W, 4°49'18"N) 2688, 17	JN642104, JN642111
CMW 25520	*P. patula*	Santa Rosa de Osos (Antioquia)	2480 (75°26'30" W 6°52'4" N) 2600, 17	JN642105, JN642112

[a] CMW = Culture Collection, Tree Protection Co-operative Programme, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa. CMW 21140 and CMW 21144 were isolated by MJ Wingfield, while CMW 25509, CMW 25510, CMW 25518, CMW 25519 and CMW 25520 were isolated by CA Rodas.

[b] GPS = Global positioning System. Elevation in meters above sea level is followed by location coordinates in parentheses, average annual rainfall (mm/year) and average temperature (°C).

[c] GenBank® accession numbers are provided for each isolate in the order TEF, β-tubulin.

Table 2: The results of a pathogenicity study with Colombian isolates of *F. circinatum* on nine-month old *P. patula* plants

Isolate/treatment	Mean lesion length in mm [a]	Standard error
CMW 21140	7.77 (a)	1.26
CMW 21144	9.23 (a)	1.29
CMW 25509	0.91 (b)	0.59
CMW 25510	6.03 (a)	1.07
CMW 25518	5.36 (ab)	1.07
CMW 25519	4.78 (ab)	0.96
CMW 25520	7.91 (a)	1.22
Control	1.86 (b)	0.91

[a] Data represent means of the 60 measurements obtained for each isolate, because similar results were obtained for all the repeats of the pathogenicity test. A one-way analysis of variance (ANOVA) indicated a significant treatment effect, where the observed *F*-value is 7.517 and the significance probability associated with the *F*-statistic is <0.0001. Individual means were compared and grouped using the Sidak method and a confidence level of 95%. Means that were not significantly different are indicated in parentheses by the same letter.

Table 3: Results of a two-way analysis of variance (ANOVA) of the pathogenicity of two isolates of *F. circinatum* on five *Pinus* families [a]

Source of variation	Degrees of freedom	Sum of squares	Mean squares	*F* statistic	*P*-value
Isolates	2	2421.85	1210.92	62.31	<0.0001
Pine families	4	6824.97	1706.24	87.80	<0.0001
Isolates X pine families	8	3692.34	461.54	23.75	<0.0001
Error	423	8220.48	19.43		

[a] Lesion length was analyzed as the response variable with fungal genotype (*i.e.*, isolate of *F. circinatum*) and plant genotype (*i.e.*, pine family) as two explanatory factors (both treated as fixed effects). The analysis was performed with SAS and employed type III sums of squares (SAS Institute 2009).

Table 4: Results of a pathogenicity study with two Colombian isolates of *F. circinatum* (CMW 21140 and CMW 21144) on eight-month old *Pinus* seedlings

Pinus family	Mean lesion length in mm (Standard error) [a]	
	CMW 21140	CMW 21144
P. patula (PPATPNEG)	17.40 (1.41) c	12.11 (1.91) b
P. tecunumanii (PTECCATA)	9.12 (0.99) b	8.48 (1.51) b
P. tecunumanii (PTECSUIZ)	0.90 (0.77) a	0.65 (0.57) a
P. maximinoi (PM1564CA)	0.57 (0.11) a	0.00 (0.00) a
P. maximinoi (PM1517CA)	0.00 (0.00) a	0.15 (0.10) a

[a] The data represent means of the 30 measurements obtained for each isolate, as similar results were obtained for all three repeats of the pathogenicity test. Individual means were compared and grouped using the Sidak method and a confidence level of 95%. Means that were not significantly different are indicated by the same letter.

Figure 1: Symptoms associated with the pitch canker fungus, *F. circinatum*, which were observed on *Pinus* species in Colombia. A. Stem discoloration on a four month-old seedling of *P. patula.* B. A seedling of *P. tecunumanii* with wilting and dieback symptoms. C. Constricted root collar on a *P. maximinoi* seedling. D. Copious resin bleeding from an infection site on the root collar of a *P. maximinoi* seedling. E. "flagging" of branches on 11-year old *P. patula.* F. pitch-soaked, resinous wood on the stem of an 18-month old *P. kesiya* tree

Figure 2: A maximum likelihood phylogeny inferred from combined TEF and β-tubulin sequence data for the so-called "American Clade" of the *Gibberella fujikuroi* complex of which *F. circinatum* is a member (O'Donnell et al. 1998a; Geiser et al. 2005). Bootstrap support values ≥75% and Bayesian posterior probabilities ≥0.95 are indicated at the branches in the order maximum likelihood/Bayesian inference, and lack of support is indicated with "−". The tree is rooted with the *Fusarium* species in the so-called "African Clade" of the *G. fujikuroi* complex
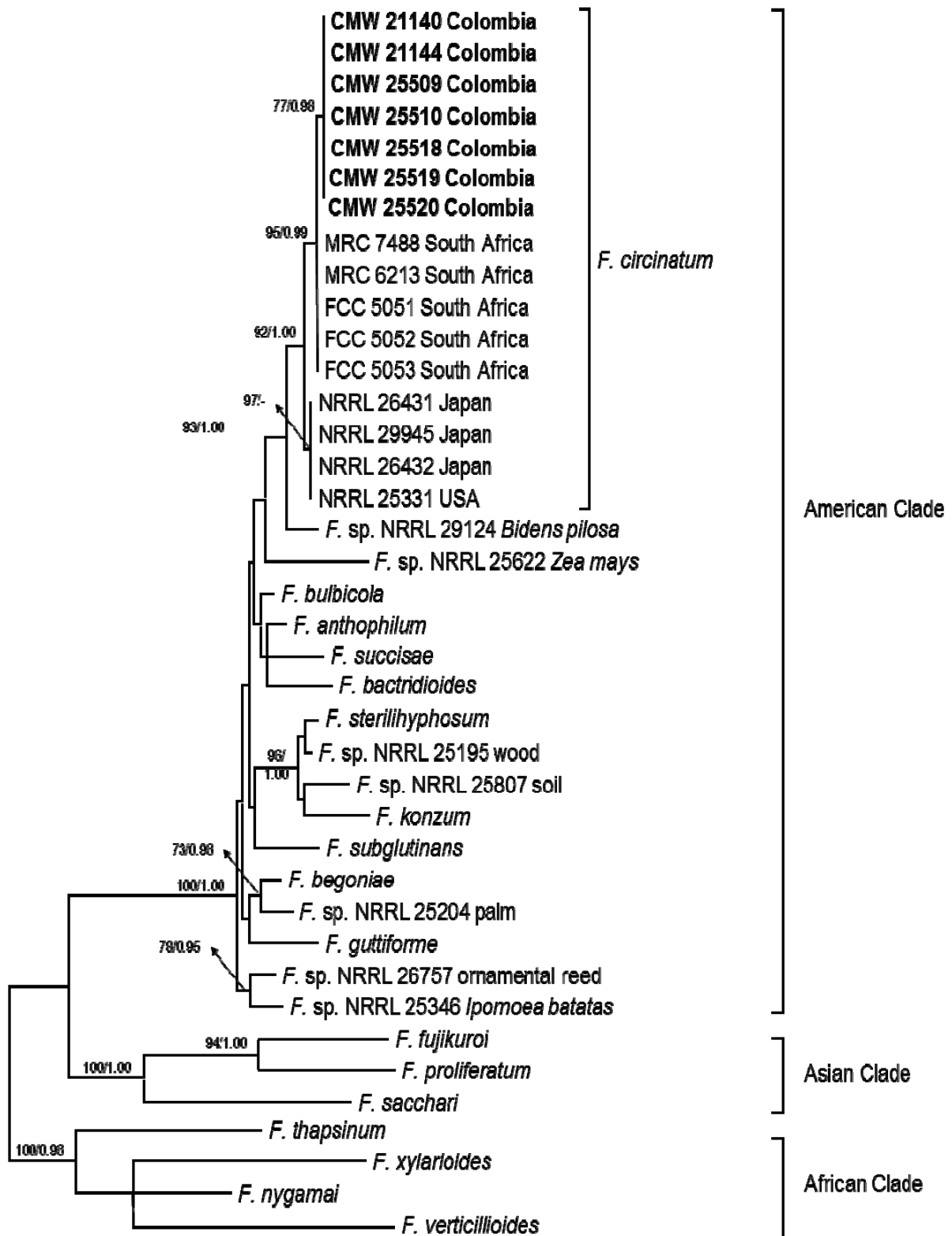
Chapter 6

Dothistroma Needle Blight: an emerging epidemic caused by *Dothistroma septosporum* in Colombia

Abstract

Plantation forestry in Colombia is based mainly on non-native species of *Pinus* and *Eucalyptus*. Since 2008, a disease, similar in symptoms to Dothistroma Needle Blight (DNB) has been found affecting large areas planted to *Pinus* spp. The aim of this study was to isolate and identify the pathogens associated with the disease and to consider patterns of its incidence and impact. Isolates from each of the three zones were compared based on rDNA sequence of the ITS regions and were conclusively identified as *Dothistroma septosporum,* the causal agent of DNB. Susceptibility was greatest on low elevation *P. tecunumanii* followed by *P. kesiya, P. oocarpa* and with minimal damage to *P. maximinoi*. A high resistance was found for high elevation provenance *P. tecunumanii*. Low-elevation *P. tecunumanii* progenies in a susceptibility trial presented 99.2% infection and an average severity of 39.7%. The levels of infection in the different zones varied significantly with the Northern Zone being the most severely affected. This constitutes the first report of the current disease distribution and susceptibility of hosts, as well as the first investigation of the relative importance of *D. septosporum* in Colombia.

Introduction

Plantation forestry in Colombia is based mainly on non-native species of *Pinus* and *Eucalyptus*. Collectively these species make up approximately 327 000 hectares (ha) of plantations (MADR 2010) that provide the raw material for pulp and solid timber products (MADR 2006). Early plantations of *Pinus* spp. were largely comprised of *P. patula* but in recent years, various other species, dominated mainly by *P. tecunumanii* and *P. maximinoi,* have increasingly been planted in order to match species more appropriately to the variable sites and altitudes found in the country.

Plantations of *Pinus* spp. in Colombia have been challenged by a number of native insect defoliating pests (Vélez 1972, Wiesner and Madrigal 1983, Mackay and Mackay 1986, Madrigal and Abril 1994, Rodas 1994) and a few diseases. In 1984, a severe infection caused by *Diplodia pinea* was recorded in *P. patula*

plantations in different areas in Colombia (Hoyos 1987, Rodas and Osorio 2008). More recently, a diverse suite of pathogens such as *Calonectria* spp. (Lombard et al. 2009), and *Fusarium circinatum* (Steenkamp et al. 2012) have affected *Pinus* plantations. These problems have increased in severity and this has resulted in an increasingly large area being planted to alternative, tolerant species.

Needle pathogens that have been reported in Colombia include *Lecanosticta acicola, Meloderma desmazierii* and *Dothistroma septosporum* (Gibson 1979, Gibson 1980, Ivory 1987). Gibson (1980) suggested that *M. desmazierii* in Colombia did not appear to be a fungus that would be of any concern in pine plantations, he did warn, however, that the new discovery of *L. acicola* in Colombia might pose a significant threat to Southern Hemisphere pine plantations. This threat was a valid concern considering the extensive disease epidemics that a similar needle pathogen, *Dothistroma septosporum* was causing in the Southern Hemisphere, especially in areas such as Chile and New Zealand (Alzamora et al. 2004, Bulman et al. 2008). Colombia was included in the distribution list of countries containing *Dothistroma septosporum* in the work by Ivory (1987), but there is no supporting information as to where it might have been found and on what host.

The similarity of symptoms between pine needle blights has created the need for tools that can contribute to the monitoring of forest diseases based on correct identification. As a result of recent molecular identification methods being developed (Ioos et al. 2010, McDougal et al. 2011), it is now relatively easy to positively identify between the two known species that cause Dothistroma Needle Blight (DNB), *D. septosporum* and *D. pini* and the pathogen that causes Brown Spot Needle Blight (BSNB), namely *L. acicola* (Barnes et al. 2004, EPPO 2008, Ioos et al. 2010, McDougal et al. 2011).

In 2008, a new and serious needle disease problem appeared in the Central Zone of Colombian pine plantations. Symptoms of the disease closely resembled those of Dothistroma Needle Blight. Within two years, the needle cast disease had spread throughout all three forestry zones in Colombia on *Pinus*

tecunumanii. Importantly, this was a tree species relatively new to forestry in the country and for which there was little knowledge of diseases and insect pests that affect it.

The aims of this study were to establish the distribution of the new and serious needle disease in Colombia, to confirm its identity based on DNA sequence data and to determine the susceptibility of different progenies of *P. tecunumanii* based on levels of infection and disease severity. In addition, the impact of the disease on *P. tecunumanii* intensively managed plantations was assessed.

Materials and Methods

Disease distribution

The extent of the needle blight epidemic in Colombia was assessed using field surveys conducted throughout all the plantation areas belonging to Smurfit Kappa Cartón de Colombia (SKCC). Initial observations commenced in 2008 and continued until 2012. The surveys covered three different geographic zones (North, Central and South) and eleven farms (Yanahuanca, Argentina, Tesalia, Cedral, Alaska, Esmeralda, La Concha, Samaria, Volconda, Unión_B and Unión_S) located in the Departments of Caldas, Risaralda, Valle del Cauca and Cauca (Table 1, Figure 1). The total area surveyed was approximately 26 730 ha and consisted of plantations of *P. patula, P. kesiya, P. maximinoi, P. oocarpa,* and two different provenances of *P. tecunumanii* namely low elevation (LE) planted between 1400 -1900 m.a.s.l. and high elevation (HE) planted at 1900 – 2500 m.a.s.l.

Pathogen identification

Sample collection, isolation and morphological characterization

To verify the identity of the pathogen responsible for the diseased symptoms in the surveyed areas, infected needles bearing distinct conidiomata were collected

from diseased *P. tecunumanii* trees in each of the three zones. Needles were placed in paper envelopes and stored at 4°C in preparation for further studies in the laboratory.

Needles were prepared for isolations by first surface-disinfesting them in 0.2% sodium hypochlorite for 1 minute, rinsing with distilled water and blotting them dry with sterile paper towels. Using a Nikon SMZ645 stereoscope, fruiting bodies were excised from the needles and placed onto MEA 2% (w/v) malt extract (Merck, Darmstadt, Germany), 1.5% (w/v) agar (OXOID, Hampshire, United Kingdom), supplemented with 1% lactic acid, and incubated for 15 days at 24°C.

Morphological characteristics of the fungus were observed using a Nikon Eclipse E200 microscope. Microscope slides were prepared by fixing conidiomata bearing conidia, excised from diseased needles, with 1% lactic acid (Carlo Erba Reagenti, Arese, Italy).

DNA sequence based comparisons

Identification was made for two cultures isolated from needles collected from each of the three forestry zones. Mycelium was scraped from the surface of the cultures on agar, freeze-dried and crushed using the Retsch MM301 mixer mill (Haan, Germany) for 3 min at 1/30 mHz. The crushed mycelium was heated to 65°C in 800 ul DEB buffer (200 mM Tris-HCL, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) for an hour and DNA was extracted using the method described by Barnes et al. (2001).

The ribosomal, Internal Transcribed Spacer (ITS) region was amplified for the selected isolates using primers ITS1 and ITS4 (White et al. 1990). The reaction mixture consisted of ± 5 ng DNA template, 200 nM of each primer, 0.2 mM of each dNTP, 1U FastStart Taq DNA Polymerase with 10× buffer (Roche Molecular Biochemicals, Mannheim, Germany) and 1.5 mM $MgCl_2$. Cycling conditions were set at 96°C for 2 min, 10 cycles of 94°C for 30s, 56°C for 30s and 72°C for 1 min. An additional 30 cycles were included with the annealing time altered to 40s and a 5s extension after each cycle with a 10 min final

elongation at 72°C. PCR amplicons were visualized on 2 % agarose gels and cleaned using Sephadex G-50 columns (SIGMA-Aldrich, Steinheim, Germany). Sequencing of the amplicons was carried out using the ABI PRISM™ Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Applied BioSystems, Foster City, California) following the manufacturer's protocols and run on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, California, USA).

Sequences were analyzed in CLC Bio (Main Workbench Version 6.6.2) and aligned using the online version of MAFFT version 6 (http://mafft.cbrc.jp/alignment/server/). Maximum parsimony phylogenetic analyses were conducted in PAUP* 4.0b10 (Swofford 2002) using the heuristic search option with random stepwise addition and tree bisection reconnection as the tree swapping algorithm. Bootstrap analyses were conducted with 1000 randomizations. The trees were rooted with sequence data for *Lecanosticta acicola* as the outgroup.

Susceptibility of *Pinus tecunumanii* progenies

Susceptibility studies were established on the farm Graminea located at 5°25'19'' N – 75°45'56'' W, (North Zone, Caldas) having an altitude of 2155 m.a.s.l., annual precipitation of 2560 mm and an average temperature of 18°C (Figure 1). At this site, 27 different *P. tecunumanii* Low Elevation (LE) progenies had been planted approximately 2 km from an area very heavily affected by needle blight. Trees in the plot were established in November of 2008 and the first disease symptoms were recorded two years later in November 2010. A total of 1260 trees in the trial were used for evaluations. These included 972 trees of *P. tecunumanii* LE progenies and they were distributed in 6 plots, each including 27 progenies and 6 trees of each progeny. In addition, four different provenances of *P. tecunumanii* [PTEBsuH1 (LE), PTEByucu (LE), PTEAcaH1 (HE) and TECASaH1 (HE)] as well as, two provenances of *P. maximinoi* (MAXcabH1 and MAXcabH2) and two of *P. kesiya* (PKcalvH1 and PKcalvH2) were included in the trial for comparative purposes. A total of 288 trees (6 trees in each of 6 plots per provenance) were used. Weather conditions such as temperature and

precipitation were monitored during the evaluation period. The trial was evaluated from November 2010 to May 2012, and data from these evaluations were used to determine the influence of tree age, climatic factors as well as the influence of progenies.

The percentage of natural infection was assessed by dividing the total number of affected trees by the total number of planted trees for each of the progenies (972 trees from 27 progenies) as well as for the additional treatments used for comparative purposes (288 trees from 8 provenances).

The disease severity level per tree was calculated using two distinct measures. Firstly, the severity was calculated in the field by dividing the total foliar area of infected trees into four quarters, each one representing 25% of the tree foliar area. Each quarter was then assessed inidividually for the amount of disease present as a percentage of the total (100%) to facilitate a relatively acurate estimate of severity (Figure 2). The data collected for each quarter were then weighted to represent the conical foliar area per quarter in which the designated value for the major quarter (bottom of the tree) corresponded to 40%, 30%, 20% and 10% (top) areas of the trees respectively (Figure 3).

Disease impact in forestry plantations

Evaluations of disease impact were made using plots of 300 m^2 (9.78 m radius), each including approximately 30 planted trees (plantation density of 3 x 3 m) (Figure 4). These plots were randomly placed within severely diseased plantations and the sampling intensity was 1% of the total affected area (1 plot per every three hectares of affected area).

The impact of the disease within affected areas was determined by evaluating the percentage of infection level as the total number of affected trees in each plot divided by the total number of trees in the plot. The severity of infection was calculated as previously described above for the Graminea trial.

A total of 90 plots were evaluated in January of 2012 in the North, Central and South Zones of the area being considered. Eleven forestry farms (Yanahuanca,

Argentina, Tesalia, Cedral, Alaska, Esmeralda, La Concha, Samaria, Volconda, Unión_B, Unión_S) that a wide range of altitudes and weather conditions were selected for evaluation in these zones (Table 1). Abiotic factors such as precipitation and altitudinal range, and age were taken under consideration at each farm.

Statistical analyses

Data for the percentage of infection and severity were analysed using descriptive statistics, and Duncan's Multiple Range test (significance level of 95%) using the programme SAS Proc Insight. These analyses were specifically also to determine whether the different geographic locations in Colombia (North, Central, and South zones) have any influence on the presence and impact of the leaf blight on *P. tecunumanii* plantations. Thus, to determine whether there was any association between the disease severity on *P. tecunumanii,* and weather conditions such as precipitation, geographic conditions such as elevation or biotic conditions such as age of diseased trees, the programme Proc GLIMMIX was used. Observations for each plot were treated with a binomial distribution for susceptibility of *P. tecunumanii* progenies in the Graminea trial as well as the disease impact in 90 plots of 300 m^2 each conducted at the North, Central and South Zones respectively.

Results

Disease distribution and host range

In June of 2008, DNB was first noticed as an important foliar disease causing significant impact on various pine species in Colombia (Figure 5A). The disease appeared in 2.5 year-old *P. tecunumanii* (Yucul provenance) plantations located at the farms Alaska (4°3′24′′N - 76°25′09′′W) and Esmeralda (4°3′27′′N - 76°25′09′′W) in the Valle del Cauca department of the Central Zone. A month later, a second report of the disease was made for 2.5 year-old *P. tecunumanii* (Suiza provenance) on the farm Argentina, (5°24′20′′N - 75°44′55′′W) in Caldas, Northern Zone.

In February of 2009 a third report of DNB emerged from the farm La Unión (Cauca, Southern Zone) at 2°17´04´´N - 76°33´56´´W in a 2.5 year-old-stand of *P. tecunumanii* (Suiza provenance). A fourth report was recorded in August of the same year where 2.1 year-old *P. kesiya* began to show signs of DNB in the Cauca Department (2°17´27´´N - 76°39´46´´W) on both farms located in the Southern Zone. In 2010, the fifth and sixth reports corresponded to infections on *P. oocarpa* and where species emerged as being highly susceptible to DNB in two localities, specifically the Campania (5°26´57´´N - 75°45´56´´W) and Angela Maria (4°49´18´´N - 75°36´21´´W) farms located in the Caldas and Risaralda Departments respectively (Northern Zone) (Figure 1).

Visual observations of the needle blight disease on different species of pines distributed in all three Zones showed that the most susceptible species were *P. tecunumanii* LE (Figure 5B), followed by *P. oocarpa* (Figure 5C), and *P. kesiya* (Figure 5D). The most tolerant species were *P. patula* (Figure 5E), followed by *P. tecunumanii* HE (Figure 5F) and *P. maximinoi* (Figure 5G).

Pathogen identification

Sample collection, isolation and morphological characterization

The early symptoms of DNB in Colombia included yellow bands on the green needles, developing into dark-red to brown bands. In the advanced stages of the disease, infection proceeded upwards from the bases of trees and irregular-shaped acervuli emerged from the necrotic needle tissues (Figure 6A-H). Hyaline, 2-5 septate, cylindrical spores, typical of *Dothistroma* were observed in the excised conidiomata from infected needles.

Isolations from infected needles yielded typical callus-like *Dothistroma* cultures of various colours from grey and pink. Some of the cultures produced a red exudate in the medium. Six cultures were made and these included two from the Northern Zone (farms Argentina and Tesalia), two from the Central Zone (farms Alaska and Esmeralda), and two from the Southern Zone (farms Don

Miguel and Unión). These isolates and representative needle samples are maintained in the Mycological Herbarium at SKCC, Colombia. The cultures are also maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria.

DNA sequence based comparisons

The six isolates amplified using ITS primers produced amplicons of approximately 500bp. All ITS sequences were identical and blasted with 100% similarity to the ITS regions of *D. septosporum* available on GenBank (e.g. AY808288 from Chile and AY808289 from Ecuador). The alignment of the ITS sequences for these isolates with those of known identity and closely related species generated a dataset of 455 characters. Of these, 35 characters were parsimony informative. Phylogenetic analyses generated eight trees with a length of 161 and a consistency and retention index of 0.870 and 0.611 respectively. One representative tree is presented in Figure 7.

Susceptibility of *Pinus tecunumanii* progenies

Infection levels

All 972 trees representing *P. tecunumanii* Low Elevation (LE) (PTLE) progenies in the Graminea trial became naturally infected with *D. septosporum* and the percentage infection ranged from 99.2% to 100% (mean 99.9%). In stands used for comparative purposes, provenances having infection percentages of 100% included *P. tecunumanii* LE (PTEBsuH1 and PTEByucu), and *P. kesiya* (PKcalvH1 and PKcalvH2). Low levels of infection were recorded on *P. maximinoi* MAXcabH1 (6.7%) and MAXcabH2 (8.3%). High levels of tolerance to infection were recorded in *P. tecunumanii* high elevation (HE) PTEAcaH1 and TECASaH1 in which no symptoms of needle blight were observed (Figure 8).

Severity levels

Pinus tecunumanii LE (PTLE) progenies differed in the range of severity level from 0% to 85% (mean 39.7%). The susceptible provenances used for comparison had mean levels of severity of 39.1% (*P. tecunumanii* LE PTEBsuH1), followed by *P. kesiya* PKcalvH2 (38.6%), *P. tecunumanii* HE PTEByucu (37.4%), and *P. kesiya* PKcalvH1 (28.2%). The lowest recorded level of severity was found in the provenances of *P. maximinoi* MAXcabH2 (0.76%) and MAXcabH1 (0.66%). *Pinus tecunumanii* HE provenance TECASaH1 and PTEAcaH1 showed no signs of infection of the disease (Table 2A). There were significant difference between the mean severity levels observed in *P. tecunumanii* LE (PTLE) progenies and the provences (MAXcabH1, MAXcabH2, PKcalvH1, PKcalvH2, PTEAcaH1, PTEBsuH1, PTEByucu, TECASaH1) used for comparative purposes (p<0.0001) (Table 2B).

Within the six plots studied, a significant association between the severity of disease and precipitation, elevation, and genetic material was observed (p < 0.05) (Table 3A and B). However, tree age did not significantly influence severity of the disease (p > 0.05). Since elevation and temperature did not vary in the Graminea farm, neither of these factors were considered in the analyses.

Diseases Impact in different forestry zones

Infection levels

The differences observed in the levels of infection between zones were statistically significant (p < 0.0001; Table 4A). The highest average infection levels for 27 plots (98.1%, *n* = 27) were recorded in the Northern Zone. This was followed by the Central Zone with 96.8% (*n* = 46) infection. The Southern Zone had the lowest levels of infection, with an average of 50.6% (*n* = 17; Table 4B).

Severity levels

Differences in levels of severity between zones and farms were statistically significant (p<0.0001; Table 5A). *Pinus tecunumanii* in the Northern Zone was the most severely affected by DNB with an average severity level of 42.4% (n = 27). This level of severity was followed by trees in the Central Zone with a 33.7% (n = 46) severity level. The lowest level of severity (15.7%; n = 17) was recorded in the Southern Zone, which had approximately half the level of severity found in the central zone (Table 5B).

For the individual farms, the highest level of severity was found in Argentina (Caldas) and Cedral (Risaralda), both in the North Zone with 51.3% (n = 6) and 50.81% (n = 6) infection respectively. The lowest level of severity, 23.3% (n = 13) and 15.7% (n = 17), were recorded for Samaria (Valle del Cauca in the Central Zone) and Unión_S (Cauca, in the Southern Zone) respectively (Table 5B).

During the four-year evaluation period, trees in the Southern Zone displayed substantial recovery of foliage and this contributed to low levels of mean infection and severity recorded. There was no significant association between the level of disease and the age or elevation of the plantation in the North, Central and South Zones on the farms included in this study ($p < 0.05$) (Table 6 and 7). However, weather conditions such as precipitation represented a significant association with the age and elevation of the plantation in the comparisons between farms (Table 6B and C), but it was not significant between zones (Table 7B and C). A minimal amount of variation in annual temperature was recorded for each zone (North = 17°C, Central = 20°C and South = 19.2°C) and for that reason, temperature was not considered in these analyses. The elevation of the plantations considered in this study ranged from 1603 to 2780 m.a.s.l. and this did not appear to impact on susceptibility of the trees although it was found to be closely linked to optimal tree growth for the species and provenances.

Discussion

Dothistroma Needle Blight first appeared as a serious foliar disease on pines in Colombia in 2008 where it resulted in very severe damage to various *Pinus* spp. Sequence comparisons based on the ITS regions of isolates from all three forestry zones confirmed the identity of the pathogen causing the epidemics as *D. septosporum* and not *D. pini*. The disease infection in Colombia appears to occur throughout the year, which is different to the situation in New Zealand, for example, where the defoliation is definitively seasonal (Bulman et al. 2008). The occurrence of the disease throughout the year and without seasonal patterns in this study is consistent with the fact that high levels of precipitation are favourable for needle infection (Brown et al. 2003).

The Colombian climate in large areas of the country where pine forestry is practiced, such as those considered in this study, is conducive to DNB outbreaks. For example, the average daily temperature throughout the year for the South, Central and Northern Zones was approximately 19°C, 20°C and 17°C respectively. A wide range of temperatures have been reported for *D. septosporum* infection (Gadgil 1974), but generally continuous needle wetness (Gadgil 1977) and warmer temperatures contribute to higher levels of disease severity. The severity of DNB is generally much greater when pine foliage retains continuous wetness for 48 hours after inoculation (Gadgil 1974). A minimum daily average temperature of 10°C and a long period of high air humidity is necessary for spore production in the pathogen (Berdnarova et al 2010, Dvorak et al. 2012). Thus, conditions present in Colombian areas considered in this study were all highly conducive to disease development and infection by *D. septosporum*.

An important element of this study was to confirm the identity of the pathogen associated with DNB in Colombia. Although the disease had been reported in the country previously (Gibson 1979, 1980) this was based on incidental reports and molecular techniques were not used to confirm the identity of the pathogen. In recent years, there has been some considerable progress in refining the identity of the pathogen (Barnes et al. 2004, Ioos et al. 2010, McDougal et al. 2011) and

confirming the differences between *D. septosporum* and *D. pini*. To date, only *D. septosporum* has been found in the Southern Hemisphere and Central America (Groenewald et al. 2007, Barnes, Personal communication) and is seemed likely that this would be the species present in Colombia. But there have been relatively uncontrolled movement of pine seed from other parts of Central America into Colombia and *D. pini* could easily also have been introduced. Confirmation of the identity of *D. septosporum* in Colombia will now allow for comparisons to be drawn from studies on the pathogen elsewhere.

During the last approximately half century, DNB has emerged as one of the most important constraints to pine plantation forestry in the Southern Hemisphere. This has almost exclusively been associated with the wide-scale plantings of *P. radiata*, which is highly susceptible to the disease (Gibson et al. 1964, Peterson 1966, Gibson 1972, Bulman et al. 2008). The disease has indeed led to the cessation of planting this tree species in many countries, notably in Africa and South America. Many new tree species have been tested and established for plantation development and it is worrying that very little is known relating to their susceptibly to pests and pathogens. The infections of DNB that has emerged in Colombia since 2008 and reported in this study keenly reflects this situation. While DNB had been documented on *P. tecunumanii, P. maximinoi* and *P. kesiya* in Central America (Evan 1985), this is the first situation were large areas of *P. tecunumanii* have been severely damaged by this, or any other serious tree pathogen (Lombard et al. 2009, Steenkamp et al. 2012).

The genetic diversity of the pathogen allows *D. septosporum* to adapt in new and changing environments (Dale et al. 2011), as well as causing loses in yields due to defoliation and necrosis (Devey et al. 2004). Breeding programs can be successful as long as *D. septosporum* is not able to reproduce sexually and generate genetic diversity (Hirst et al. 1999). It is important to identify species and progenies that present natural tolerance to *D. septosporum*, in order to avoid high infection levels in the future. This is especially important due to the increasing dispersal and impact of *D. septosporum* in the Northern Hemisphere (Gadgil 1974), as well as other continents (Watt et al. 2009).

Dothistroma Needle Blight poses a serious threat to the future of pine forestry in Colombia. This is because one of the pine species *P. tecunumanii*, considered to be important for the future of this industry in the country, is clearly highly susceptible. In addition, other species of importance such as *P. maximinoi, P. oocarpa* and *P. kesiya* also display varying levels of susceptibility to infection. This is of concern, especially considering that planting of the previously important species *P. patula* has been substantially reduced in some areas due to the negative impact of another pathogen, *Diplodia pinea* (Rodas and Osorio 2008). However, there are also good prospects to resolve the DNB problem through breeding and selection. For example, some provenances of *P. tecunumanii* show higher levels of resistance than others and hybrids between tolerant genotypes of these provenances and *P. patula* hold promise for the future.

Clearly a great deal of work will need to be done to resolve the DNB problem in Colombia. In addition to breeding and selection for disease tolerance, it will be necessary to better understand the genetics of the pathogen in the country. *Dothistroma septosporum* is known to be heterothallic and to have two mating types (Barnes et al. 2004, Groenewald et al. 2007). The durability of selected disease-tolerant planting stock will be negatively affected if both are present in Colombia and sexual reproduction can occur between them. Such studies and others including those aimed at better understanding the biology of the pathogen in Colombia should be important goals in the future.

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Table 1: Needle blight diseases of *Dothistroma septosporum* and its distribution in Colombia on planted provenances of *P. tecunumanii*

Zone	Department	Forestry Farm	Number of plots in each farm	Coordinates		m.a.s.l.[*]	Precipitation (mm/year) 2011	Planted provenance elevation	Year of Plantation
				Latitude	Longitude				
North	**Caldas**	Yanahuanca	3	5º 26' 12'' N	75º 45' 46'' W	2350	4389	*P. tec* Catan HE[**]	Dec-05
		Argentina	6	5º 24' 20'' N	75º 44' 55'' W	2244	3027	*P. tec* Suiza LE[***]	Dec-05
	Risaralda	Tesalia	5	4º 48' 15'' N	75º 36' 31'' W	1986	4082	*P. tec* Suiza LE[***]	Dec-04
			7	4º 48' 15'' N	75º 36' 31'' W	1908	4082	*P. tec* Suiza LE[***]	Dec-05
		Cedral	6	4º 42' 57'' N	75º 38' 15'' W	1902	4346	*P. tec* Yucul LE[***]	Oct-06
Central	**Valle del Cauca**	Alaska	4	4º 3' 24'' N	76º 25' 09'' W	1763	2276	*P. tec* Yucul LE[***]	Dec-05
			8	4º 3' 24'' N	76º 25' 09'' W	1950	2276	*P. tec* Yucul LE[***]	Feb-06
		Esmeralda	4	4º 3' 27'' N	76º 25' 53'' W	1763	2276	*P. tec* Yucul LE[***]	Dec-05
		La Concha	1	4º 0' 46'' N	76º 25' 24'' W	1741	2712	*P. tec* Arcad LE[***]	Jul-04
			6	4º 0' 46'' N	76º 25' 24'' W	1603	2712	*P. tec* Yucul LE[***]	Dec-06
		Samaria	2	4º 1' 47'' N	76º 26' 30'' W	1680	2712	*P. tec* Suiza LE[***]	Oct-07
			6	4º 1' 47'' N	76º 26' 30'' W	1603	2712	*P. tec* Suiza LE[***]	Oct-07
			5	4º 1' 47'' N	76º 26' 30'' W	1745	2712	*P. tec* Suiza LE[***]	May-08
		Volconda	5	4º 1' 47'' N	76º 26' 06'' W	1754	2712	*P. tec* Yucul LE[***]	Jun-06
		Unión_B	5	4º 25' 13'' N	76º 15' 24'' W	1630	2902	*P. tec* Yucul LE[***]	Aug-06
South	**Cauca**	Unión_S	8	2º 17' 04'' N	76º 33' 56'' W	2701	3102	*P. tec* Suiza LE[***]	Dec-04
			9	2º 17' 04'' N	76º 33' 56'' W	2780	3102	*P. tec* Suiza LE[***]	Dec-04
Total 3	**4**	**11**	**90**						

m.a.s.l.: meters above sea level

[**] HE.: High Elevation, [***] LE.: Low Elevation

Table 2: Results of the levels of **severity** on different progenies of *P. tecunumanii* LE and other pine species to *D. septosporum* in the susceptibility Graminea trial. A. Percentage of severity in the different pines species. B. Analysis of all pine species as a group.

(A)

Pine species	Progeny / Provenance	Mean	SD[1]	CV[2]	n[3]	Duncan grouping		
P. tecunumanii LE	Test: **PTLE**	**39.72**	**20.45**	**51.48**	**633**	**A**		
	Comparative treatments:							
P. kesiya	PKcalvH1	28.15	27.20	96.63	33		B	
	PKcalvH2	38.55	26.23	68.04	29	A	B	
P. tecunumanii LE	PTEBsuH1	39.10	19.57	50.05	29	A	B	
	PTEByucu	37.37	17.54	46.49	19	A	B	
P. maximinoi	MAXcabH2	0.76	2.33	303.89	26			C
	MAXcabH1	0.66	2.77	416.02	27			C
P. tecunumanii HE	PTEAcaH1	0	0	0	23			C
	TECASaH1	0	0	0	29			C
	Mean	34.29						
	CV	57.29						
	R-Square	0.31						

[1]Standard Deviation
[2]Coefficient of Variation
[3]Sample number

(B)

Source	DF	MS	F value	P value
matgen*	8	17780.96	46.05	<0.0001
Error	839	386.1		

* 9 levels: MAXcabH1, MAXcabH2, PKcalvH1, PKcalvH2, PTLE, PTEAcaH1, PTEBsuH1, PTEByucu, TECASaH1

Table 3: Results of the relationship of level of severity with precipitation and age of diseased trees distributed in six plots of the Graminea trial on susceptibility of *Pinus tecunumanii* progenies. A. Covariance Parameter Estimates. B. Solution for Fixed Effects. C. Type III Tests of Fixed Effects.

(A)

Cov Parm	Estimate	Standard Error
Residual (VC)	0.06931	0.002806

(B)

Effect	matgen	BLO*	Estimate	Standard Error	DF	t value	Pr > [t]
Intercept			-22.8874	3475.75	1220	-0.01	0.9947
BLO*		1	0.08571	0.05807	1220	1.48	0.1402
		2	0.04682	0.05790	1220	0.81	0.4189
		3	-0.2189	0.05862	1220	-3.73	0.0002
		4	0.1104	0.05732	1220	1.93	0.0543
		5	-0.2104	0.05857	1220	-3.59	0.0003
		6	0	.	.		.
matgen	**PTLE**		21.8701	3475.75	1220	0.01	0.9950
	PKcalvH1		22.1262	3475.75	1220	0.01	0.9949
	PKcalvH2		22.2074	3475.75	1220	0.01	0.9949
	PTEBsuH1		22.0437	3475.75	1220	0.01	0.9949
	PTEByucu		22.0078	3475.75	1220	0.01	0.9949
	MAXcabH1		17.4955	3475.75	1220	0.01	0.9960
	MAXcabH2		17.6039	3475.75	1220	0.01	0.9960
	PTEAcaH1		0.000232	4915.14	1220	0.00	10.000
	TECASaH1		0	.	.	.	.
Precipitation			0.000179	0.000042	1220	4.29	<0.0001
Age			0.003122	0.003141	1220	0.99	0.3205

*BLO: 6 plots

(C)

Effect	Num DF	Den DF	F Value	Pr > F
BLO*	5	1220	12.42	<0.0001
matgen	8	1220	19.24	<0.0001
Precipitation	1	1220	18.42	<0.0001
Age	1	1220	0.99	0.3205

*BLO: 6 plots

Table 4: Disease impact based on the **infection** percentage level in plots of 300 m^2; A. Indicates analyses of infection percentages between **zones** (p ≤ 0.05). B. Infection between forestry zones with Duncan's Test grouping.

(A)

Source	DF	MS	F value	P value
Zone	2	14995.61	219.14	<0.0001
Error	79	68.42		

(B)

Forestry Area		Mean	*n*	Duncan Grouping
Zone	**North**	98.09	27	A
	Central	96.77	46	A
	South	50.64	17	B
	Error	**DF**	**MS**	
		79	68.42	

Table 5: Disease impact based on the **severity** percentage level in plots of 300 m^2; A. Indicates analyses of severity percentages between **zones and farms** (p ≤ 0.05). B. Severity between zones and forestry farms with Duncan's Test grouping for severity.

(A)

Source	DF	MS	F value	P value
Zone	2	3746.21	77.47	<0.0001
Farm	6	491.21	10.16	<0.0001
Error	79	48.35		

(B)

Forestry Area		Mean	n	Duncan Grouping
Zone	North	42.38	27	A
	Central	33.65	46	B
	South	15.68	17	C
	Error	DF	MS	
		79	48.35	
Farms	Yanahuanca	37.97	3	B
	Argentina	51.33	6	A
	Tesalia	34.80	12	C
	Cedral	50.81	6	A
	Alaska	37.86	12	C
	Esmeralda	34.29	4	C
	Volconda	35.05	5	C
	Concha	35.98	7	C
	Samaria	23.34	13	D
	Unión_B	45.16	5	B
	Unión_S	15.68	17	D
	Error	DF	MS	
		79	48.35	

Table 6: Results of the relationship of the severity with elevation, precipitation and age of trees in plots of 300 m^2 per zone. A. Covariance Parameter Estimates; B. Solution for Fixed Effects; C. Type III Tests of Fixed Effects.

(A)

Cov Parm	Subject	Estimate	Standard Error
UN (1,1)	Plot	0.07490	0.003426

(B)

Effect	Zone	Estimate	Standard Error	DF	t value	Pr > [t]
Intercept		-4.2636	0.3557	956	-11.99	<0.0001
Zone	North	1.8785	0.1109	956	13.46	<0.0001
	Central	1.8074	0.1343	956	16.94	<0.0001
	South	0	.	.	.	.
Elevation		0.000593	0.000132	956	4.49	<0.0001
Precipitation		0.000146	0.000068	956	2.16	0.0311
Age		0.01145	0.001188	956	9.64	<0.0001

(C)

Effect	Num DF	Den DF	F Value	Pr > F
Zone	2	956	155.86	<0.0001
Elevation	1	956	20.12	<0.0001
Precipitation	1	956	4.66	0.0311
Age	1	956	92.96	<0.0001

Table 7: Influence of elevation, precipitation and age of trees on levels of severity calculated in plots of 300 m^2 per farm. A. Covariance Parameter Estimates; B. Solution for Fixed Effects; C. Type III Tests of Fixed Effects.

(A)

Cov Parm	Subject	Estimate	Standard Error
UN (1,1)	Plot	0.05316	0.002442

(B)

Effect	Farm	Estimate	Standard Error	DF	t value	Pr > [t]
Intercept		13.1113	1.5443	948	8.49	<0.0001
Farm	Alaska	-2.6611	0.2863	948	-9.30	<0.0001
	Argentin	0.8733	0.1024	948	8.53	<0.0001
	Cedral	-1.6667	0.2814	948	-5.92	<0.0001
	Concha	-3.5603	0.4066	948	-8.76	<0.0001
	Esmerald	-2.0931	0.2487	948	-8.42	<0.0001
	Samaria	-3.8972	0.4113	948	-9.48	<0.0001
	Tesalia	-2.4612	0.2539	948	-9.69	<0.0001
	Unión_B	-0.6170	0.1679	948	-3.67	0.0003
	Unión_S	1.4767	0.2999	948	4.92	<0.0001
	Volconda	-3.3932	0.3854	948	-8.80	<0.0001
	Yanahuan	0	.	.	.	.
Elevation		-0.00658	0.000680	948	-9.68	<0.0001
Precipitation		0.000104	0.000059	948	1.77	0.0772
Age		0.02238	0.001438	948	15.56	<0.0001

(C)

Effect	Num DF	Den DF	F Value	Pr > F
Farm	10	948	83.47	<0.0001
Elevation	1	948	93.76	<0.0001
Precipitation	1	948	3.13	0.0772
Age	1	948	242.26	<0.0001

Figure 1: Geographic distribution of Dothistroma Needle Blight (DNB) in Colombia in the North (red dots) Central (blue dots) and Southern Zones (green dots), on planted *P. tecunumanii.* The coloured dots indicate the area in which farms are located and numbers represent the order in which DNB was cronologically reported. The green dot indicates the Graminea farm where the susceptibility trial was conducted.
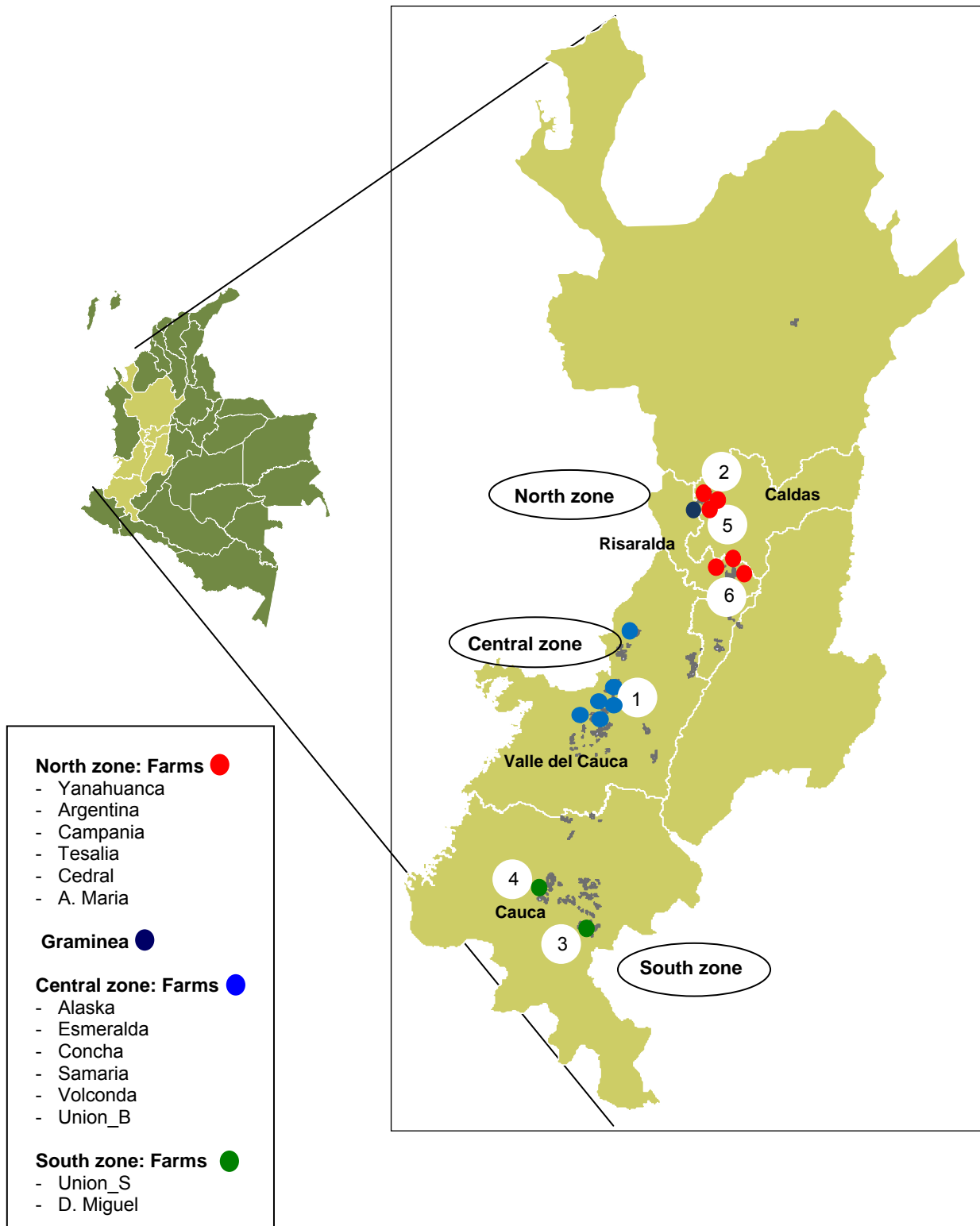
North zone: Farms 🔴
- Yanahuanca
- Argentina
- Campania
- Tesalia
- Cedral
- A. Maria

Graminea ⚫

Central zone: Farms 🔵
- Alaska
- Esmeralda
- Concha
- Samaria
- Volconda
- Union_B

South zone: Farms 🟢
- Union_S
- D. Miguel

North zone

Central zone

South zone

Caldas

Risaralda

Valle del Cauca

Cauca

Figure 2: The **percentage of infection level** of DNB. This is calculated by dividing the total foliar area of each tree into quarters that represent 25% each and then assessing how many of these quarters show the presence of the disease.
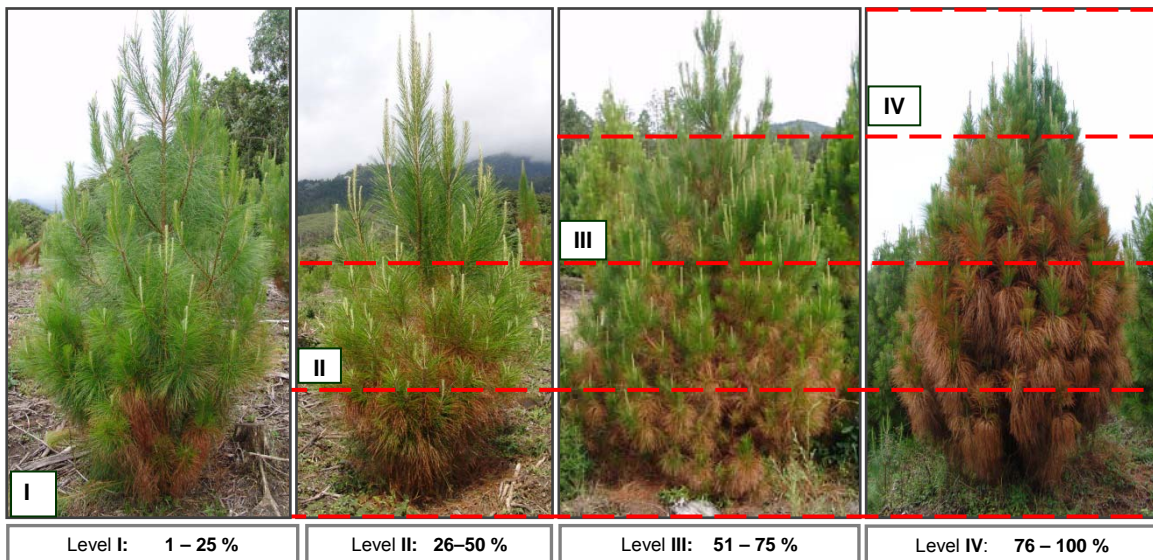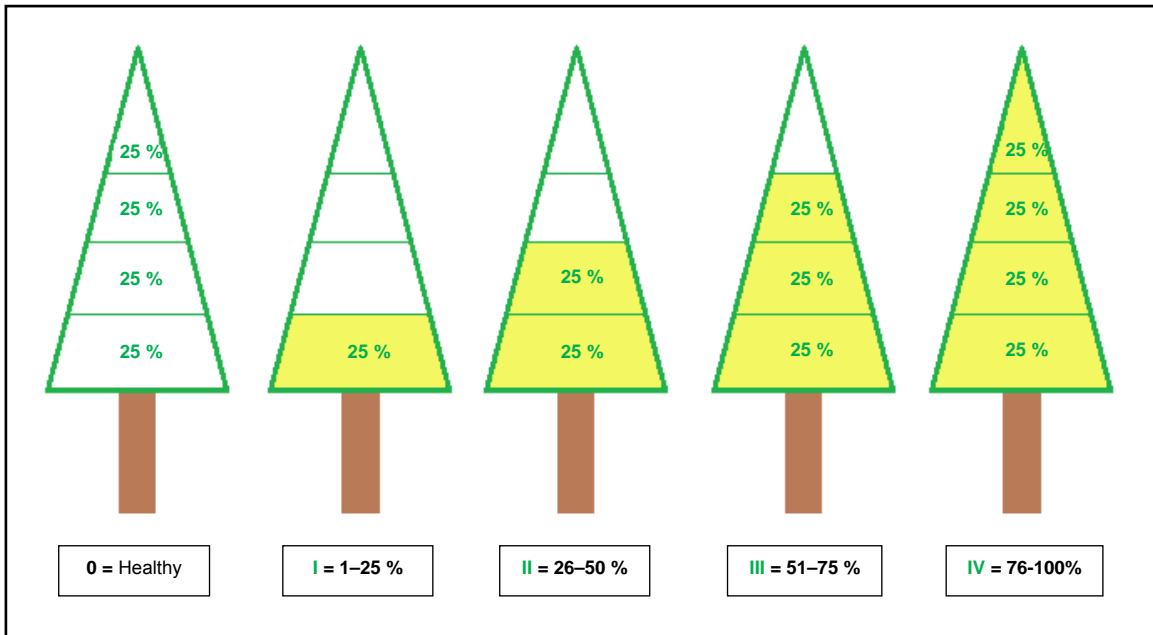
| 0 = Healthy | I = 1–25 % | II = 26–50 % | III = 51–75 % | IV = 76-100% |

| Level I: 1 – 25 % | Level II: 26–50 % | Level III: 51 – 75 % | Level IV: 76 – 100 % |

Figure 3: The **percentage of severity** of DNB. This is calculated by dividing the total foliar area of a tree into quarters and then estimating the percentage (out of 100%) of infected area per each quarter. These values are then statistically analyzed using a weighted mean average model based on the conical foliar area distribution of *Pinus* in which the designated value for the major quarter (bottom of the tree) corresponds to 40%, next quarter with 30%, 20%, and the top, 10%, respectively.

Each Quarter is the equivalent of a **25%** of the tree foliar area

Quarter: **II – 10%**

Quarters: **I – 80%**
II – 30%

Quarters: **I – 40%**
II – 90%
III – 10%

Quarter: **II – 10%**

Quarters: **I - 80 %**
II - 30 %

Quarters: **I - 40 %**
II - 90 %
III - 10 %
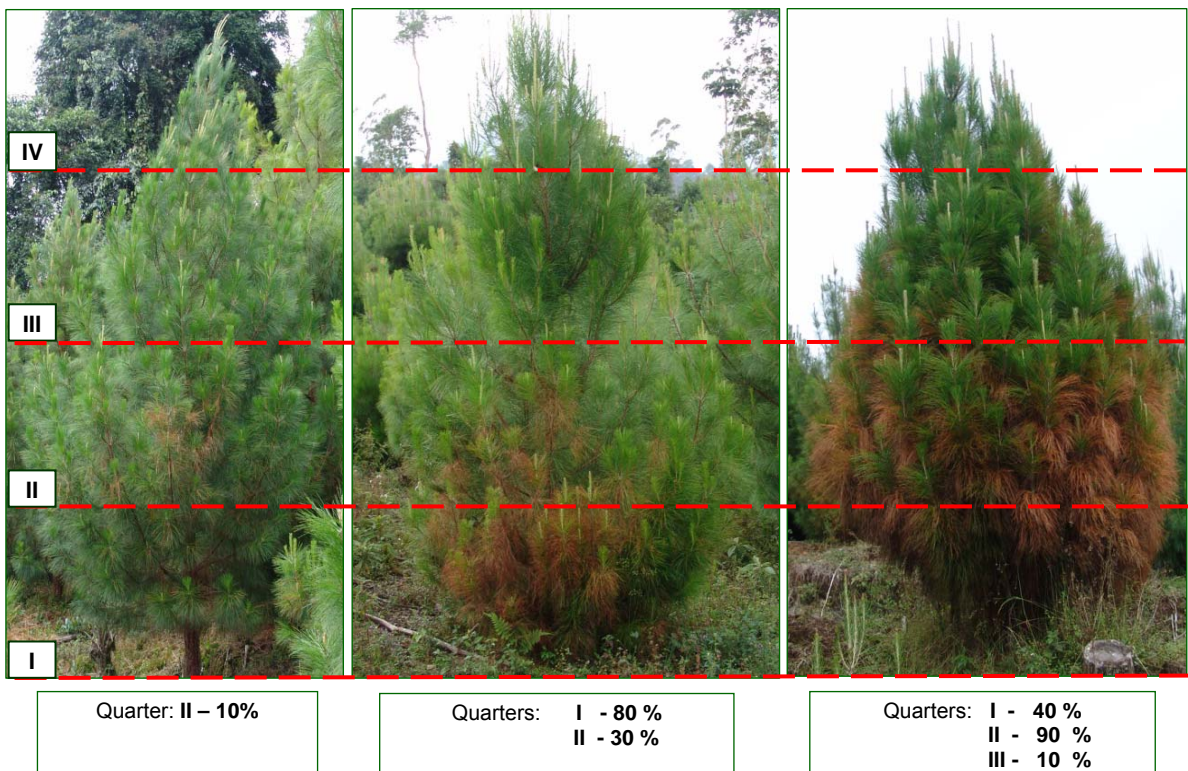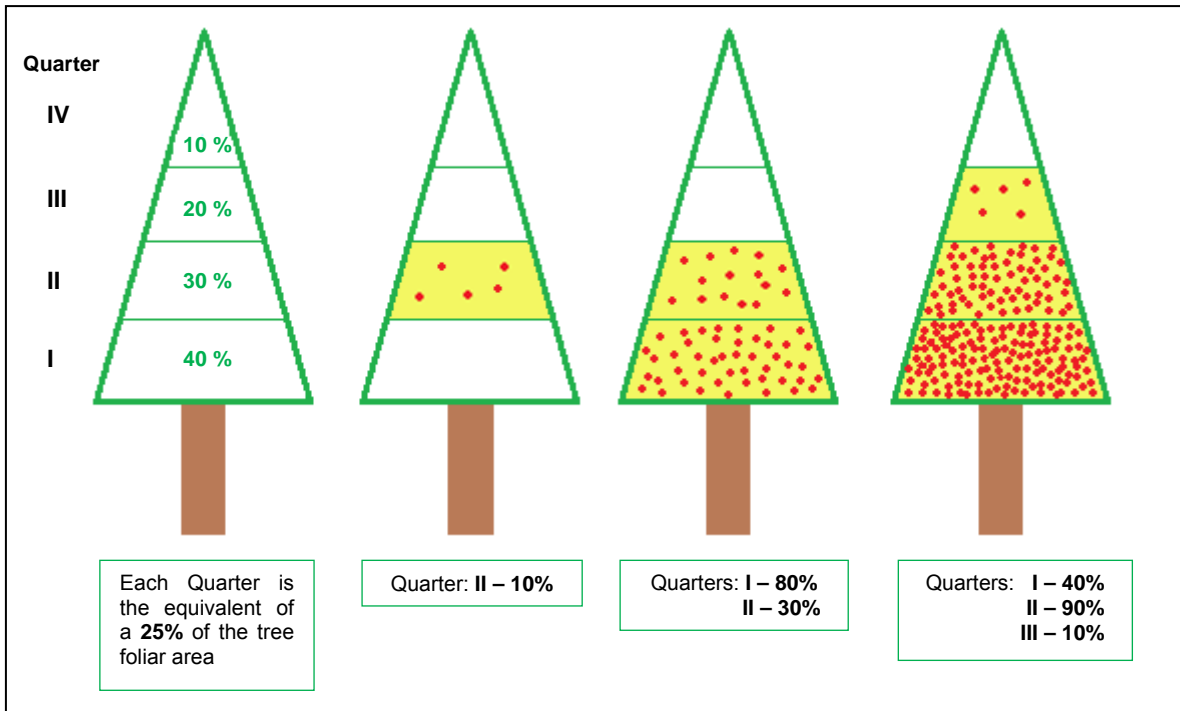
Figure 4: Evaluation of the impact of DNB. **Percentage of infection levels** per plot calculated based on the number of affected trees divided by the total number of trees in a circular area of 300 m^2 (including approximately 30 planted trees) with a radius of 9,8 m (1 plot of 300 m^2 per 3 ha). The sampling intensity was 1% of the total affected area in each site.

Plot of 300 m²

9,78 m
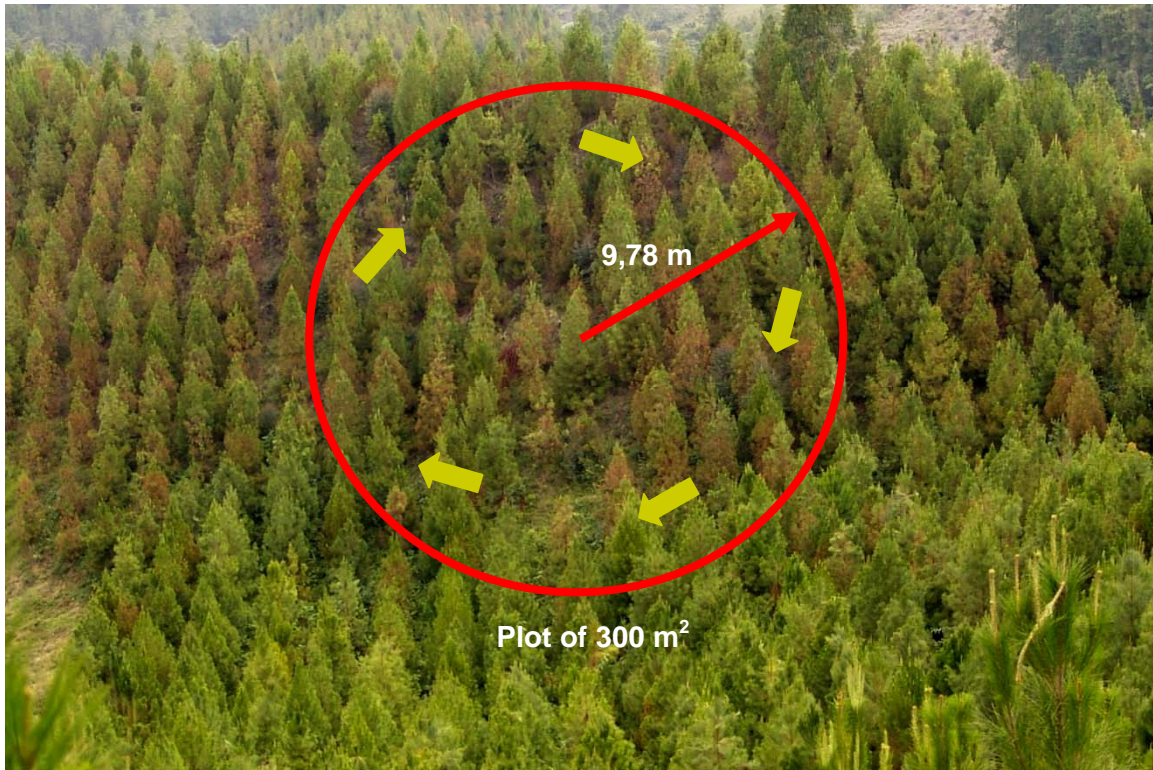
Figure 5: Visual observation of DNB symptoms on different pine species in three different zones in Colombia. A. Tolerant and susceptible *Pinus* species to DNB. Susceptible species of *Pinus* to DNB: B. *P. tecunumanii* from low elevation (LE). C. *P. oocarpa.* D. *P. kesiya.* Tolerant species to DNB: E. *P. patula.* F. *P. tecunumanii* high elevation (HE) and G. *P. maximinoi.*

Figure 6: Different symptoms of DNB on *P. tecunumanii* on needles (B-D and H) and branches (E-G) with symptoms expressed as density of red bands. A. High level of infection. B. High level of infectiion. C. Medium level of infection. D. Low level of infection. Different symptoms of the disease infection on branches: E. Low . F. Medium, and G High infection. H. Abundant presence of acervuli of DNB on *P. tecunumanii* needles.
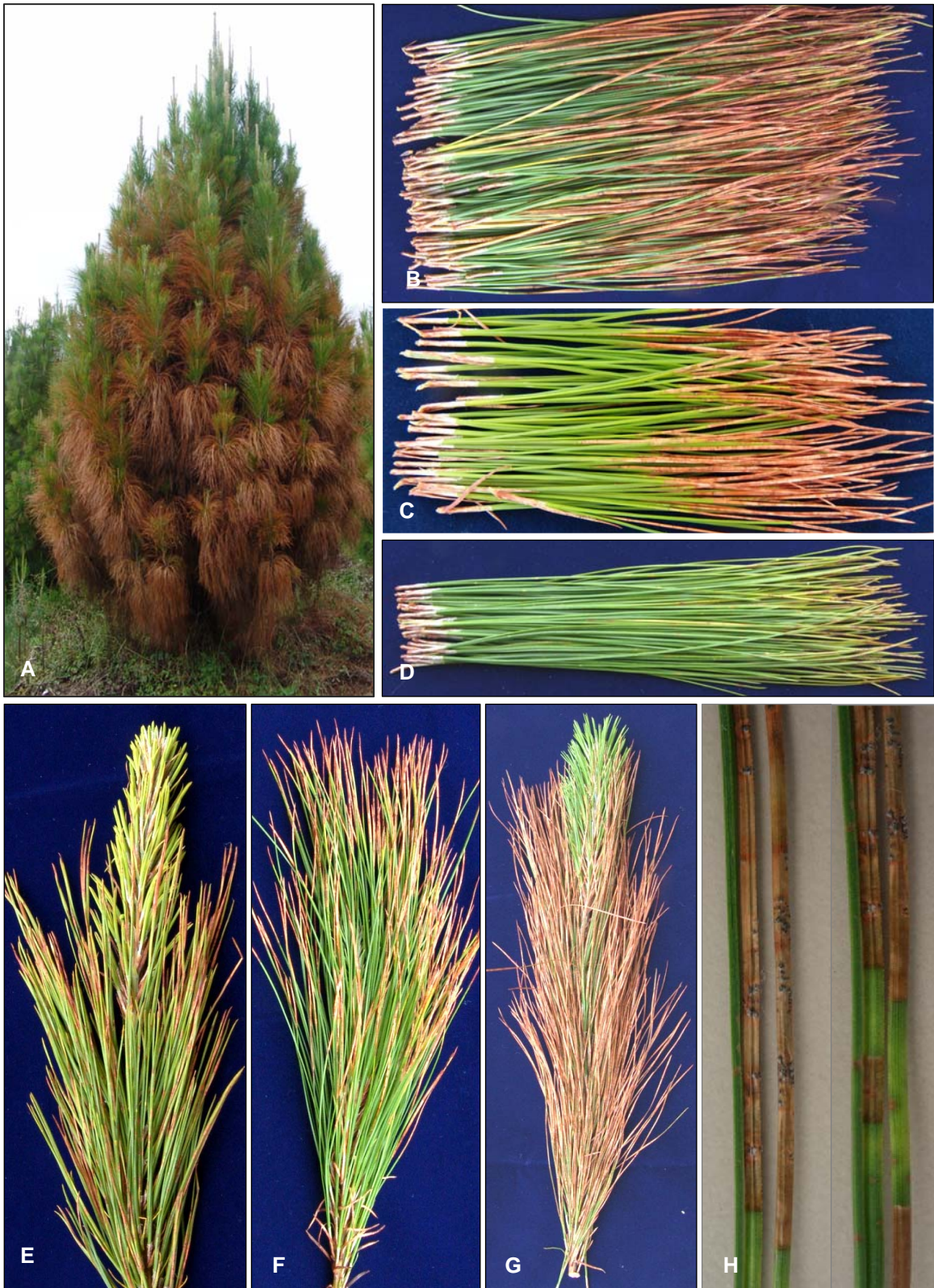
Figure 7: Phylogenetic tree showing the placement of the isolates from Colombia in the *Dothistroma septosporum* clade. From a data set of 455 aligned characters of the ITS region, 35 were parsimony informative and phylogenetic analysis generated 8 trees with length of 161 and a consistency and retention index of 0.870 and 0.611, respectively.
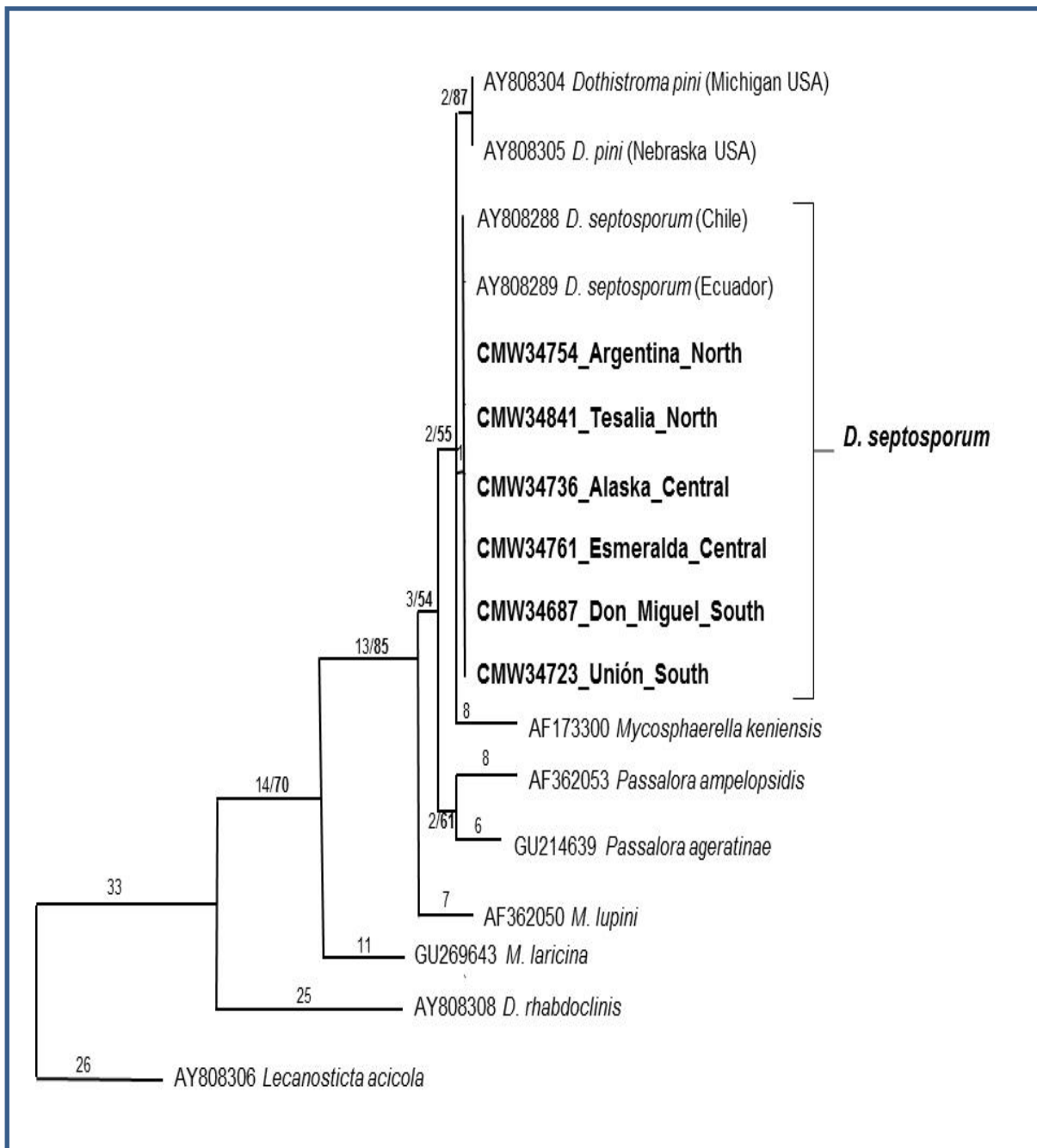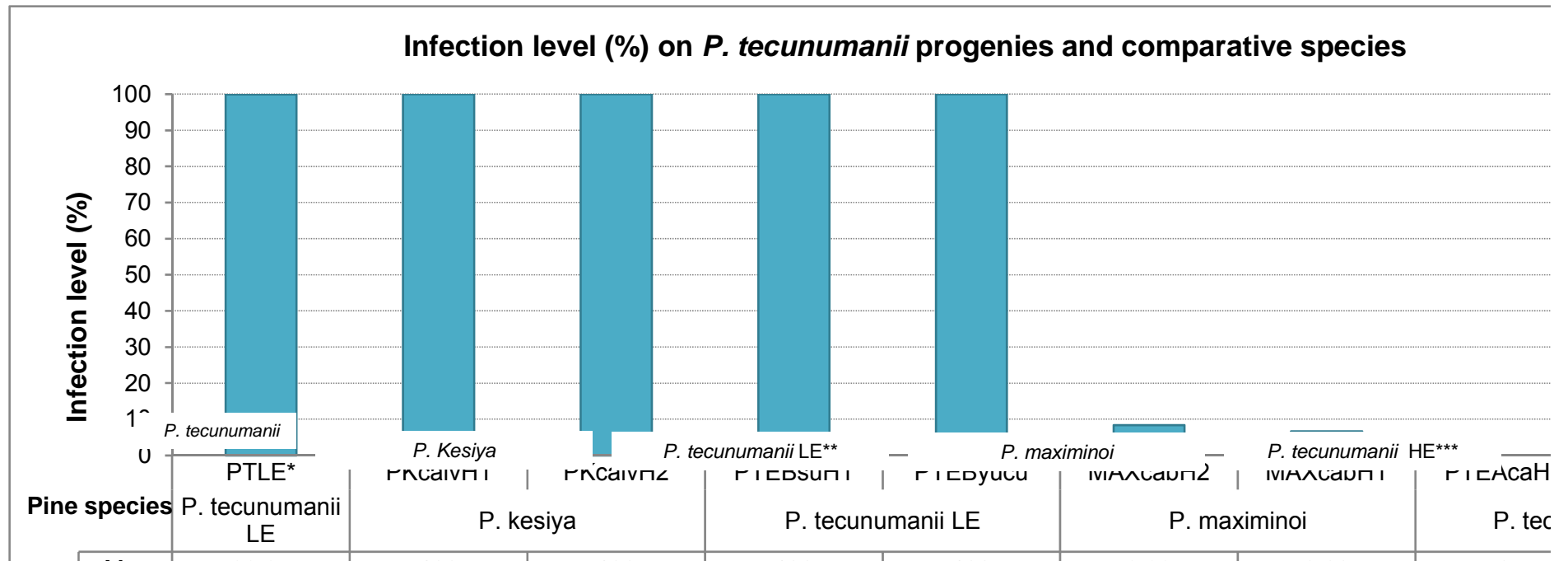
Figure 8: **Percentage of infection** by *D. septosporum* on different progenies of *P. tecunumanii* LE and other pine species in the Graminea susceptibility trial in the North Zone.

Infection level (%) on *P. tecunumanii* progenies and comparative species

*PTLE. Represented by 27 progenies of *P. tecunumanii* from low elevation (PTLE)

**LE. *P. tecunumanii* from low elevation

***HE. *P. tecunumanii* from high elevation

Chapter 7

Ceratocystis neglecta **sp. nov., infecting** *Eucalyptus* **trees in Colombia**

Abstract

Commercial plantation forestry utilising species of non-native *Eucalyptus* trees forms an important industry in Colombia. These trees are, however, threatened by fungal diseases. In recent years a number of reports of *Ceratocystis fimbriata* s.l., causing wilt and death of *Eucalyptus* spp. have emerged from African and South American countries. In Colombia, the fungus is a serious pathogen of coffee, cacao and citrus where it enters wounds and causes a severe canker stain disease. *Ceratocystis fimbriata* has, however, not been found on *Eucalyptus* spp. in Colombia and the aim of this study was to consider whether it might infect wounds on these trees in the country. *Eucalyptus grandis* trees were artificially wounded in three different geographic zones of Colombia and a *Ceratocystis* sp. was commonly isolated from these wounds. Isolates of the fungus were identified based on morphology and through comparisons of sequences for the ITS regions of the rDNA operon. Morphological and DNA sequence comparisons showed that isolates from *E. grandis* in Colombia represent a new species of *Ceratocystis*, closely resembling *C. fimbriata* s.s. and for which the name *C. neglecta* sp. nov. is given. To determine the possible impact of *C. neglecta* on commercial forestry operations, two isolates were used in field pathogenicity trials on different clones of *E. grandis*. Isolates were shown to differ in their ability to cause lesions on *E. grandis*, with one isolate being highly pathogenic. The different clones of *E. grandis* also differed in their susceptibility to infection by the fungus.

Introduction

Colombia has a rapidly growing forestry industry supporting the production of solid wood and paper products. In the past, native trees have been exploited to produce these products, but recent trends are to grow trees for this purpose in intensively managed plantations. Non-native species of *Eucalyptus* and *Pinus* are the most commonly grown trees, and these currently make up approximately 150 000 hectares of plantations. In the case of *Eucalyptus*, large areas have been planted to clones of *E. grandis* Maiden and hybrids of this

species with *E. urophylla* S.T. Blake, also known as *E. "urograndis"*. Little is, however, known regarding diseases of these *Eucalyptus* trees in Colombia.

Ceratocystis fimbriata Ellis & Halst. s.l. includes some of the most important pathogens of woody plants, causing canker stain and vascular wilt diseases (Kile 1993). During the course of the last decade *C. fimbriata* s.l has been reported from dying *Eucalyptus* trees with increasing frequency. *Ceratocystis fimbriata* s.l. was first reported as a pathogen of *Eucalyptus* trees in the 1990's after it was isolated from diseased and dying *Eucalyptus* trees in the Republic of Congo (Roux et al. 1999). At approximately the same time, it was found killing *Eucalyptus* trees in Brazil (Laia et al. 1999, Roux et al. 1999). It has since also been reported from dying *Eucalyptus* trees in Uganda (Roux et al., 2001), Uruguay (Barnes et al. 2003a) and most recently from artificially wounded *Eucalyptus* trees in South Africa (Roux et al. 2004).

Ceratocystis fimbriata s.l. is a serious pathogen of coffee (*Coffea arabica* L.) (Pontis 1951, Mourichon 1994, Marin et al. 2003) and cacao (*Theobroma cacao* L.) (Garces 1944, Marin et al. 2003) in Colombia. It has also been isolated from the Brazalian fire tree *Schizolobium parahyba* (Vell.) S.F. Blake, in the country (Marin et al. 2003). Because of the importance of coffee to the Colombian economy, *C. fimbriata* s.l. is recognised as one of the most important agricultural pathogens in this country (Castro 1998). The fungus infects trees via wounds made at the bases of coffee trees during farming operations (Marin et al. 2003). Its common occurrence in soil as chlamydospores (Kile 1993) provides ample opportunity for infection of wounds that are generally at the bases of trees.

The taxonomy of *C. fimbriata* has been a source of considerable debate since its first description in the late 1800's. This confusion commenced with the misidentification of its fruiting bodies as pycnidia when the fungus was first found associated with sweet potato black rot (Halsted 1890). Subsequent reports of *C. fimbriata* from a wide range of hosts and geographic areas led to further confusion because there is substantial variation in the morphology and pathogenicity of isolates. This led Webster and Butler (1967) to conclude that *C.*

fimbriata "is a large and diverse species which consists of numerous strains". The advent of DNA sequencing and other less subjective techniques than morphological comparisons have shown that isolates of *C. fimbriata* s.l. represent phylogenetically distinct and cryptic species. In recent years several new species have thus been reported, all of which would earlier have been identified as *C. fimbriata* (Wingfield et al. 1996, Barnes et al. 2003b, Van Wyk et al. 2004, Baker Engelbrecht and Harrington 2005, Johnson et al. 2005).

Ceratocystis fimbriata s.s. is now recognised to represent the sweet potato black rot pathogen (Baker Engelbrecht and Harrington 2005) and not the many described and undescribed cryptic species in the *C. fimbriata* s.l. species complex. Many species in this group can be recognised in phylogenetic trees, representing large numbers of isolates, and these have yet to be described. In a recent study, Marin et al. (2003), for example showed that a large collection of *C. fimbriata* isolates from coffee, cocoa and citrus trees in Colombia reside in two distinct phylogenetic clades. These fungi are currently referred to as representing *C. fimbriata* s.l., but they apparently represent undescribed species.

There is growing evidence that *C. fimbriata* s.l. should be considered an important constraint to *Eucalyptus* plantation forestry. The wide spread occurrence of this pathogen on coffee and other crops in Colombia, often in areas in close proximity to *Eucalyptus* plantations, is a matter of concern. The aim of this study was, therefore, to determine whether *C. fimbriata* s.l. might occur on *Eucalyptus* spp. in this country. Furthermore, the potential threat of this fungus to *Eucalyptus* forestry in Colombia was considered in artificial inoculation experiments.

Materials and Methods

Collection of isolates

Wounds were made on trees at eight farms in three different forestry zones of Colombia. These were at the San Jose and Vanessa farms in the Cauca zone, the Suiza, Cecilia and Samaria farms located in the Valle zone, and the Carolina, Cedral and Angela Maria farms in the Andina zone. At each of the farms in the three zones, twenty to forty trees were selected to be wounded. Wounds were made in June 2002, February 2004 and November 2005 by cutting a patch of bark (10 cm^2) from the stems of trees, to expose the cambium, similar to the method described by Barnes et al. (2003b).

Two to eight weeks after wounding wood and bark samples were collected from the wounds, placed in paper packets and transported to the laboratory for analyses. Isolations were made from discoloured wood using a combination of the carrot baiting technique (Moller and DeVay 1968) and moisture chambering of material. For the carrot baiting technique pieces of wood (~ 2 cm^2) showing staining symptoms were wrapped tightly between two slices (~ 1 cm thick) of carrot that had been surface disinfested with 70% ethanol. These carrot baits were incubated at 25ºC for up to two weeks and regularly inspected for the presence of *Ceratocystis* ascocarps. When present, the ascospore masses were removed from the apices of the ascocarps and transferred to 2% malt extract agar plates (MEA: 20g malt extract, 15g agar; Biolab Diagnostics Ltd, Midrand, South Africa), containing 100mg Streptomycin sulfate (SIGMA-ALDRICH CHEMIE Gmbh, STEIN-HEIM, Germany) and incubated at 25ºC. Half of the material collected was placed in plastic bags with moist tissue paper and incubated at 25ºC to induce fungal growth and sporulation. Ascospore drops and mycelium of a *Ceratocystis* sp. was transferred from the moisture chambers to MEA for purification. All isolates obtained were lodged in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1). Microscope slides bearing structures and dried down cultures have also been lodged in the National Collection of Fungi (PREM), Pretoria, South Africa and representative isolates

deposited with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands.

Morphological characteristics

The *Ceratocystis* isolates collected from wounds were grown on 2% MEA and identified based on morphological characteristics (Upadhyay 1981). A representative of these isolates (CMW17808) was chosen and fifty measurements of all morphological structures were made after mounting the structures in lactophenol on microscope slides. Measurements are presented as (min-) (average- std. dev.) - (average + std. dev.) (-max). Rayner's (1970) colour charts were used to note the colour of the mycelium and other structures. To determine the optimum growth temperature of the isolates from Colombia, cultures (CMW18194, CMW11284) were grown on 2% MEA for seven days. Mycelial plugs (5 mm) were taken from the actively growing margins of these cultures and transferred to 2% MEA plates for incubation at temperatures ranging from five to 35ºC at 5ºC intervals. Five plates were prepared for each isolate tested at each temperature. Two measurements, perpendicular to each other, were made on the seventh day. The trial was repeated once.

DNA sequence comparisons

Four *Ceratocystis* isolates (CMW11285, CMW11284, CMW17808, CMW18194) from *Eucalyptus* in Colombia were used for DNA sequence comparisons. For DNA extraction, cultures were grown on 2% MEA for two weeks. Mycelial masses were scraped off, placed in an Eppendorf tube and freeze dried overnight. DNA extraction was performed as described by Van Wyk et al. (2006).

The Internal Transcribed Spacer regions (ITS1, 2), including the 5.8S rDNA operon were amplified using the polymerase chain reaction (PCR) and using primers ITS1 and ITS4 (White *et al.*, 1990). Five ng of the DNA template was added to a 25 µl polymerase chain reaction (PCR) mixture containing 0.2 mM of each dNTP, 0.4 µM of each primer, 1 X FastStart buffer containing 1.5 mM

MgCl2 (supplied with the enzyme) and FastStart *Taq* enzyme (2 U) (Roche Diagnostics, Mannheim, Germany). The PCR amplification consisted of an initial denaturation step at 96°C for 4 min. This was followed by 10 cycles denaturation at 95°C for 40 s, annealing for 40 s at 55°C and an elongation step for 45 s at 70°C. Subsequently, 30 cycles consisting of 94ºC for 20 s, 55ºC for 40 s with a 5 s extension step, after each cycle and 70ºC for 45s were performed with a final step of 10 min at 72ºC. PCR products were visualized using UV light after separation on a 2% agarose gel containing ethidium bromide. The products were then purified using 6% Sepha-dex G-50 columns (Steinheim, Germany).

PCR products were sequenced using an ABI PRISM Big DYE Terminator Cycle Sequencing Ready Reaction Kit version 3.0 (Applied Biosystems, Foster City, CA) and the same primers as used in the PCR reactions. Sequencing reactions were run on an ABI Prism 3100 DNA sequencer (Applied Bio-Systems).

Sequences for the *Ceratocystis* sp. from *E. grandis* in Colombia were compared with those of closely related *Ceratocystis* spp. obtained from the GenBank [NCBI (National Centre for Biotechnology Information)] nucleotide database (http://www.ncbi.nlm. nih.gov/) or published in previous studies (Barnes et al. 2003a, Marin et al. 2003, Van Wyk et al. 2004, Johnson et al. 2005) (Table 1). Sequences were manually aligned using the program Sequence Navigator version 1.0.1 (Applied Biosystems). The alignments were analysed using Phylogenetic Analysis Using Parsimony (PAUP) software, version 4.0b10 (Swofford 2002). The heuristic search option based on parsimony with random stepwise addition and tree bisection reconnection (TBR) was used. Gaps were treated as fifth character and confidence intervals using 1000 bootstrap replicates were calculated. *Ceratocystis virescens* (R.W. Davidson) C. Moreau was included in the analyses as the out-group.

Pathogenicity tests

Three inoculation trials, using the *Ceratocystis* sp. collected from *Eucalyptus* in Colombia, were conducted in commercial *E. grandis* plantations in the Valle zone of Colombia. The three plantations were situated on the Buenos Aires Farm, Trujillo, Valle (1994 masl, 1740 mm/y of precipitation, located at 76º21'24" W, 4º 13'60" N), the Cedral farm in Darien, Valle (1692 masl, with an average precipitation of 1422 mm/y, located at 76º25'29" W, 4º1'05" N) and the La Suiza farm in Restrepo, Valle (precipitation 1279 mm/y, located at 1553 masl, 76º29'37" W, 3º51'35" N). At each of the three sites, 50 trees each of two clones (clone 301, clone 02) and one seed source (seed lot 211) of *E. grandis* were inoculated with two isolates (CMW 11284, CMW11285) of the *Ceratocystis* sp. from *E. grandis*. At each site, 50 trees of each of the *E. grandis* clones or trees representing the seed source were also inoculated with sterile agar to serve as a negative control.

The two different clones and the trees representing the seed source were not present uniformly at the three different farms. At La Suiza and Buenos Aires, 50 trees of each of the *E. grandis* clones 301 and 02 were inoculated with the two *Ceratocystis* isolates and the control respectively. At Cedral, the same number of trees was inoculated but only clone 301 was available. Thus, instead of clone 02, 50 trees generated from seed belonging to the seed lot *E. grandis* 211 were used together with clone 301. In all cases, the trees were one-year- old and they were distributed in five blocks with 10 trees of each of the clones or the seed lot, selected for inoculation.

Inoculations were made on the stems of trees ~1 m above the ground using a six millimeter diameter cork borer. This instrument was used to remove a piece of bark from each stem to expose the cambium. A disc of the same size was taken from the edge of a rapidly growing 11-day-old *Ceratocystis* colony and placed into the exposed wound with the mycelium facing the cambium. In order to prevent desiccation, the inoculation sites were covered with tissue paper moistened with sterile water and secured with masking tape.

Internal lesion lengths were recorded in mm after 12 weeks. Data analysis was performed using SAS® (SAS Institute Inc., SAS Campus Drive, Cary, NC 27513, Version 8.2, running under VM/ESA on the University of Pretoria mainframe computer). The lack of normality of the data indicated that a nonparametric approach be followed. Kruskal/ Wallis determinations were thus performed with appropriate grouping variables and by specifying the "compare" option.

Results

Morphological characteristics

A *Ceratocystis* sp. (Figure 1), resembling *C. fimbriata* s.l. in culture, was collected from wounds on *E. grandis* trees in La Suiza and Buenos Aires. Morphological characteristics of these isolates were typical for the genus *Ceratocystis* (Upadhyay 1981), but some differences were observed. Compared to *C. fimbriata* s.s., the *Ceratocystis* sp. from *Eucalyptus* in Colombia had longer ascomatal necks and two morphological forms of phialides were observed, where isolates of *C. fimbriata* s.s. has only one phialide type. Furthermore, the Colombian isolates produced barrel-shaped conidia in chains, as well as cylindrical hyaline conidia. This is in contrast to isolates of *C. fimbriata* s.s. that do not produce barrel shaped conidia (Baker Engelbrecht and Harrington 2005).

Cultures from *E. grandis* covered the plates in eight to 15 days and had a strong fruity aroma characteristic of many *Ceratocystis* spp. Growth studies in culture showed that the Colombian isolates have a growth optimum at 25°C, with colonies reaching 35 mm in seven days at this temperature. No growth was observed at 5, 10 or 35°C.

DNA sequence comparisons

The phylogenetic data set consisted of 33 in-group taxa, with the sequence of *C. virescens* defined as a monophyletic sister out-group taxon (Figure 2). This data set consisted of 583 sequence characters of which 62 were parsimony-

uninformative and 185 were parsimony-informative. Thirty trees were obtained from the heuristic search and one was selected for presentation (Figure 2). The tree had a length of 439 steps, with a Consistency Index (CI) of 0.7312, a Retention Index (RI) of 0.8590 and Re-scaled Consistency Index (RC) of 0.6281 (TreeBase SN3474).

The *Ceratocystis* isolates from *E. grandis* in Colombia grouped alone in a well-resolved clade, separate from all other lineages, including those published previously for other Colombian isolates (Marin et al., 2003) (Figure 2, Bootstrap support 93%). *Ceratocystis fimbriata* s.s. from sweet potato (type) grouped separately from all other isolates considered in this study (Bootstrap support 72%). The other lineages represent *C. platani* (J.M. Walter) Engelbr. & T.C. Harr. (Bootstrap support 98%), *C. albifundus* M.J. Wingf., De Beer & M.J. Morris (Bootstrap support 100%), *C. pirilliformis* I. Barnes & M.J. Wingf. (Bootstrap support 100%), *C. polychroma* M. van Wyk, M.J. Wingf. & E.C.Y. Liew (Bootstrap support 84%), *C. variospora* (R.W. Davidson) C. Moreau (Bootstrap support 55%), *C. smalleyi* J.A. Johnson & T.C. Harr. (Bootstrap support 83%), *C. caryae* J.A. Johnson & T.C. Harr. (Bootstrap support 0%), *C. cacaofunesta* Engelbr. & T.C. Harr. (Bootstrap support 93%), *C. populicola* J.A. Johnson & T.C. Harr. (Bootstrap support 100%) and *C. atrox* M. van Wyk & M.J. Wingf. (Bootstrap support 96%).

Taxonomy

Comparison of the DNA sequence data for the ITS regions, including the 5.8S rDNA, confirmed morphological observations that the *Ceratocystis* isolates from *Eucalyptus* trees in Colombia are related to, but different from *C. fimbriata* s.s. The fungus thus represents a new and previously undescribed species of *Ceratocystis* and it is described as follows:

Ceratocystis neglecta M. van Wyk, Jol. Roux & C. Rodas, **sp. nov.** (Figure 1)
MycoBank: 10947.
Anamorph: *Thielaviopsis* sp.

Etymology: The name neglecta refers to the fact that this fungus has been overlooked in the past.

Coloniae glaucovirides. *Hyphae* laeves, non segmentatae, (2-)4-6 μm latae. *Bases ascomatum* atrobrunneae vel nigrae, globosae, (173-)202-244(-281) μm latae, (153-)178-228(-250) μm longae, (163-)190-236(-266) μm diametro. *Colla ascomatum* basi atrobrunneae, apicem versus pallescentia, basi (27-)31-39 (-46) μm, apice (14-)16-20(-22) μm lata, (691-)745-840(-889) μm longa. *Hyphae ostiolares* divergentes, hyalinae, (35-)41-49(-54) μm longa. *Asci* non visi. *Ascosporae* in massa alba vel flavobubalina in apicibus collorum crescunt; lateraliter visa cucullata vel pileata, non septata, hyalina, vaginata, 3-6 μm longa, 4-7 μm lata.

Anamorpha Thielaviopsis: Conidiophorae biformes, in mycelio singulae, primaria hyalina, (75-)80-114(-152) μm longae, basi (4-)5-7(-8) μm, apice (3-)4-6(-7) μm lata; secondaria hyalina phialida primaria brevior, (38-)48-76(-89) μm longa, basi apiceque (3-)5-7(-8) μm lata. Evolutio *conidiorum* phialidica pern formatione parietum annularium, *conidia* biformia, singula vel concatenata, primaria cylindrica (11-)15-27(-30) μm longa, (3-)5-6 μm lata; secondaria orculiformia (6-)10-11 μm longa, (4-)5-7(-9) μm lata. Chlamydosporae adsunt, (8-)10-12(-13) μm lata, (9-)10-14(-16) μm longa.

Colony color greenish glaucous (33'''f). *Hyphae* smooth, non-segmented, (2-)4-6 μm wide. *Ascomatal bases* dark brown to black, globose, (173-)202-244(-281) μm wide, (153-)178-228(-250) μm in length, (163-)190-236(- 266) μm in diameter. *Ascomatal necks* dark brown at base becoming lighter towards apex, (27-)31-39(-46) μm wide at base of neck, (14-)16-20(-22) μm wide at tip of neck, (691-)745- 840(-889) μm in length. *Ostiolar hyphae* divergent, hyaline, (35-)41-49(-54) μm in length. *Asci* not observed. *Ascospores* accumulate in a round, white to yellow (yellow-buff 19d) mass at the apices of the ascomatal necks, hat-shaped in side view, aseptate, hyaline, invested in sheath, 3-6 μm in length and 4-7 μm in width.

Thielaviopsis anamorph: Conidiophores of two types occurring singly on mycelium, primary conidiophores hyaline, long, (75-)80-114(-152) µm in length, (4-) 5-7(-8) µm wide at base, (3-)4-6(-7) µm wide at tip, secondary conidiophores hyaline, shorter than primary phialides, (38-)48-76(-89) µm in length, (3-)5-7(-8) µm wide at base, (3-)5-7(-8) µm wide at tip. *Conidium* development phialidic through ring wall building, *conidia* of two types formed singly or in chains; primary conidia, bacilliform cylindrical, (11-)-15-27(-30) µm in length, (3-)5-6 µm wide, secondary conidia, doliiform (barrel-shaped) (6-)10-11 µm in length, (4-)5-7(-9) µm wide. *Chlamydospores* present, (8-)10-12(-13) µm wide and (9-)10-14(-16) µm in length.

Habitat: On bark and cambium of wounded living *Eucalyptus* spp.

Known distribution: Colombia

Material examined: COLOMBIA, from artificial wounds on *Eucalyptus grandis* trees, 2004, C. Rodas & J. Roux (**holotype** Herb. PREM 59616); culture ex-type CMW 17808. Colombia, artificial wounds on *E. grandis* trees, 2004, C. Rodas & J. Roux, **paratype** PREM 59617; culture ex-paratype CMW 18194. Colombia, from artificial wounds on *E. grandis* trees, 2002, C. Rodas & J. Roux, **paratype** PREM 59618; culture ex-paratype CMW 11284 = CBS 121349. Colombia, from artificial wounds on *E. grandis* trees, 2002, C. Rodas & J. Roux, culture ex-paratype CMW 11285.

Pathogenicity tests

Ceratocystis neglecta gave rise to lesions of varying length on inoculated *E. grandis* trees (Figure 3-5). Isolate (CMW11285) from wounds on *E. grandis* at La Suiza, was highly pathogenic and produced extensive lesions significantly different (P = 0.0001) to that of the control and other isolate used (Figure 3-5).

Eucalyptus grandis clone 301 planted at all three sites was most susceptible with lesions extending up to 350 mm in length (Figure 3-5). Clone 02 was clearly more tolerant to *C. neglecta* (P < 0.05). Trees representing the seed lot *E. grandis* 211 collectively had a level of susceptibility to *C. neglecta* intermediate between that of clones 301 and 02 (Figure 5).

Some differences were observed in the results between the different farms. For CMW11284, Clone 301 did not differ significantly in susceptibility at the Cedral and La Suiza farms. However, at the Buenos Aires farm significantly larger lesions were produced when Clone 301 was inoculated with CMW 11284 (P < 0.05). For isolate CMW11284, Clone 02 differed significantly in susceptibility at the Buenos Aires and La Suiza Farms (P < 0.05). In all cases the control lesions were significantly less than those of the *C. neglecta* isolates.

For isolate CMW11285, Clone 301 did not differ significantly at the Buenos Aires and Cedral farms, but at the La Suiza farm it was significantly more tolerant to infection that at the former two farms (P < 0.05). For isolate CMW11285 and Clone 02, there were no statistically significant lesions between Buenos Aires Farm and La Suiza Farm (P > 0.05). In all cases the control lesions were significantly less than those of the *C. neglecta* isolates.

Discussion

Results of this study gave rise to the discovery of a new *Ceratocystis* sp. now known as *C. neglecta*, which is closely related to, but distinct from *C. fimbriata* s.s. This fungus was isolated from artificially inflicted wounds on *E. grandis* trees in Colombia. These infections, however, appear not to be common and occurred on only a few trees occurring in the three different climatic zones considered in this study. Although wounds became infected with *C. neglecta*, no indication was found of trees dying due to these infections. This may be due to the fact that trees were inspected only once after wounding, which might not have been sufficiently long for symptoms to develop. Another explanation could be that trees wounded in this study were not highly susceptible to infection by *C. neglecta*.

Morphologically, *C. neglecta* is distinct from *C. fimbriata* s.s. and all other closely related species previously considered as *C. fimbriata*. When *C. neglecta* is compared with *C. fimbriata* s.s., differences can be observed in the length of the ascomatal necks, with those of *C. neglecta* being longer than those of the latter species. Furthermore, the ostiolar hyphae of *C. neglecta* are much shorter

than those of *C. fimbriata* s.s., *C. platani* and *C. cacaofunesta*. The conidiophores of *C. neglecta* are also shorter than those of *C. fimbriata*, while they are longer than those of *C. platani* and *C. cacaofunesta*. The ascomatal necks of *C. cacaofunesta* are much longer than those of *C. neglecta* and other species in this group, except for *C. polychroma*, which also has long necks. Similar to other species in the *C. fimbriata* s.l. complex, *C. neglecta* has divergent ostiolar hyphae, which distinguishes it from *C. pirilliformis* which has convergent ostiolar hyphae.

In phylogenetic studies, *C. neglecta* grouped separately from all other described species of *Ceratocystis* in the *C. fimbriata* s.l. species complex. Comparison with isolates from Colombia that have previously also been studied using DNA sequence comparisons (Barnes et al. 2003a, Marin et al. 2003) also showed that *C. neglecta* represents a different species from these lineages. A study of *C. fimbriata* s.l. isolates from coffee growing regions in Colombia previously showed that at least two distinct phylogenetic lineages exist for those isolates (Marin et al. 2003). This suggests that additional, currently undescribed species in *C. fimbriata* s.l. exist in Colombia.

Although *C. neglecta* was not found associated with naturally infected and dying *Eucalyptus* trees in this study, we were able to show that isolates of this fungus can give rise to distinct lesions when inoculated onto susceptible *Eucalyptus* trees. The fungus is evidently a potentially important pathogen of these trees. Furthermore, pathogenicity tests showed clearly that one *E. grandis* clone deployed in Colombian plantations is highly susceptible to infection by *C. neglecta*. Previously unexplained deaths of trees in plantations could well have been due to this fungus, which can also be difficult to isolate.

An important and interesting outcome of this study was the fact that different clones of *E. grandis* differ substantially in their susceptibility to infection by *C. neglecta*. Thus, Clone 301 was highly susceptible to infection by the most pathogenic isolate of *C. neglecta,* at all three sites where this clone was tested. This is in contrast to Clone 02 that was considerably less susceptible to the isolates tested. The fact that the trees generated from seed were more

susceptible to infection by the most pathogenic isolate than one of the clones, is typical of results found in other studies (Zauza et al. 2004). Thus, seedling material harbours a wide range of susceptibility to pathogens and it displays a wide variability in response to infection.

In this study, artificially inflicted wounds were made on trees to determine whether these might become infected by isolates of *C. fimbriata* s.l. Similar wounding studies have previously been used on *Eucalyptus* trees in Australia (Barnes et al. 2003b, Kile et al. 1996) and South Africa (Roux et al. 2004) and these have led to the discovery of new species of *Ceratocystis* spp. as well as the detection of *C. fimbriata* s.l. where it was previously not known to occur. *Ceratocystis* spp. are well-known to infect wounds on trees and these infections probably originated from infected sap-feeding insects visiting wounds (Hinds 1972, Juzwik and French 1983, Teviotdale and Harper 1991). We believe that *C. neglecta* infection of the wounds made on *Eucalyptus* in this study originated from insects visiting these wounds, although further studies are needed to confirm this.

Inoculations in this study showed that one isolate of *C. neglecta* from wounds on *Eucalyptus,* was significantly more pathogenic than the other isolate chosen for inoculation trials. Variability in virulence of individuals of a pathogen is a well-recognised phenomenon and emphasises the importance of choosing appropriate isolates when screening planting stock for resistance. If this isolate had not been included in the trials, *C. neglecta* would not have been recognised as a potentially important pathogen of *Eucalyptus* in Colombia.

Results of this study have shown that *C. neglecta* is a potentially important pathogen of *Eucalyptus* in Colombia. Where trees die due to wilt and where vascular discoloration is noted, this fungus should be included amongst the possible causes of death. In these cases, isolation techniques suitable for recognising *Ceratocystis* infections should be included. Results have also shown that clones differ markedly in their susceptibility to infection. If *C. neglecta* becomes an important pathogen in the future, there will be excellent

opportunities to reduce losses through the selection of disease tolerant planting stock.

Acknowledgments

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Table 1: *Ceratocystis* isolates used in DNA sequence comparisons and inoculation studies

Species	Culture number	Host	Country	GenBank accession number	Collectors
C. albifundus	CMW 4068b	*Acacia mearnsii*	South Africa	DQ520638	J. Roux
C. albifundus	CMW 5329	*A. mearnsii*	South Africa	AF388947	J. Roux
C. atrox	CMW 19383	*Eucalyptus* sp.	Australia	EF070414	M.J. Wingfield
C. atrox	CMW 19385	*Eucalyptus* sp.	Australia	EF070415	M.J. Wingfield
C. cacaofunesta	CMW 15051	*Theobroma cacao*	Brazil	DQ520636	A.J. Hansen
C. cacaofunesta	CMW 14809	*T. cacao*	Ecuador	DQ520637	C. Suarez
C. caryae	C 1829	*Carya* sp.	USA	AY907035	J.A. Johnson
C. caryae	C 1827	*Carya* sp.	USA	AY907034	J.A. Johnson
C. fimbriata s.s	CMW 15049	*Ipomoea batatas*	USA	DQ520629	C.F. Andrus
C. fimbriata s.s	CMW 1547	*I. batatas*	Papua New Guinea	AF264904	E.H.C. McKenzie
C. fimbriata	CMW 4844b	*Coffea* sp.	Colombia	AF395691	B.L. Castro
C. fimbriata	CMW 4824b	*Coffea* sp.	Colombia	AF395692	B.L. Castro
C. fimbriata	CMW 4829b	*Citrus*	Colombia	AF395688	B.L. Castro
C. fimbriata	CMW 4835b	*Coffea* sp.	Colombia	AF395689	B.L. Castro
C. fimbriata	CMW 8858b	*Schizolobium parahyba*	Colombia	AY233865	B.L. Castro
C. fimbriata	C 1750	Cacao	Colombia	AY157955	N.Ac
C. fimbriata	CMW 8860b	Cacao	Colombia	AY233869	B.L. Castro
C. neglecta	CMW 11284b	*E. grandis*	Colombia	EF127988	C. Rodas & J. Roux
C. neglecta	CMW 11285b	*E. grandis*	Colombia	EF127989	C. Rodas & J. Roux
C. neglecta	CMW 17808b	*E. grandis*	Colombia	EF127990	C. Rodas
C. neglecta	CMW 18194b	*E. grandis*	Colombia	EF127991	C. Rodas
C. platani	CMW 1896	*Platanus* sp.	Switzerland	AF395681	O. Petrini
C. platani	CMW 2242	*Platanus* sp.	Italy	AF264903	A. Pauconesi
C. pirilliformis	CMW 6569	*E. nitens*	Australia	AF427104	M.J. Wingfield
C. pirilliformis	CMW 6579	*E. nitens*	Australia	AF427105	M.J. Wingfield
C. polychroma	CMW 11424	*Syzygium aromaticum*	Indonesia	AY528970	M.J. Wingfield
C. polychroma	CMW 11436	*S. aromaticum*	Indonesia	AY528971	M.J. Wingfield
C. populicola	C 995	*Populus* sp.	Poland	AY907029	Gremmen
C. populicola	C 685	*Populus* sp.	Canada	AY907028	E. Smalley
C. smalleyi	C 1828	*Carya* sp.	USA	AY907032	J.A. Johnson
C. smalleyi	C 1410	*Carya* sp.	USA	AY907031	T.C. Harrington
C. variospora	C 1965	*Prunus* sp.	USA	AY907045	J.A. Johnson
C. variospora	C 1707	*Betula* sp.	Japan	AY907044	Matsuya
C. virescens	CMW 11164b	*Fagus americana*	USA	N.A	D. Houston

[a] CMW = Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. ATCC = American Type Culture Collection (ATCC), Manassas, Virginia, USA. CBS = the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. C = from the collection of T.C. Harrington.
[b] Isolates sequenced in this study.
Dried cultures representing CMW1185 (PREM57511) and CMW11284 (PREM57512) have also been deposited in the National Collection of Fungi (PREM), Pretoria, South Africa.
[c] N.A. = not available.

Figure 1: Morphological features of *Ceratocystis neglecta* from *Eucalyptus grandis* in Colombia. A. Ascomata with globose bases. B. Hat-shaped ascospores. C. Divergent ostiolar hyphae. D. Primary phialide. E. Secondary phialide. F. Chlamydospores. G. Barrel-shaped conidia. H. Cylindrical conidia. Bars: A = 100 µm, C-H = 10 µm; B = 5 µm
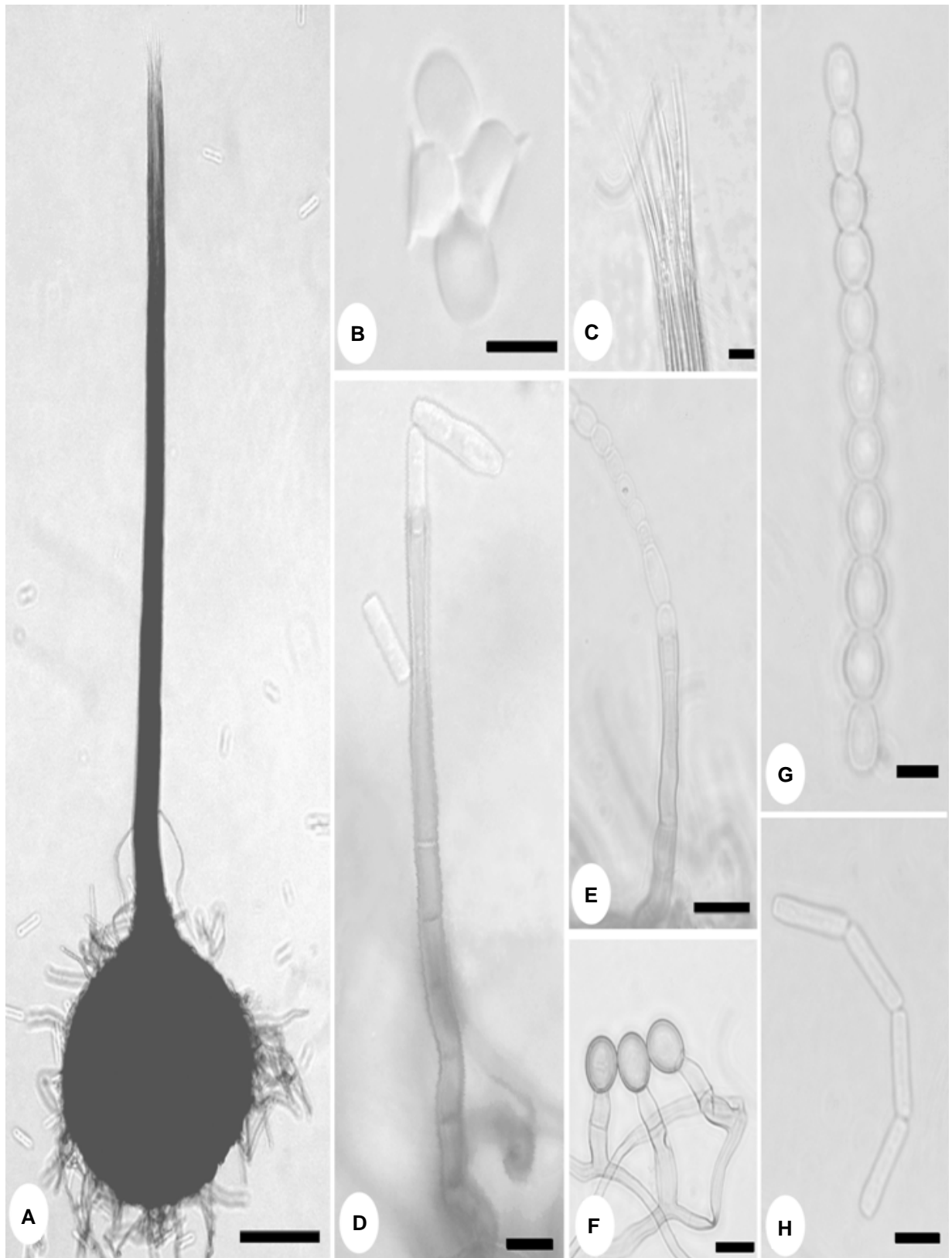
Figure 2: A phylogenetic tree (tree length = 439 steps, CI =0.7312, RI =0.8590, HI = 0.2688, RC = 0.6281) generated from DNA sequences of the ITS1/2 regions of the rDNA for various *Ceratocystis* species. Bootstrap values (1000 replicates) are indicated in bold. An isolate of *C. virescens* was used as the out-group taxon.
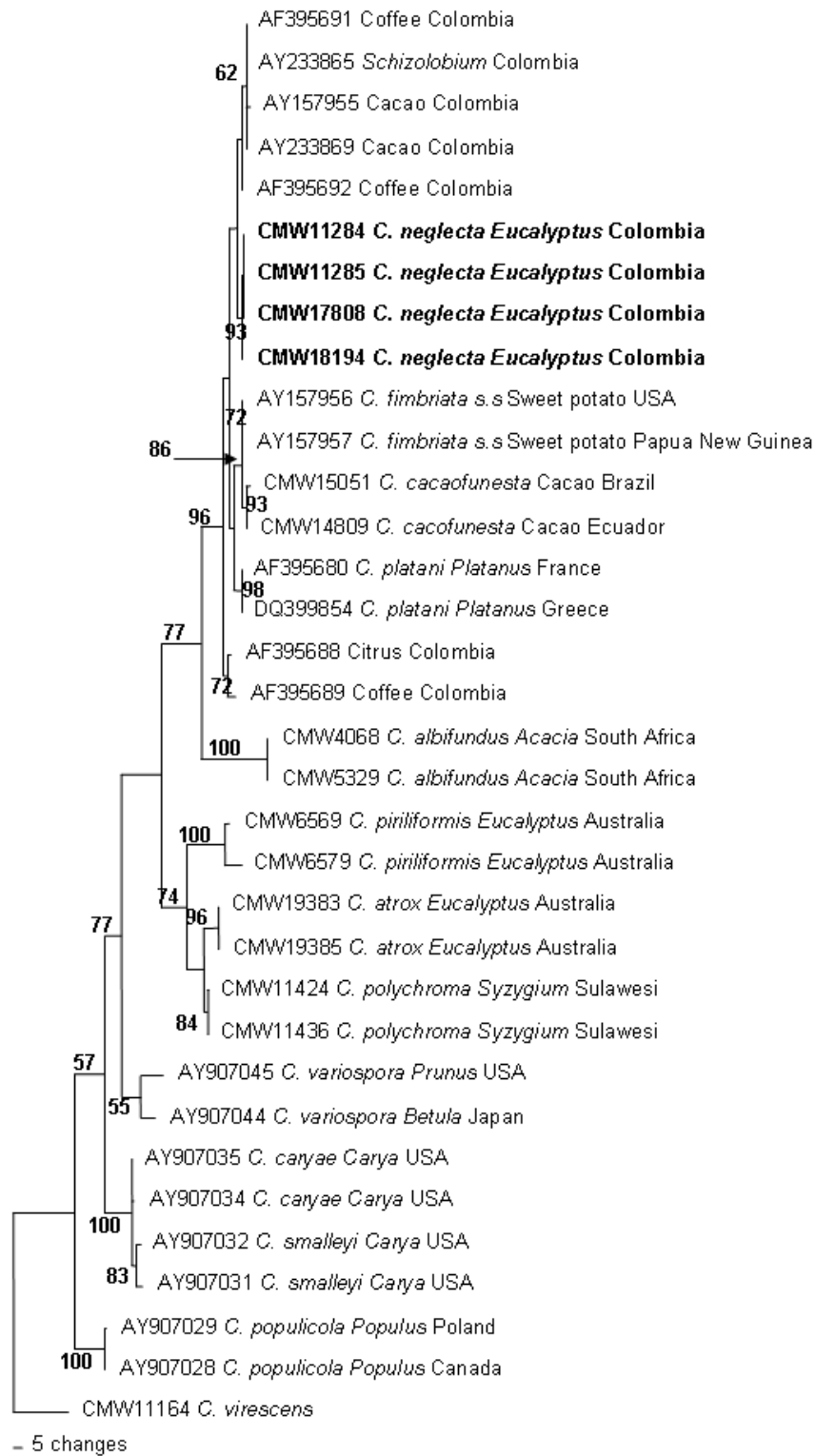
AF395691 Coffee Colombia
AY233865 *Schizolobium* Colombia
AY157955 Cacao Colombia
AY233869 Cacao Colombia
AF395692 Coffee Colombia
CMW11284 *C. neglecta Eucalyptus* Colombia
CMW11285 *C. neglecta Eucalyptus* Colombia
CMW17808 *C. neglecta Eucalyptus* Colombia
CMW18194 *C. neglecta Eucalyptus* Colombia
AY157956 *C. fimbriata s.s* Sweet potato USA
AY157957 *C. fimbriata s.s* Sweet potato Papua New Guinea
CMW15051 *C. cacaofunesta* Cacao Brazil
CMW14809 *C. cacofunesta* Cacao Ecuador
AF395680 *C. platani Platanus* France
DQ399854 *C. platani Platanus* Greece
AF395688 Citrus Colombia
AF395689 Coffee Colombia
CMW4068 *C. albifundus Acacia* South Africa
CMW5329 *C. albifundus Acacia* South Africa
CMW6569 *C. piriliformis Eucalyptus* Australia
CMW6579 *C. piriliformis Eucalyptus* Australia
CMW19383 *C. atrox Eucalyptus* Australia
CMW19385 *C. atrox Eucalyptus* Australia
CMW11424 *C. polychroma Syzygium* Sulawesi
CMW11436 *C. polychroma Syzygium* Sulawesi
AY907045 *C. variospora Prunus* USA
AY907044 *C. variospora Betula* Japan
AY907035 *C. caryae Carya* USA
AY907034 *C. caryae Carya* USA
AY907032 *C. smalleyi Carya* USA
AY907031 *C. smalleyi Carya* USA
AY907029 *C. populicola Populus* Poland
AY907028 *C. populicola Populus* Canada
CMW11164 *C. virescens*

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Figure 3: Results of an inoculation trial with isolates of *Ceratocystis neglecta* from *Eucalyptus grandis* (CMW11285 and CMW11284) from Colombia and a negative control. Inoculations were done on *E. grandis* clones 301 and 02 at Buenos Aires farm, Trujillo Valle. Different letters above bars indicates statistical differences between isolates and clones at Buenos Aires Farm (P < 0.05)
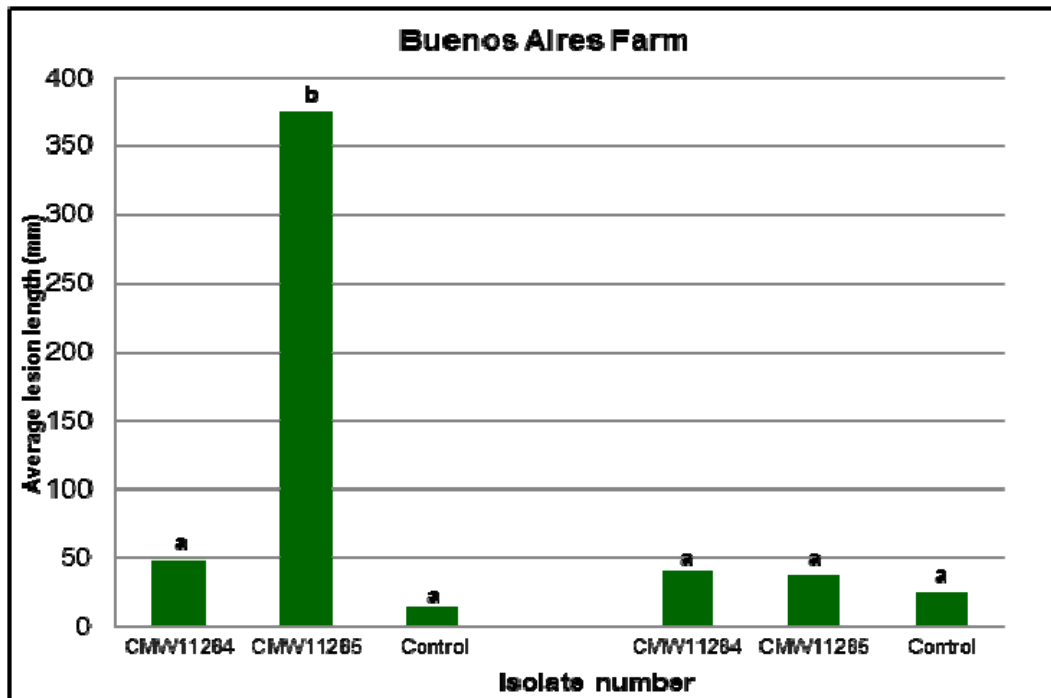
Figure 4: Results of an inoculation trial with isolates of *Ceratocystis neglecta* from *Eucalyptus grandis* (CMW11285 and CMW11284) from Colombia and a negative control. Inoculations were done on *E. grandis* clones 301 and 02 at La Suiza farm, Restrepo Valle. Different letters above bars indicates statistical differences between isolates and clones at La Suiza Farm (P < 0.05)
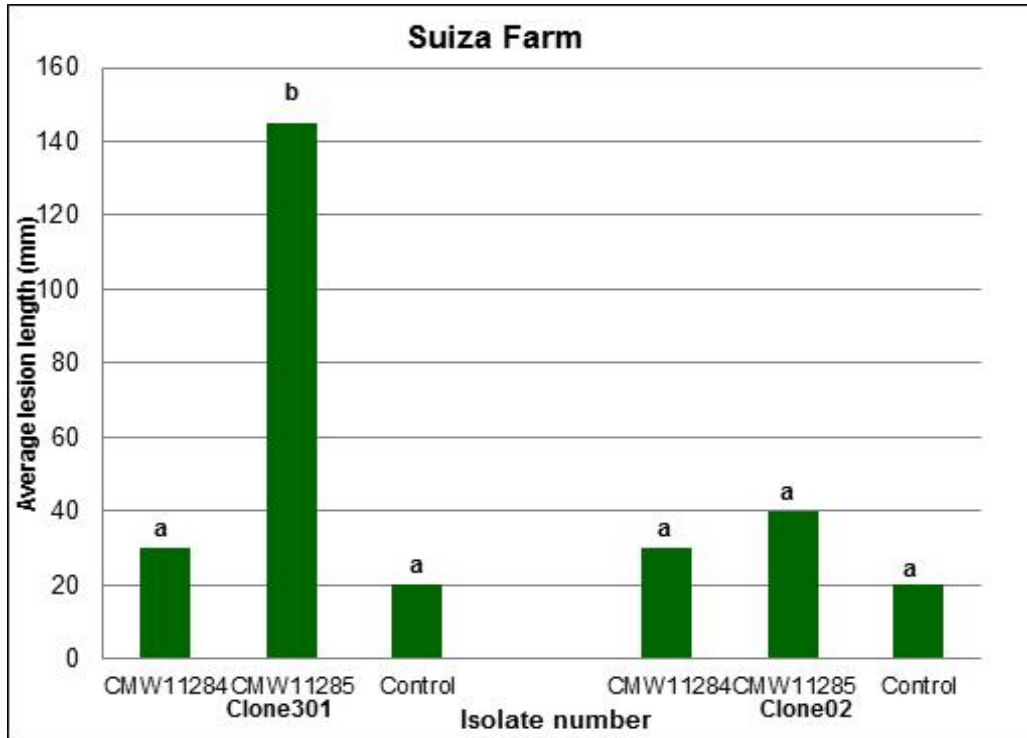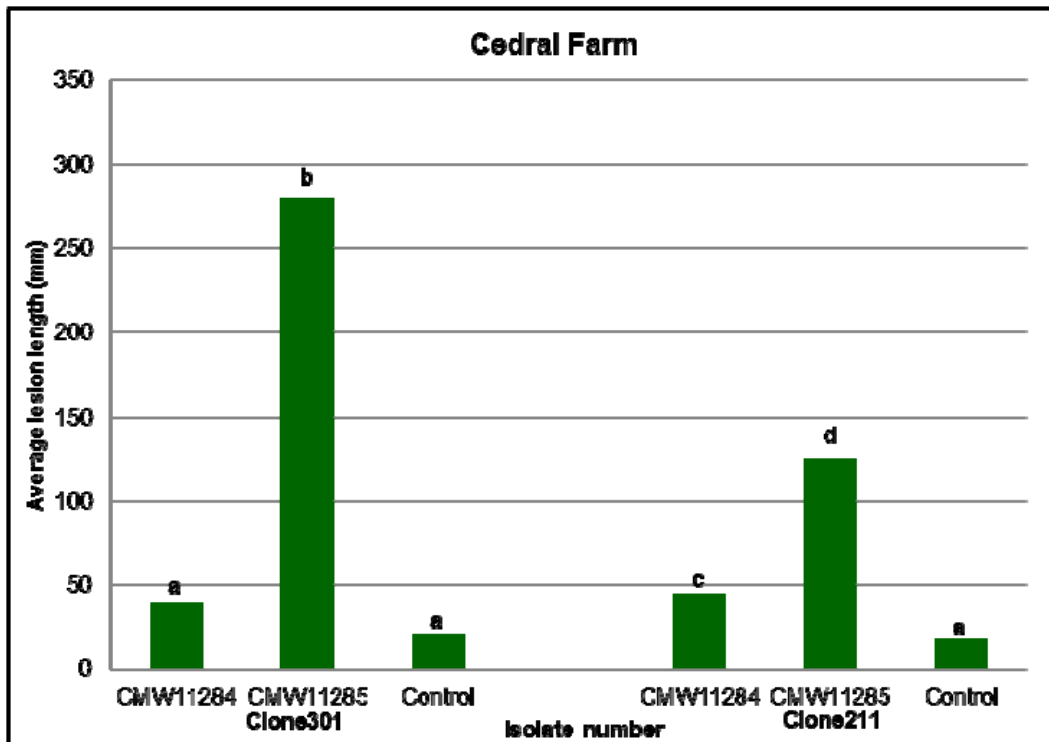
Figure 5: Results of an inoculation trial with isolates of *Ceratocystis neglecta* from *Eucalyptus grandis* (CMW11285 and CMW11284) and a negative control. Inoculations were done on clone 301 and seed lot 211 at Cedral farm, Darien, Valle. Different letters above bars indicates statistical differences between isolates and clones at Cedral Farm (P < 0.05)

Summary

Colombia has established approximately 327 000 ha of plantations of native species, as well as those of *Pinus* and *Eucalyptus*. These plantations are seriously threatened by pests and pathogens, including those that are native and others that have and will be introduced into the country from elsewhere in the world. In order to reduce the impact that these pathogens and insect pests will have on productivity and sustainability, the studies presented in this thesis were undertaken. In order to establish a foundation for further investigations, a review of the the Colombian commercial forestry sector is presented against a backdrop of all recorded pests and pathogens found in plantations of *Eucalyptus* and *Pinus* species. The main group of defoliator insects that cause economic losses include the native Geometridae, Phasmatodea and Formicidae. Various directed studies in this thesis include those on the biology and control of the Phasmid, *Litosermyle ocanae* where natural parasitism by the parasitoid wasp *Adelphe* sp. has been shown to be effective. Likewise, the defoliator, *Chrysomima semilutearia* (Lepidoptera: Geometridae) was recognized as important in1991 and a study on its life history as well as that of the introduced egg-parasitoid *Telenomus alsophilae* (Hymenoptera: Scelionidae) was undertaken. A further part of this thesis included a study of a *Pineus* sp., which is a newly emerging pest in Colombia, and the predatory effect of *Ceraeochrysa* sp. on the life stages of this *Pineus* sp. were characterized and shown to be an effective control agent. Studies in this thesis also included the first reports of the pitch canker pathogen *Fusarium circinatum* and *Dothistroma septosporum* in Colombia and their impact on different families and provenances of *Pinus* was considered. Both pathogens were characterized for the first time in Colombia using DNA-based techniques. On *Eucalyptus*, the fungus *Ceratocystis neglecta* was shown to be a potentially important pathogen in plantations and inoculation trials showed that *Eucalyptus* clones differed markedly in their susceptibility to infection. Other less detailed studies included the recent appearance of various important and damaging new pathogens and insect pests including *Monalonion velezangeli* (Hemiptera:Miridae), *Glycaspis brimblecombei* (Hemiptera:Psyllidae) and the rust pathogen *Puccinia psidii,* all three occurring on *Eucalyptus.* Prospects for future management strategies are described for all the reported pests and

pathogens and it is hoped that these will provide a strong basis to promote the future sustainability of plantation forestry in Colombia.

Appendix 1

Three new and important insect pests recorded for the first time in Colombian plantations

Abstract

Subsequent to 1950 in Colombian plantations, commercially propagated and non-native trees, including *Pinus*, *Eucalyptus* and *Cupressus* species, have been damaged by several native defoliating insects, residing in the Lepidoptera (Geometridae); Phasmatodea (Heteronemiidae) and the Hymenoptera (Formicidae) order. Severe defoliation has occurred and during the last 7 years, three new insect pests have appeared for the first time. These include *Monalonion velezangeli* (Hemiptera: Miridae); *Glycaspis brimblecombei* (Hemiptera: Psyllidae) and *Pineus* sp. (Hemiptera: Adelgidae). This report provides details of these new pests, their hosts, areas of occurrence, likely origin and prospects for their management in the future.

Introduction

Plantation forestry, mainly established based on non-native species of *Pinus* and *Eucalyptus,* supports major solid wood and paper industries world-wide and this is also true in Colombia. As is true in other parts of the world, insect pests and pathogens (Wingfield et al. 2011a) pose a very serious threat to plantation forestry in Colombia. At present, there are 327 000 ha of trees established in plantations in the country and this area is likely to expand in future (MADR 2010). Of this area, 75 000 ha are planted with *Pinus* species, about 46 000 ha of *Eucalyptus* spp. Have been established and native species cover about 206 000 ha (Annonymous 2011).

As in many other countries of the tropics and southern hemisphere, plantations of *Pinus* spp. and *Eucalyptus* spp. were initially separated from their natural enemies. Pests and diseases have gradually begun to appear and in some cases, these are threatening the sustainability of plantation resources (Wingfield 2003, Wingfield et al. 2010, 2011a). The pests and pathogens include those that are native to the areas where they occur and others that have been introduced from the areas where they are native or elsewhere where they have become established as invasive aliens. This is a trend that is likely to

continue as has been suggested in a number of recent reviews (Wingfield 2003, Wingfield et al. 2010, 2011a, 2011b, 2013).

Colombian plantations of *Pinus* and *Eucalyptus* have been challenged by various native insects, particularly defoliators residing in the Lepidoptera (Geometridae) (Gallego 1959, Vélez 1972, Bustillo 1976, Rodas 1994, 1996), Hymenoptera (Formicidae) (Mackay and Mackay 1986), and Phasmatodea (Heteronemiidae) (Madrigal and Abril 1994). These problems have been dealt with largely through the implementation of biological control strategies (Bustillo 1976, Bustillo and Drooz 1977, Bustillo 1978, Rodas 1997, Madrigal 2003, Ortiz and Guzmán 2007). An exception has been the very serious infestations of native leaf cutting ants that have required chemical control options (Ortiz 1998, Forti et al. 2007, Rodas CA. unpublished data).

The monitoring of Colombian *Pinus* and *Eucalyptus* plantations for new pests and pathogens is an ongoing and important activity. During the last decade, this monitoring process has led to the discovery of three serious insect pests not previously known in Colombia. The aim of this study is to formally report these pests, one on *Pinus* spp. and two on *Eucalyptus* spp., and to provide some background regarding their biology and relative importance.

Monalonion velezangeli (Hemiptera: Miridae)

Monalonion velezangeli (Hemiptera: Miridae) was first recorded on avocado in Colombia in 1984 and identified in 1988 (Carvalho and Costa 1988). The origin of the insect is believed to be Central and South America where it is known on various crop plants (Vélez 1997). In recent years, *M. venezangeli* has become a significant pest on a number of Colombian tree crops including avocado (Carvalho and Costa 1988, Londoño and Vargas 2010a), coffee (Ramírez et al. 2007) guava and cocoa (Giraldo et al. 2009, Londoño and Vargas 2010b).

For approximately 15 years, very serious damage has been noted on young *Eucalyptus* trees in Colombian plantations but the causal agent of the problem has not been known. During 2011, adults of a sap-sucking mirid were found in

damaged plantations and these have been identified as *M. velezangeli* (Figure 1A-F). The main damage caused by this insect is sap-sucking on young tissues such as buds and twigs. This result in the rapid appearance of dark necrotic lesions after one hour, apparently due to the production of toxic enzymatic saliva by the insect (Figure 1G-I). Damage includes the loss of apical dominance, dried leaves, and decreased growth. Careful observation of the trees has shown that the main damage occurs on *Eucalyptus* during the night and it is this nocturnal behavior of *M. velezangeli* that resulted in it not being identified for many years. Both nymphs and adults are responsible for the damage to young *Eucalyptus* species.

Severe damage can be observed in trees with relatively low levels of infestation by *M. velezangeli*. This is due to secondary infections by the fungal pathogen *Neofusicoccum ribis* (Rodas et al. 2009). This is consistent with observations of similar symptoms associated with damage due to *Heliopeltis* spp. (Miridae) on *Eucalyptus*, where Botryosphaeriaceae have been found causing secondary infections (Wingfield MJ unpublished data).

The first collections of *M. venezangeli* on *Eucalyptus* in Colombia were made in February of 2011, where it was found in a young (2.2-years-old) *E. grandis* seedling plantation on the La Tigresa Forestry Farm (3°49′44′′ N - 76°34′54′′ W) near Restrepo (Valle del Cauca). In the same year, a 1.5-year-old *E. grandis* clone was seriously affected by the insect and secondary *Botryosphaeria* infections on the La Mesa Farm (3°43′59′′ N - 76°12′16′′ W) near Palmira (Valle del Cauca). The insect was also detected in a 6-month-old *E. grandis* seedling plantation on the Buenavista2 Forestry Farm (2°23′03′′ N - 76°35′02′′ W) Salinas (Cauca).

Monalonion venezangeli is clearly an insect pest of emerging importance on *Eucalyptus* in Colombia. Damage is most severe on young trees growing in moist and shaded sites. While trees appear to recover from infestation as they become older, they can be very seriously malformed and this is also connected to secondary infections by *N. ribis* (Rodas et al. 2009). There is good evidence to show that clones differ in their susceptibility to damage (Rodas CA.

unpublished data) and this is linked to the differing susceptibility of clones to infection by *N. ribis* (Rodas et al. 2009). Thus, planting clones tolerant to infestation holds substantial promise. In addition, the use of natural enemies requires further investigation.

Glycaspis brimblecombei (Hemiptera: Psyllidae)

Glycaspis brimblecombei has recently been found in Colombia and is commonly known as the Eucalyptus red gum lerp psyllid (Paine et al. 2006). This name emerges from the white conical lerp that serves as a cover for the insect on leaves. *Glycaspis brimblecombei* feeds on a wide range of *Eucalyptus* species; however, it shows strong preference for the species commonly referred to as the river red gums including *E. camaldulensis* (Paine et al. 2000). The insect disperses and becomes established very rapidly and for this reason it is difficult to control. Furthermore, chemical control is both expensive and environmentally undesirable (Santana and Buckhardt 2007).

Glycaspis brimblecombei is a native pest in Australia (Moore 1964) and is rapidly moving to new areas globally. The first report outside Australia was in 1998 in Los Angeles, USA (Dahlsten and Rowney 2000). Three years later, *G. brimblecombei* was recorded in Hawaii, USA (Nagamine and Reu 2001) and by that time, it had already also reached Northern Mexico (Castillo 2003). The pest was recorded for the first time in South America (Chile) in 2002 (Sandoval and Rothman 2002); it was recorded in Brazil in 2003 Wilcken et al. (2003); Madagascar in 2004 (Hollis 2004); in Argentina in 2005 (Bouvet et al. 2005); and it was found in Venezuela in 2007 (Rosales et al. 2008). The pest was first detected in Europe in 2007 when it was found on the border between Portugal and Spain (Valente and Hodkinson 2009). It was later reported in the Iberian Peninsula and Morocco in 2008 and Italy in 2010 (Laudonia and Garonna 2010, Peris-Felipo et al. 2011). Most recently, *G. brimblecombei* was reported from South Africa in 2012 (Hurley BP, personal communication).

In Colombia, *G. brimblecombei* was noted for the first time in 2005 on various *Eucalyptus* species. This insect was firstly discovered by a private Forestry

Company (LYR personal communication) in Villanueva, Casanare department where it was found affecting *E. teriticornis* and to a lesser extent *E. pellita* and *E. urophylla.* Between, 2009 and 2012, low levels of *G. brimblecombei* have been recorded in young (6 months - 2.6-year-old) *E. urograndis* plantations on the La Estancia Forestry Farm (3°41′31′′ N - 76°32′48′′ W) Yumbo, (Valle del Cauca). It has also been recorded on 2.3-year-old *E. grandis* on the El Nogal Forestry Farm [4°42′60′′ N - 75°36′26′′ W] in Pereira, (Risaralda). The most recent collections of *G. brimblecombei* were on 6-month-old *E. teriticornis* and *E. camaldulensis* in La Guamo [5°48′38′′ N - 75°41′02′′ W], Pintada, (Antioquia) where a severe infestation was recorded (Figure 2E). Currently recorded hosts include *E. teriticornis, E. pellita, E. urophylla, E. urograndis, E. grandis,* and *E. camaldulensis* and the pest is considered a serious threat to forestry in the country.

Glycaspis brimblecombei is characterized by a white lerp, composed principally of honeydew and wax, which protects the nymphs (Figure 2A-C). Once they reach the adult stage, the wings are fully developed and these insects colonize new substrates (Figure 2D). Damage is principally on the foliage, shoots and branches where sap-sucking from young tissues results in defoliation, branch dieback, and in the case of heavy levels of infestation, trees can die. Defoliation and reduction in leaf area most likely also contributes to a decrease in wood production (Figure 2E).

The best option to reduce the impact of *G. brimblecombei* is classical biological control (Figure 2F and G). This has been very effective using the parasitoid *Psyllaephagus bliteus* (Hymenoptera: Encyrtidae), which is a host-specific parasitoid of *G. brimblecombei* and also very useful for biological control programs in other parts of the world (Brennan et al. 1999, Dahlsten and Rowney 2000, Paine et al. 2006). Other reported families or insects that might be useful for biological control include Anthocoridae, Chrysopidae, Coccinellidae, Hemerobiidae and Syrphidae (Dahlsten and Rowney 2000).

Pineus **sp. (Hemiptera: Adelgidae)**

Pineus spp. are well-known sap-sucking insects that have caused serious damage on *Pinus* spp., such as in Christmas tree plantations (Triplehorn and Johnson 2005), and in commercial plantations (Havill and Foottit 2007, Petro and Madoffe 2011). The Adelgidae are known to feed exclusively on conifers (Scholtz and Holm 1985, Triplehorn and Johnson 2005) and this is true for species of *Pineus*, which are commonly referred to as pine adelgids, pine woolly aphids or common pine aphids (FAO 2007). The name "wooly aphid" reflects the white wool-like masses of wax that are produced by the insects on the branches and between needles where they live and feed in (Scholtz and Holm 1985).

The first appearance of a *Pineus* sp. in plantations of non-native pines was when *Pineus pini* was recorded simultaneously in Kenya and Zimbabwe in 1968 and it where it was believed to have been introduced from Australia on *Pinus taeda* in 1962 (Barnes et al. 1976, Petro and Madoffe 2011). This insect has subsequently spread to South Africa and the species involved is believed to be *P. boerneri* (Blackman and Eastop 1994, Lazzari and Cardoso 2011). *Pineus* sp. has been recorded in plantations of *Pinus* spp. in Africa, Europe, North and South America, Australia and New Zealand (FAO 2007). In general, aphids such as *Cinara* spp. on *Pinus* species in Brazil (Lázzari et al. 2004), *Cinara cupressi* in Chile (Montalva et al. 2010), *P. pini* in Spain (Soria et al. 1996), *P. pini* in Kenya (Odera 1974), *C. cedri* and *Pineus orientalis* in Turkey (Toper Kagín and ÇANAKÇIOĞLU 2003), among other countries, can result in very serious losses.

The only adelgid known on *Pinus* spp. in Colombia is the recently encountered *Pineus* sp. The insect has not been identified to species level but it appears to be the same as *P. boerneri* known in Brazil (Lazzari and Cardoso 2011) and it is the species that is found on *Pinus* spp. in Southern Africa. The symptoms of infestation become obvious when populations are high and these include masses of eggs, nymphs and adults covered by white cottony tufts of a wax-like substance that the insects exude to cover themselves. This results in

chlorosis of the needles and a reduction of tree growth, followed by shoot death; die-back of branches, and in extreme cases, death of trees (Figure 3A-C). In Colombia, microscopic examination of infested branches has shown that adult males are not present (Figure 3D).

The first report of *Pineus* sp. in Colombia was in 2008 on 2.1-year-old *Pinus kesiya* on the Aguaclara Forestry Farm (3°41′31′′ N - 76°32′48′′ W), La Cumbre (Valle del Cauca). The insect has subsequently been found on other *Pinus* spp. including 2.2 year-old *P. tecunumanii* on the Potrerito Farm (2°58′11′′ N - 76°43′34′′ W) Timba, Cauca in January of 2009 and by May of the same year, two additional plantations of 3.4-year-old *P. maximinoi* on the La Gaviota Farm (3°47′43′′ N - 76°35′36′′ W) near Restrepo, Valle del Cauca and on the La Quebrada Farm (3°47′30′′ N - 76°34′26′′ W). In 2009, 2.3-year-old *P. oocarpa* was also found infested on the Parcela2 Farm (2°59′02′′ N - 76°43′29′′ W), Timba (Cauca). Thus, all *Pinus* spp. grown in Colombia appear to be susceptible to infestation by *Pineus* and the pest is also found widely in the country.

Individual trees in plantations differ in their susceptibility to infestation by *Pineus* sp. In many cases, there are sufficiently high numbers of uninfected trees to enable productive forestry to continue, other than in the case of highly susceptible species such as *P. kesiya*. Biological control found for *Pineus* sp. includes different species of the Chrysopidae such as *Ceraeochrysa* sp. and *Chrysoperla* sp., and some of the Coccinelidae such as *Harmonia axyridis* (Brown et al. 2011).

Conclusion

This study has recorded the relatively recent appearance of three important and damaging new insect pests of plantation-grown *Pinus* and *Eucalyptus* spp. in Colombia. Prior to the appearance of these pests, all entomological problems affecting plantation forestry in the country were caused by native insects. This situation appears to be changing with two of the three insects recorded in this study being non-natives. There is a large number of seriously damaging insect

pests of *Eucalyptus* and *Pinus* already in South America but not in Colombia. These insects include the Eucalyptus gall wasp *Leptocybe invasa* (Costa et al. 2008), the Eucalyptus winter bronze bug *Thaumastocoris peregrinus* (Carpintero and Dellapé 2006, Wilcken et al. 2010) as well as the Sirex wood wasp *Sirex noctilio* (Hurley et al. 2007) and various bark beetles (Marvaldi and Lanteri, 2005) on *Pinus.* The imminent arrival of these pests represents a huge threat to plantation forestry in Colombia and every effort must be made to establish early detection systems and management strategies.

The continuous expansion of global trade in plants, manufactured and other wood products, increases the risk of new pest introductions, and consequently an establishment of pests in new areas. This situation demands the need for strict phytosanitary measures, which is the most effective means to reduce the global spread of pests and pathogens. Since these insects have been increasing in importance and they represent a threat to Colombian plantations, quarantine programmes are in the process of being formulated. The hope is that they will reduce the economic impact of new pests and pathogens to the forestry industry.

While pests and pathogens represent a very substantial challenge to sustainable plantation forestry in Colombia and globally (Wingfield et al. 2010, Wingfield et al. 2011b) there are many opportunities to deal with these problems. Breeding and selection for disease and pest tolerant planting stock provides one such opportunity that has already been very effective. There are also numerous examples of substantial success in reducing populations of insect pests using biological control (Garnas et al. 2012). Furthermore, new technologies are constantly emerging including those linked to DNA based diagnostics and breeding tools (Wingfield et al. 2012). Investment in these opportunities will allow progressive forestry companies to remain competitive.

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Figure 1: Life stages of *Monalonion velezangeli* (Hemiptera: Miridae)*.* A-B. Appendices of the egg inserted into branch tissue. C. Egg with two exposed appendices. D. Third nymphal stage feeding on young leaf tissue. E-F. Adult Mirid. G-H. Early damage symptoms. I. Advanced damage caused by the insect
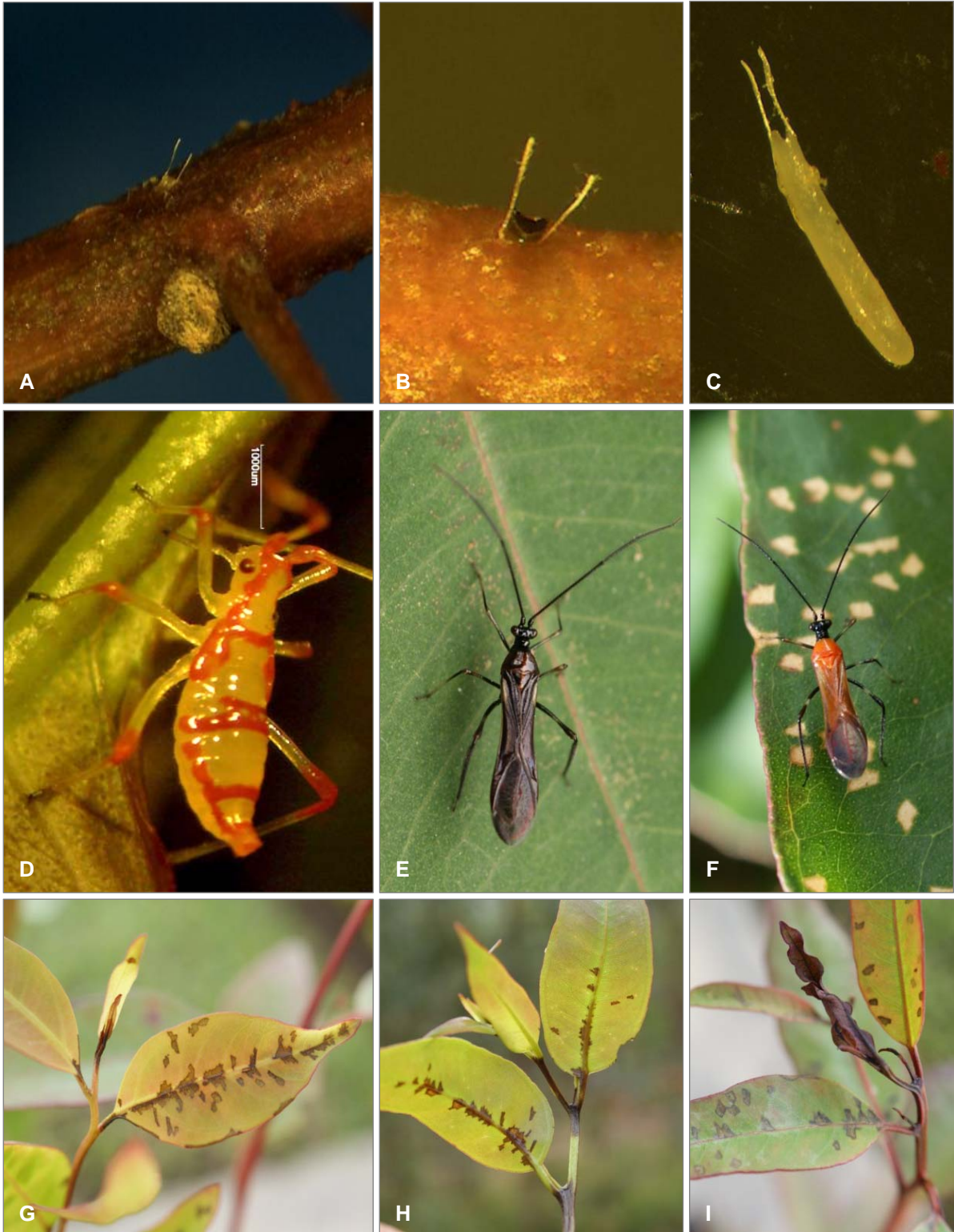
Figure 2: Life stages of the lerp psyllid *Glycaspis brimblecombei* (Hemiptera: Psyllidae) and biological control agents. A. Eggs on leaf surface. B-C. Uncovered nymphs. D. Adult male and female and conical lerps of nymphs. E. Abundant presence of nymphs on *E. camaldulensis.* F. Unidentified *G. brimblecombei* parasitoid (Hymenoptera). G. Unidentified Reduviidae (Hemiptera) as predators

Figure 3: Life stages and symptoms damage caused by *Pineus* sp. (Hemiptera: Adelgidae). A. Affected *Pinus kesiya* plantation. B. Severe infestation on bark of *P. kesiya*. C. Infested cones of *P. maximinoi.* D. (1). Microscopy of eggs, and (2) female adults (indicated by arrows)

Appendix 2

First report of *Puccinia psidii* **on** *E. citriodora, E. grandis* **and** *E. urograndis* **species in Colombia**

Puccinia psidii causes serious damage to *Eucalyptus* spp. plantations, and the disease is commonly referred to as guava, *Eucalyptus* or myrtle rust. It is found on many members of the Myrtaceae, occurring widely in Central and South American countries (Coutinho et al. 1998, Old et al. 2003). The importance of this pathogen is amplified by the fact that it has an extensive host range including at least 17 genera and 70 species of Myrtaceae (Simpson et al. 2006, Glen et al. 2007, Zauza et al. 2010). *Puccinia psidii* is been reported as a pathogen on trees in *Eucalyptus* plantations in numerous countries (Coutinho et al. 1998) including Brazil (Joffily 1944), Uruguay (Telechea et al. 2003), Japan (Kawanishi et al. 2009), Australia (Carnegie et al. 2012) and recently South Africa (Roux et al. 2013)

Myrtle rust has been known in Colombia since the late 1930´s (Kern and Toro 1935, Kern and Thurston 1940, 1954) where it has been noted on non-native Myrtaceae including *Syzygium jambos* (Figure 1A) and *Psidium guajava* (Figure 1B). It has very recently (2010) appeared for the first time in plantations of *Eucalyptus citriodora* (Figure 1C) and *E. grandis* (Figure 1D). Records now include infections on *E. citriodora* trees in Restrepo (Valle del Cauca department) located at 3°51´45´´ N - 76°29´49´´ W, 1-year-old *E. grandis* at La Suiza Farm 3°50´55´´ N - 76°29´33´´ W and on cuttings and seedlings of *E. grandis* in Rancho Grande Nursery of SKCC (3°51´43´´ N - 76°30´48´´ W).

In Colombia, as is true elsewhere, the primary symptoms on *Eucalyptus* are on young tissues such as leaves and shoots. These include masses of bright orange urediniospores commonly resulting in deformation of the infected tissue, necrotic lesions and shoot death in the advanced stages of disease. Urediniospores are ellipsoid to globose in shape with echilunate hyaline cell walls. Uredinospores are an average of 21.1 (13.8 – 26.9) μm long and 16.4 (10.4 – 23.1) μm wide ($n = 48$). Inoculations have been carried out using 2 x 10^5 urediniospores/ml taken from *S. jambos* and applied to *E. grandis* and *E. urograndis*, maintained at a constant temperature of 24°C and a humidity of 78%. These inoculations showed greater levels of susceptibility on the selected *E. urograndis* than on the *E. grandis* clones.

To confirm the identity of the pathogen, uredinospores were taken from the *S. jambos* tree that served as a source of inoculum. DNA isolation, PCR and sequencing techniques were as described in previously (Maier et al. 2003, 2007). We amplified and sequenced the 5' end of the nuclear large subunit of the ribosomal DNA (1061 bp, GenBank accession EU711425), and the full ITS (575 bp, GenBank accession EU711423) region. BLAST search revealed that the LSU sequence was 99% identical to other sequences of *P. psidii* deposited in GenBank, while the ITS sequence showed 99-100% similarity, thus supporting the identification based on symptomatology and light microscopic observations. In addition, morphological characterization was made from *E. grandis* samples and these were consistent for those of the fungus (Salazar and Buriticá 2012).

This is the first report of *P. psidii* on *Eucalyptus* in Colombia, thus far including *E. citriodora, E. urograndis* hybrids and *E. grandis* as hosts. Studies are now being conducted to screen clones of *E. grandis* and *E. urograndis* clones for resistance to *P. psidii* in Colombia. These will then inform future planting strategies and aim to minimize damage due to the pathogen.

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Figure 1: Rust infection associated with *Puccinia psidii* on different hosts. A. *Syzygium jambos.* B. *Psidium guajava.* C. *E. citriodora.* D. *E. grandis*