INDUCING RESISTANCE OF SPANISH CEDAR *Cedrela odorata* L. AND MAHOGANY *Swietenia macrophylla* King AGAINST *Hypsipyla grandella* (Zeller) BY GRAFTING

A Dissertation

Presented in Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

with a

Major in Plant Sciences

in the

College of Graduate Studies

University of Idaho

and with an Emphasis in

Tropical Agriculture

In the

Graduate School

Centro Agronómico Tropical de Investigación y Enseñanza

by

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November 2006

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AUTHORIZATION TO SUBMIT DISSERTATION

This dissertation of Julián Pérez Flores, submitted for the degree of Ph.D. with a major in Plant Sciences and titled "Inducing resistance of Spanish cedar *Cedrela odorata* L. and mahogany *Swietenia macrophylla* King against *Hypsipyla grandella* (Zeller) by grafting", has been reviewed in final form. Permission, as indicated by the signatures and dates given below, is now granted to submit final copies to the College of Graduate Studies, and to the Graduate School at CATIE for approval.

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ABSTRACT

A viable management program to prevent the shootborer *Hypsipyla grandella* (Zeller) from damaging *Swietenia macrophylla* King and *Cedrela odorata* L., which are the most valuable timber species in Latin America, is currently needed to establish commercial plantations. Grafting effects of both species onto resistant *Khaya senegalensis* (Desr.) A. Juss. or *Toona ciliata* M. Roem for resistance against *H. grandella* was investigated. Micropropagation techniques for *C. odorata* and *S. macrophylla* useful for cloning selected resistant trees were also examined. Grafting effects were determined by inoculating whole plants with *H. grandella* eggs or instar III larvae and leaf disks with instar II larvae. Crude leaf extracts and alkaloid, limonoid, and phenolic fractions extracted from the four species and *C. odorata* grafted onto *T. ciliata* plants were tested on *C. odorata* leaf disks inoculated with *H. grandella* instar II larvae. Damage of whole plants (i.e., number of frass piles, tunnels or damaged leaves, tunnel lengths and apical bud damage), larval feeding (i.e., leaf consumption and weight gain) and larval performance (i.e., time to pupation and adulthood, pupal weight and length, mortality and normal development of wings) were assessed. Resistant rootstocks conferred resistance to susceptible scions. Larvae feeding on disks from *K. senegalensis* grafted onto *S. macrophylla* extended by eight days the time to pupation and adulthood, and caused abnormal wings on 40% of adults. *Toona ciliata* intact plants or used as rootstocks, decreased leaf consumption and weight gain by 95% and increased larval mortality up to 100%. Crude extracts from resistant and grafted plants affected larval performance and were more detrimental to larvae than those from susceptible species. Putative alkaloids from *C. odorata* grafted on *T. ciliata* reduced leaf consumption and weight gain by 35% and caused abnormal wings on 20% of adults, whereas alkaloids from *S. macrophylla* reduced time to pupation and adulthood. For *in vitro* establishment, initial contamination was reduced from 90% to 30% by using NaOCl or plant preservative mixture, and benzylaminopurine improved bud break on shoot explants of *S. macrophylla* by more than double compared to *C. odorata* shoots.

VITA

Julián Pérez Flores was born in Córdoba, Veracruz (México) in 1971. In 1986, he began studying Agronomy at the Universidad Autónoma Chapingo in México State (México), receiving an Agronomist Engineer degree in 1993. From 1993 to 1995, he worked as agricultural extension practitioner with producers of ornamental, vegetable and fruit crops in the states of Guanajuato, Puebla, and Veracruz. In Veracruz, he also worked for the Instituto Nacional Indigenista developing agricultural production projects for native people. From 1995 to 1999, he worked in the Colegio de Postgraduados en Ciencias Agrícolas – Campus Tabasco, in the area of Plant Genetic Resources and Productivity.

In 2000-01, he completed a M.Sc. study at CATIE in Ecological Agriculture with emphasis on Plant Genetic Resources and Biotechnology. His thesis dealt with the micropropagation of *Cedrela odorata* L. (Spanish cedar). In 2002, he started a Ph.D. study in Plant Sciences within a Joint Program between the University of Idaho and CATIE. In his dissertation, he investigated the use of grafting of *C. odorata* L. and *Swietenia macrophylla* onto *Toona ciliata* and *Khaya senegalensis* to prevent damage due to the shootborer *Hypsipyla grandella*, and the investigated methods to establish a micropropagation protocol for both *C. odorata* and *S. macrophylla* King.

ACKNOWLEDGEMENTS

I want to acknowledge my advisors María Elena Aguilar and Robert R. Tripepi, who supported me all the time. I also thank Sanford D. Eigenbrode, Luko Hilje and Francisco Mesén, members of my Graduate Committee, for sharing their knowledge and patience with me. I am grateful to the personnel of the Biotechnology, Entomology and computer laboratories at CATIE for their friendship and aid to complete my research, especially to Arturo Ramírez, Edgar Esquivel, Alexis Pereira, Gustavo López, Frank López, Nelly Vázquez, Juan Luis Ortíz and Maritza Loaiza. I also want to thank to the library personnel at CATIE and University of Idaho for helping me obtain the information needed to complete my dissertation. I also thank the institutions that gave me financial support for this research: The Consejo Nacional de Ciencia y Tecnología and the Colegio de Postgraduados en Ciencias Agrícolas from México and the Department of Plant, Soil and Entomological Sciences at the University of Idaho. Finally, I want to acknowledge all the people that I met during the time I was completing my dissertation. I will remember them always.

DEDICATION

To God, who is always with me and my family.

To my dear wife Ana María and my children Yuliana and Julián.

To my parents, sisters and brothers, who always have supported me.

To my friends at both CATIE and at the Univertiy of Idaho.

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INTRODUCTORY CHAPTER

Introduction

Spanish cedar (*Cedrela odorata* L.) and mahogany (*Swietenia macrophylla* King) trees are of major significance in the economies of many tropical countries (Patiño, 1997); however, natural populations of these species are being reduced quickly due to selective harvest (Albert *et al*., 1995). On the other hand, the shootborer *Hypsipyla grandella* (Lepidoptera: Pyralidae), causes problems for Spanish cedar and mahogany trees by feeding mainly on the apical bud of the main shoot causing forks in the main trunk and giving rise to non-commercial trees. Larvae also can feed on fruits, leaves and bark. The problem is so serious that it has limited the establishment of commercial plantations of these species in Latin America (Newton *et al*. 1993).

Exotic Meliaceae have often been found to be less susceptible than the indigenous ones to attacks of native *Hypsipyla* spp. (Cunningham *et al*., 2005). *Toona ciliata* M. Roem., a species closely related to *Cedrela*, is heavily attacked by *H*. *robusta* when grown in its native habitat (Asia, Africa and Australia), but it is not attacked by *H*. *grandella* when planted in Central America (Whitmore, 1976). In Australia, *C. fissilis* Vell. is not attacked by *H*. *robusta* (Bygrave & Bygrave, 1998).

A biochemical basis to the resistance (antibiosis) of *T. ciliata* to *H*. *grandella* was initially suggested by Grijpma (1976), who also showed that such resistance was acquired by susceptible *C. odorata* when it was grafted onto *T. ciliata*, as the scion showed resistance to the attack. Moreover, this author suggested that substances conferring resistance were alkaloids. Resistance by grafting Meliaceae plants against *H. robusta* was confirmed by

Bygrave and Bygrave (1998, 2001), but they did not determine whether it involves translocation of substances from rootstocks to scions. However, De Paula *et al*. (1997) and Da Silva *et al*. (1999) on *C. odorata* grafted on *T. ciliata* determined that triterpenoids, limonoids and phenolics were transfered to the former species, but none of these substances could be determined as responsible to confer resistance to *H. grandella*.

Trees from a number of Meliaceae species, such as *Trichilia havanensis* Jacq. *Aglaia* sp. F. Allam., *Azadirachta indica* A. Juss, *Melia azedarach* L., and *Cedrela toona* Roxb (*Toona ciliata*) have been shown to contain alkaloids (Smolenski *et al*., 1974). In addition, Grijpma & Roberts (1975) speculated that alkaloids are responsible for resistance of *T. ciliata* to *H. grandella* and they can be transported to *C*. *odorata* shoots grafted onto *T. ciliata* rootstocks so that *C. odorata* shoots can become toxic to larvae. *T. ciliata* also contains limonoids (derivatives of triterpenes), many of which are either powerful insecticides or feeding deterrents (Kubo & Klocke, 1986). However, limonoids seem unrelated to the induced resistance of *C*. *odorata* grafted onto *T*. *ciliata* against *H*. *grandella* (De Paula *et al*., 1997); instead, these authors stated that cycloartanes, catechin and proanthocyanidins (phenolic compounds) are likely to be responsible for such resistance, as all of them were absent from *C*. *odorata*, but present in *T*. *ciliata*. Nevertheless, after chemical analysis of plants, Newton *et al*. (1999) reported a high content of proanthocyanidins in some *C. odorata* plants from different localities of Costa Rica.

Grafting may improve some characteristics of the resulting plants, such as resistance to diseases, insects, or herbicides. Serial grafting (i.e., sucessive grafting using scions of grafted plants on rootstocks of the same species of original graft combination) may also rejuvenate several woody perennial species (Perrin *et al.*, 1994). These positive influences from grafting might be explained by transmissibility of one or several biochemical signals through cells in the graft union, implying a small-sized molecule(s) and its/their replication maintaining restored traits (Huang *et al*., 1992), such as insect resistance. Thus, grafting

can result in resistant plants, which could be reproduced in a large scale by micropropagation (Hartmann *et al*., 2002).

In micropropagation, the use of mature plant material to initiate *in vitro* cultures is advantageous because the phenotype of the stock plant is known and the plantlets have a short juvenile period when transferred to the field (Hackett & Murray, 1993). However, mature explants, particularly those from tree species, are hard to work with in micropropagation (Rouse-Miller & Duncan, 2000). An alternative source of plant material for *in vitro* cultures might be new shoots produced from grafted plants which are young and easily respond to micropropagation compared to mature plants.

The Problem

Hypsipyla grandella is the main factor limiting the extensive planting of *C. odorata* and *S. macrophylla*. In spite of considerable research completed to date, practical control methods are lacking because of the low damage threshold of one larva per tree (Hilje & Cornelius, 2001), and also because the larva is encrypted, i.e., larva penetrates the stem and pupates inside.

In the 1970's, grafting *C. odorata* onto *T. ciliata* showed to be a promising alternative, as grafted plants were resistant to *H. grandella* (Grijpma, 1976). However, this alternative did not receive enough attention, and the pest has remained as serious as ever. Nonetheless, the conclusion of an international workshop on *Hypsipyla* spp. was that the integrated pest management for *Hypsipyla* spp. should be centered on the prevention of pest damage rather than in its cure (Floyd & Hauxwell, 2001). Such prevention could certainly be based on using resistant trees, which have been detected both in native and exotic Meliaceae species to Latin America.

The best way to take advantage of the differential host preference by *Hypsipyla* spp. would be by grafting native susceptible Meliaceae species onto exotic resistant ones. So far, grafting has been completed using *Cedrela* spp. and *T. ciliata* (Grijpma, 1976; De Paula *et al*, 1997; Bygrave & Bygrave, 1998, 2001), but use of *S. macrophylla* and *K. senegalensis*, which possess similar economic importance and face equivalent problems with *Hypsipyla* spp. has yet to be tested.

Once obtained, resistant plants could be cloned to obtain sufficient plants for field-testing before establishing commercial plantations. Such propagation should be accomplished by *in vitro* culture of nodal explants to obtain true-to-type genotypes.

An efficient technique for micropropagation of *C*. *odorata* or *S*. *macrophylla* is lacking. So far, both species have been micropropagated by shoot tips or nodal segments taken from seedlings of *in vitro* germinated seeds (Orellana, 1997; Pérez *et al*., 2002), which is unsuitable for cloning elite trees (for example, resistant plants to *H. grandella* larvae). For cloning elite trees, micropropagation by nodal explants taken from mature trees is needed. However, explants from mature trees fail to respond easily to *in vitro* culture due to high levels of contaminants and age (Kane, 2000). The successful establishment of explants is essential in the micropropagation process to proceed to the multiplication and following stages.

General objectives

1. To investigate the use of grafting of *C. odorata* and *S. macrophylla* onto *T. ciliata* and *K. senegalensis* to prevent damage by *H. grandella* larvae.

2. To establish a micropropagation method for *C. odorata* and *S. macrophylla* by nodal explants.

Specific objectives

1.1. To assess the effect of grafting on damage by *H. grandella* to *C. odorata* and *S. macrophylla*, as well as on larval performance and mortality.

1.2. To determine the effect of crude extracts and alkaloid, limonoid, and phenolic fractions from susceptible and resistant Meliaceae species, as well from a grafted combination of plants, on *H. grandella* larval performance and mortality.

2.1. To develop disinfection methods for *C. odorata* and *S. macrophylla* in order to establish successfully *in vitro* cultures of nodal explants.

2.2. To improve the growth of *C. odorata* and *S. macrophylla* nodal explants in the *in vitro* establishment.

Background

Importance of Meliaceae species

The family Meliaceae is of major importance in the economies of many countries. It is comprised of 51 genera and approximately 550 species, some of which are among the world's tallest woody plants, reaching 70 m in height and 15 m at diameter breast height (dbh) (Styles & Khosla, 1976). Most of the Meliaceae species grow in tropical areas. There, the most important genera containing commercial species are *Cedrela and Swietenia* in America, and *Toona* and *Khaya* in the paleotropics (Australia, Africa and Asia) (Chalmers *et al*., 1994). However, constant selection of the best trees in logging operations lead to severe

genetic erosion among species of the four genera cited (Styles & Khosla, 1976). Therefore, Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) included both *C. odorata* and *S. macrophylla* in November 2002. During the same year, The International Union for the Conservation of Nature and Natural Resources (IUCN) included *K. senegalensis* as a vulnerable species in the Red List of Threatened Species.

Cedrela odorata, *S*. *macrophylla, T*. *ciliata* and *K. senegalensis* are members of the subfamily Swietenioideae (Pennington, 1981). They are among the most valuable tropical timber trees found worldwide (Bygrave & Bygrave, 2005). Timber is used for manufacturing high quality furniture, carvings, decorative panels, veneers, flooring, special boxes, musical instruments, housing banisters, and even boats (Edmods, 1993; Blundell & Gullison, 2003). They are also planted for both landscaping, as shade trees in urban areas, and for agroforestry (Bygrave & Bygrave, 2005). Moreover, each species has important particular properties.

***Cedrela odorata* L.**, known as Spanish cedar, true cedar or cedro (due to its similar odor to true genera *Cedrus*), is distributed from northern Mexico (26^0 N) through Central America and the Caribbean to Brazil and Argentina (28^0 S) (Pennington, 1981). The species prefers an annual rainfall of 1000 to 3500 mm and grows well from sea level up to 3000 m (Cintrón, 1990).

Trees are fast-growing and become large trees at maturity, reaching 40 m height and 2 m dbh (Cintrón, 1990). It is a suitable species for forest plantations due to its adaptability, fast growth, valuable timber, and branchless habit (Cornelius & Watt, 2003). Geographic distribution of the species is similar to that for S. *macrophylla* (Cintrón, 1990).

***Swietenia macrophylla* King**, known as Honduran mahogany, big-leaf mahogany, or American mahogany, grows in lowland humid regions from about 23^0 N on the Atlantic watershed of Mexico through Central and South America to about 18^0 S in Brazil, Bolivia, and Peru (Figueroa, 1994). The trees can reach up to 70 m height and 3.5 m dbh, although the most common size is 40 m height and 1 m dbh, and the average commercial timber is 20 to 30 m height with 60 to 120 cm dbh (Cornelius *et al*., 2004).

Currently, *S. macrophylla* trees are the main source of mahogany wood since *S. mahagoni* (Cuban, Dominican, West Indian, or small-leaf mahogany) and *S. humilis* (dry-zone, Pacific, Pacific Coast, or Mexican mahogany) have been depleted from natural forests (Blundell & Gullison, 2003; Cornelius *et al*., 2004). For international trade, *S. macrophylla* timber is still extracted from natural forests, as it has been for 400 years (Grogan *et al*., 2002). In natural forests, the extremely low density of 0.1 to 3.0 commercial trees ha^{-1} increases its risk of depletion (Navarro *et al*., 2004).

Swietenia macrophylla is widely distributed and planted both in native and exotic locations in over 40 countries across the tropics (Nair, 2002). More than 190,000 ha have been established in Southeast Asia and Oceania along with some plantations in Mexico and Guatemala (Nair, 2002). However, the establishment of extensive plantations has been precluded by *H. grandella* attack.

***Toona ciliata* M. Roem**, known as Australian red cedar, is one of the most important tropical and subtropical timber-producing species (Edmonds, 1993), distributed from India and Southern China, through southeast Asia and New Guinea to the east coast of Australia (Chowdhury, 2004). This species is also considered as a multipurpose tree: ornamental, medicinal, and edible; leaves are used as a vegetable in Malaysia and China, or as animal fodder in India (Edmonds, 1993). Flowers contain nyctanthin, quercitin, and a flavone used in red and yellow dye production in India; and the bark is used in tanning and leather work.

Also, cut branches hanging in storage facilities are used to discourage pests (Edmonds, 1993).

The trees are extremely fast-growing, attaining up to 3.3 m per year in 4.5 years in Costa Rica (Otárola *et al*., 1976) and can grow fast under shade. Trees can reach 50 m height with a 3 m dbh. The stem is straight and 75% without branches, with basal buttresses present.

***Khaya senegalensis* (Desr.) A. Juss.**, known as African mahogany, is an important tropical timber species used for centuries in the making of furniture and indoor decoration providing the best surface finishing of all the African mahoganies. The strength of this species compares favorably with that of American mahoganies (*Swietenia* spp.). Besides timber, the species is important as medicinal (bark), fodder (leaves), and edible plant (oil from seeds for cooking) (Joker & Gaméné, 2003).

Khaya senegalensis is naturally distributed from Senegal to Sudan and Uganda in the African rainforest zone and grows in riparian forests and scattered in high-rainfall savannah woodland. This species grows from 0 to 800 m altitude in areas with 700 to1750 mm annual precipitation and a dry season of 4 to 7 months.

Hypsipyla spp. as pests

The shootborer problem has confronted tropical foresters for as long as they have tried to grow mahoganies (*Swietenia* spp. and *Khaya* spp.) (Speight & Wylie, 2001), cedars (*Cedrela* spp. and *Toona* spp.) and other valuable Meliaceae species, primarily in the subfamily Swietenioideae, in their native areas (Newton *et al*., 1993). Once the insect invades a mahogany plantation, it can increase quickly and all trees in the plantation can be attacked within a year or so (Matsumoto & Rubinsin, 1990).

Hypsipyla spp. are of great economic importance because they attack trees during the time of maximum growth (Grijpma, 1974a). The larva deforms young saplings by boring into the main shoot, killing the apical meristem (Grijpma, 1974b). Infestation by the shootborer does not kill the trees, but attacking the apical shoot results in multiple shoot growth and forked trunks on the trees (Grijpma, 1976). Multiple trunks cause poorly shaped trees to develop, which are of little use for lumber.

***Hypsipyla* spp. biology and ecology**

Eleven species of *Hypsipyla* have been recognized in the World (Horak, 2000), of which *H. grandella*, *H*. *ferrealis*, *H*. *dorsimacula*, and *H*. *fluviatella* are indigenous in America (Becker, 1976); *H. grandell*a (Zeller) is found throughout Central and South America (except Chile), and inhabits on many Caribbean Islands and the southern tip of Florida (Becker, 1976). Among the seven *Hypsipyla* species reported in the Old World (Horak, 2000), only two are well identified. *H. robusta* (Moore) is widely distributed throughout West and East Africa, India, Indonesia, Australia and South East Asia (Roberts, 1968), whereas *H. albipartalis* is found in Uganda (Horak, 2000).

Hypsipyla grandella and *H*. *robusta* have a wide geographic distribution, damaging native Meliaceae species and preventing their normal growth (Yamasaki *et al*., 1990). Although their biology is similar to a large extent, hereafter I will refer to *H. grandella.*

The *H. grandella* female is much larger than the male, and its abdomen can distend to contain up to 1000 eggs (Grijpma & Roberts, 1975). Eggs are laid singly (Holsten, 1976), or occasionally in clusters of two to seven (Roovers, 1971), usually in concealed positions in shoots, stem, and leaf axils. Oviposition occurs during the evening or early morning, and eggs hatch at night three days later (Holsten, 1976).

Larvae can develop through five to six stages, lasting about 30 days in total (Taveras *et al*., 2004). The young larva inhabits the main shoot, whereas older larvae inhabit the bottom of the stem, in young trees. They rarely colonize the roots (Yamasaki *et al*., 1990). Larvae are also known to feed on bark, flowers, fruits and seeds of their hosts (Cibrián *et al*., 1995). When starving, the stronger larvae become cannibalistic, devouring their weaker or less matured siblings (Ramírez-Sánchez, 1964).

Larval development takes longer at temperatures lower than 20^0C (Taveras *et al*., 2004a). Larvae pupate in cocoons within stem tunnels (Ramírez-Sánchez, 1964). Pupation lasts about 10 to 12 days (Taveras *et al*., 2004a) depending on temperature.

In Turrialba Costa Rica, Taveras *et al*. (2004b) determined that *H. grandella* was present in the field all year round, and its abundance mainly depended on shoot availability, predation, parasitism and cumulative temperature. Therefore, when shoots production decreases in their host trees, population drastically declines (Yamasaki *et al*., 1990; Newton *et al*., 1998).

Management of *Hypsipyla grandella*

Research efforts looking for management methods of this insect pest have been considerable (Lamb, 1966; Grijpma, 1974a, b; Whitmore, 1976; Newton *et al*., 1993, 1999; Mayhew & Newton, 1998).

After more than eight decades of research in some 23 tropical countries, an economically viable and environmental-friendly chemical management approach to prevent economic damage by *Hypsipyla* spp. is still lacking.

Regarding chemical control, Wylie (2001) suggested that it may be applicable in nurseries, where the size of plants allows such control, making it operational and economically viable. Since instar I larva feeds on leaves for a brief period before boring into

the main shoot or fruits (Ramírez-Sánchez, 1964), insecticide applications could reduce its populations. However, some inconveniencies are the high cost associated with repeated applications over many years, as well as operative factors such as fast penetration of larva on shoots after egg hatch, leaching of the product by rainfall, and unsuitable application methods when trees grow very tall (Newton *et al*., 1993).

In fact, the main conclusion of an international workshop on *Hypsipyla* spp. held in Sri Lanka in 1996 was that this pest must be managed by an integrated program aiming at its prevention (Floyd & Hauxwell, 2001), which agreed with the suggestions of Grijpma (1974b) and Whitmore (1976). Therefore, an integrated *H. grandella* management should comprise biological control, tree breeding and silvicultural practices.

Regarding biological control, about 40 insect species have been identified as natural enemies of *H. grandella* in America (Sands & Murphy, 2001). In Turrialba, Costa Rica, Taveras *et al*. (2004b) found that 48% of larval mortality was due to natural mortality agents, both biotic and abiotic. Although they regulate *H. grandella* populations to some degree, natural enemies seem to lack promise as a control alternative (Sands & Murphy, 2001).

Swietenia macrophylla and *C. odorata* breeding has been based on tests of provenances and progenies, which have resulted in some selected materials which show considerable variation in growth as well as in resistance to *H. grandella* (Cornelius & Watt, 2003; Navarro *et al*., 2004). Research to identify genetic strains of these resistant trees or from trees that overcome attack is being developed by molecular biology (Gillies *et al*., 1997, 1999; Novick *et al*., 2003).

Silvicultural practices used in the management of *H. grandella* are focused in reducing the incidence and severity of attacks in plantations, and begin with the choice of planting site (Newton *et al*., 1993). Planting at high densities or under the shade of natural forest promotes apical growth (Cornelius *et al*., 2004). Also, pruning of damaged trees improve the form and increase the growth of trees (Cornelius, 2001).

Resistance in Meliaceae against *Hypsipyla* spp.

Certainly, potential for host-plant resistance by exotic plant species exists. For instance, *T. ciliata* from Australia and *K*. *ivorensis* from Africa are not attacked by *H*. *grandella* in Central America where native *Cedrela* spp. and *Swietenia* spp. are seriously affected (Grijpma, 1976). Moreover, *Chukrasia tabularis* A. Juss. was susceptible, although *T*. *ciliata* (both exotic) was immune to attacks of *H*. *grandella* in Puerto Rico (Grijpma, 1976). This author also stated that resistance differs among native species. *Hypsipyla grandella* prefers *C*. *odorata* over *Swietenia* spp., whereas *H. robusta* prefers *T. ciliata* and *K. senegalensis* over the other *Khaya* spp. (Roberts, 1968).

Specific physical host defenses. In the case of *H. grandella* attack, the tree's physical size and bark characteristics may be important to reduce, or to recover from, damage (Newton *et al*., 1999). Due to size and leaf biomass, older trees can sustain a level of insect infestation that would deform or kill younger trees. The main problem with mature trees is that they are able to act as pest reservoirs for neighboring younger trees (Speight, 1997).

Other physical features to prevent plant consumption are stem number and rigidity, leaf toughness, and pubescence (Speight & Wylie, 2001). For Meliaceae, stem number and rigidity may also affect the probability that stem boring insects cause stems to break or fall over when damaged (Matsumoto & Rubinsin, 1990).

The amount of foliar pubescence has been found to be important for oviposition in a number of instances. Some insect species require relatively glabrous surfaces, whereas others prefer heavily pubescent oviposition sites (Beck, 1980). Species in the genus *Cedrela* have many trichomes on the leaves and mainly on the apical bud, whereas species in the genus *Swietenia* have glabrous leaves and shoots (Pennington, 1981).

Specific chemical host defenses. For some Meliaceae species, limonoids, alkaloids and phenolics have been identified to affect several insect pests including *H. grandella*. The family Meliaceae is recognized by the common characteristic of having oxidized triterpenes, which have been known as meliacions, triterpenoids or limonoids (Taylor, 1981).

Studies on the biological activity of meliaceous limonoids are limited because of their complex chemical structures (Champagne *et al*., 1992), but in general they serve as deterrents to insect feeding or oviposition (Gershenzon & Croteau, 1992). These compounds have been found in 51 species from 20 genera in eight tribes (Chatterjee *et al*., 1971). Some examples are: gedunin in *Entandrophragma angolense* Tiama; dihydrogedunin in *Guarea thompsonii* Sprague & Hutch.; havanensin and prieurianin in *Trichilia* spp.; obacunol in *Lovoa trichilioides* Harms; mexicanolide in *C*. *odorata*; cedrelone in *T. ciliata*; and azadirachtin in *Melia azederach* and *Azadirachta indica* species (neem tree).

The best well-known limonoid is azadirachtin, which is toxic or growth disrupting against nearly 200 insect and mite species (Saxena, 1989; Champagne *et al*., 1992). Also, commercial neem products (Azatin and Nim 80) proved to be toxic to *H*. *grandella* when incorporated in diet mixtures (Mancebo *et al*., 2002).

Other chemicals found in Meliaceae are phenolics, such as the furanocoumarin bergapten in *T*. *ciliata* and the flavonoids quercitin and kaenferol in *C*. *odorata* and *T*. *ciliata* (Chatterjee *et al*., 1971). Since furanocoumarins are potent feeding deterrents to certain insect species, bergapten might cause resistance of *T*. *ciliata* to *H*. *grandella*, but not to *H*. *robusta* (Schoonhoven, 1972).

Differences in relative plant susceptibility may be attributed to genetic variability either in the insects or in the introduced Meliaceae species, as well as to adaptation of insects to introduced host plants (Grijpma, 1976). Also, the presence or absence of certain plant chemicals at an intraspecific level may be a principal cause for resistance or

susceptibility (Chatterjee *et al*., 1971), as happens for *Cedrela* spp. and *T. ciliata* (Grijpma, 1976).

For *T*. *ciliata,* the chemical compounds responsible for the resistance to *H*. *grandella* may be alkaloids (Grijpma, 1976), which have been detected in aqueous ethanolic extracts (Smolenski *et al*., 1974). Such extracts have been observed to deter feeding, retard growth, and have toxic effects (Grijpma & Roberts, 1975). Moreover, after force-feedig of instar VI *H*. *grandella* larvae with aqueous extracts of *T. ciliata* leaves, larvae spit and regurgitate a brown liquid, which gave an alkaloidal precipitate when mixed with a solution of tannin (Grijpma, 1976).

Grafting

Grafting is a widely used means of plant propagation and plant development control (such as blooming and growth) that is of considerable economic and horticultural importance (Hartmann *et al*., 2002).

Grafting is the combination of two or more plants: the rootstock provides the root system, and the scion produces the commercial crop (Errea *et al*., 2001). However, grafting is a tedious procedure, and it may impose limitations when performed on a commercial scale. In addition, some grafts fail to work due to possible incompatibility, especially for interspecific combinations of plants (Errea *et al*., 2001). Intergeneric combination of plants has even lower chances or success (Hartmann *et al*., 2002)

Generally, the more closely related the plants are botanically, the better the chances are for the graft union being successful due to similarities in their cambia (Hartmann *et al*., 2002). A graft union is considered successful and complete when several functional phloem and xylem connections form across the graft interface (Gebhart & Goldbach, 1988; Schöning & Kollmann, 1997). Although incompatibility could be expressed months or years

later (Hartmann *et al*., 2002), for woody species phenolic content in callus tissues *in vitro* could be a good system to early detection of such incompatibility of grafts, such as those grafts using apricot *Prunus armeniaca* on *Prunus* spp. (Errea, 2001). This author stated that different phenolic content in graft partners might imply metabolic disfunctions at the graft union and diverse phenolics have been implicated in process of cell division intensity, development and differentiation in new tissues on the graft union.

The process to form a successful graft union is as follows (Hartmann *et al*., 2002):

- Establishment of an intimate contact between the cambial regions of rootstock and scion and production of callus tissue (parenchymatic cells) by both components of the graft in the cambial region.
- Differentiation of certain parenchyma cells of the callus into new cambial cells connected to the original cambium both in the rootstock and in the scion.
- Production of new vascular tissues (xylem and phloem) by the new cambium, forming the vascular connections necessary for a successful graft union.
- Formation of a new secondary vascular cambium to continue production of new secondary xylem and phloem cells.

The transport of assimilates across the graft union has been demonstrated with whole-plant autografts and heterografts using ^{14}C-labelling techniques (Rachow-Brandt & Kollmann, 1992). Also, alkaloids are translocated between stock and scion for intergeneric grafts of Solanaceae (Lowman & Kelly, 1968).

The rate of tomato seed germination and the size and vigor of resulting seedlings are influenced by the rootstock (various species of Solanaceae) upon which tomato scions have been grafted (Detjen, 1943). Even though these results could be attributed to increased plant vigor affecting the quality of seeds, graft-induced genetic changes have been obtained

for some crops such as pepper, eggplant, tomato, tobacco, soybean and mulberry in Japan (Hirata *et al*., 2002). They have demonstrated the existence of gene transfer from rootstocks to scions by molecular analysis of graft hybrid lines and intact plants and attributed such genetic modification to cytoplasmic variation, which would constitute a new gene introduction approach for plant species.

For *Prunus cerasifera* grafted onto *P. armeniaca*, Li *et al*. (2002) by using RAPD (randomly amplified polymorphic DNA) markers, detected scion DNA in plants produced from rootstocks indicating direct transfer of DNA from the scion to the rootstock causing changes in several characteristics such as leaf color and shape, as well as flowering habit. Such characteristics induced by grafting were stable when branches taken from the new plants were grafted on peach and plum (*Prunus* spp.) rootstocks.

Concerning grafting in Meliaceae species. *Toona ciliata* introduced and grown in Costa Rica was not attacked by *H. grandella* (Grijpma, 1974b), whereas *Cedrela* spp. grown in the New South Wales, Australia, were not attacked by native *H*. *robusta* (Bygrave & Bygrave, 1998). In the latter place, *C*. *fissilis* scions grafted on *T*. *ciliata* rootstock did not show evidence of *H*. *robusta* attack for about eight years, whereas *C. odorata* grafted on *T. ciliata* and *T*. *ciliata* control trees were attacked to varying degrees (Bygrave & Bygrave, 2001).

Grafted plants with *C. odorata* on *T. ciliata* were found to be resistant to *H*. *grandella* although to a lesser extent than the intact *T. ciliata* plants (Grijpma, 1976). The resistance transferred in grafted *C. odorata* suggested that the plant constituent(s) responsible for this resistance could be translocated from the *T. ciliata* rootstock to the *C. odorata* scion (Grijpma & Roberts, 1975).

Grafted plants with *C*. *odorata* on *T*. *ciliata* were used in Brazil to analyze chemical compounds that might be responsible for the transferred resistance of *C*. *odorata* against *H*. *grandella* (Da Silva *et al*., 1999). Substances directly responsible for rootstock resistance to

the insect are currently unknown, but some secondary compounds, possibly limonoids are candidates (Agostinho *et al*., 1994). However, limonoids seemed dissociated with the resistance of *C*. *odorata* grafted onto *T*. *ciliata* against *H*. *grandella* since the type of limonoids detected on the scion are produced by both *C. odorata* and *T. ciliata* plants (De Paula *et al*., 1997). Instead, they attributed the resistance of the grafted plants to cycloartanes and catechin (precursors of proantocyanidins, which are phenolic compounds) since both of them were found on the *C*. *odorata* scion and are commonly produced by *T*. *ciliata* but not by *C. odorata*. However, differencial damage by *H. grandella* among several *C. odorata* provenances from Costa Rica was attributed to proantocyanidins content by Newton *et al*. (1999)

Micropropagation of tropical trees

Methods of vegetative propagation for mature trees must be developed for forest tree improvement, because the potential for obtaining better trees for plantations through sexual breeding is limited (Bonga, 1985).

For most tree species, variation in the wild as well as in genetically improved populations is relatively high. This large variation can be exploited in clonal propagation of juvenile as well as mature trees (Bonga & Von Aderkas, 1992). Nevertheless, vegetative propagation of woody plants large enough to have demonstrated their economic potential or pest resistance is often difficult or impossible with the traditional methods of rooting stem cuttings or grafting (Bonga, 1985), although exceptions exist such as eucalypts which grow really fast.

Woody species in the juvenile phase are generally easy to clone by conventional techniques; when *in vitro*, the micronutrient requirements of the cultures become more specific with explant maturity (Monteuuis, 1991). When natural sources of juvenile tissues

are absent, the adult plant must be rejuvenated (i.e., caused to express juvenile characteristics) for example by pruning (Pliego-Alfaro & Murashige, 1987).

Research on plant resistance to insects is changing with the advent of biotechnology, molecular biology, genetic engineering and tissue culture techniques (Cheliak & Rogers, 1990). Genetic engineering currently depends on plant tissue culture (PTC) and micropropagation methods in order to regenerate transformed (resistant) plants.

Plant tissue culture is the process whereby small pieces of living tissue (explants) are isolated from the plant and grown aseptically for indefinite periods on a nutrient medium. One technique of PTC is callus culture, which permits regenerating many propagules of callus from a single explant. A change in plant growth regulators added to the culture medium can result in callus cultures forming shoots (organogenesis). Callus culture has potential use for developing insect resistant plants. However, the callus culture technique has yet to be achieved for *C. odorata*, but it was achieved for *S. macrophylla* (Maruyama, 2006).

An alternative is micropropagation by axillary shoot culture. This technique possess a key role in the cloning of selected woody plants that have demonstrated their potential (Bonga, 1985), such as insect resistance. However, woody plants are difficult to establish *in vitro*, mainly when explants are taken from mature plants. Thus, juvenile explants are beneficial for successful micropropagation (Oria Coria & Villalobos, 1985).

Genetic engineering

Genetic engineering technologies have been increasingly used, either to modify the expression of specific genes of interest in plants or to introduce specific new traits that are unavailable within the breeding population or genetic resource (Walter, 2004). So far,

breeding for *C. odorata* and *S. macrophylla* has comprised the selection of trees among progenies of selected parents. Nevertheless, this selection process is time-consuming due to the long biological span of forest species (Cheliak & Rogers, 1990). Actually, the forest breeding goals are mainly oriented towards the introduction of pest resistance genes (Leple *et al*., 1992).

Genes providing resistance against insects and pathogens can be transferred to forest trees (Walter, 2004). The gene for the *Bacillus thuringiensis* toxin (CryIAc), for example, has been used in tree genetic engineering and has rendered trees resistant to attacks by certain lepidopteran pests (Shin *et al*., 1994; Tang & Tian, 2003).

The use of genetic engineering to introduce resistance genes into *C. odorata* or *S. macrophylla* would be important to reduce the time required for the creation of new resistant individuals. Irrespective of the DNA transfer method used, the rate-limiting step in the development of transgenic plants is the ability to regenerate them from transformed material under selection (MacRae & van Staden, 1999). Since shoot regeneration of both species from *in vitro* germinated seedlings has been established (Orellana, 1997; Pérez *et al*., 2001), and somatic embryogenesis has been reported for *S. macrophylla* (Maruyama, 2006), genetic transformation (e.g., *Bt* gene introduction) for both species could be possible. In this way, transformed plants could be used in an integrated management program to face the *H*. *grandella* problem.

With basic knowledge of the problem and its background, and to address the objectives of the present dissertation, several bioassays were completed in greenhouse and laboratories at CATIE. In the following chapters 2 and 3, I report the study of the effect of grafting on damage by different *H. grandella* larval as well as the effect on larval performance and mortality, by using whole plants in greenhouse and a leaf disk bioassay. In chapter 4 I report the effects of crude extracts and alkaloids, limonoids and phenolics

fractions on larval mortality and performance of *H. grandella*, detected by leaf disk bioassays. In the chapter 5, several experiments are presented on efficiently establishing *C. odorata* and *S. macrophylla in vitro*. Finally in the chapter 6, the main findings of this dissertation are summarized, followed by some suggestions for using these results and possible future research to improve the knowledge obtained from this research and focused on control of *H. grandella*.

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CHAPTER 2

A grafting approach to prevent *Hypsipyla grandella* (Zeller) (Lepidoptera: Pyralidae) damage to New World Meliaceae species. I. Tests on whole plants

Running head: Grafting approach to prevent H. grandella damage: tests on whole plants.

Key words *Cedrela, Swietenia, Khaya, Toona,* grafting, mahogany shootborer, damage, larval performance.

Abstract

1 *Cedrela odorata* and *Swietenia macrophylla*, either susceptible to *Hypsipyla grandella*, were grafted onto resistant species (*Khaya senegalensis* and *Toona ciliata*). Four-month-old grafted plants were then compared to their reciprocal graft and to both intact and autografted plants for damage due *to H. grandella* larvae and for their effects on larval performance.

2 Damage was assessed as the number of frass piles, number and length of tunnels, number of damaged leaves, and damage to the apical bud. Larval performance was evaluated in terms of time to reach pupation and pupal weight and length.

3 Two types of experiments were conducted, with the main plant shoot inoculated on the apical bud either with eggs or instar III larvae. In both cases, plant damage differed significantly ($P \leq 0.03$), few larvae reached pupation, and pupal weight and length of surviving larvae was similar each other.

4 Resistance conferred was by resistant rootstocks to susceptible scions and, autografted susceptible species and susceptible grafted on resistant species had similar damage. In both experiments, grafting by itself, regardless of the rootstock and scion combination, also reduced damage caused by *H. grandella* larvae.

5 Damage on autografted *C. odorata* was intermediate between that on both intact *C. odorata* and *C. odorata* grafted onto *T. ciliata* plants. The grafting procedure appeared to inhibit *H. grandella* larval performance even for susceptible autografted species.

Introduction

High-quality timber from Spanish cedar *Cedrela odorata* L. and mahogany *Swietenia macrophylla* King is of major significance for economies in many neotropical countries (Newton *et al*., 1993). Unfortunately, natural populations of these species are being reduced quickly due to selective harvest (Albert *et al.*, 1995). In addition, the mahogany shootborer, *Hypsipyla grandella* (Zeller) (Lepidoptera: Pyralidae), has limited their establishment in commercial plantations in Latin America, as its larva mainly feeds on apical shoots, inducing branching on the trees and rendering the timber unmarketable (Grijpma, 1971).

Exotic Meliaceae species are less susceptible than indigenous ones to attacks by native *Hypsipyla* spp. (Cunningham *et al*., 2005). For example, Australian red cedar *Toona ciliata* M. Roem., closely related to *Cedrela* spp., is heavily attacked by *H. robusta* Moore when growing in its Old World native habitats (Bygrave & Bygrave, 1998) but is not attacked by *H*. *grandella* when planted in Central America (Grijpma, 1976). Conversely, in Australia, imported *Cedrela* spp. are not attacked by *H*. *robusta* (Bygrave & Bygrave 2001). Moreover, *S. macrophylla* and the African mahogany *Khaya senegalensis* (Desr.) A. Juss., have been reported to suffer less damage by either *H. robusta* or *H. grandella*, respectively.

This reduced susceptibility of exotic Meliaceae to native *Hypsipyla* species (Manso, 1974; Grijpma, 1976; Agostinho *et al.*, 1994) may allow generation of resistant native Meliaceae trees to *H*. *grandella* by grafting susceptible scions onto resistant, exotic rootstocks. For instance, in Costa Rica, *C. odorata* shoots grafted onto *T. ciliata* were shown to be resistant, although resistance was lower than that for *Toona* trees themselves

(Grijpma, 1976). Also, in New South Wales, *C*. *odorata* and *C*. *fissilis* scions grafted onto *T*. *ciliata* differed in attacks by *H*. *robusta*, being more damaged the scions from *C. odorata* than scions from *C. fissilis*, which indicated different level of resistance between species of the same genus (Bygrave & Bygrave, 2001). Resistance of grafted plants strongly indicates that some substances are translocated from the *Toona* rootstock to the *Cedrela* scion (Grijpma & Roberts, 1975). Such translocation was confirmed by Da Silva *et al*. (1999) who found cathechin (i.e., a phenolic compound) in scions of *C. odorata* grafted onto *T. ciliata* rootstocks even though catechin is synthesized only in *T. ciliata* plants.

So far, grafting has been examined by using combinations of only *Cedrela* spp. and *T. ciliata*, disregarding important species such as *S. macrophylla* and *K. senegalensis*, the four of them belonging to the subfamily Swietenioideae (Pennington & Styles, 1975). In addition, the effects of reciprocal grafting and autografting of these four species are unknown.

Therefore, the objective of this study was to determine the effect of grafting different combinations of susceptible and resistant Meliaceae species to damage by *H. grandella*. The hypotheses tested were: 1) exotic species (*K. senegalensis* and *T. ciliata*) are resistant to *H. grandella* attack; 2) autografting confers resistance; 3) resistant rootstocks confer resistance to susceptible scions; and 4) susceptible rootstocks do not affect the resistance of exotic resistant scions.

Methods

Site description

Research was conducted at the Cabiria Experiment Station, within the premises of the Tropical Agricultural Research and Higher Education Center (CATIE), in Turrialba, Costa Rica. CATIE is located in the Caribbean watershed of this country, at 602 m altitude, within

the premontane wet forest life zone (Tosi, 1969). Average annual values for climatic variables are 2600 mm rainfall, 22^0C, 88% RH, and 17 MJ m^{-2} of solar radiation (Salas, 2000).

Plants and grafting treatments

Seeds of the susceptible species *C. odorata* and *S. macrophylla* from Pococí, Costa Rica, as well as the resistant *K. senegalensis* from Burkina Faso, and *T. ciliata* from Australia, were provided by the Forest Seed Bank at CATIE. Seeds were sown at CATIE's nursery, and then plants were kept inside a greenhouse used to acclimatize coffee plants. Six- to 12-month-old plants were grafted at the nursery by the wedge grafting technique (Bygrave & Bygrave, 1998). The grafting point was 20 to 30 cm above the soil. Rootstocks were less than 1 cm in diameter at the grafting point, whereas the scions were slightly thinner. Grafted plants were maintained in the same greenhouse until the scions grew and developed at least 10 leaves (ca. four months).

Species susceptible to *H. grandella* were grafted onto resistant ones. Also, reciprocal combinations (i.e., resistant scion onto susceptible rootstock) and autografting (scion and rootstock from the same species) were completed. Intact (i.e., non-grafted) and autografted plants were used as controls. The aim was to have a full set of combinations, but some grafts were unsuccessful or unready (e.g., *C. odorata* onto *K. senegalensis*, *K. senegalensis* onto *C. odorata*, or *S. macrophylla* onto *T. ciliata*) at the time when experiments were completed.

Experiments

Two types of experiments were carried out, with the main plant shoot inoculated either with eggs or instar III larvae (8 to 16 mm long). Instar III larvae were used since they are easily handled for experimental purposes. Instar I larvae emerging from eggs are more

representative of the initial response of host plants to *H. grandella*, either intact or altered by grafting, but are more difficult to manipulate.

Eggs and larvae for experiments were taken from a colony kept at the Entomology Laboratory at CATIE, and established in 1998 from field-collected larvae feeding on *C. odorata*. Larvae in the colony are normally fed with tender *C. odorata* leaves from instars I to III, and then placed onto an artificial diet (Vargas *et al*., 2001) until pupation. Pupae are then moved to a metal framed cage covered with fine mesh, kept at a greenhouse, where adults emerge, mate and oviposit. Eggs are collected and taken to the laboratory to sustain the colony.

Experiment I: *H. grandella* eggs. The experiment was conducted from April 23 through June 15, 2004. Plants were carefully inspected to preclude predation by ants, wasps or spiders. Predation was prevented by closing the sides of the greenhouse with a plastic shade net (50 to 60% of full sunlight) and by smearing a sticky substance, Tanglefoot (The Tanglefoot Co., Grand Rapids, MI) around tree stems 10 to 15 cm above the ground).

Four-day-old *H. grandella* eggs were placed on the main shoot, using a thin paintbrush, between 16:30 to 17:00, which is the time of oviposition in nature (Ramírez-Sánchez, 1964). Egg age was appraised by color, as they are white just after oviposition and turn to red before hatching on the fifth day (Taveras *et al*., 2004).

The experiment consisted of a completely randomized design, with three to five replications depending on the availability of plants. The experimental unit was an individual plant with three *H. grandella* eggs. Three eggs were used to ensure the presence of at least one larva per plant.

From days 2 to 15 damage was appraised daily by counting the number of frass piles (mounds of sawdust and silk), number and length of tunnels in the main or lateral buds and in shoots, number of damaged leaves due to larval feeding (whether on petioles or leaflets)

and damage to the apical bud (scored at 0 for intact and, 1 for either partially or fully consumed bud). Tunnel length was determined by dissecting the buds or the shoots, and measuring them; an average tunnel length per plant was calculated afterwards.

In addition, on day 15, the number of surviving larvae was recorded and individual larvae were transferred to vials with artificial diet (Vargas *et al.*, 2001). Vials were kept inside an environmental chamber Percival I-35L (Boone, Iowa) at 25^0C, 80 to 90% RH, and 12:12 L:D, to detect any sublethal effects, by recording the time to reach the pupal stage (days from oviposition to pupation), as well as pupal length (mm) and weight (mg). Pupation was considered completed when pupae turned dark brown, so that they could be weighed and measured without stress or injury.

Experiment II: *H. grandella* third-instar larvae. The experiment was conducted from November 25, 2004 through January 18, 2005. Plants were 16 months old, including the four months from grafting. Predation was prevented as in experiment I. Two instar III larvae were placed on the main plant shoot by using a thin paintbrush. The experiment consisted of a completely randomized design, with three to six replications depending on the availability of plants. The experimental unit was an individual plant with two larvae. Variables appraised were the same as those in experiment I.

Statistical analysis

Since the number of eggs or larvae was higher than the one required by *H. grandella* to cause damage, the number of surviving larvae was considered as a covariate for all the other variables. In both experiments, data were examined for conformity to assumptions required for analysis of covariance (ANCOVA). If necessary, data were transformed by Y = sqrt(Y + 0.5) to meet these assumptions.

Analysis of covariance, was completed using the GLM procedure in SAS (SAS, 2001) and followed by orthogonal contrasts. Contrasts for both experiments were: 1) intact susceptible vs. intact resistant species; 2) autografted susceptible vs. autografted resistant species; 3) autografted susceptible vs. susceptible grafted on resistant species; 4) autografted resistant vs. resistant grafted on susceptible rootstock species.

Apical bud damage was analyzed by a Chi-square test to examine the hypothesis that its occurrence was similar among the species tested.

Results

Damage on plants

In both experiments, plant species, whether grafted or intact, significantly affected the number of frass piles, tunnel length and number of damaged leaves, whereas tunnel number differed only in experiment I (Table 2.1). Results from the ANCOVA and orthogonal contrasts for each variable are summarized below.

The number of frass piles in both experiments was much lower for resistant than for susceptible species, being almost nil for the resistant ones (Fig. 2.1). The same trend held when comparing frass piles on the autografts, although the difference was reduced for the previous comparison for both experiments.

In both experiments none of the other contrasts were significant. In the first case, autografting susceptible species reduced the number of frass piles to a level as low as that of the susceptible species grafted onto the resistant ones (Fig. 2.1). In the second case, the number of frass piles was almost nil for both autografted resistant species and resistant species grafted onto susceptible ones.

Even though the number of frass piles was almost negligible on the resistant species, larvae occasional attacked them, especially *K. senegalensis*. On the contrary, for

susceptible species, despite the lack of statistical differences in any of the experiments, the number of frass piles consistently tended to be lower on mahogany plants compared to cedar.

Table 2.1 Probability values of orthogonal contrasts and analysis of covariance for variables evaluated in experiments with *H. grandella* and four intact and grafted Meliaceae species.

Experiment I: Eggs placed on plants

Contrast between species		No. frass piles	Tunnel		No. damaged leaves
			No.	Length	
		Probabilities			
Intact susceptible vs. intact resistant species		0.0009	0.0681	0.0053	0.0020
Autografted susceptible vs. autografted resistant species		0.0004	0.0566	0.1502	0.4416
Autografted susceptible vs. susceptible grafted on resistant		0.3869	0.6174	0.4018	0.5486
Autografted resistant vs. resistant grafted on susceptible		0.5959	0.3416	0.9521	1.0000
ANCOVA Statistics	P	0.0001	0.0384	<0.0001	0.0001
	F value	4.12	2.03	4.78	4.16

Experiment II: Third-instar larvae placed on plants

		Probabilities			
Intact susceptible vs. intact resistant species		< 0.0001	0.3727	< 0.0001	0.0002
Autografted susceptible vs. autografted resistant species		0.0309	0.8880	0.0035	0.0486
Autografted susceptible vs. susceptible grafted on resistant		0.9040	0.1891	0.2282	0.4603
Autografted resistant vs. resistant grafted on susceptible		0.8485	0.9949	0.9223	0.6650
ANCOVA Statistics	P	<0.0001	0.3382	< 0.0001	0.0134
	F value	4.21	1.17	3.90	2.28

The covariate for each variable was the mean number of surviving larvae per treatment. d.f. = 12, 1 and 14, 1 for experiment I and II, respectively. Experiment I, intact susceptible species: *Cedrela odorata (C), Swietenia macrophylla (S)*; intact resistant species: *Khaya senegalensis (K), Toona ciliata (T)*. Autografted susceptible species: *C/C*, *S/S*; Autografted resistant species *K/K, T/T*. Susceptible species grafted onto resistant species: *C/T*, *S/K*. Resistant species grafted onto susceptible species: *K/S*, *T/C*, *T/S*. Experiment II, treatments identical as above plus *C/K*, *K/C*.

Even though resistant plants clearly had fewer tunnels than susceptible plants (both intact and autografted), the difference was not significant in statistical terms in experiment I; in fact, the resistant species completely lacked tunnels (Fig. 2.2A). In experiment II, none of the other contrasts was significant (Table 2.1, Fig. 2.2B).

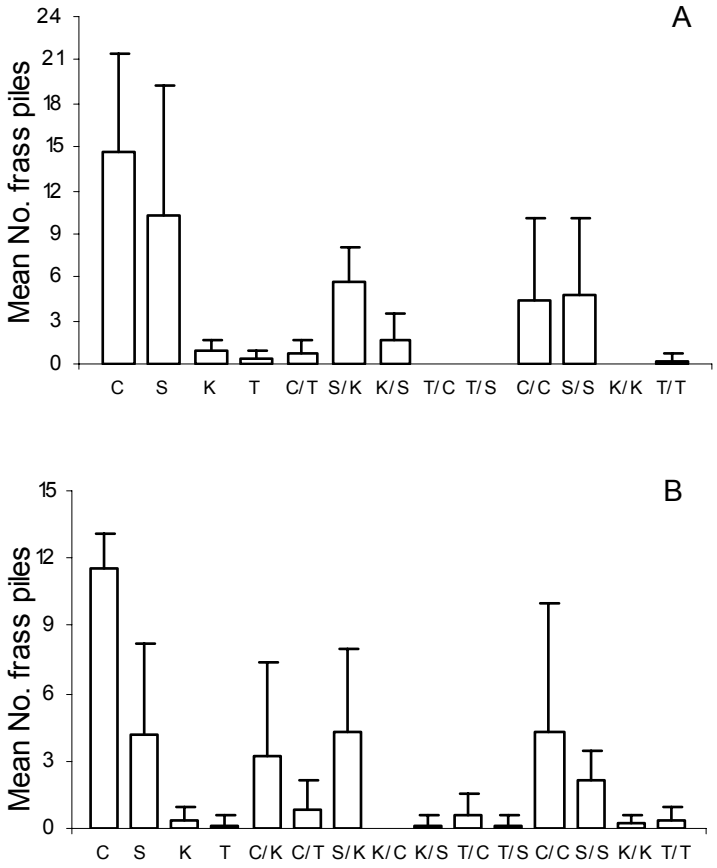

Figure 2.1 Mean number of frass piles per plant for intact and grafted Meliaceae species after inoculation with *H. grandella* eggs (A) or third instar larvae (B). Intact plants: C = *Cedrela odorata*, S = *Swietenia macrophylla*, K = *Khaya senegalensis*, T = *Toona ciliata;* grafted plants: C/K, C/T, S/K, K/C, K/S, T/C, T/S, C/C, S/S, K/K, T/T. Error bars indicate SE, (n = 3 to 6).

On the contrary, tunnel length differed significantly between susceptible and resistant plants in both experiments (Table 2.1, Fig. 2.3), with *C. odorata* having much longer tunnels than *S. macrophylla*; likewise, autografted susceptible plants had longer tunnels than autografted resistant ones only in experiment II. None of the other contrasts were significant. Moreover,

intact *K. senegalensis* and *T. ciliata* intact or used as scions for *C. odorata* and *S. macrophylla*, had only shallow tunnels (i.e., incomplete penetration of the stem).

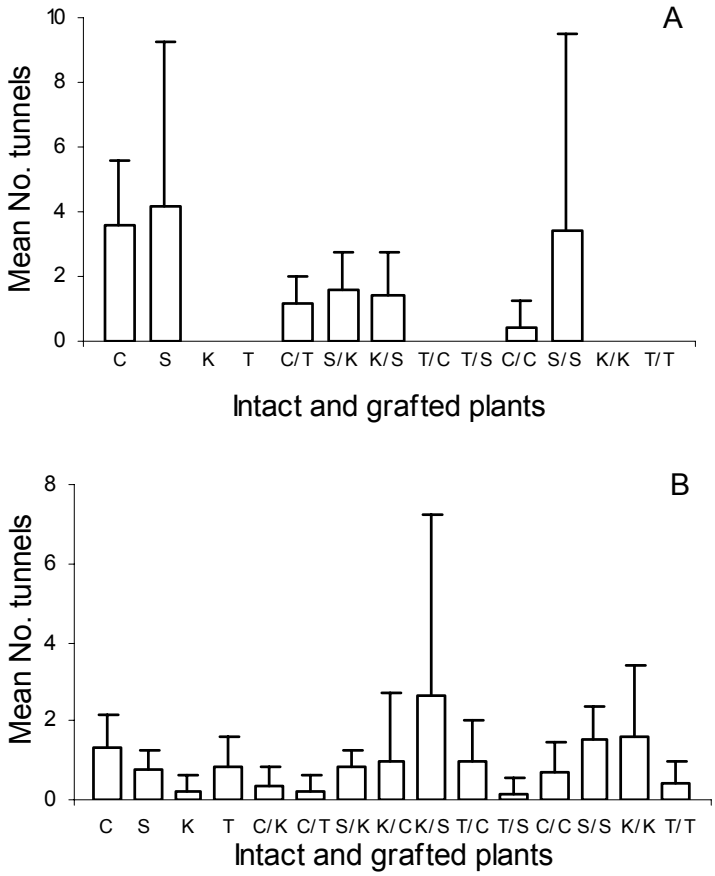

Figure 2.2 Mean number of tunnels per plant for intact and grafted Meliaceae species after inoculation with *H. grandella* eggs (A) or third instar larvae (B). Intact plants: C = *Cedrela odorata*, S = *Swietenia macrophylla*, K = *Khaya senegalensis*, T = *Toona ciliata;* grafted plants: C/K, C/T, S/K, K/C, K/S, T/C, T/S, C/C, S/S, K/K, T/T. Error bars indicate SE, (n = 3 to 6).

The number of damaged leaves varied markedly between susceptible and resistant plants for both experiments (Table 2.1, Fig. 2.4), with *C. odorata* having more damaged foliage than *S. macrophylla*; also, autografted susceptible plants had more damaged leaves than autografted resistant ones only in experiment II. None of the other contrasts were significant.

In both experiments, autografted *K. senegalensis* and *T. ciliata* lacked leaf damage as when they were intact in experiment I (Fig. 2.4).

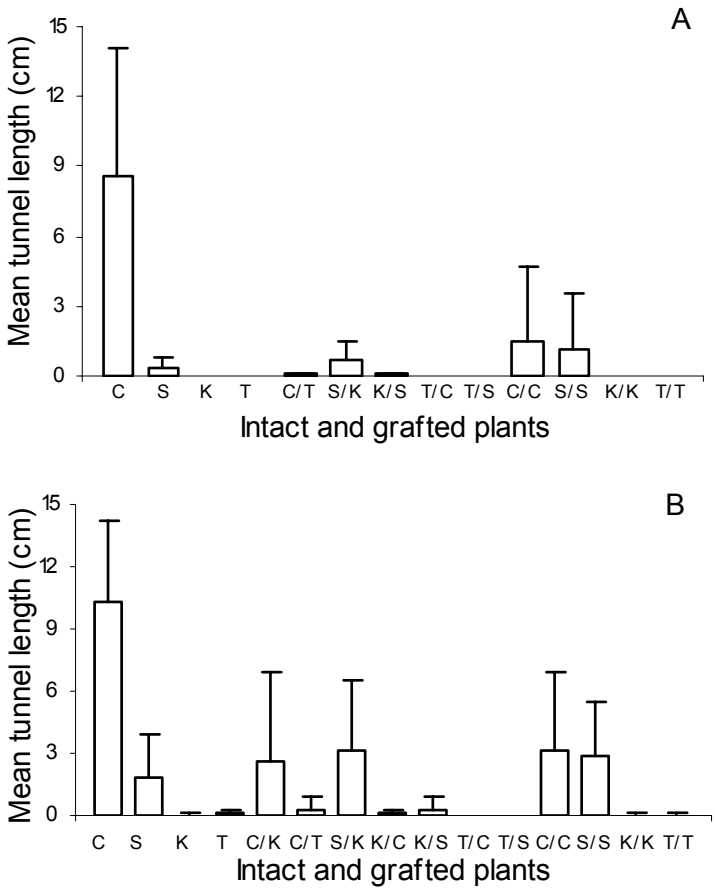

Figure 2.3 Mean tunnel length per plant for intact and grafted Meliaceae species after inoculation with *H. grandella* eggs (A) or third instar larvae (B). Intact plants: C = *Cedrela odorata*, S = *Swietenia macrophylla*, K = *Khaya senegalensis*, T = *Toona ciliata;* grafted plants: C/K, C/T, S/K, K/C, K/S, T/C, T/S, C/C, S/S, K/K, T/T. Error bars indicate SE, (n = 3 to 6).

Apical bud damage differed among treatments (χ^2 = 41.13, 39.29; P < 0.0001, 0.0003; d.f. = 13, 14) for experiments I and II, respectively. Both intact and autografted *K. senegalensis* and *T. ciliata* plants completely lacked damage (Fig. 2.5), whereas intact *C. odorata* and *S. macrophylla* suffered 90 and 100% and 40 and 80% damage in experiments I and II, respectively.

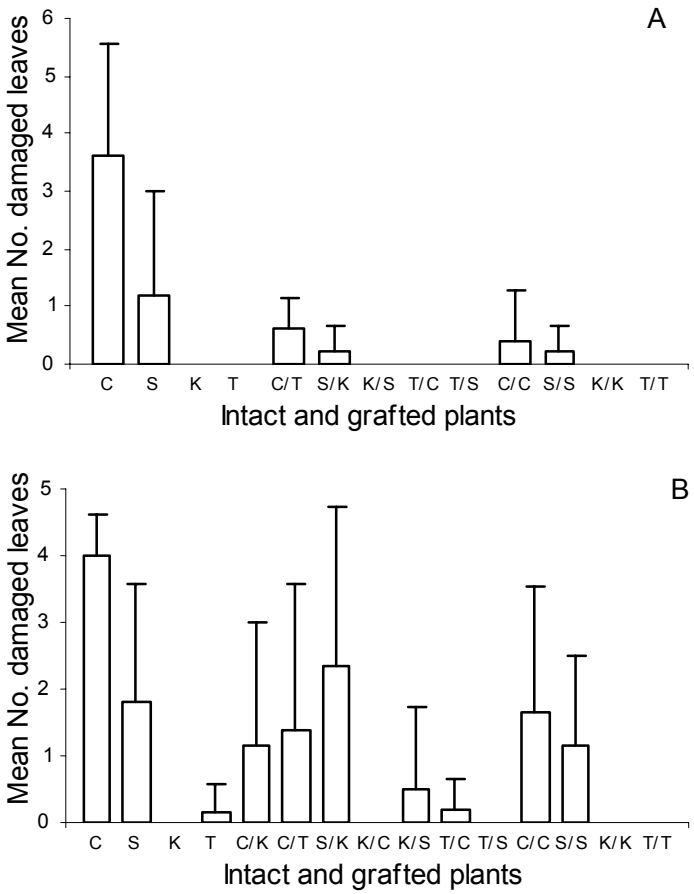

Figure 2.4 Mean number of damaged leaves per plant for intact and grafted Meliaceae species after inoculation with *H. grandella* eggs (A) or third instar larvae (B). Intact plants: C = *Cedrela odorata*, S = *Swietenia macrophylla*, K = *Khaya senegalensis*, T = *Toona ciliata*; grafted plants: C/K, C/T, S/K, K/C, K/S, T/C, T/S, C/C, S/S, K/K, T/T. Error bars indicate SE, (n = 3 to 6).

In experiment I, apical bud damage on autografted *C. odorata* was reduced by 78%, but autografting failed to prevent damage of *S. macrophylla*. When the latter was grafted onto *K. senegalensis*, damage was reduced by 50% with respect to intact *S. macrophylla* plants. Moreover, apical bud damage was reduced by 100% for *C. odorata* scions grafted on *T. ciliata* with respect to damage on intact *C. odorata* (Fig. 2.5A). In experiment II, apical bud damage was reduced by 50 and 16% on the respective autografts of *C. odorata* and *S. macrophylla* with respect to intact plants. Moreover, *C. odorata* grafted onto *T. ciliata* and *T. ciliata* grafted onto *C. odorata*, reduced the apical damage by up to 80% as compared with

intact *C. odorata*. *Cedrela odorata* grafted onto *K. senegalensis* and *K. senegalensis* grafted onto *C. odorata* reduced the apical damage by 67% and 100%, respectively, compared to intact *C. odorata* (Fig. 2.5B).

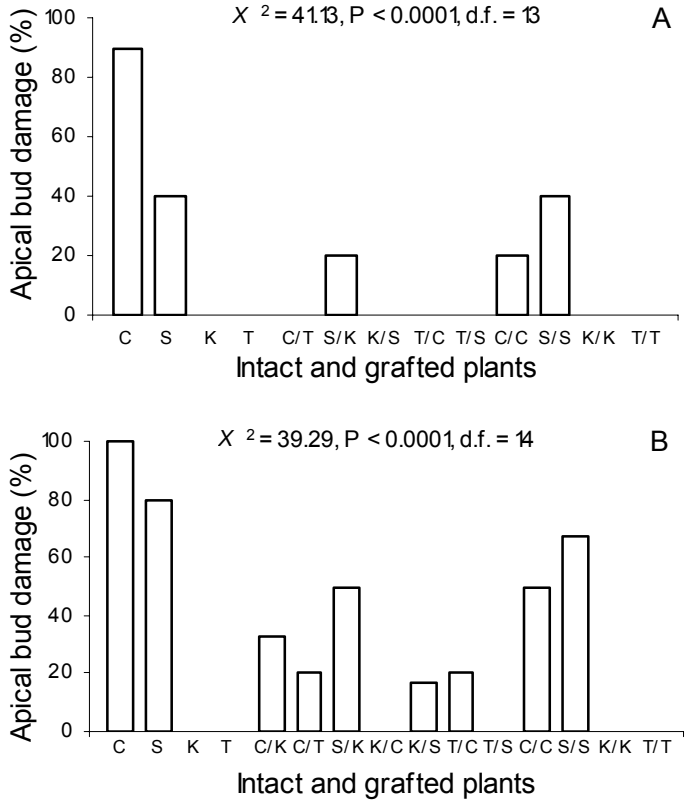

Figure 2.5 Percentage of apical bud damage per treatment for intact and grafted Meliaceae species after inoculation with *H. grandella* eggs (A) or third instar larvae (B). Intact plants: C = *Cedrela odorata*, S = *Swietenia macrophylla*, K = *Khaya senegalensis*, T = *Toona ciliata;* grafted plants: C/K, C/T, S/K, K/C, K/S, T/C, T/S, C/C, S/S, K/K, T/T.

Larval performance

In experiment I, only larvae on intact *C. odorata* plants developed to the pupal stage (i.e., 29%, 7 out of 24 surviving larvae), requiring 32 days. In experiment II, 67% (i.e., 6 out of 9) surviving larvae on intact and autografted *C. odorata,* as well as on the grafted *C. odorata* or *S. macrophylla* onto *K. senegalensis* plants developed to pupa requiring 30 days to do so. Therefore, the statistical analysis was completed only for pupal weight and length in the

experiment II, and these variables were similar among plant species (F = 0.26, 0.39; d.f. = 4, 1; P > F = 0.88, 0.81, respectively). Orthogonal comparisons were not completed due to high larval mortality mainly on *T. ciliata* plants (Fig. 2.6).

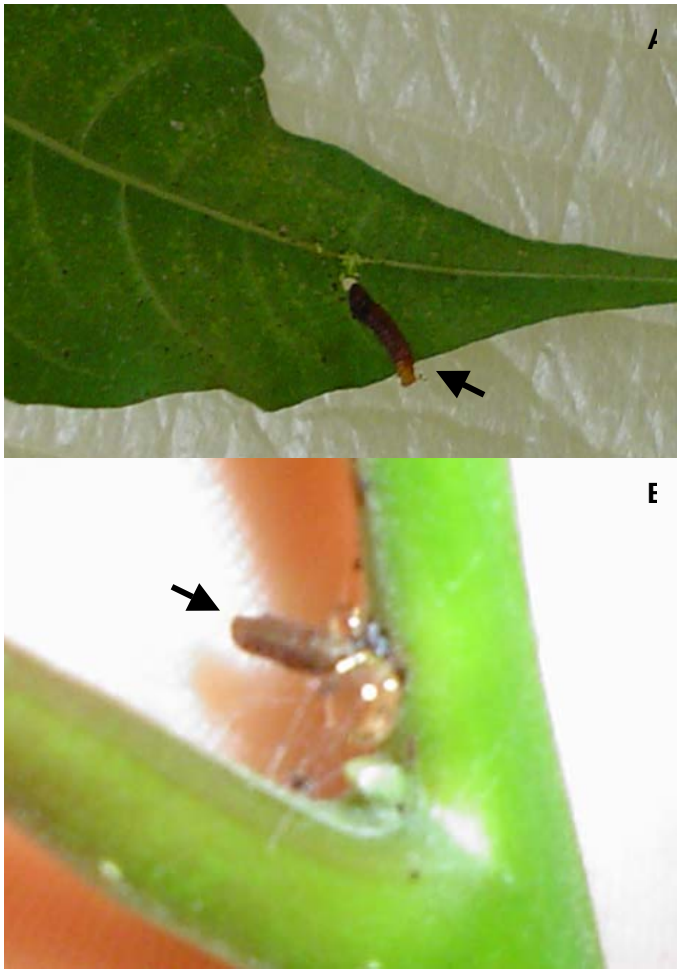

Figure 2.6 Mortality of *H. grandella* larvae due to toxic effects of leaves (A) and resins (B) on *Toona ciliata* plants. Arrows show dead larvae.

Discussion

Although the grafting technique has proven to be successful for a number of Meliaceae species (Bygrave & Bygrave, 2005), some grafts failed to work. Grafts of *C. odorata* onto *K. senegalensis*, *K. senegalensis* onto *C. odorata*, and *S. macrophylla* onto *T. ciliata* seemed to be incompatible under our conditions. Intergeneric grafts such as these typically have low success rates (Hartmann *et al*., 2002), as was noted by Bygrave and Bygrave (1998) trying

to graft *T. ciliata* onto *C. odorata*. Fortunately, the successful grafted and intact plants provided enough combinations of susceptible and resistant species to test my hypotheses regarding plant damage and larval performance.

In both experiments, the exotic species (*K. senegalensis* and *T. ciliata*) were clearly resistant to attack by *H. grandella* larvae, whereas the native species (*C. odorata* and *S. macrophylla*) were susceptible. These results possibly are of the lack of coevolution between this New World insect species and Old World Meliaceae species, as was also demonstrated for neem *Azadirachta indica*, whose metabolites showed either direct insecticidal or growth-disrupting on *H. grandella* (Mancebo *et al.*, 2002). Also, longer-term chronic toxicity (e.g., Cornell *et al*., 1998) apparently contributed to *H. grandella* mortality of resistant plants (comparing experiment I where only intact *C. odorata* plants allowed surviving larvae developed to pupa and experiment II where more treatments allowed pupation).

In addition to the expected resistance to *H. grandella* by intact (Bygrave & Bygrave, 2001) and autografted resistant plants, grafting alone provided some degree of resistance even for the susceptible species. This improved resistance could be attributed to plant-induced defenses resulting from the mechanical (wounding to make the graft) damage. Plants respond to mechanical wounding with the induction of numerous genes (Reymond *et al*., 2000; Lorenzo *et al*., 2003) and may prevent insect feeding by decreasing nutritional value or increasing concentrations of defensive secondary compounds in new foliage (Schoonhoven *et al*., 2005). However, in a leaf disk bioassay mechanical damage lack evidence of induced resistance in autografted plants (Pérez-Flores *et al*. unpublished).

Cedrela odorata and *S. macrophylla* appeared to respond differently to grafting, as shown by their respective autografts. For autografted *C. odorata* plants, the number of frass piles was reduced by 66% by autografting, whereas for *S. macrophylla* the reduction was only 51%. In both experiments, autografting diminished tunnel length in *C. odorata* stem but

not in *S. macrophylla*. Intact *S. macrophylla* plants are less susceptible than intact *C. odorata* (Speight & Wylie, 2001), probably making the autografting effect to attack by *H. grandella* larvae more difficult to detect.

An autografting effect was also detectable based on the amount of apical damage, and this was most evident in experiment I. This finding is important since apical bud damage is the type of injury that leads to loss of apical dominance and causes branching of the main stem, which results in a noncommercial tree (Grijpma, 1976).

In both experiments, intact susceptible plants had the most damaged apical buds. In agreement with Speight and Wylie (2001), damage by *H. grandella* larvae was more severe on *C. odorata* than on *S. macrophylla* plants.

Susceptibility of intact *C. odorata* plants also was detected in both experiments by a longest tunnel length, almost six times longer than the average tunnel length in the other species or combination of grafted plants, even though the number of tunnels differed only in experiment I.

The reduction of apical shoot damage on either autografted or any rootstock/scion combination plant was encouraging since it indicated that even attacked autografted trees could overcome shootborer damage and still grow into economically useful trees in commercial plantations.

Plant damage by *H. grandella* was noticeably influenced by larval age (neonates responses differed from those of instar III larvae). For example, susceptible species had more damage as indicated by the number of frass piles and tunnels made by younger larvae than by older ones. Resistant species were free from damage indicated by both tunnel number and length in experiment I.

Resistant intact plants, as well as grafted plants using *T. ciliata* either as rootstock or scion had shallow perforations, which were soon sealed by the plant. The shorter tunnels in experiment I compared to experiment II could be due to the fact that *H. grandella* neonates

fed first on petiole and leaf surface and then on the apical bud which agrees with Grijpma (1971). This author reported that feeding by neonate larvae was confined to the main shoot before boring into apical or lateral buds.

The trend for the number of damaged leaves was generally similar to that of tunnel length. Intact *C. odorata* plants had the most leaf damage in both experiments, and leaf damage for intact plants and plant combinations was higher in experiment II. This result could be explained because leaves were mainly damaged as the shoot was perforated, and tunnel length was longer in experiment II.

Differences in plant damage between experiments (e.g., the 21.7 and 30.8% of apical damage for experiment I and II, respectively), were expected due to the different larval stages tested. Also, neonate lepidopterans (experiment I) can detect diets that could be toxic to them, whereas later instars (experiment II) seem to lack that ability (Zalucki *et al*., 2002).

Hypsipyla grandella has limited establishment of commercial plantations of *C. odorata* and *S. macrophylla* species in the American tropics. Due to the low damage threshold of one larva per plant and the susceptibility of native species (Hilje & Cornelius, 2001), *H. grandella* infestations approach 100% in many plantations (Cornelius *et al*., 2004). Therefore, the resistance detected in exotic species and in *C. odorata* grafted onto *T. ciliata*, or *S. macrophylla* grafted onto *K. senegalensis*, as well as on the autografted susceptible species, could be economically important and exploit internal defenses of the trees against *H. grandella*.

Therefore, as resistance from *K. senegalensis* and *T. ciliata* can be transferred to native species by grafting, either toxins or feeding deterrents from these rootstocks may be translocated across the graft union and reach the main shoots of the scions. Bioactive compounds may be alkaloids (Smolenski *et al*., 1974), limonoids (Koul & Isman, 1992; Maia

et al., 2000), or phenolics (Newton *et al*., 1999; Da Silva *et al*., 1999), all of which are produced in these species and have been implicated as defensive compounds.

I observed that once in contact with *T. ciliata* main shoots, instar III larvae ballooned to lower leaves and tried to feed on them instead of the main shoot, and this resulted in the death of the larvae. If they bit a lateral bud, an exudate was produced, in which larvae became entrapped. Both susceptible species grafted onto resistant rootstocks, autografted susceptible species, and resistant *K. senegalensis* plants lacked an exudate that could trap larvae. The exudates produced by *T. ciliata* are apparently part of the defenses of this species, as are resinous exudates in other plant species (Lewinshon, 1991; Phillips & Croteau, 1999).

Resistant trees could be deployed in three ways. First, *T. ciliata* trees could be used as trap plants in mixed plantations with *S. macrophylla* or *C. odorata* species. *Hypsipyla grandella* moths readily oviposit on *T. ciliata* (Grijpma & Roberts, 1975; Maia *et al*., 2000) since essential oils from leaves of this resistant species and both *C. odorata* and *S. macrophylla*, are similar (Maia *et al*., 2000; Soares *et al*., 2003), although the foliage and resins of stem of *T. ciliata* are toxic to larvae.

Second, entire plantations of *C. odorata* grafted onto *T. ciliata* plants could be established. This approach has been successful for *T. ciliata* grafted onto *C. fissilis* in Australia where after eight years the trees maintained their resistance against *H. robusta* (Bygrave & Bygrave, 2005). Taking into account that, depending on the site, 5 to 8 years are span required by *C. odorata* and *S. macrophylla* plants to achieve a commercially valuable bole (Cibrián *et al*., 1995), long-term evaluation of the grafted plants should be completed to know if resistance is maintained and if they are adapted to field conditions. Grafted plants grown in the field must also be evaluated to record their phenotypic development since sometimes the growth of grafted plants differs from growth of intact plants (Zobel & Talbert, 1984).

Third, autografted *C. odorata* and *S. macrophylla* providing some level of resistance could lead to simple methods for enhancing resistance in the trees based on wounding only. This avenue needs to be explored more thoroughly, although a good indicator is that damaged *T. ciliata* trees by *H. robusta* (Cunningham & Floyd, 2006) or *S. macrophylla* and *C. odorata* damaged by *H. grandella* (Pers. obs.) grow more quickly and produce more biomass than undamaged trees.

The approach to transfer resistance to native Meliaceae trees by grafting their susceptible scions onto resistant rootstocks was pursued in the present research including a wider range of species than those used by Grijpma (1976). Moreover, the effect of autografted and reciprocal grafted plants could be assessed in order to demonstrate that the plant grafting might be used in an integrated pest management of *H. grandella*.

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CHAPTER 3

A grafting approach to prevent *Hypsipyla grandella* (Zeller) (Lepidoptera: Pyralidae) damage to New World Meliaceae species. II. Tests using leaf disks

Running head: Grafting approach to prevent H. grandella damage: leaf disk bioassay.

Key words *C. odorata, S. macrophylla, K. senegalensis, T. ciliata,* grafting, leaf disks, *Hypsipyla*, mortality, performance.

Abstract

1 Leaf disks were taken from susceptible species (*Cedrela odorata* and *Swietenia macrophylla*), scions grafted onto resistant ones (*Khaya senegalensis* and *Toona ciliata*), from their reciprocal grafts, and from both intact and autografted plants, to determine their effects on growth and mortality of instar II *Hypsipyla grandella* larvae. Mortality was evaluated 2, 5, 21 and 45 days after starting the bioassay. Leaf area consumed and weight gain per larvae were assessed two days after starting bioassay. Time to reach pupation and pupal weight and length a day after pupation, and time to adult stage and appearance of wings were determined at the end of the bioassay.

2 Plant species significantly affected mortality ($P \leq 0.04$) throughout the test. Eighty to 100% of larvae fed leaf disks from intact *T. ciliata* and its autograft, or *C. odorata* onto *T. ciliata* and its reciprocal graft died in the first two days of evaluation.

3 All other factors measured, except pupal weight and length, were also affected ($P \leq 0.01$) by the leaf disks. Intact resistant and reciprocal grafted plants reduced leaf consumption and caused to loose weight by larvae. Larvae fed on *K. senegalensis* grafted onto *S. macrophylla* extended by eight days the time to pupa and to adult stages compared to larvae fed on intact *C. odorata*, and induced abnormal wing formation.

Introduction

Spanish cedar *Cedrela odorata* L. and mahogany *Swietenia macrophylla* King trees are valuable trees commonly attacked by *Hypsipyla grandella* (Zeller) (Lepidoptera: Pyralidae), with cedar and mahogany plantations frequently 100% effected (Cornelius & Watt, 2003). A single *H. grandella* female can oviposit over 1000 eggs under laboratory conditions, although field observations have shown that over 50% of the trees in a young *C. odorata* plantation contained only 1 to 3 eggs per plant (Grijpma & Roberts, 1975). Nonetheless, a single borer larva can destroy the apical bud, deforming trees and making them useless for timber (Schabel *et al*., 1999), since its damage threshold corresponds to one larva per tree (Hilje & Cornelius, 2001).

Hypsipyla grandella-resistant genotypes could be a key component for developing an integrated management program for this pest (Newton *et al*., 1993; Speight & Wylie, 2001). Unfortunately, genotypes resistant to *H. grandella* for native Meliaceae are unknown (Newton *et al*., 1999; Cornelius & Watt, 2003; Navarro & Hernández, 2004), although seedlings from some specific regions, for example *C. odorata* from the Caribbean watershed of Costa Rica, somehow tolerate damage by producing a single secondary shoot that achieves apical dominance after attack (Newton *et al*. 1999, Navarro & Hernández, 2004).

On the other hand, some exotic Meliaceae species do have resistance against *H. grandella* maybe due to the absence of coevolution between these species (Grijpma, 1976; Agostinho *et al*., 1994). For instance, in Costa Rica, introduced Australian red cedar (*Toona ciliata* M. Roem.) trees were able to attract *H. grandella* and even permit oviposition, but larvae died when attempting penetration of its shoots (Grijpma & Roberts, 1975). Also, in Cuba, introduced African mahogany *Khaya senegalensis* Desr. A. Juss, has been reported resistant to *H. grandella* attack (Manso, 1974).

Fortunately, such resistance can be transferred to native Meliaceae trees by grafting their susceptible scions onto resistant rootstocks (Da Silva *et al*., 1999), as was shown by

Grijpma (1976) for *C. odorata* on *T. ciliata* in Costa Rica. This approach was not pursued again until recently, when Pérez-Flores *et al*. (2006) conducted more in-depth studies, including a wider range of species and experimental methods.

They tested whole intact and grafted Meliaceae small trees artificially infested with *H. grandella* eggs or larvae inside a greenhouse. In both cases, plant damage differed significantly among the species, whether intact or grafted. Larvae died when inoculated on resistant species, whereas pupal weight and length of surviving larvae were similar. Resistance was conferred from resistant rootstocks to susceptible scions and autografted susceptible and susceptible species grafted on resistant species were similar in terms of plant damage. Damage on autografted *C. odorata* was intermediate between that on both intact (non-grafted) *C. odorata* and *C. odorata* grafted onto *T. ciliata* plants. Also, regardless of the rootstock and scion combination, grafting by itself reduced damage caused by *H. grandella*.

Although bioassays with intact plants as performed by Pérez-Flores *et al*. (2006) closely mimic field conditions, they are complicated by differences in leaf age, damage, disease, or water content which all may cause differential feeding by insects (Hare, 1998). These sources of variation can hamper attempts to determine resistance mechanisms. Therefore, results of a bioassay using leaf disks are presented here and compared to previously reported results with whole plants. Although excision of leaf tissue may cause changes in physiology substantial enough to alter the outcome of the bioassay (Hare, 1998), leaf disks are uniform in size and shape, easy to manipulate, require little space and, since they can be kept inside environmental chambers, external conditions can be easily controlled, to obtain more reliable measurements of insect response than on greenhouse or on field by using whole plants.

The objective of this research was to use a leaf disk bioassay to determine effects of both intact and grafted Meliaceae species on the mortality and performance of *H. grandella*

larvae. Hypotheses tested were: 1) leaf disks from exotic species *K. senegalensis* and *T. ciliata* cause greater mortality and impair performance of *H. grandella* as compared to disks from native species *C. odorata* and *S. macrophylla*; 2) feeding and survival of *H. grandella* larvae are reduced on leaf disks from autografted susceptible species as compared with disks from autografted resistant species; 3) resistant rootstocks confer resistance to susceptible scions, as measured by *H. grandella* feeding and survival on leaf disks; and 4) susceptible rootstocks do not affect the resistance of exotic scions as measured by *H. grandella* feeding and survival on leaf disks.

Methods

The bioassay was completed in an environmental chamber Percival I35-L (Boone, Iowa) at 25 ^{0}C, 80-90% RH, and 12:12 L:D, at the Entomology Laboratory at the Tropical Agricultural Research and Higher Education Center (CATIE) in Turrialba, Costa Rica, from March 3 through April 20, 2005.

Plant material preparation

Seeds of the susceptible species *C. odorata*, *S. macrophylla*, from Costa Rica, as well as the resistant *K. senegalensis* from Burkina Faso, and *T. ciliata* from Australia, were provided by the Forest Seed Bank at CATIE. Seeds were grown in a nursery at the Cabiria Experiment Station, within the premises of the Tropical Agricultural Research and Higher Education Center (CATIE), in Turrialba, Costa Rica.

Six- to 12-month-old potted plants were used for grafting. The susceptible species were grafted onto resistant ones. Also, reciprocal combinations (i.e., resistant scions onto susceptible rootstocks) and autografted (scion and rootstock from the same species) plants were made. Intact and autografted plants were used as controls, grafted plants to test whether resistant species used as rootstock conferred resistance to scions susceptible

ones, and reciprocal grafted plants to test whether susceptible species used as rootstocks influenced *H. grandella* responses to scions of resistant species. The aim was to have a full set of combinations, but grafts of *K. senegalensis* onto *C. odorata* and *T. ciliata* onto *S. macrophylla* were unsuccessful due to incompatibility.

The grafting technique used was the wedge graft (Bygrave & Bygrave, 1998). The graft union was made 20 to 30 cm above of the soil. The rootstocks were less than 1 cm in diameter at the graft point, and the diameters of the scions were slightly less. Grafted plants were maintained in a greenhouse during eight months until the scions grew.

The first new and fully developed leaf on each plant was excised early in the morning, placed individually in a plastic bag, and taken to the laboratory. There, two disks per leaf were cut with a cork borer (2.3 cm diameter) from the two central leaflets; the cork borer was cleaned after cutting disks from each species.

Experimental procedure

Larvae for bioassays were taken from a colony kept at the Entomology Laboratory at CATIE, and established in 1998 from field-collected larvae feeding on *C. odorata*. Larvae in the colony are normally fed with tender *C. odorata* leaves from instars I to III, and then placed onto an artificial diet (Vargas *et al*., 2001) until pupation. Pupae are then moved to a metal framed cage covered with fine mesh, kept at a greenhouse, where adults emerge, mate and oviposit. Eggs are collected and taken to the laboratory to sustain the colony.

In contrast to a previous study (Pérez-Flores *et al*., 2006) instar II larvae were selected for bioassay because they are less sensitive to handling than instar I but approximate what would occur in nature regarding initial plant attack by *H. grandella* larvae. Instar II bores into the apical bud only after feeding on tender petioles and foliage (Grijpma, 1971). To initiate a test, a leaf disk was placed, with the abaxial side up, inside a 30-ml glass vial. Then an instar II *H. grandella* larva (4 to 8 mm length) was placed onto the leaf disk,

after being deprived of food for 3 hours. A wet piece of paper towel was fastened with the lid of each vial to avoid excessive desiccation of leaf disks. The vial was then turned over so the larva was below the leaf disk. The vials were arranged in a completely randomized design for 16 combinations of susceptible and resistant species, i.e., 16 sources of leaf disks (four grafts, four reciprocal grafts, as well as four autografts and four intact plants as controls). Larvae without leaf disks served as relative controls. Each treatment was replicated ten times, each one consisting of two leaf disks per plant with one larva per vial.

Measurements

All the larvae used per treatment were weighed before and two days after starting the bioassay, then an average weight gain per larva per plant species was calculated by subtracting initial from final weight. Also at two days, larval mortality was assessed and leaf consumption estimated. To estimate leaf consumption, the disk was glued to a transparent film and then overlayed onto graph paper (1 mm^2 grid size) to count the leaf area eaten. Living larvae were individually transferred by means of a thin paintbrush into a vial containing ca. 6 ml of artificial diet (Vargas *et al*., 2001), and then reared until adult emergence. Mortality at 5, 21 and 45 days after starting bioassay, time (days) to achieve the pupa and adult stages; pupal weight (mg), and length (mm), and wing shape were all measured for these larvae.

Larval and pupal mortality were recorded as 0 and 1 for dead and live larvae, respectively, since just one larva was used per leaf disk. Larvae were classified as dead if they were immobile or blackened. Pupae were classified as dead if they failed to emerge after 45 days or if they appeared blackened or shriveled (Mancebo *et al*., 2002). Pupal weight and length were determined one day after pupation. On the day of adult emergence, wing shape was recorded once their expansion and drying were completed. Normal and abnormal wings were recorded as 0 and 1, respectively. Wings were considered normal

when both forewings were similar in length and covered the whole abdomen longitudinally (Sliwa & Becker, 1973). Abnormalities were absence of or rudimentary forewings and shortened forewings exposing the abdomen. To exclude possible effects of the artificial diet alone on these characteristics, 100 pupas were selected at random from the colony and reared to adults. None of these insects had abnormal wings.

Statistical analysis

Data were examined for conformity to assumptions required for analysis of variance (ANOVA). If necessary, data were transformed by Y = sqrt(Y + 0.5) to meet these assumptions. ANOVA was completed using the GLM procedure in SAS (SAS, 2001). Orthogonal contrasts were used to test the species and graft combination effects on larval performance and mortality. The contrasts were as follows: 1) intact susceptible vs. intact resistant species; 2) autografted susceptible vs. autografted resistant species; 3) autografted *C. odorata* vs. *C. odorata* grafted onto resistant species rootstocks; 4) autografted *S. macrophylla* vs. *S. macrophylla* grafted onto resistant species 5) autografted resistant species vs. resistant grafted onto susceptible rootstock.

Larval and pupal mortality and the proportion of insects with normal wings were analyzed by the Chi-square test to examine plant species effects on larval performance. Also, Pearson's correlation coefficients (Proc Corr, SAS, 2001) were completed for consumed leaf area and weight gain with the other variables of performance (larval and pupal mortality, time to pupa and adult stages, pupal weight and length, and proportion of normal wings) by using the mean from each treatment.

Results

Larval performance

Leaf area consumption, time to reach pupation, and time to adult stage by *H. grandella* differed among the species tested (P < 0.0001, 0.005, and 0.01, respectively), whereas pupal weight and length were similar (P = 0.5, and 0.3, respectively) (Table 3.1). All the orthogonal comparisons for leaf area consumption were significant, but comparisons of developmental parameters were precluded because of high larval mortality on the majority of plant species or grafted combinations (Table 3.1).

Table 3.1 Probability values for orthogonal contrasts and ANOVA for several variables assessed in a bioassay with *H. grandella* larvae on foliar disks from combinations of intact and grafted Meliaceae species.

Contrast and ANOVA		Consumed leaf area	Days to		Pupa	
			Pupa	Adult	Weight	Length
		Probabilities				
Intact susceptible vs. intact resistant species		<0.0001	0.7280	0.7380	0.8587	0.9138
Autografted susceptible vs. autografted resistant species		<0.0001	-	-	-	-
Autografted *Cedrela* vs. *Cedrela* grafted on resistant		<0.0001	0.1829	0.5181	0.9811	0.8450
Autografted *Swietenia* vs. *Swietenia* grafted on resistant		0.0062	-	-	-	-
Autografted resistant vs. resistant grafted on susceptible		0.8315	-	-	-	-
ANOVA	*P*	<0.0001	0.005	0.01	0.5	0.3
	F	23.35	2.92	2.65	0.89	1.08
	d.f.	13, 100	10, 52	10, 46	10, 52	10, 52

Treatments: intact susceptible species: *Cedrela odorata (C), Swietenia macrophylla (S)*; intact resistant species: *Khaya senegalensis (K), Toona ciliata (T)*. Autografted susceptible species: *C/C*, *S/S*; Autografted resistant species *K/K, T/T*. Susceptible species grafted onto resistant species: *C/K, C/T*, *S/K, S/T*. Resistant species grafted onto susceptible species: *K/S*, *T/C*.

Leaf area consumed. Leaf area consumed by *H. grandella* larvae differed on leaf disks from intact and autografted susceptible and intact and autografted resistant plants. Consumption also differed on leaf disks from susceptible species grafted onto resistant ones

as compared with disks from autografted *C. odorata* or *S. macrophylla* plants (i.e., resistant species conferred resistance to susceptible scions). Feeding by *H. grandella* on leaf disks from autografted resistant *K. senegalensis* or *T. ciliata* was similar to that on leaf disks from resistant scions grafted onto susceptible rootstocks (i.e. susceptible species failed to confer susceptibility) (Table 3.1, Fig. 3.1).

Intact and autografted species produced the same trend regarding leaf area consumed. In decreasing order, leaf disk area consumed follwed the trend *C. odorata* > *S. macrophylla* > *K. senegalensis* > *T. ciliata*. Leaf disk consumption was significantly less for *T. ciliata* intact and autografted plants and any other plant combinations with *T. ciliata* involved as scion or rootstock as compared with all the other graft combination plants using *K. senegalensis* (Fig. 3.1). *Hypsipyla grandella* larvae almost barely fed on leaf disks from some graft combinations such as *S. macrophylla* on *T. ciliata*, *K. senegalensis* on *S. macrophylla* or *T. ciliata* on *C. odorata* and lost weight or died after two days of exposure.

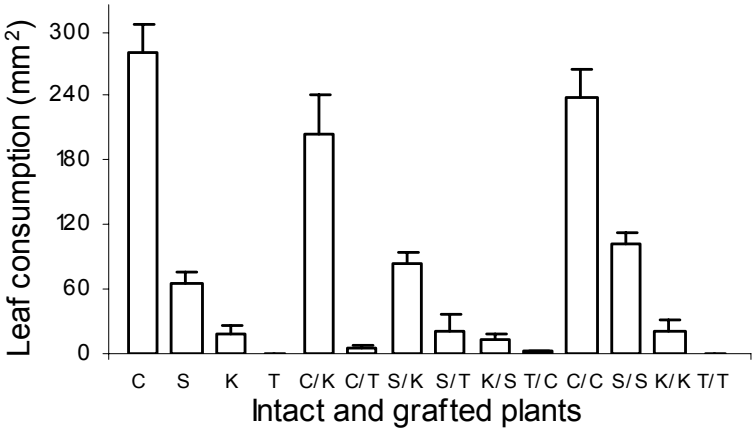

Figure 3.1 Consumed leaf area (mm^2) per instar II *H. grandella* larvae reared on leaf disks from four intact and grafted Meliaceae species. Data were taken two days after starting the bioassay. Intact plants: C = *Cedrela odorata*, S = *Swietenia macrophylla*, K = *Khaya senegalensis*, T = *Toona ciliata*. Grafted plants: C/K, C/T, S/K, S/T, K/S, T/C, C/C, S/S, K/K, T/T. Data are means ± SE, n = 20.

Weight gain. Larval weight gain on the leaf disks from intact plants, in decreasing order followed the trend *C. odorata* > *S. macrophylla* > *K. senegalensis* > *T. ciliata* (Fig. 3.2) and the trend was similar for autografted plants of these species.

Figure 3.2 Weight gain of instar II *H. grandella* larvae after two days exposed on leaf disks from four intact and grafted Meliaceae species. Data are means from 20 larvae. Intact plants: C = *Cedrela odorata*, S = *Swietenia macrophylla*, K = *Khaya senegalensis*, T = *Toona ciliata*. Grafted plants: C/K, C/T, S/K, S/T, K/S, T/C, C/C, S/S, K/K, T/T. ND = No disk.

Weight gain by larvae feeding on leaf disks from *C. odorata* grafted either onto *K. senegalensis* or *T. ciliata* was lower than on intact or autografted *C. odorata* plants. The same trend held for grafted and intact *S. macrophylla*, and larvae exposed to leaf disks from *S. macrophylla* grafted on *T. ciliata* lost rather than gained weight (Fig. 3.2). Larvae also lost weight when exposed to intact *T. ciliata* as well as for the reciprocal grafts *K. senegalensis* onto *S. macrophylla* and *T. ciliata* onto *C. odorata*. Larvae deprived of leaf disks to feed upon also lost weight.

Time to reach the pupal and adult stages. Some comparisons of development times were not completed, because of insufficient data due to mortality after feeding on leaves from some species or graft combination plants (Table 3.1). Nevertheless, the effects of leaf disks from resistant plants were similar to those from susceptible ones regarding time to reach

pupal and adult stages, and autografted *C. odorata* was similar to *C. odorata* grafted onto resistant *K. senegalensis* plants.

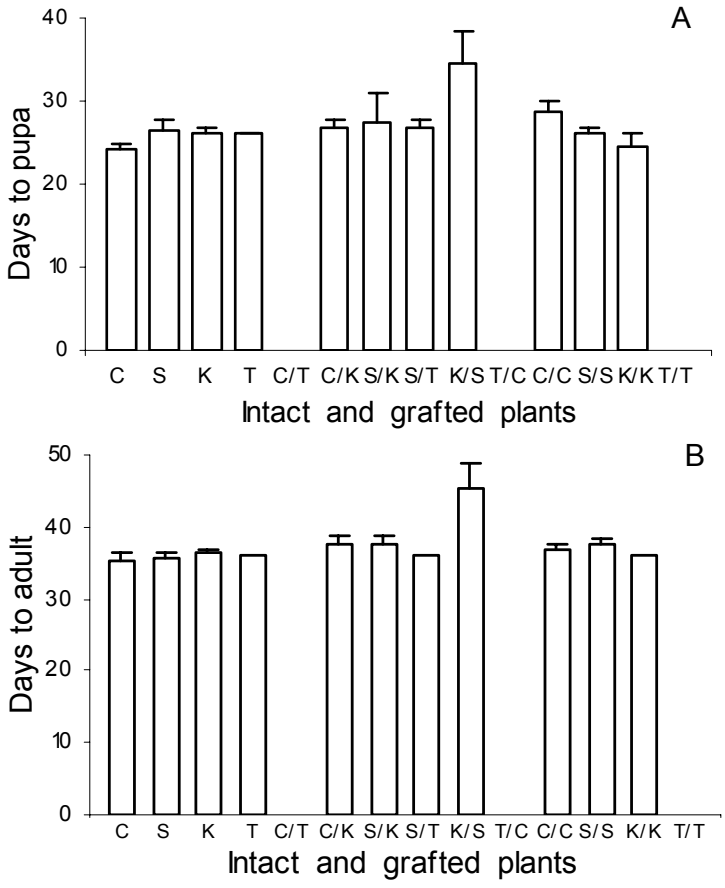

Figure 3.3 Time to pupa (A) and to adult (B) stages for instar II *H. grandella* larvae reared on leaf disks from four intact and grafted Meliaceae species. Intact plants: C = *Cedrela odorata*, S = *Swietenia macrophylla*, K = *Khaya senegalensis*, T = *Toona ciliata*. Grafted plants: C/K, C/T, S/K, S/T, K/S, T/C, C/C, S/S, K/K, T/T. Values are means ± SE, n = 20.

Among the other graft combinations, larvae fed leaf disks from reciprocal graft *K. senegalensis* onto *S. macrophylla* extended by 10 days the time to pupation in comparison with larvae fed intact *C. odorata* (34.3 ± 3.8 days vs. 24.3 ± 0.5 days). All larvae fed leaf disks from *C. odorata* grafted onto *T. ciliata*, *T. ciliata* onto *C. odorata*, or autografted *T. ciliata,* and starved larvae (larvae without leaf disk) died before pupating (Fig. 3.3A). Larvae fed leaf disks from *K. senegalensis* grafted onto *S. macrophylla* also extended by 10 days

the time to adult compared with larvae fed on intact *C. odorata* leaf disks (45.2 ± 2.8 days vs. 35.2 ± 1.3 days) (Fig. 3.3B).

Pupal length and weight. Larvae fed leaf disks from any of the four plant species or their tested combinations that permitted development to pupation had similar pupal length and weight (Table 3.1).

Wing development. The proportion of *H. grandella* with normal wing development differed significantly among the leaf disks from plant species (χ^2 = 18.85, P = 0.04, d.f. = 10). Abnormal wings formed only on larvae fed leaf disks from four treatments, all involving *K. senegalensis*: intact plants of this species (3 out of 5 moths), *K. senegalensis* grafted onto *S. macrophylla* (3 out of 5 moths), and *K. senegalensis* as a rootstock for either *C. odorata* (2 out of 7 moths) or *S. macrophylla* (1 out of 13 moths). Abnormalities included slightly short forewings (Fig. 3.4A), which leave the abdomen exposed, or rudimentary forewings (Figs. 3.4B, 3.4C).

Larval mortality

Plant species significantly affected the mortality of *H. grandella* on the four evaluation dates (P < 0.0001). Most larvae died if they were fed leaves from *T. ciliata* (intact, autografted, or *C. odorata* on *T. ciliata* and its reciprocal graft *T. ciliata* on *C. odorata*). Almost all larvae, near 100%, died after just two days of exposure (Fig. 3.5). Mortality on *K. senegalensis* leaf disks was lower, ranging from 70% on intact plants to nearly 90% on autografted ones.

Mortality on *C. odorata* and *S. macrophylla* leaf disks was lower than on leaf disks from resistant species both for intact and autografted plants; by the end of the test, mortality reached about 50% for larvae fed disks from intact plants, and only 10% and 40% of larvae on autograted *S. macrophylla* and *C. odorata*, respectively, died. Grafting *C. odorata* or *S.*

macrophylla each one to *K. senegalensis* rootstocks decreased mortality of *H. grandella* larvae mainly for *Swietenia* scions compared to intact plants.

Figure 3.4 *Hypsipyla grandella* female with normal forewings (left) and male adult with abnormal forewings (right) (A); and 43 day-old moths with abnormal wings in dorsal (B) and ventral views (C).

Larval mortality was negatively correlated to consumed leaf area and weight gain. In both cases, correlation decreased with the age. For consumed leaf area, the coefficients corresponded to -0.78, -0.75, -0.66 and -0.63, ($n = 14$, $P \leq 0.01$) at 2, 5, 21 and 45 days after starting the bioassay, respectively; whereas for weight gain coefficients corresponded to -0.80, -0.78, -0.70 and -0.68 ($n = 14$, $P \leq 0.005$), respectively.

Consumed leaf area and weight gain were not correlated to time to pupa and adult stages, pupal weight and length, and to proportions with normal wings (data not shown).

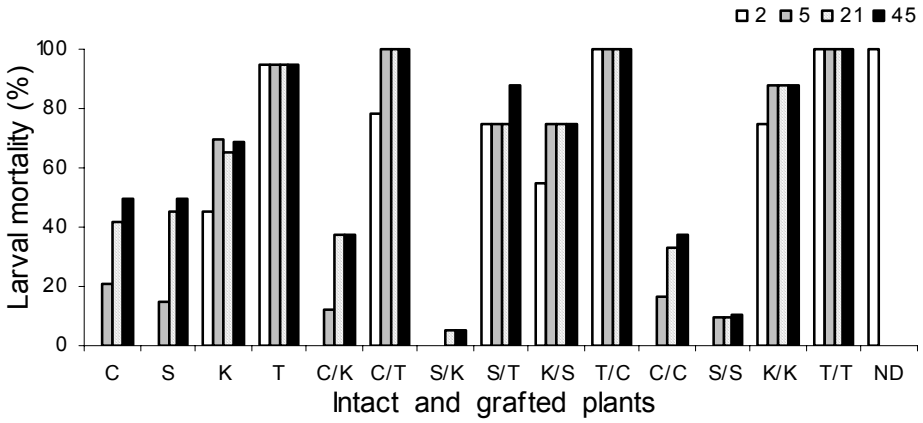

Figure 3.5 Cumulative mortality of instar II *H. grandella* larvae reared on leaf disks from four intact and grafted Meliaceae species. Mortality was determined 2, 5, 21 and 45 days after starting the bioassay. Data are means from 20 larvae. Intact plants: C = *C. odorata*, S = *S. macrophylla*, K = *K. senegalensis*, T = *T. ciliata*. Grafted plants: C/K, C/T, S/K, S/T, K/S, T/C, C/C, S/S, K/K, T/T. ND = No disk.

Discussion

Performance and survival of *H. grandella* differed on leaf disks from the Meliaceae species and grafting combinations tested. Toxic and deterrent effects of exotic resistant species on *H. grandella* larvae were confirmed (Da Silva *et al*., 1999). Moreover, leaf consumption and weight gain was larger and larval mortality reduced for larvae exposed to leaf disks from susceptible autografted species than in those exposed to resistant ones. Larval mortality was approximately 50% on susceptible intact *C. odorata* and *S. macrophylla* but was 80% to 100% on resistant *K. senegalensis*, *T. ciliata* and grafted plants with *K. senegalensis* or *T. ciliata* as rootstocks or scions.

Based on the mortality rates in my bioassay, leaves from *C. odorata* grafted onto *T. ciliata* was as resistant against *H. grandella* as were leaves from intact *T. ciliata*. Grijpma and Roberts (1975) reported that *C. odorata* grafted onto *T. ciliata* was less resistant than 4-month-old *T. ciliata* plants. The difference in my results and those of Grijpma and Roberts

(1975) could be caused by the nature of the bioassay, since they used whole leaflets to feed groups of 10 instar I larvae, whereas I used individual leaf disks and one instar II larvae for each disk. In addition they used 4-month-old plants, whose foliage is more tender than that from eight month-old plants used in my study.

Khaya senegalensis and *T. ciliata* had different effects on the insects. Larval mortality of *H. grandella* was higher on leaf disks from plants of intact *T. ciliata* or plants with *T. ciliata* as rootstock or scion than on comparable plants from *K. senegalensis*. The resistance could be due to differences in specific substances or concentrations of the same or different substances (Da Silva *et al*., 1999). *Khaya senegalensis* used as rootstock or scion or autografted allowed some larvae to develop to adult, but all treatments involving *K. senegalensis* caused some incidence of abnormal wing development. The toxicity of *T. ciliata* prevented 95% of the arvae from developing into adults, limiting an assessment of the effect of this host plant species on wing development.

The transmissibility of resistance across grafts suggests translocated chemical defenses are involved. In a prior test, Pérez-Flores *et al*. (2006), showed that resistance could be independent of rootstock or scion identity and partly inducible by autografting since whole plants of autografted *C. odorata* and *S. macrophylla* exhibited some resistance to *H. grandella* larvae. In contrast, in the present study autograft-induced resistance was absent for the susceptible species. Pérez-Flores *et al*. (2006) used plants from the same cohort as that used for the present study, but the plants were four months younger, indicating that autografting-based resistance is transient. In the present study, resistance only occurred in plants including scions or rootstocks from the resistant species. Such resistance could be due to combinations of toxic, antifeedant or growth-inhibitory substances.

Antifeedant and growth-inhibitory activities of *K. senegalensis* have been reported against instar II larvae of *Spodoptera littoralis* (El-Aswad *et al*., 2003). Moreover, antifeedant

activity of *T. ciliata* against *Epilachna varivestis* (Veitch *et al*., 1999) and *Spodoptera litura* (Gopalakrishnan *et al*., 2000) has been reported.

Toona ciliata leaf consumption by larvae was less than that for *K. senegalensis,* either because of differences in the amount or in the composition of substances translocated between both plants. Leaf disks from *K. senegalensis* grafted onto *S. macrophylla* delayed pupation, maybe due to food stress caused by the starvation of larvae during the two days of exposure to these leaves. Starvation for 24 h significantly delayed pupation of the tropical butterfly *Bicichus anynana* (Bauerfeind & Fischer, 2005). However, the duration of the pupal stage for larvae fed *C. odorata* or *K. senegalensis* leaf disks were similar (10.9 days and 10.7 days, respectively). The mean time to reach adulthood agreed with that reported by Grijpma (1971) rearing *H. grandella* on artificial diet (37.2 ± 0.4 days vs. 37 days, respectively). Also, the time required to reach adulthood of larvae reared on *C. odorata*, in the present bioassay, (35.2 ± 1.3 days) was similar to that reported by Grijpma (1971) (35 days).

Delayed pupation is important since if grafted trees with *T. ciliata* or *K. senegalensis* rootstocks are deployed to prevent *H. grandella* damage, the net effects could be enhanced due to the combined effects of resistance and biological control, by providing more time for action by larval predators. Such effects, however, would be limited in the case of *H. grandella* to those natural enemies that attack the early instars before they enter the stem.

Similar larval response to leaf disks from autografted resistant species and those from grafted on susceptible ones could be attributed to substances synthesized by resistant species (scions) (Da Silva *et al*., 1999), masking any substance from susceptible species (rootstocks). Another reason could be that susceptibility to insect pests also depends on physical characteristics of plant tissues (Cunningham & Floyd, 2004), such as trichomes or leaf toughness and hardness (Lucas *et al*., 2000), and resistant scions could have maintained their physical characteristics after grafting.

The results of the leaf disk bioassays agreed with the hypothesis that exotic species increased the mortality and decreased larval growth of *H. grandella*. Larvae consumed less leaf tissue or none at all, and fewer survived on leaf disks from *K. senegalensis* and *T. ciliata* than on *C. odorata* and *S. macrophylla*. Pupal weight and length from larvae fed leaf disks from resistant and susceptible species were similar, possibly because the 48 h exposure of larvae to the resistant species was too short to upset growth characteristics.

The leaf disk bioassay in this study confirmed that resistant species transfer resistance to susceptible species via grafting and that resistant species used as scions of susceptible ones maintained their resistance, as had been demonstrated by inoculating shoots of intact plants from these treatments with eggs or instar III larvae (Pérez-Flores *et al*., 2006).

The utility of leaf disk bioassays was also confirmed by Mancebo *et al*. (2000). They tested plant extracts on *C. odorata* against *H. grandella* and found similar results from greenhouse tests on whole plants and laboratory leaf disk bioassays. However, their extracts were from plant species different to Meliaceae and they did not test grafted plants.

In conclusion, the use of grafted resistant plants could be the best way to prevent *H. grandella* attack since the resistance should be constitutive instead of induced as Hannover (1980) suggested would be preferable for control of insect pests. Also, Newton *et al*. (1993) and Speight and Wylie (2001) suggested the use of resistant Meliaceae plants to prevent *H. grandella* attack. Nevertheless, grafted resistant plants should be tested in the field to confirm their resistance and to determine their commercial suitability before establishing commercial plantations.

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CHAPTER 4

Leaf extracts from Meliaceae species can decrease survival and performance of *Hypsipyla grandella* (Zeller) (Lepidoptera: Pyralidae) larvae

Running head: Meliaceae extracts affect H. grandella survival and performance

Key words Extracts, alkaloids, limonoids, phenolics, *Cedrela, Swietenia, Khaya, Toona*, shootborer, leaf disk bioassay.

Abstract

1 Crude leaf extracts and putative alkaloid, limonoid, and phenolic fractions from the extracts from *Cedrela odorata*, *Swietenia macrophylla*, *Khaya senegalensis*, *Toona ciliata*, and *C. odorata* grafted on *T. ciliata* plants, were tested on *C. odorata* leaf disks to determine their effects on performance and survival of *Hypsipyla grandella* larvae.

2 Larval performance was assessed by determining leaf area consumption and weight gain per larvae two days after starting the bioassay, days to reach pupation, pupal weight and length one day after pupation, and days to adult stage and moth wing appearance. Survival of *H. grandella* larvae was assessed 2, 10 and 25 days after starting the bioassays.

3 Crude extracts from resistant plants equally affected larval survival and performance compared to crude extract from *C. odorata* grafted on *T. ciliata* plants, and these extracts were more detrimental to larvae than those from susceptible species.

4 In the fraction bioassay, alkaloids decreased leaf consumption and weight gain of larvae, as well as time to reach pupa and adult stages, whereas limonoids reduced larval survival on the three dates of evaluation.

5 The best fractions to reduce leaf consumption and weight gain was alkaloid extracted from *C. odorata* grafted on *T. ciliata* and phenolics extracted from *C. odorata*. Moreover, the alkaloid

fraction extracted from the grafted plants caused 20% of adults *H. grandella* to form abnormal wings. The best fractions to reduce pupal weight and length as well to reduce the time to reach pupal and adult stages were alkaloids extracted from *S. macrophylla*.

Introduction

Internal chemicals in tree tissues may exert their effect in the volatile state causing an insect to avoid the tree completely, or they may deter the insect after it contacts the tree or ingests tissue (Cunningham & Floyd, 2004). Research on biochemical basis for resistance to *Hypsipyla grandella* (Zeller) in Meliaceae has been completed on limonoids (De Paula *et al.*, 1997, Da Silva *et al*., 1999), while Grijpma (1976) suggested that the biochemical basis for resistance of *T. ciliata* may be due to alkaloids. Further, Newton *et al*. (1999) suggested that proanthocyanidins (i.e., phenolic compounds) may reduce susceptibility of *C. odorata* to attack by *H. grandella* larvae.

The family Meliaceae is distinguished by the common occurrence of triterpenoids known as limonoids or meliacions (Taylor, 1981), which possess antifeedant, toxic, or growth-reducing properties to different species of insects (Gershenzon & Croteau, 1992; Champagne *et al*., 1992). For example, azadirachtin, the most well-known limonoid, is toxic against nearly 200 insect and mite species (Saxena, 1989). In preliminary experiments, azadirachtin proved to be toxic to *H. grandella* larvae when incorporated in diet mixtures (Grijpma, 1976); moreover, such toxic effect plus growth-disruptant activity was reported later by Mancebo *et al*. (2002) for Azatin and Nim 80, respectively, both mainly containing azadirachtin. Limonoids, however, seemed unrelated to the induced resistance of *C*. *odorata* grafted onto *T*. *ciliata* against *H*. *grandella* larvae (De Paula *et al*., 1997); instead, these authors stated that cycloartanes, catechin and proanthocyanidins (phenolic compounds) are likely responsible for such resistance, as all of them were absent from *C*. *odorata* scions or intact (non-grafted) plants but present in *T*. *ciliata*.

Other phenolic compounds, such as methylcoumarins and the furanocoumarin bergapten, have been found in *T*. *ciliata* (Chowdhury, 2004), proanthocyanidins in *C. odorata* (Newton *et al*., 1999), and the flavonoids quercitin and kaenferol in *C*. *odorata* and *T*. *ciliata* (Chatterjee *et al*., 1971). Furanocoumarins are potent feeding deterrents to certain insect species (Schoonhoven, 1972), and bergapten might promote the resistance of *T. ciliata* against *H*. *grandella* larvae.

Other chemicals found in Meliaceae species and should provide resistance against *H. grandella* are alkaloids. Alkaloids are nitrogen compounds that function as plant defenses against herbivores (Caporale, 1995) and were detected in ethanolic extracts from *T. ciliata* (Smolenski *et al*., 1974). These extracts had toxic effects and reduced growth on *H. grandella* larvae (Grijpma & Roberts, 1975). Therefore, alkaloid compounds could be responsible for the resistance of *T*. *ciliata* against *H*. *grandella* larvae.

Therefore, the objective of the present research was to detect the effects of crude, alkaloid, limonoid, and phenolic extracts from foliage of susceptible and resistant Meliaceae species, as well from *C. odorata* grafted on *T. ciliata* plants, on *H. grandella* larval mortality and performance. The hypotheses to test were: 1) crude extracts from leaves of resistant species negatively affect survival and larval performance more than extracts from susceptible species, 2) crude extract from leaves of *C. odorata* grafted onto *T. ciliata* affect survival and larval performance at the same level as leaves from resistant species, and 3) since the three classes of compounds have been reported in Meliaceae species, all of them negatively affect the survival and performance of *H. grandella* larvae.

Methods

Extract preparation

The extracts were prepared in the Animal Nutrition Laboratory at CATIE, from Meliaceae species and *C. odorata* scion grafted on *T. ciliata* rootstock. Plants were grown from seeds in a nursery at the Cabiria Experiment Station, within the premises of the Tropical Agricultural Research and Higher Education Center (CATIE), in Turrialba, Costa Rica. Seeds of the susceptible species *C. odorata* and *S. macrophylla*, from Pococí, Costa Rica, as well as the resistant *K. senegalensis* from Burkina Faso, and *T. ciliata* from Australia, were provided by the Forest Seed Bank at CATIE.

Crude extracts*.* Fresh leaves and shoots (500 g) from 1-year-old plants were cut into 2 to 5 cm pieces, and then ground in liquid nitrogen to 0.05 mm in a mill (Model 3 Wiley Mill®, Thomas Co., Philadelphia). Later, the ground material was extracted with 2 l 70/30 methanol/water by volume. Extraction was completed at room temperature (20^0C) for eight days. Each extract was filtered through Whatman paper No. 4, and the extract was concentrated to a small volume (200 ml) by a rotary evaporator (40^0C) (so all methanol was removed). One part of each concentrated crude aqueous extract was then used in a bioassay and the other was partitioned among ether and dichloromethane (CH_2Cl_2) to produce extracts likely to contain predominantly alkaloids, phenolics and limonoids, respectively.

Alkaloid and phenolic fractions. Alkaloid and phenolic compounds were isolated by the acid-base separation method (Millar & Sims, 1998). Back extraction of the ether extract with 0.5 M HCl removed amine bases such as alkaloids. A second extraction of the remaining ether extract with 0.5 M NaOH removed phenols, which are ionizable at high pH. The basic

and phenolic compounds were recovered by adjusting the pH of each extract to the point where the compounds were present in their uncharged forms (alkaloids, pH ≅ 11; phenols, pH ≅ 7).

Limonoid fractions. Twenty-five ml of crude extract were separated on a silica gel column (400 mm x 8 mm, Silica Gel grade 60, 254 g gravity flow), and then eluted with dichloromethane (Céspedes *et al*., 2000). This dichloromethane fraction was then concentrated to a small volume (50 ml) by a rotary evaporator and then used in the bioassays.

Experimental procedures

Larvae were taken from a colony kept at the Entomology Laboratory at CATIE, established in 1998 from field-collected larvae feeding on *C. odorata*. Larvae are normally fed with *C. odorata* tender leaves from instars I to III, and then placed into an artificial diet (Vargas *et al*., 2001) until pupation. Pupae are then moved to a cubic metal frame covered with fine mesh, kept at a greenhouse, where adults emerge, mate and oviposit. Eggs are collected and taken to laboratory to rear the colony.

Cedrela odorata leaf disks were taken from plants which are the food source for rearing the *H. grandella* colony at CATIE's Entomology Laboratory. Leaf disks were cut from central leaflets, by using a cork borer (2.3 cm diameter, 4.15 cm^2).

Ten leaf disks per treatment were put on a Petri dish and then sprayed with 1 ml (12 µl cm^{-2}) of each extract added with 0.03% Citowett (BASF, Canada, Inc.) as surfactant agent. The application of extracts to leaf disks was done by using a De Vilbiss 15 sprayer (The De Vilbiss, U.S.A.) connected to a vacuum pump (GAST™ DOA-P104-AA, GAST Manufacturing Corp. Benton Harbor, Michigan), with 0.7 kg/cm^2 constant pressure.

Sprayed leaf disks were allowed to dry and were placed individually, with the abaxial side up, inside a 30-ml glass vial. An instar II *H. grandella* larva (4 to 8 mm length) was then placed onto the leaf disk after being deprived of food for 3 h (Mancebo *et al*., 2002). A wet piece of paper towel was fastened to the lid of each vial to avoid excessive desiccation of leaf disks. The vial was then turned over so the larva was below the leaf disk and vials were arranged in a completely randomized design.

The crude extract bioassay consisted of eight treatments (i.e., five crude extracts, with one each from *C. odorata*, *S. macrophylla*, *K. senegalensis*, *T. ciliata*, and *C. odorata* grafted on *T. ciliata* plants; distilled water (absolute control) and methanol (extract solvent) and larvae deprived of food as relative controls. Bioassay with extract fractions consisted on 19 treatments (five limonoid, alkaloid and phenolic extracts with one each from the four normal Meliaceae species and the grafted plants, dichloromethane and ether solvents as well as larvae without leaf disk as relative controls, and water as absolute control).

Ten leaves were treated with a crude extract or fraction in the bioassays. For both types of extracts (crude or fractions), the experimental unit was a leaf disk with one instar II *H*. *grandella* larva in a capped vial. Instar II larvae were selected because they are less sensitive to handling than instar I, while yet resembling what would occur in nature regarding initial plant attack by *H. grandella* larvae. The fractions bioassay was completed three times each one by using ten replicates larvae on leaf disks.

Bioassays were completed in an environmental chamber Percival I35-L (Boone, Iowa) at 25^0C, 80 to 90% RH, and 12:12 L:D, at the Entomology Laboratory at the Tropical Agricultural Research and Higher Education Center (CATIE) in Turrialba, Costa Rica, from October 15 through December 6, 2005.

Assessments

All larvae were weighed before and two days after starting the bioassay to determine weight gain per larva. Also at two days, larval survival was assessed and leaf area consumption was estimated. To estimate leaf consumption, the disk was glued to a transparent film and then overlayed onto graph paper (1 mm^2 grid size) to count the area of leaf removed. Living larvae were individually transferred by means of a thin paintbrush into a vial containing ca. 6 ml of artificial diet (Vargas *et al*., 2001), and then reared until adult emergence. Larval survival at 10 and 25 days after starting bioassay, time (days) to achieve the pupa and adult stages, pupal weight (mg) and length (mm), and wing shape were all measured for these larvae.

Larvae were classified as dead if they were immobile or blackened. Pupae were classified as dead if they failed to emerge after 45 days or if they appeared blackened or shriveled (Mancebo *et al*., 2002). Data for pupa were determined one day after pupation. On the day of adult emergence, wing shape was recorded after wing expansion and drying were completed. Wings were considered normal if both forewings were similar in length and covered the whole abdomen longitudinally (Sliwa & Becker, 1973). Abnormalities were considered to be absence of or rudimentary forewings or shortened forewings exposing the abdomen.

Statistical analysis

Data were examined for conformity to assumptions required for analysis of variance (ANOVA). If necessary, data were transformed by Y = sqrt(Y + 0.5) to meet these assumptions. ANOVA was completed using the GLM procedure in SAS (SAS, 2001) for all bioassays. For bioassays with fractions, orthogonal contrasts were used to test the species and graft combination effects on larval performance. The contrasts were: 1) limonoid vs. phenolic fraction; 2) alkaloid vs. limonoid fraction; 3) alkaloid vs. phenolic fraction.

Larval survival as well as proportion of insects with normal wings in the crude extract bioassay was analyzed by the Chi-square test to examine plant species effects on these qualitative data.

Results

Crude extracts

Larval performance. The extracts significantly affected ($P \leq 0.05$) leaf area consumption and larval weight gain, as well as time to reach the pupal and adult stages, but pupal weight and length were unaffected by the extracts (Table 4.1). The proportions of *H. grandella* with normal wings were also similar to each other ($\chi^2 = 5.46$, $P = 0.36$, d.f. = 5) since abnormal wings developed only on one adult that had fed on a leaf disk sprayed with extract from *C. odorata* grafted onto *T. ciliata*.

Crude extract from *C. odorata* was similar to the water control allowing feeding by *H. grandella* larvae but differed in its effects compared to the other crude extracts and methanol (Table 4.1, Fig. 4.1). The extract from *C. odorata* grafted to *T. ciliata* rootstocks as well as extract from *T. ciliata* plants decreased leaf disk consumption compared to consumption of disks sprayed with *C. odorata* extract. Also, extracts from resistant *K. senegalensis* did not decrease leaf consumption as drastically compared to *T. ciliata* extracts. Moreover, the extract from *K. senegalensis* allowed similar feeding than *S. macrophylla* (Table 4.1).

All of the larvae fed leaf disks sprayed with *T. ciliata* crude extract died before pupating. The other extracts and controls were similar in their effects on the larva although *S. macrophylla* extract delayed pupation by 3.6 days compared to the water control. All larvae fed leaf disks from *T. ciliata* and the starved larvae (larvae without leaf disk) died

before pupating. On another hand, *C. odorata* leaf disks sprayed with *C. odorata* extract induced the least time to achieve the adult stage by *H. grandella*. The longest time needed to reach adult stage was for larvae fed leaf disks sprayed with *S. macrophylla* crude extract (Table 4.1).

Also, larvae fed leaf disks sprayed with *T. ciliata* extract had the least weight gain. Moreover, extracts from *C. odorata* grafted onto *T. ciliata* as well as methanol and the two days off diet (without leaf disk) caused larval weight loss. On another hand, the least pupal weight (from larvae fed on leaf disks sprayed with *C. odorata* grafted onto *T. ciliata* extract) and the largest one (from larvae reared with leaf disks sprayed with *S. macrophylla* extract) differed by 21.4 mg (Table 4.1).

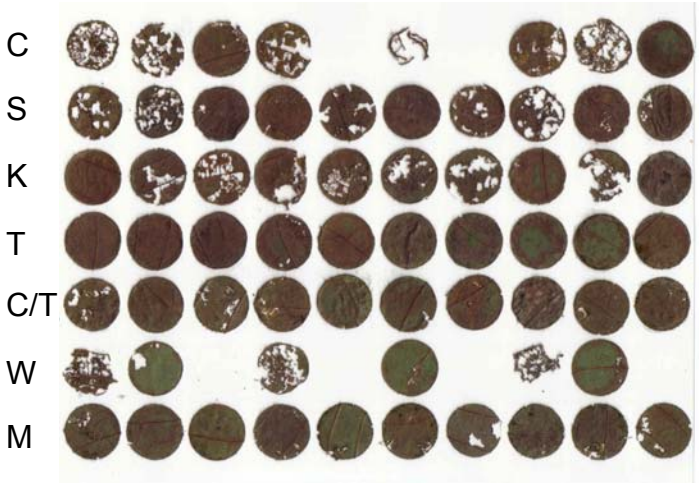

Figure 4.1 Leaf consumption for instar II *H. grandella* larva in *C. odorata* leaf disks treated with crude extracts from four Meliaceae species and a graft combination. C = *C. odorata*, S = *S. macrophylla*, K = *K. senegalensis*, T = *T. ciliata*, C/T = *C. odorata* grafted on *T. ciliata*, W = water, M = methanol. Data were taken two days after starting bioassay. Missing leaf disks were due to complete tissue consumption by the larvae.

Larval survival. Treatments differed significantly (χ^2 = 33.13, 23.51, 17.09; P < 0.0001, 0.001, 0.02; d.f. = 7) for larval survival at 2, 10, and 25 days after starting the bioassay. Ninety percent of larvae reared on disks sprayed with *T. ciliata* extract died during the first two days, and 100% mortality was reached during the first 10 days (Fig. 4.2).

Table 4.1 Analysis of variance and means (± SE) for variables of *H. grandella* performance evaluated in the bioassay with crude extracts from Meliaceae plants.

Source of extract	Consumed leaf area (mm^2)[a]	Weight gain (mg)[b]	Time (days) to pupa[c]	Time (days) to adult[d]	Pupa weight (mg)[e]	Pupa lenght (mm)[f]
Cedrela odorata	265.1 ± 46.5	116.1 ± 12.9	21.5 ± 0.3	29.8 ± 0.6	147.4 ± 12.7	15.0 ± 0.5
Swietenia macrophylla	82.7 ± 27.8	7.2 ± 0.8	24.6 ± 1.5	34.5 ± 1.4	163.4 ± 10.6	15.2 ± 0.7
Khaya senegalensis	106.9 ± 27.6	24.8 ± 4.1	23.2 ± 0.9	33.7 ± 0.6	146.0 ± 4.6	14.8 ± 0.2
Toona ciliata	5.1 ± 3.2	0.9 ± 0.1	-	-	-	-
C. odorata / T. ciliata	31.5 ± 11.0	-15.0 ± 1.9	22.0 ± 0.6	32.0 ± 1.0	142.0 ± 10.7	14.3 ± 0.8
Methanol	28.5 ± 5.9	-23.0 ± 0.0	21.3 ± 0.2	31.5 ± 1.8	155.3 ± 6.5	15.5 ± 0.2
Water	268.5 ± 56.1	53.0 ± 1.9	21.0 ± 2.0	32.0 ± 1.0	148.5 ± 22.5	15.5 ± 0.5
No disk	-	-13.0 ± 0.0	-	-	-	-
ANOVA *P*	<0.0001	<0.0001	0.05	0.05	0.81	0.36
F	1.2	8.11	2.3	3.6	4.2	6.5
d.f.	6, 63	7, 72	5, 24	5, 19	5, 24	5, 24

For standard error (SE): a, n = 70; b, n = 80; c, e, f, n = 30; d, n = 25; a, b were scored two days after larval exposure on leaf disks; e,f, were scored one day after pupation.

The effect of *T. ciliata* extract was similar to that of two days starvation with 100% larval mortality. Moreover *T. ciliata* extract had a more potent and faster toxic effect than the control methanol. Half of the larvae exposed to leaf disks treated with methanol died two days after starting the bioassay, and a 70% and 90% of cumulative mortality was recorded on the other two dates of evaluation. None of the larvae exposed to leaf disks treated with the extract from *S. macrophylla* died during the first two days of bioassay, but 30% and 60% died 10 and 25 days after starting bioassay. The extract from grafted *C. odorata* on *T. ciliata* caused a higher and faster larval mortality compared to the extract from intact *C. odorata* plants (Fig. 4.2).

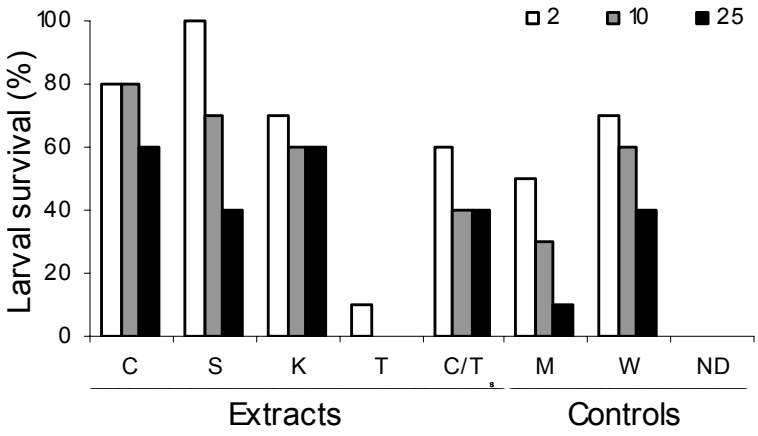

Figure 4.2 Survival of instar II *H. grandella* larvae reared on *C. odorata* leaf disks treated with crude extracts from four Meliaceae species and a graft combination. Data were taken 2, 20, and 25 days after starting bioassay. C = *C. odorata*, S = *S. macrophylla*, K = *K. senegalensis*, T = *T. ciliata*, C/T = *C. odorata* grafted onto *T. ciliata*, M = methanol, W = water, ND = No disk. Values are means (n = 10). For the first, second, and third date of evaluation, P ≤ 0.02, d.f. = 7; χ^2 = 33.1, 17.1, and 23.5, respectively.

Fractions

Fractions from the four Meliaceae species and the grafted combination significantly affected (P ≤ 0.02) larval leaf consumption and weight gain, time to pupa and to adult stages, and normal wing development as well as survival in the three dates of evaluation, but pupal weight and length were similar regardless of the extract fraction used (Table 4.2).

Table 4.2 Probability values of orthogonal contrasts and analysis of variance for *H. grandella* performance and survival on leaf disks treated with fractionated plant extracts from Meliaceae species.

Contrast	Probabilities									
	Consumed leaf area (mm^2)	Weight Gain (mg	Time (days) to Pupa				Normal wings	Larval survival		
			pupa	adult	weight (mg	length (mm)		a	b	c
Alkaloids vs limonoids	0.71	<0.0001	0.59	0.34	0.04	0.02	0.02	0.39	0.09	0.84
Alkaloids vs phenolics	0.90	<0.0001	0.44	0.46	0.04	0.004	0.06	0.13	0.41	0.07
Limonoids vs phenolics	0.80	0.91	0.19	0.84	0.96	0.50	0.67	0.02	0.01	0.05
ANOVA										
P	<0.0001	<0.0001	0.007	0.001	0.78	0.48	0.0003	<0.0001	0.0008	0.02
F	3.15	97.94	2.12	2.51	0.72	0.98	2.81	4.94	2.58	1.86
d.f.	17, 162	18, 171	18,161	18,154	18, 160	18, 160	18,153	18,171	18,170	18,170

a, b, and c means 2, 10 and 25 days after starting the bioassay respectively. Data are from three bioassays combined into one data set analysis.

The putative fractions of extracts had similar effects on consumed leaf area and time to reach pupa and adult stages. Alkaloids compared to limonoids or phenolics fractions had a similar trend for all larval performance and survival variables except for normal wing development. Alkaloid fraction effect on larval weight gain and pupa weight and length differed from that of putative limonoid or phenolic fractions, but the fractions had similar effects on larval survival. Limonoid and phenolic fractions differed only for larval survival but not for larval performance variables. Normal wing development only differed between alkaloids and limonoid compounds (Table 4.2).

Alkaloid and phenolic fraction effects depended on the plant source for leaf consumption. The alkaloid fraction from *C. odorata* grafted on *T. ciliata* and phenolic fraction from *C. odorata,* reduced *H. grandella* leaf consumption compared to the three fractions from *S. macrophylla* and also compared to controls, specifically to the control water. Phenolic fractions effect on leaf consumption was more notable between *C. odorata* compared to S. *macrophylla* or the grafted plants. Contrarily, the limonoid fractions from the four species and the grafted combination were similar each other in their effect on leaf consumption (Fig. 4.3).

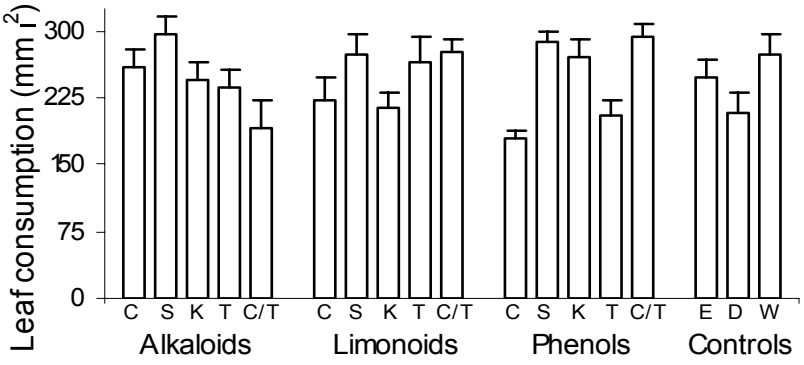

Figure 4.3 Leaf consumption for instar II *H. grandella* larva reared on *C. odorata* leaf disks treated with alkaloid, limonoid and phenolic fractions from four Meliaceae species and a graft combination. C = *C. odorata*, S = *S. macrophylla*, K = *K. senegalensis*, T = *T. ciliata*, C/T = *C. odorata* grafted onto *T. ciliata*, E = Ether, D = Dichloromethane, W = Water. Data were taken two days after starting bioassay. Data are means (± SE, n = 30) from three bioassays combined into one data set analysis.

Except for phenolic fractions from *C. odorata* or *T. ciliata*, which reduced weight gain of larvae, the other phenolic fractions were similar each other and also were similar to the control water to allow weight gain of larvae. Larval weight gain differed significantly also for larvae fed alkaloids fractions from the four Meliaceae species and the *C. odorata* grafted on *T. ciliata* plants compared to the controls ether and water. Alkaloid fraction effect on weight gain was more notable between *S. macrophylla* and *T. ciliata* or the grafted combination (Fig. 4.4).

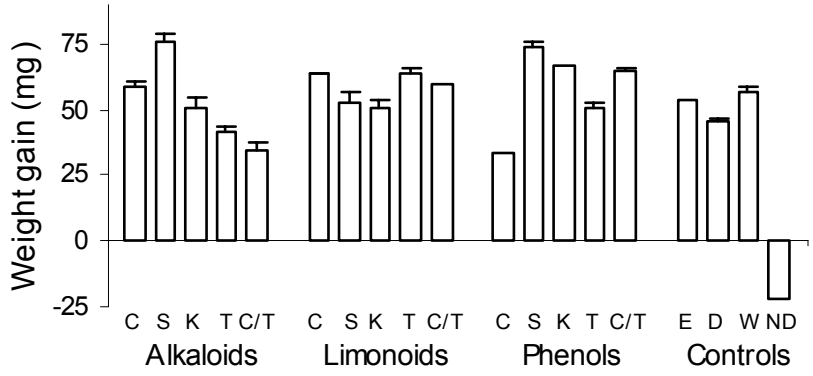

Figure 4.4 Weight gain for instar II *H. grandella* larvae reared on *C. odorata* leaf disks treated with alkaloid, limonoid and phenolic fractions from four Meliaceae species and a graft combination. C = *C. odorata*, S = *S. macrophylla*, K = *K. senegalensis*, T = *T. ciliata*, C/T = *C. odorata* grafted onto *T. ciliata*, E = Ether, D = Dichloromethane, W = Water, ND = No disk. Data were taken two days after starting bioassay. Data are means (± SE, n = 30) from three bioassays combined into one data set analysis.

Alkaloid, limonoid, or phenolic fractions from *S. macrophylla*, as well as limonoid fraction from the grafted *C. odorata* on *T. ciliata* or the phenolic fraction from *T. ciliata* plants, reduced the time to pupation by two and four days compared to the controls water and no-food, respectively, but their effect was similar to the other controls and fractions from the various species (Fig. 4.5A). All fractions that reduced the time to pupation, also had less time to the adult stage by five days compared to the no-food, but their effect was again similar to all the other controls and extracts from the various species (Fig. 4.5B).

The alkaloid fraction from *C. odorata* grafted onto *T. ciliata* and limonoid fractions from *C. odorata* or from *K. senegalensis* seemed to delay pupation compared to the other fractions (Fig. 4.5A). Such treatments and the phenolic fraction from *C. odorata* also seemed to delay adulthood compared to all the other fractions (Fig. 4.5B).

Fractions affected normal development of wings (Table 4.2) and such negative effect was given by the alkaloid fraction from the *C. odorata* grafted on *T. ciliata* plants and by phenolic fraction from *T. ciliata* plants causing 20% and 4% of adults with abnormal wing shape, respectively. All larvae fed leaf disks treated with any other fraction or the controls formed normal wings when they developed into adult moths.

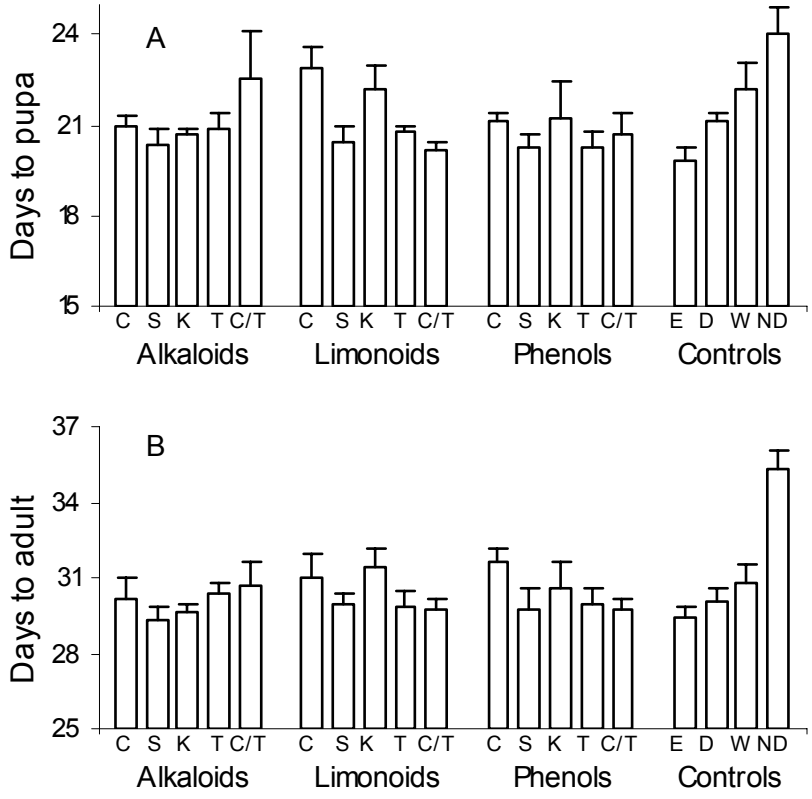

Figure 4.5 Time to pupa (A) and to adult (B) stages of *H. grandella* larvae reared on *C. odorata* leaf disks treated with alkaloid, limonoid, and phenolic fractions from four Meliaceae species and a graft combination. C = *C. odorata*, S = *S. macrophylla*, K = *K. senegalensis*, T = *T. ciliata*, C/T = *C. odorata* grafted onto *T. ciliata,* E = Ether, D = Dichloromethane, W = Water, ND = No disk. Data are means (± SE, n = 30) from three bioassays combined into one data set analysis.

Larval survival. Extract fractions affected larval survival differently (Table 4.2) at 2, 10 and 25 days after starting bioassay. Fewer larvae reared on disks sprayed with limonoid extracts survived compared to larvae reared on disks sprayed with alkaloid or phenolic extracts.

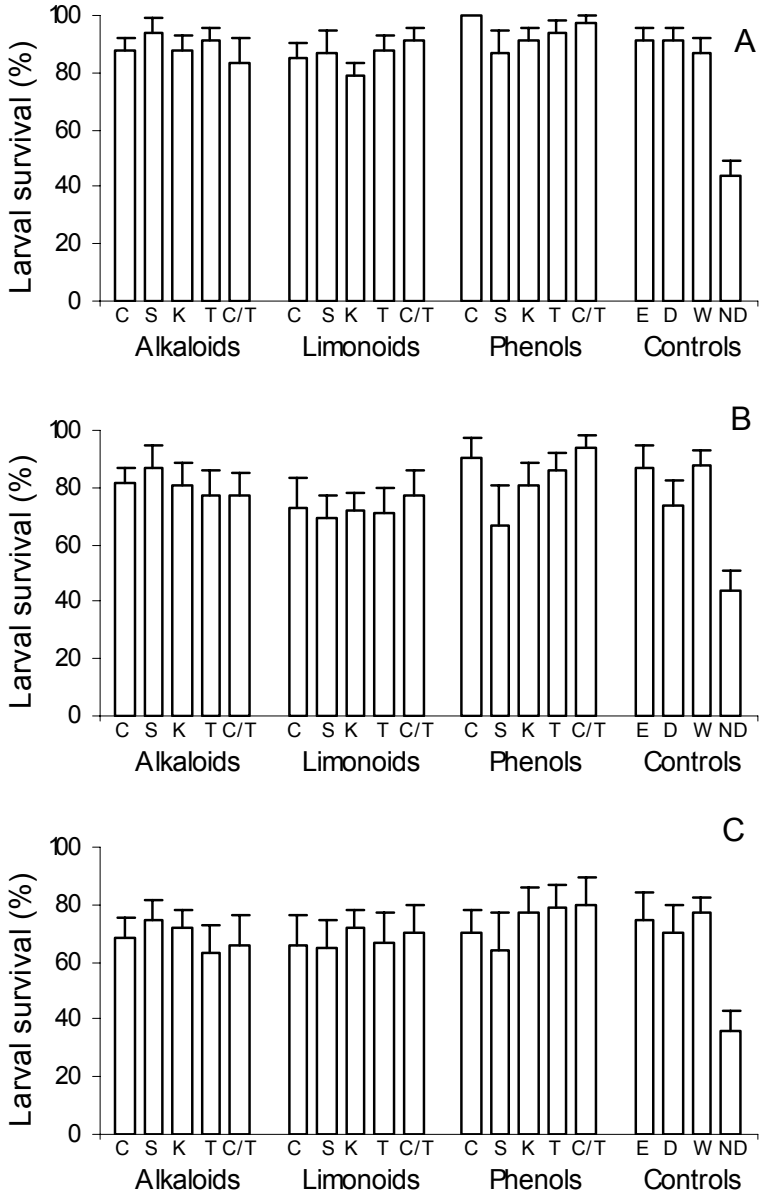

Figure 4.6 Survival of instar II *H. grandella* larvae exposed during two days on *C. odorata* leaf disks treated with alkaloid, limonoid, and phenolic fractions from four Meliaceae species and a graft combination. Data were taken 2, 10 and 25 days after starting bioassay (A, B, and C, respectively). C = *C. odorata*, S = *S. macrophylla*, K = *K. senegalensis*, T = *T. ciliata*, C/T = *C. odorata* grafted onto *T. ciliata,* E = Ether, D = Dichloromethane, W = Water, ND = No disk. Values are means ± SE, n = 30.

The reduction for larval survival at two days after starting bioassay was most notable for limonoid extracts from *K. senegalensis* compared to alkaloids from S*. macrophylla* and phenolics from *C. odorata* or *C. odorata* grafted on *T. ciliata* (Fig. 4.6A). Ten days after starting bioassay, larval survival was reduced by limonoids from *S. macrophylla* and *K. senegalensis* compared to alkaloids from *S. macrophylla* and phenolics from *C. odorata* and *C. odorata* grafted on *T. ciliata* (Fig. 4.6B). However, in the first two dates of evaluation the reduction of survival by fractions was not at the same level caused by the control starved larvae. The effect of starvation during two days also was notable after 25 days starting the bioassay when all fractions had a similar effect on larval survival (Fig. 4.6C).

Discussion

In previous greenhouse tests using whole plants of the four species cited and their combinations by grafting, *T. ciliata* and *C. odorata* species had the highest and the lowest damage, respectively due to *H. grandella* larvae, whereas the grafted *C. odorata* onto *T. ciliata* was similar in resistance to normal *T. ciliata* plants. Such results were confirmed by a leaf disk bioassay in laboratory (Pérez-Flores *et al*., 2006b).

In the present study, both crude extracts and fractions from leaves and shoots of the four Meliaceae species and the grafted combination, affected larval survival and performance of *H. grandella*. *Toona ciliata* crude extract was toxic to *H. grandella* larvae. Larvae ate very little of the *C*. *odorata* leaf disks sprayed with this extract and 90% died after two days. Such a deleterious effect of *T. ciliata* crude extract agreed with the previous results (Grijpma & Roberts, 1975) of larvae reared directly on either *T. ciliata* leaf disks or intact plants (Pérez-Flores *et al*., 2006a, b). Grijpma and Roberts (1975) did not find mortality of instar II *H. grandella* larvae reared on an artificial diet mixed with a methanol extract from *T. ciliata* leaves but found 53% mortality when diet was mixed with aqueous leaf extract of *T. ciliata*.

Cedrela odorata crude extract seemed to have a phagostimulatory effect on *H. grandella* larvae since larvae exposed to *C. odorata* leaf disks sprayed with this species crude extract consumed three times more leaf area than when exposed to leaf disks treated with the extract from *S. macrophylla* (i.e., the other susceptible species). Moreover, *C. odorata* crude extract caused to larvae gain weight twice and four times more than larvae exposed to water treated disks (absolute control) or leaf disks treated with *S. macrophylla* crude extract, respectively (Table 4.1).

Swietenia macrophylla extract decreased larval weight gain compared to *C. odorata* extract and the water control and delayed pupation and pupa weighed more compared to all the other extracts. Such results could explain the preference of *Cedrela* spp. over *Swietenia* spp. reported by Speight and Wylie (2001). Rodríguez and Vendramin (1996) found that *Cedrela fissilis* extract at 5% in artificial diet failed to affect survival and length of larvae or pupae weight of *Spodoptera frugiperda*, but *S. macrophylla* leaf extract, also at 5% in artificial diet, deterred feeding and inhibited growth.

Toona ciliata seemed to have a deterrent effect both as intact (non-grafted) plant and as a rootstock for *C. odorata*. The crude extracts from these plants decreased leaf area consumption to a similar level of methanol and less than water control treatments. Weight loss caused by relative controls was expected since methanol dehydrated plant tissues and made the leaf disks less appealing to the larvae, so they reduced leaf consumption, whereas larvae without leaf disk used their own reserves to survive.

The leaf crude extract from *C. odorata* grafted onto *T. ciliata* also caused larvae to lose weight similar to the relative control treatments methanol and no-food control (Table 4.1). Weight loss caused by the grafted plant could be attributed to alkaloids (Grijpma, 1976), limonoids (Da Silva *et al*., 1999), or phenolics (De Paula *et al*., 1997) from the rootstock translocated to the *C. odorata* scion and present in the crude extract. My results indicated that alkaloids from the grafted *C. odorata* scions on *T. ciliata* rootstocks decreased

larval weight gain at the same level of the intact (non-grafted) *T. ciliata* plants and such fractions differed to limonoids and phenolics fractions as well as to the control treatments. Alkaloid fractions from the grafted *C. odorata* on *T. ciliata* also reduced leaf consumption by *H. grandella* (Fig. 4.3). These findings caused by the *C. odorata* grafted onto *T. ciliata* alkaloid fraction agree with the hypothesis of Grijpma (1976) about the transfer of alkaloids from *T. ciliata* to *C. odorata* to confer resistance in this susceptible scion.

Consumed leaf area and weight gain had an inverse relation when leaf disks were sprayed with limonoid extracts. In other words, with exception of limonoid fraction from *K. senegalensis*, the limonoid fractions from the other species and grafted *C. odorata* on *T. ciliata* seemed to have a phagoestimulatory effect on *H. grandella* larvae, but after consumption larvae weight gain was lower than the expected, or larvae died. For *Spodoptera frugiperda* neonates, limonoids from *C. odorata* species mixed in artificial diet also showed a postdigestive toxicity reducing growth and causing significant mortality after rearing (Céspedes *et al*., 2000).

The difference between crude extract and fraction bioassays regarding larval mortality seems consistent with a probable synergistic effect of multiple defenses (Warthen, 1990). Here I found that besides causing deterrence, crude extracts caused a high mortality, specifically those extracts from *T. ciliata* species (Fig. 4.2).

In summary my hypotheses were confirmed since crude extracts from the resistant *T. ciliata* species reduced larval survival and performance more than the leaf extracts from susceptible species. In addition, the crude extract from *C. odorata* grafted on *T. ciliata* affected equally larval survival and performance compared to the control methanol and its effects were more deleterious compared to the crude extract from *K. senegalensis*. Substances in the resistant species *T. ciliata* acted as direct toxicants to *H. grandella* larvae similar to in the neem-derived Azatin (Mancebo *et al*., 2002). These authors reported that

only small amounts of ingested *C. odorata* leaf disks dipped in Azatin 10% were enough to kill almost 100% of larvae in a 24 h period of exposure.

Taking in account that an efficient control for *H. grandella* is currently lacking due to the low damage threshold of one larva per plant (Hilje & Cornelius, 2001), the present findings are important because they demonstrated that *H. grandella* is affected by crude plant extracts of resistant Meliaceae species as well as by the alkaloid fraction extracted from *C. odorata* scions grafted on *T. ciliata* rootstocks. These extracts might become an important resource to be used for an integrated pest management approach for this insect.

Chemicals that reduce *H. grandella* growth and survival seemed to be translocated from roots or stems to leaves, as seen for leaf disks (Pérez-Flores *et al*., 2006b) and results of crude extract from grafted plants (this study). Therefore the extracts represent potentially useful raw material for developing microinjections or implants into tree stems as slow-release formulations, increasing their persistence; a further step would be to identify the specific substances in the alkaloid, limonoid, and phenolic fractions that act against *H. grandella* in order to synthesize, combine, and incorporate them in commercial products. They could be deployed to protect *Cedrela* spp. and *Swietenia* spp. trees for 5 to 8 years (critical period to *H. grandella*), which is the time required to achieve a commercial trunk for these species, depending on the site where they grow (Cibrián *et al*., 1995).

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CHAPTER 5

In vitro establishment of *Swietenia macrophylla* King and *Cedrela odorata* L. by nodal explants

Running head: Establishment of mahogany and Spanish cedar nodal explants

Key words Spanish cedar, mahogany, Meliaceae, disinfection, *in vitro* response.

Abstract

In order to establish nodal explants taken from *Swietenia macrophylla* and *Cedrela odorata* 10-year-old plants, sodium hypochlorite (NaOCl) and plant preservative mixture (PPM^{TM}) were tested as surface disinfectants and/or added to the culture media. Also, N^6-benzylaminopurine (BAP), silver nitrate ($AgNO_3$), activated charcoal, vented caps and Micropore (3M) paper tape were tested to determine explant response from both species in micropropagation. Sodium hypochlorite at 15% for 20 min, during surface sterilization and NaOCl added to the culture medium at 0.2% w/v, was the best treatment to reduce contamination for both species, and this combined treatment reduced contamination in *S. macrophylla* explants more than that of *C. odorata* explants. Plant preservative mixture^{TM} added to the medium worked better on *S. macrophylla* than on *C. odorata* for reducing contamination and increasing bud break. On *S. macrophylla*, PPM^{TM} also favored the growth of sprouted buds without impairing bud break or growth of sprouted buds. For surface sterilization PPM at 5% failed to reduce contamination, prevented bud break and was toxic to subsequent growth of *C. odorata* nodal explants. Toxicity increased as the PPM treatment time increased. For *S. macrophylla*, the highest BAP concentration resulted in higher percentages of bud break than for *C. odorata* (64% and 25%, respectively). Neither activated charcoal nor $AgNO_3$ alone or combined prevented defoliation. Silver nitrate at 3 mg l^{-1} decreased contamination but also increased defoliation. Instead, activated charcoal prevented defoliation. Bud break was twofold higher in nodal explants established

on vessels using vented caps than in normal caps. The results for the type of sealant were similar to results for the type of caps. The response of *S. macrophylla* to disinfection and BAP treatments was better than for *C. odorata* in the establishment phase of micropropagation.

Introduction

In vitro propagation of *Swietenia macrophylla* King (mahogany) and *Cedrela odorata* L. (Spanish cedar) was completed by using shoot tips (Rodríguez *et al*., 2003; Maruyama, 2006) or nodal explants excised from young seedlings taken from *in vitro* germinated seeds (Maruyama *et al*., 1989; Orellana, 1997; Valverde *et al*., 1998; Pérez *et al*., 2002). Explants from mature trees are more prone to contamination and recalcitrance (i.e., lack of response) to *in vitro* manipulation than are explants from young plants such as seedlings (Merkle & Dean, 2000; Rouse-Miller & Duncan, 2000).

Recalcitrance of mahogany and cedar in micropropagation is characterized by lack of ability to break bud, absence or negligible growth of sprouted buds, leaf abscission on new shoots and presence of only one elongated petiole (Lee & Rao, 1988). In addition, explants sometimes become necrotic due to the presence of phenolics and die without apparent contamination (Flores, 2001). Nevertheless, contamination is the other essential problem to overcome in order to micropropagate forest trees successfully (Maruyama *et al*. 1989).

Specifically, for mahogany and cedar the difficulty in obtaining microbe-free cultures starting with explants from mature trees has been related to surface and endogenous contamination in donor plants (Maruyama, 2006). This author did not observe contamination on initial culture or cultures, but contamination was present after the fifth or later subcultures of mahogany shoots from pruned trees of 3-year-old.

During micropropagation of *Toona ciliata* (Meliaceae) nodal explants from 2-year-old plants, between 17 and 35% of the cultures were contaminated during establishment, even

though explants were surface sterilized by soaking in 70% ethanol for 1 min followed by immersion in 1.8% w/v sodium hypochlorite (NaOCl) for 30 minutes. For *S. macrophylla* nodal explants from 2-year-old plants, losses due to contamination during *in vitro* establishment were 44% even after double surface sterilization in calcium hypochlorite [$Ca(OCl)_2$] at 10% w/v for 20 min and then 8% w/v for 10 min) (Flores, 2001). In the same study, surface sterilization of explants by immersion in 50% w/v NaOCl for 15 min reduced contamination loss by 45%. Such results indicate that sodium or calcium hypochlorite as surface sterilizants could reduce contaminants in Meliaceae nodal explants.

Contamination of explants can also be prevented by adding NaOCl to the culture medium (Teixeira *et al*., 2006). These researchers report that the addition of NaOCl (0.0003% active chlorine) sterilized the culture media and doubled biomass and the number of new shoots on *Ananas comosus* explants.

Niedz (1998) suggests Plant Preservative Mixture (PPM^{TM}, Plant Cell Technology, Washington DC, USA) be used to reduce endogenous and surface contamination of explants. This product is a heat stable biocide, which kills bacteria and fungi cells, prevents germination of spores, and can eliminate endogenous contaminants without impairing the response of explants in tissue culture (Guri & Patel 1998). These authors state that the active ingredients (isothiazolones—methylchloroisothiazolinone and methylisothiazolinone) in PPM^{TM} interfere the citric acid cycle and the electron transport chain and also inhibit transport of monosacharides and amino acids in fungus and bacterium cells, improving the response of explants.

The response of explants *in vitro* also could be improved by adding plant growth regulators (PGR) and other substances to the culture medium and also modifying the atmosphere inside the vessels. N^6-benzylaminopurine (BAP) is the typical PGR used to induce bud break, whereas activated charcoal and silver nitrate ($AgNO_3$) have been used to improve the response of explants by modifying both the culture medium and the atmosphere

in culture vessels. Silver nitrate is a regulator of ethylene biosynthesis, one of the main gases affecting explant response in culture vessels (Fuentes *et al*., 2000) and has bactericidal activity (Kuvshinov *et al*., 1999). Activated charcoal is often added to the culture medium to absorb inhibitory substances released by the explant into the medium and vessel headspace, and has the additional role for enhancing air movement in the culture vessels (Kitaya *et al*., 2005).

The atmosphere in vessels also can be modified by air exchange as it is practiced in photoautotrophic micropropagation (Zobayed *et al*., 2004). Air exchange inside of the vessels depends on the type of sealant and closures. For genetic transformation of *Brassica rappa*, cultures sealed with Micropore 3M paper tape increased regeneration from 0 to 5% up to 60 to 80% compared to parafilm mainly by allowing air interchange (Kuvshinov *et al*., 1999).

Recalcitrance and contamination in *S. macrophylla* and *C. odorata* are the main causes of ineffective micropropagation of both species *in vitro* by nodal explants excised from mature donor plants. Therefore, in order to establish nodal explants taken from 10-year-old plants S*. macrophylla* and *C. odorata* successfully, NaOCl and PPM^{TM} were tested as surface disinfectants and/or added to the culture media. Also, BAP, AgNO3, activated charcoal, vented caps and Micropore paper tape were tested to determine the response of such explants from both species during micropropagation.

Methods

Plant material. Single node explants were taken from stems in 10-year-old *C. odorata* and *S. macrophylla* plants, which were maintained in a greenhouse. The shoots were always cut early in the morning (ca. 6 a.m.) to obtain turgid tissues. Inside the greenhouse, leaves were severed, and shoots were put in a plastic bag and taken to the biotechnology laboratory at the Tropical Agricultural Research and Higher Education Center (CATIE, Costa Rica).

There, plant material was washed with soap 0.03% (FADIS-Quimisol, Costa Rica), under running water, before cutting single node explants of about 2 cm in length. Nodal explants were then pre-disinfested as follows unless stated otherwise: 1 hour in Benomyl (Piscis, Costa Rica; 2 g l^{-1}) plus Manzate (Pfizer, México; 3 g l^{-1}) plus Agrimycin (Pfizer, México; 1 g l^{-1}) plus Tween 20 (Sigma, St. Louis, MO, USA; one drop for 100 ml of solution); 20 min in $Ca(OCl)_2$ at 10% w/v + one drop of Tween 20 for 100 ml of solution, and 15 min in $Ca(OCl)_2$ at 8% w/v plus one drop of Tween 20 for 100 ml of solution. Explants were treated with $Ca(OCl)_2$ inside the laminar flow hood. Explants were rinsed three times for 30 seconds each with sterile double-distilled water after surface sterilization.

1. Establishment of aseptic cultures

1.1. Use of NaOCl for surface sterilization and added to the culture media. a) Pre-disinfection in $Ca(OCl)_2$ was changed by using NaOCl (commercial bleach at 3.5% w/v active ingredient) at 15, 30, or 45% for 10 or 20 min and then factorial combined with NaOCl at 0, 2 or 4% v/v added to the medium. Each one of the 18 treatments consisted of three replications, each one with ten *C. odorata* nodal explants.

b) For *S. macrophylla* nodal explants, NaOCl at 15% as for pre-disinfection procedure for 10 or 20 min was factorial combined to NaOCl at 2 or 4% v/v added to the medium. Each treatment consisted of five replications, each one with ten explants.

c) From the two latter experiments, the best combination reducing explant contamination and increasing bud break was NaOCl at 2% v/v added to the medium and NaOCl 15% v/v for 10 min during surface sterilization. Therefore, for *S. macrophylla* another experiment consisting of three introductions of nodal explants 15 days after each other, was completed in order to confirm the effect of such treatment. Each introduction consisted of four replications. The number of explants introduced depended on the availability of plant

material. Therefore, the first introduction had 25 nodal explants per replication, the second had 70, and the third one had 45 nodal explants per replication.

In the three experiments, the medium used was Schenk and Hildebrant (SH medium) supplemented with BAP 1 mg l^{-1}. Containers and caps were autoclaved at 1.46 kg cm^{-2} for 20 min, and the culture medium boiled for 5 to10 min after heating for ten min per liter in a microwave previous to dispense it in the containers. This procedure of culture media sterilization and autoclaving of containers and caps were used for all experiments excepting PPM™ ones where media and recipients were autoclaved at 1.46 kg cm^{-2} for 20 min.

1.2. Use of PPM™ added to the culture media and combined to the pre-disinfection treatment. a) In order to test the effect of PPM™ added to the culture medium, media supplemented with 0, 1, 2, 3, or 4 ml l^{-1} of this biocide and were placed in a chamber. Each dose (treatment) contained five replications, with ten explants in each one. b) In a parallel experiment, the pre-disinfection in $Ca(OCl)_2$ was changed by rinsing nodal explants in PPM™ at 0 or 2 ml l^{-1}; later, explants were rinsed with sterile double distilled water and inoculated directly into the culture medium containing PPM™ at 0, 0.5, 1.0, 1.5, or 2 ml l^{-1}. Each treatment had five replications, with five explants each one.

1.3. Use of PPM™ instead of the pre-disinfection treatment. To reduce endogenous contamination in *C. odorata* nodal explants, PPM™ at 5% v/v in Murashige and Skoog (MS, Murashige and Skoog, 1962) salts was used instead of the pre-disinfection treatment (Benomyl + Agrimycin, and then $Ca(OCl)_2$). Nodal explants were treated for 6, 12, 18, or 24 hours, with pre-disinfection treatment as control. Gentle shaking was applied continuosly during the PPM™ treatment in a shaker (GyromaxTM 721, HOTECH Instruments Corp.). Each one of the four treatments and control had ten replications, each one with ten explants per replication. The culture medium was also supplemented with indolebutyric acid (IBA) 0.5

mg l^{-1}, isopentenyladenine (2-iP) 1 mg l^{-1} and PPM^{TM} 2 ml l^{-1}. Explants receiving the control treatment were rinsed three times in sterile double-distilled water after surface sterilization; explants disinfected with PPM^{TM} at 5% v/v were not rinsed.

2. Response of *C. odorata* and *S. macrophylla* to *in vitro* culture

2.1. Effect of BAP on bud break

Cedrela odorata and *S. macrophylla* nodal explants were disinfected by rinsing in fungicides as described above and then treated for ten min in NaOCl 15% v/v. One experiment was established to evaluate the effect of BAP at 0, 0.5, 1, 2, or 4 mg l^{-1}, on bud break by both species. Each one of these five treatments had four replications for *C. odorata* and five for *S. macrophylla*, each one with ten explants. The MS culture medium was supplemented with NaOCl 2% v/v.

2.2. Improvement of the culture atmosphere

a) Use of silver nitrate and activated charcoal. The objective of the experiment was to prevent the leaf abscission by shoots from *C. odorata* nodal explants, by adding activated charcoal or $AgNO_3$ to the culture medium. The explants used had been introduced 20 days before on half strength MS medium, supplemented with sucrose 1.5% w/v, 2-iP 1 mg l^{-1}, NaOCl 2% (v/v), and Agrimycin 1 g l^{-1}.

Activated charcoal at 1 g l^{-1} and $AgNO_3$ (3 mg l^{-1}), were tested alone and combined (three treatments) along with a control, which lacked these substances. Each one of these four treatments had five replications with ten explants per replication.

b) Use of vented caps. The objective of the experiment was to compare the effects of normal and vented caps (Magenta Corp., Sigma Chemical) on leaf abscission of *S.*

machrophylla during *in vitro* establishment. Three replications were used for each treatment (either a normal or a vented cap on a glass jar), each one with 50 explants.

c) Use of Micropore as a vessel sealant. The objective of the experiment was to compare the effect of polyethylene food wrap (Glad, Costa Rica) which is a common vessel sealant and Micropore tape (3M Corporation, St. Paul, MN) as sealants during establishment of *C. odorata* nodal explants. Six replications were used for both treatments, each one with 12 nodal explants.

Half-strength MS culture medium was used in the experiments for the response of explants *in vitro*. Media was supplemented with 2-iP 0.05 mg l^{-1}, indoleacetic acid (IAA) 2 mg l^{-1}, sucrose 1.5% w/v, activated charcoal 1 g l^{-1}, streptomycin 300 mg l^{-1}, and NaOCl 2% v/v.

The variables evaluated for aseptic establishment of cultures were the percentages of contamination by fungi and bacteria as well as bud break (shoots with axillary bud expanded at least 3 mm), shoot length and damage to explants (in the case of PPM^{TM}), whereas the responses of explants to *in vitro* culture that were recorded were bud break, the mean number of dropped leaflets per shoot, the mean number of total leaflets produced per shoot, and the percentage of leaf abscission (mean of fallen leaflets divided per mean total leaves multiplied by 100).

Culture conditions. All culture media were supplemented with sucrose 3% w/v and gelled with agar 7 g l^{-1}, pH was adjusted to 6.0 or 5.8 regarding to the addition or absence of NaOCl in the medium. Cultures were incubated at 25^0C under 12 h photoperiod provided by Phillips cool-white fluorescent light tubes.

Experimental design. All experiments were established in a completely randomized design. The experimental unit was an individual nodal explant in a 50 ml vial with 10 ml of culture medium for the establishment of aseptic cultures or in 200 ml glass jar with 30 ml of culture medium for the response of nodal explants to *in vitro* culture.

Statistical analysis. Data were examined for normal distribution and homogeneity of variances required for analysis of variance (ANOVA). Contamination and bud break data were transformed by Y = arcsin(sqrt(Y)), and shoot length was transformed by Y = sqrt (Y + 0.5) before statistical analysis. ANOVA was completed (Proc GLM; SAS, 2001) and, if F test was significant, means were compared using the Duncan's multiple range test or the least mean squares test at the 5% level.

Results and discussion

Although donor plants were maintained in a greenhouse and fungicides and bactericides were applied routinely every 15 days, in a preliminary experiment for *in vitro* establishment the incidence of contamination was high and bud break was low by using the pre-disinfection procedure. Ninety percent of C*. odorata* and 84% of *S. macrophylla* nodal explants taken from 10-year-old stock plants were contaminated. Contamination was mainly due to bacteria in *C. odorata* explants and due to fungi in *S. macrophylla* explants. Moreover, bud break was lower than 17% for both species.

Such contamination was the main cause of losing explants since contamination of cultures resulted in plant death (Leifert *et al*., 1991). This finding agreed with the results of Sharma and Ramamurthy (2000) for the micropropagation of elite 4-year-old *Eucalyptus tereticornis*. They found the highest contamination of explants without bud break during the hot and humid months of late summer and such conditions are permanent at CATIE (Salas, 2000) where the research was completed.

1. Establishment of aseptic cultures

1.1. Use of NaOCl during surface sterilization and added to the culture media.

Contamination of *C. odorata* nodal explants differed among levels of NaOCl used during surface sterilization (d.f. = 17, 36; F = 10.03, P < 0.0001), between the two times of sterilization (d.f. = 1, 36; F = 17.33, P = 0.0002), and among levels of NaOCl added to the culture medium (d.f. = 2, 36; F = 66.32, P < 0.0001) (Table 5.1). However, a three-way interaction between the factors was absent (P = 0.1).

Table 5.1 Effect of NaOCl as a surface sterilizant and added to the culture medium on contamination and bud break percentages of *C. odorata* nodal explants.

Factor of NaOCl	Percentage	
	Contamination	Bud break
Min of pre-disinfection		
10	57.4 a	10.4 a
20	42.2 b	7.4 a
Concentration (%) used for pre-disinfection		
15	52.2 a	11.1 a
30	52.8 a	7.8 a
45	44.4 a	7.8 a
Added to the culture medium (%)		
0	78.9 a	5.6 a
2	40.6 b	11.7 a
4	30.0 b	9.4 a

For each factor within a column, means with the same letter are similar as determined by Duncan test at 5% level.

The two-way interaction of exposure time during surface sterilization and levels of NaOCl added to the medium affected bud break of explants (d.f. = 2, 36; F = 3.45, P = 0.04), More time of exposure and high level of NaOCl during surface sterilization, as well high level of NaOCl added to the medium reduced explant contamination, but also reduced bud break (Table 5.1) perhaps due to toxic effects. During the sterilization treatments deep penetration of sterilizing agents into the explant tissue could have caused toxic effects, which result in

delayed and reduced growth responses by explants (Thakur & Sood, 2006), such as sprouting of buds.

The combination of NaOCl at 15% v/v for 20 min during surface sterilization and NaOCl added to the culture medium at 2% v/v reduced contamination to 43% and allowed 20% bud break (data not shown), which resulted in lower than average explant contamination (49.8% ± 1.5%) but higher than average bud break (8.9% ± 1.1%).

Contamination of *S. macrophylla* nodal explants differed among NaOCl treatments (d.f. = 3, 16; F = 4.15, P = 0.02) (Table 5.2). The NaOCl concentration in the medium affected explant contamination (d.f. = 1, F = 12.09, P = 0.003), but an interaction between the factors was absent (d.f. = 1, F = 0.25, P = 0.6). The mean (± SE) for explant contamination was 29.8% ± 3.4%.

Treatments also affected bud break differently (d.f. = 3, 16; F = 7.67, P = 0.0004). The NaOCl concentrations in the medium affected bud break (d.f. = 1, F = 28.06, P = 0.0001), but an interaction between NaOCl treatment time during surface sterilization and NaOCl concentration in the medium was absent (d.f. = 1, F = 4.10, P = 0.06). The mean (± SE) bud break for explants in all treatments was 34.1% ± 2.2%.

The least squares means indicated statistical differences among treatments for both contamination and bud break (Table 5.2). The low NaOCl concentration in the medium reduced contamination and increased bud break significantly more than the high concentration. Such results agreed with those of Teixeira *et al*. (2006) who reported active chlorine concentration as low as 0.0003% used to sterilize the culture medium and improve the response of *Ananas comusus* (pineapple) by doubling biomass and number of new shoots.

Ten minutes of surface sterilization reduced contamination while increasing bud break compared to the 20 min, but bud break was similar when NaOCl treatment time was combined with the low concentration of NaOCl added to the medium (Table 5.2). Therefore,

the best combination for explant treatment was NaOCl at 2 ml l^{-1} added to the medium and 15% NaOCl used for 10 min during surface sterilization.

Table 5.2 Effect of NaOCl as a surface sterilizant and added to the culture medium on contamination and bud break percentages of *S. macrophylla* nodal explants.

NaOCl		Percentage	
15% surface sterilization (min)	In the medium (% v/v)	Contamination	Bud break
10	2	14.0 a	50.0 a
20	2	20.0 a	42.0 a
10	4	43.2 b	18.6 b
20	4	42.0 b	26.0 b

Means with the same letter within columns are similar as determined by least squares means at the 5% level (n = 50).

In the follow up test of the best treatment combination for *S. macrophylla* introductions, nodal explants had low contamination and a 53% ± 3.5% bud break, although bud break was similar among introductions (d.f. = 2, 9; F = 3.86, P = 0.06). The low explant contamination for the experiment (19.8% ± 1.8%) and differences among introductions (d.f. = 2, 9; F = 12.45, P = 0.003) could be explained by environmental conditions. Twenty-three and 28.2% nodal explants were contaminated in the first and third introductions, respectively, whereas only 8.2% in the second, maybe because the first and third introductions of nodal explants were made during periods of heavy rainfall, and the experiment was conducted in December when temperature is lower than in summer, although rainfall amounts are higher (Salas, 2000). For the micropropagation of *Eucalyptus tereticornis* at various times through out the year, incidence of endogenous fungal or bacterial contaminants was most severe during the hot and humid months of late summer (Sharma & Ramamurty, 2000).

1.2. Use of PPM^{TM} added to the culture medium and then combined with PPM^{TM} treatment during surface sterilization. When PPM^{TM} was added to the medium, the concentrations effects were similar on contamination (d.f. = 4, 20; F = 0.27, P = 0.9) and bud break percentage (d.f. = 4, 20; F = 1.49, P = 0.2) for *C. odorata*; the mean (± SE) for contamination and bud break were 49.6% ± 3.6% and 71.6% ± 2.5%, respectively, and contamination of nodal explants was mainly due to bacteria. For *S. macrophylla*, PPM^{TM} in the culture medium significantly reduced contamination, and also significantly favored shoot elongation, but its effect on bud break was similar (d.f. = 4, 20; F = 0.55, P = 0.7). The mean (± SE) bud break was 81.2% ± 2.4%. Contamination of nodal explants was mainly due to fungi.

The difference of contaminants between *S. macrophylla* (32.4% fungi, 5.2% bacteria) and *C. odorata* (8.8% fungi, 40.8% bacteria), agreed with the fact that different plant species grown *in vitro* create distinct environments inside the culture vessels, which in turn allow or prevent the growth of different contaminants (Leifert & Waites, 1992). Such difference of contaminants between both species also could be attributed to different efficacy of PPM^{TM} treatments. The PPM^{TM} added to the medium worked better for *S. macrophylla* than *C. odorata* reducing contamination and increasing bud break. Compared to the preliminary experiment, contamination was reduced by 46.4% and 40%, whereas bud break was increased by 68.2% and 54.6% for *S. macrophylla* and *C. odorata*, respectively. Besides reducing contamination and increasing bud break, PPM^{TM} at 2 ml l^{-1} also favored the growth of sprouted buds on *S. macrophylla* (Table 5.3).

For *C. odorata* PPM^{TM} doses were similar to the control for contamination and bud break percentages. The mean contamination varied from 42% for PPM^{TM} at 4 ml l^{-1} to 54% for PPM^{TM} at 3 ml l^{-1}. The bud break mean percentage varied from 64% on PPM^{TM} at 3 ml l^{-1} to 84% for PPM^{TM} at 1 ml l^{-1}. For the control, contamination and bud break were 50% and 72%, respectively.

Table 5.3 Effect of PPM^{TM} added to the culture medium on contamination and shoot length of *S. macrophylla* nodal explants.

PPM^{TM} (ml l^{-1})	Contamination (%)	Shoot length (cm)
0	54 a	0.4 b
1	44 ab	0.6 ab
2	32 bc	0.7 a
3	38 ab	0.5 ab
4	20 c	0.5 ab
Mean	37.6	0.5
SE	2.6	0.03
F	4.07	2.84
P	0.01	0.05

n = 25 for standard error (SE); degree of freedom for F values equal to (4, 20). Means with the same letter within columns are similar as determined by Duncan test at the 5% level.

When PPM^{TM} was used during surface disinfestations and added to the culture medium, treatment effects on *C. odorata* nodal explants were similar to each other for contamination (d.f. = 9, 40; F = 1.05, P = 0.42) and bud break (d.f. = 9, 40; F = 0.65, P = 0.74). Although the bud break (67.2% ± 3.3%) was similar to that of the previous experiment, the contamination was higher (79.2 ± 2.6%, with 71.5% contaminated explants by bacteria, and 7.7% by fungi). This result could be explained by the lack of a complete pre-disinfection in the half of introduced explants because PPM^{TM} at 0 or 2 ml l^{-1} was applied instead of $Ca(OCl)_2$ in the surface sterilization procedure. Jiménez *et al*. (2006) reported that the use of pre-disinfection with NaOCl combined with PPM^{TM} in the culture medium was an efficient way to establish *in vitro* explants of the neotropical giant bamboo (*Guadua angustifolia*). Another reason would be that three out of four concentrations of PPM^{TM} added to the medium, were lower than the level suggested by the manufacturer (2 ml l^{-1}), which also was the best in the previous experiment to reduce contamination, to increase bud break, and enhance shoot elongation in *S. macrophylla*.

For *S. macrophylla*, PPM^{TM} treatments used during surface sterilization and in the medium affected explant contamination and bud break differently (Table 5.4), but shoot elongation appeared unaffected (d.f. 9, 37; F = 1.01, P = 0.4). The mean (± SE) shoot

elongation was 0.5 cm ± 0.2 cm. If used alone, PPM^{TM} at 1 or 2 ml l^{-1} added to the medium, controlled the contamination better and increased bud break compared to all the other treatments.

Table 5.4 Effect of PPM^{TM} used during surface sterilization and added to the culture medium on contamination and bud break percentages of *S. macrophylla* nodal explants.

PPM^{TM} (ml l^{-1}) concentration		Contamination (%)	Bud break (%)
Surface sterilization	In the medium		
0	0	44 abc	52 b
0	0.5	32 bc	72 ab
0	1	20 c	96 a
0	1.5	32 bc	56 b
0	2	20 c	72 ab
2	0	70 a	55 b
2	0.5	52 abc	64 b
2	1	70 a	55 b
2	1.5	60 ab	50 b
2	2	44 abc	44 b
Statistics	Mean	43.0	62.1
	SE	3.0	2.9
	F	2.90	2.28
	P	0.01	0.03

n = 47 for standard error (SE); degree of freedom for F values equal to (9, 37). Means with the same letter within columns are similar as determined by Duncan test at the 5% level.

The combination of PPM^{TM} used during surface sterilization and added to the culture medium increased contamination and reduced bud break, compared to the previous experiment. In this experiment, PPM^{TM} again worked better for *S. macrophylla* than for *C. odorata* (see the above experiment), maybe due to the different microbial contaminants in each species. *Swietenia macrophylla* was contaminated by fungi and *C. odorata* by bacteria. Such specificity of contaminants could be attributed to plant species-specific bacteria being introduced into culture together with the explants (Fisse *et al*., 1988).

1.3. Use of PPM^{TM} during surface sterilization. The time of treating explants with PPM^{TM} at the level suggested by the manufacturer affected explant contamination, bud break, and damage by necrosis differently (Table 5.5). Compared to the control, PPM^{TM} at 5% during surface sterilization failed to reduce contamination and bud break but was toxic to subsequent growth of *C. odorata* nodal explants. Toxicity increased as the PPM^{TM} treatment time increased. Such toxicity could be due to PPM^{TM} being an acid (Guri & Patel, 1998). So, PPM^{TM} damaged the nodal explants preventing bud break and increasing explant susceptibility to contaminants. Nevertheless, the biocides (methylchloroisothiazolinone and methylisothiazolinone) used in PPM^{TM} are reported by the manufacturer (Plant Cell Technology) to be nonphytotoxic at concentrations of 4 to 5% during 4 to 12 hours for the prophylactic control of endogenous contaminants in plant tissue cultures for woody plants.

Table 5.5 Effect of rinsing time with PPM^{TM} on percentage of contamination, bud break, and necrosis of *C. odorata* nodal explants.

Rinsing with PPM^{TM} 5% (h)	Contamination	Bud break	Necrosis
Control	56 b	30 a	1 c
6	80 a	0 b	4 c
12	59 b	1 b	26 b
18	45 b	0 b	45 a
24	60 b	1 b	55 a
Statistics			
Mean	60	6.4	25.8
SE	2.2	6.4	1.8
F	6.28	33.18	45.55
P	0.0004	<0.0001	0.0001

n = 50 for standard error (SE); degree of freedom for F values equal to (4, 45). Means with the same letter within columns are similar as determined by Duncan test at the 5% level.

Both *S. macrophylla* and *C. odorata* responded differently to PPM^{TM} treatments. Although PPM^{TM} contains a mixture of two isothiazolones, which are a broad-spectrum industrial biocides (Niedz, 1998), the level of contamination recorded for both species was similar to that reported by Flores (2001) for nodal explants from 5-year-old *S. macrophylla* plants

when using $Ca(OCl)_2$ and NaOCl during surface sterilization. He recorded 44% of contamination with double surface sterilization treatment with $Ca(OCl)_2$ (10% for 20 min then 8% for 10 min) and 45% of contamination with NaOCl at 50% for 15 min. However, donor plants used in this research were 5 year older than that used for Flores (2001), and donor plant age is related to the incidence of contamination.

The results indicated that PPM^{TM} can be added at 2 ml l^{-1} to the culture medium during *in vitro* establishment of *S. macrophylla* to control contamination without impairing bud break or shoot elongation. For *C. odorata*, however, more experiments with this product are necessary to reduce contamination. Niedz (1998) indicated that PPM^{TM} can be routinely added to tissue culture medium to control air- and waterborne bacterial and fungal contaminants effectively.

2. Response of *C. odorata* and *S. macrophylla* to *in vitro* culture

2.1. Effect of BAP in the bud break. Various concentrations of BAP failed to affect bud break of *C. odorata* (d.f. = 4, 15; F = 0.91, P = 0.5) and *S. macrophylla* (d.f. = 5, 23; F = 1.83, P = 0.1) similarly. Nevertheless, both species responded differently to BAP treatments (d.f. = 5, 39; F = 4.94, P = 0.001), and in general bud break was almost twofold higher for *S. macrophylla* compared to *C. odorata* (44.7% ± 3.7% and 23.5% ± 2.1%, respectively). Rodríguez *et al*. (2003) also found a better response *in vitro* bud break of a *Swietenia* hybrid compared to *C. odorata* after BAP treatment. Although they found that stem explants from both species responded to increasing concentrations of BAP by sprouting more buds, in this study only *S. macrophylla* responded to the highest BAP concentration (Table 5.6). The difference in the results of Rodríguez *et al*. (2003) and this study might be due to the origin of explants, since they used nodal explants from *in vitro* seedlings but I used nodal explants from 10-year-old plants.

Table 5.6 Effect of BAP on bud break of *C. odorata* and *S. macrophylla* nodal explants.

BAP (mg l^{-1})	Bud break (%)	
	C. odorata	*S. macrophylla*
0	25.0 a	41.3 a
0.5	27.5 a	49.3 ab
1.0	25.0 a	29.3 b
2.0	15.0 a	37.3 ab
4.0	25.0 a	64.0 a

Each value is the mean for n = 40 in *C. odorata*, and n = 50 in *S. macrophylla*. Means with the same letter within columns are similar as determined by Duncan test at 5% level.

For *S. macrophylla*, BAP at 4 mg l^{-1} seemed to favor the bud break, which disagreed with the result of Mroginski *et al*. (2003) using nodal explants from 2-year-old *Toona ciliata* (Meliaceae) plants but agreed with the same researchers when they forced bud break for nodal explants from 10-year-old *T. ciliata* trees by using BAP at 5 mg l^{-1}. However, no two species have the same behavior *in vitro* (Kane, 2000), so the highest BAP concentration resulted in higher percentages of bud break for *S. macrophylla* than for *C. odorata*. This result could be explained because although both species were the same age, S. *macrophylla* tissues were woodier than *C. odorata*.

2.2. Improvement of the culture atmosphere. In all the previous experiments, some shoots grew ca. 1 to 1.5 cm and produced leaves. Leaves stayed on the explants until the 15^{th} day after inoculation and then fell off. So, three experiments were conducted in an attempt to prevent abscission by improving the culture atmosphere.

Effect of silver nitrate and activated charcoal on *C. odorata*. Neither activated charcoal nor $AgNO_3$ alone or combined prevented defoliation. $AgNO_3$ has shown to improve *in vitro* culture of some herbaceous species (Ozudogrl *et al*., 2005). In the present experiment,

although $AgNO_3$ at 3 mg l^{-1} lacked a statistical significant effect, it tended to decrease contamination while increasing defoliation (Table 5.7).

The effect of $AgNO_3$ on explant contamination could be explained because $AgNO_3$ at 1% is suggested as a surface disinfectant for explants (Kane, 2000) since Ag^+ can have bactericidal activity (Kuvshinov *et al*., 1999); therefore, using a higher concentration may prevent contamination of inoculated explants. The effect of $AgNO_3$ increasing defoliation could be explained by a phytotoxic effect of Ag^+, which is a heavy metal (Pua, 1999). For *Petunia hybrida*, $AgNO_3$ also inhibited shoot proliferation (Dimasi-Theriou *et al*., 1992).

Activated charcoal also helped to prevent defoliation, although treatments were statistically similar (Table 5.7). This positive effect could be explained because it is typically an absorbent for gases (e.g., ethylene) (Pan & van Staden, 1998). Therefore, it could have improved the vessel atmosphere and the explant response.

Table 5.7 Effect of silver nitrate and activated charcoal on contamination and leaf abscission in *C. odorata* nodal explants.

$AgNO_3$ (mg l^{-1})	Activated charcoal (g l^{-1})	Contamination (%)	Leaflets		Abscission (%)
			dropped No.	total No.	
0	0	60.0 ± 13.0	1.8 ± 0.6	14.0 ± 3.4	10.5 ± 3.2
0	1	60.0 ± 15.3	0.4 ± 0.2	12.2 ± 2.3	3.2 ± 2.0
3	0	58.6 ± 16.3	4.6 ± 3.2	16.6 ± 4.0	18.8 ± 10.7
3	1	30.4 ± 11.2	2.2 ± 0.8	13.0 ± 1.4	16.5 ± 6.6
Statistics	Mean	52.2	2.2	13.9	12.3
	SE	6.5	0.8	1.3	3.0
	F	1.07	1.1	0.42	1.12
	P	0.39	0.37	0.74	0.36
	d.f.	3, 16	3, 16	3, 16	3, 16

Each value is the average (± SE) of 50 nodal explants.

The interaction of both factors prevented contamination but also increased defoliation. Activated charcoal prevented defoliation more than $AgNO_3$ but not statistically (Table 5.7). Activated charcoal provided a dark environment, which could have prevented the photodegradation of NaOCl in the culture medium and favored controlling the contaminants.

Also, activated charcoal can absorb deleterious substances (e.g. ethylene) from *in vitro* culture. However, activated charcoal combined with $AgNO_3$ could have increased defoliation since absorption of ethylene by activated charcoal and Ag^+ interference with the receptor systems of the cells, may have resulted in a lower level of reception, which may be interpreted by the cells as an absence of ethylene. The perceived absence of ethylene thereby may have to lead overproduction (Theologist, 1992), which caused the defoliation. For *in vitro* rooting of *Prunus salicina*, activated charcoal induced chlorosis and severe leaf drop negatively affecting rooted shoots (Rosati *et al*., 1980). Moreover, activated charcoal improved shoot length of *Ficus carica* (Barbosa *et al*., 1992), although also reduced shoot proliferation by the same species (Fráguas *et al*., 2004), and rooting of *C. odorata* shoots (Pérez *et al*., 2006).

Use of vented caps in *S. macrophylla*. The types of caps used on cultures failed to affect contamination (d.f. = 1, 10; F = 0.09, P = 0.77) and bud break (d.f. = 1, 10; F = 4.04, P = 0.11) of *S. macrophyla* nodal explants. Nevertheless, the culture atmosphere seemed to be improved since fewer contaminated nodal explants were in vessels covered with vented caps than in normal caps (33.3 ± 16.2% and 43.2 ± 20.8%, respectively). Also, bud break was over double for nodal explants established in vessels with vented caps than for those with normal caps (52.7 ± 13.0% and 25.3 ± 4.4%, respectively). These results could be explained by a positive effect of ventilation in the environmental atmosphere, specifically by the filter of vented caps that might have allowed the exchange of air without microbial contaminant entering to the vessel. In photoautotrophic micropropagation, ventilation improved growth and quality, as well as lowered the percentage of contamination due to the manipulation of environmental conditions inside to the culture vessels (Zobayed *et al*., 2004). Such conditions allowed that on shoots about 2 cm in length, abscission was absent

at the day 20 (Fig. 5.1A). Leaves remained on shoots up to day 35, and then they started to drop from the shoot (Fig. 5.1B).

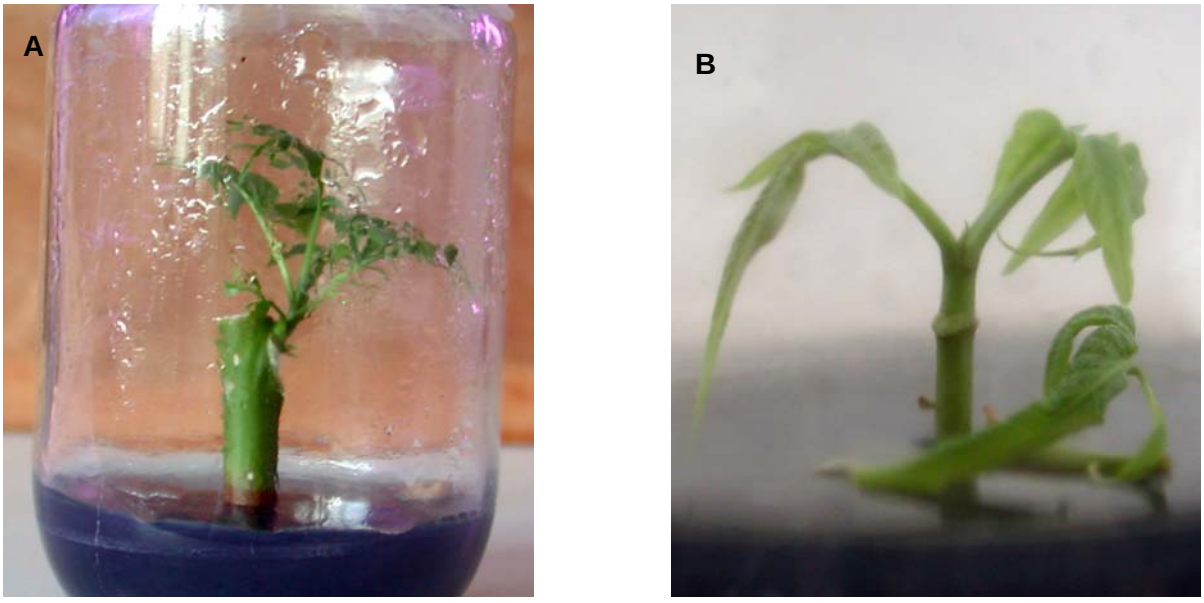

Figure 5.1 New shoot on *C. odorata* nodal explants maintaining leaves and leaflets at 20 days (A) and starting defoliation at 35 days after inoculation (B).

Use of Micropore as sealant in *C. odorata.* The results for the type of sealant were similar to results for the type of caps. Contamination was similar for vessels sealed with either type of sealant (d.f. = 1, 10; F = 1.57, P = 0.24), and bud break was also unaffected by the type of sealant (d.f. = 1, 10; F = 0.41, P = 0.54). Micropore, however, was numerically better than the normal sealant since the contamination percent was lower (30.1 ± 11.0% compared to 50.0 ± 11.4%) and the bud break percentage higher (52.8 ± 10.0% compared to 43.0 ± 11.5%). This effect could be due to the better control of humidity inside the container allowed by the Micropore sealant. Micropore tape is a breathable paper tape that allows gas exchange but keeps the culture atmosphere free from airborne bacteria and contaminants (Burne, 2006).

Gaseous substances such as CO_2 and ethylene in the headspace of cultured explants could affect their *in vitro* response (Demeester *et al*., 1995). Sealing the containers with closure materials that allow gas exchange may be essential for bud break. Therefore, Micropore would be combined with vented caps in order to get a better control of the *in vitro* atmosphere to improve responses by *C. odorata* and *S. macrophylla* nodal explants.

In conclusion, the use of PPM^{TM} or NaOCl added to the medium and combined with NaOCl as surface sterilization after a pre-disinfection treatment with fungicides and bactericides, decreased contamination in both *C. odorata* and *S. macrophylla*. The response for *S. macrophylla* to disinfection treatments was better than for *C. odorata*. For both species, the combination of vented caps and Micropore as sealant might improve the *in vitro* response of nodal explants in the establishment phase of micropropagation.

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CHAPTER 6

CONCLUDING CHAPTER

Introduction

After more than three decades of research, viable management approaches to prevent *H. grandella* from damaging *S. macrophylla* and *C. odorata* (Cornelius & Watt, 2003), which are the most valuable timber species of Latin America (Navarro & Hernández, 2004), has yet to be devised to establish commercial plantations. Management of this pest should be attainable through the combined use of tactics such as development of pest resistant planting stocks, pruning and vegetative propagation by grafting or micropropagation of selected resistant or tolerant trees, as well as other promising approaches currently being explored (Hilje & Cornelius, 2001).

Since *Hypsipyla* spp. prefer endemic Meliaceae spp. in their respective centers of origin (Cunningham *et al*., 2005), and grafting seemed to improve resistance on *Cedrela* spp. placed onto *T. ciliata* (Grijpma, 1976; De Paula *et al*., 1997; Da Silva *et al*., 1999; Bygrave & Bygrave, 2005), I completed research on the potential of grafting to induce resistance against *H. grandella* in *C. odorata* and *S. macrophylla*. Also I examined micropropagation techniques that will be useful for cloning selected resistant trees of both species.

As was presented in the chapter 2 of this dissertation, grafting experiments to date have disregarded important species such as *S. macrophylla* and *K. senegalensis*, even though both of them are of high economic importance, as *C. odorata* and *T. ciliata*, and face the same problem with *Hypsipyla* spp. In addition, the effects of reciprocal grafting and autografting of these four species on damage caused by *H. grandella*, as well as on larval performance and mortality were unknown.

Although the most realistic bioassays should employ whole plants, they are complicated by differences in leaf age, damage, disease, or water content which all may cause differential feeding by insects (Hare, 1998). Therefore, in the search for a more convenient and practical method, a bioassay using leaf disks was described in chapter 3 to determine effects of both intact and grafted Meliaceae species on the mortality and performance of *H. grandella* larvae. Such kind of bioassay by using leaf disk from susceptible, resistant, and grafted Meliaceae plants had not been completed previously.

In chapter 4, I reported the use of the leaf disk bioassays in order to test the effects of crude extracts and alkaloid, limonoid and phenolic fractions from leaf extracts on larval mortality and performance of *H. grandella*. Research on biochemical basis for resistance to this insect pest in Meliaceae has been completed only on limonoids (De Paula *et al*., 1997; Da Silva *et al*., 1999), even though Grijpma (1976) suggested that the biochemical basis for resistance in *T. ciliata* may be alkaloids and Newton *et al*. (1999) suggested that proanthocyanidins (i.e., phenolics) may reduce susceptibility of *C. odorata* to *H. grandella*.

In chapter 5, I presented micropropagation research that involved using nodal explants from *C. odorata* and *S. macrophylla*, as an alternative to clone selected plants as it is practiced for other forest species, such as *Eucalyptus* spp. (Glocke *et al*., 2006) and teak *Tectona grandis* (Goh & Galiana, 2000).

In the present chapter I will summarize the principal findings of the dissertation following the specific targeted objectives. Then I will describe the potential use of my results, and finally I will describe possible future research in order to expand the knowledge obtained from my study.

Objectives and principal findings

General objective 1. To investigate the potential to prevent damage due *H. grandella* by grafting *C. odorata* and *S. macrophylla* onto *T. ciliata* and *K. senegalensis*.

Specific objective 1.1. To determine the effect of grafting *C. odorata* or *S. macrophylla* on damage by *H. grandella* neonates and instar III larvae, larval performance and mortality.

Effect of Meliaceae species

In two experiments, the exotic species *K. senegalensis* and *T. ciliata* were partial or totally resistant to damage caused by *H. grandella* neonates or instar III larvae, whereas the native species *C. odorata* and *S. macrophylla* were susceptible to larval attack. These results were consistent whether young whole plants were used or leaf disks were tested in bioassays. Regardless of the method, *K. senegalensis* and *T. ciliata* decreased to different degrees the larval feeding and performance and increased larval mortality as compared to native species *C. odorata* and *S. macrophylla.* Larvae died after feeding on *T. ciliata* or *K. senegalensis* whole plants. On the leaf disk bioassay, 90% and 100% of larvae fed on leaf disks from intact and autografted *T. ciliata* died. Also, larvae consumed less leaf tissue or none at all, and fewer survived on leaf disks from *K. senegalensis* and *T. ciliata* than on the susceptible species. Pupal weight and length from larvae fed leaf disks from resistant and susceptible species were similar, perhaps because the 48 h of exposure in the bioassay may have been insufficient to alter larval growth processes. However, when neonates or instar III larvae where exposed during 15 days on whole resistant plants all larvae died.

Whole plants or leaf disks of *K. senegalensis* used as rootstock or scion or autografted allowed some larvae to develop to adults, but all treatments involving *K.*

senegalensis caused abnormal wing development on some *H. grandella* adults. The toxicity of *T. ciliata* prevented most larvae from developing to adults, precluding an assessment of this species effect on wing development.

These results could be an expression of the lack of coevolution between this New World insect species and Old World Meliaceae species, as was also demonstrated for neem *Azadirachta indica* A. Juss., whose metabolites showed either direct insecticidal or growth-disrupting on *H. grandella* (Mancebo *et al.*, 2002). *Toona ciliata* was more resistant to larva feeding than *K. senegalensis* either because of differences in the amounts or composition of antifeedant and toxic compounds.

Autografting of susceptible species induced resistance to *H. grandella*

Autografted plants *C. odorata* and *S. macrophylla* inoculated with eggs or instar III larvae, exhibited some resistance against *H. grandella* as measured by reduced feeding and survival of larvae exposed to leaves of autografted plants. This resistance may be partly inducible and dependent of the species used as rootstock and scion. However, in the leaf disk bioassay autografting-induced resistance was absent for the susceptible species. These results could be attributed to plant-induced defenses resulting from either mechanical (cut to graft or to get leaf disks) or insect damage inoculation (herbivore attack), and indicate that the translocated chemicals were more active in stem tissues or they were labile and broke down in leaf tissues.

Damage on autografted *C. odorata* was intermediate between that on both intact *C. odorata* and *C. odorata* grafted onto *T. ciliata* plants inoculated with eggs or larvae. Autografting diminished tunnel length on *C. odorata* but had no effect on *S. macrophylla*. Intact *S. macrophylla* is less susceptible than *C. odorata* (Speight & Wylie, 2001) probably making the autografting effect more difficult to detect.

Autografting also seemed to limit the amount of apical damage most strongly for whole plants inoculated with eggs, which is important since this type of damage in most plants results in the break apical dominance, which induces lateral branching and causes a tree to lose commercial value.

Resistant rootstocks improved insect resistance of susceptible scions

Khaya senegalensis and *T. ciliata* plants used as rootstocks for *C. odorata* and *S. macrophylla* scions decreased larval feeding and performance as well as increased mortality of larvae compared to larvae placed on intact *C. odorata* and *S. macrophylla* plants. These results indicate that substances responsible for resistance in the rootstock species are translocated to the scions. Resistant rootstocks conferred resistance to susceptible scions as determined by *H. grandella* feeding and survival on foliage from grafted plants. Therefore, the transmissibility of resistance across graft union indicated translocated chemical defenses are involved. Also for whole plants inoculated with either eggs or instar III larvae, the effect of grafting susceptible species on resistant ones was detected mainly for *C. odorata* grafted onto *T. ciliata*, which had reduced damage on the main shoot and leaves. Leaf disk bioassays confirmed results from the whole plants tests. Moreover, 75 and 100% of the larvae fed leaf disks from *S. macrophylla* or C*. odorata* grafted onto *T. ciliata* respectively, died. Based on these results, *C. odorata* scions grafted onto *T. ciliata* rootstocks were as resistant to *H. grandella* as were intact *T. ciliata* plants.

Susceptible rootstocks were neutral to resistant scions

Susceptible rootstocks failed to affect the properties of resistant scions as determined by *H. grandella* feeding and survival on foliage from grafted plants. Intact resistant plants and

plants grafted using *T. ciliata* or *K. senegalensis* as a scion had shallow tunnels, which were soon sealed by the plant. Also, 100% larvae fed leaf disks from *T. ciliata* grafted onto *C. odorata* died. These results suggest chemicals giving resistance in exotic plants may be synthesized in foliage (scions) and mask any chemical translocated from susceptible rootstocks that could confer susceptibility to *H. grandella* larvae. However, Bygrave and Bygrave (1998) reported transference of susceptibility for *C. odorata* against *H. robusta*, but not for *C. fissilis* both grafted on *T. ciliata* rootstocks.

Susceptibility to insect pests depends both on chemical composition (Cunningham & Floyd, 2004) and on physical characteristics of plant tissues such as lack or presence of trichomes, and leaf toughness and hardness (Lucas *et al*., 2000). The latter two leaf characteristics are more typical for *K. senegalensis* than for *T. ciliata* whereas thricomes are present in *T. ciliata* but absent in *K. senegalensis* (Pers. obs.). Although such physical characteristics of foliage could have been maintained on resistant scions after grafting on susceptible species and so conserve its resistance to *H. grandella* larvae, a morphological characterization of these reciprocal grafted plants is needed to a better understand of the neutral effect of susceptible Meliaceae species as rootstocks for resistant ones.

Specific objective 1.2. To determine the effect of crude extracts and alkaloid, limonoid, and phenolic fractions from susceptible and resistant Meliaceae species, as well as from a grafted combination of plants, on *H. grandella* larval survival and performance.

Crude extracts affected survival and performance of *H. grandella* larvae

Crude extracts from resistant plants decreased larval survival and performance more than that extracts from susceptible species. Crude leaf extracts caused a high mortality of *H.*

grandella instar II larvae, specifically those extracts from *T. ciliata* plants. The crude extract from this resistant species was toxic to *H. grandella* larvae. Larvae ate very little treated tissue, and 90% died after two days of exposure to *C. odorata* leaf disks sprayed with this extract. This effect of *T. ciliata* crude extract agreed with the previous results of larvae reared directly on either *T. ciliata* leaf discs or on intact plants (Chapters 3 and 4).

The extract from *C. odorata* grafted onto *T. ciliata* equally decreased larval survival as extracts from resistant plants. Both extracts caused larval weight loss similar to the relative controls methanol and larvae without a source of food (leaf disk) and therefore starved during two days.

Alkaloid, limonoid and phenolic fractions decreased survival and performance of *H. grandella* larvae

Alkaloid and phenolic fractions decreased leaf consumption and weight gain of larvae, as well as time to reach pupa and adult stages, whereas limonoids reduced larval survival. The best fractions to reduce leaf consumption and weight gain was alkaloid extracted from *C. odorata* grafted on *T. ciliata* and phenolics extracted from *C. odorata*. Moreover, alkaloid fraction extracted from the grafted plants induced 20% of adults *H. grandella* with abnormal wing shape. The best fractions to reduce pupal weight and length as well to reduce the time to reach pupa and adult stages were alkaloids extracted from *S. macrophylla*.

In general limonoids decreased survival of *H. grandella* instar II larvae, whereas alkaloids reduced leaf consumption and weight gain per larva compared with the other two fractions and also reduced time to pupa and to adult stages compared to the starved larvae (absolute control) during two days. Moreover, alkaloids reduced pupal weight and length compared to both fractions and controls (Appendix 1).

These findings are particularly important since they also were caused by alkaloid fraction extracted from the *C. odorata* scions grafted onto *T. ciliata* indicating the possible use of grafted plants in commercial plantations to overcome damage from *H. grandella* larvae.

In nature, the concentration of alkaloids, limonoids and phenolics in plants differs with their age, and such compounds in Meliaceae could affect *H. grandella* from larval stage by causing mortality and delaying pupation through later stages of the adult moths by causing abnormal wing formation, as I found in this research.

General objective 2. To establish a micropropagation method for older seedlings of *C. odorata* and *S. macrophylla* by nodal explants.

Specific objective 2.1. To develop disinfection methods for *C. odorata* and *S. macrophylla* in order to establish *in vitro* cultures of nodal explants.

The 90% and 84% initial contamination in explants taken from 10-year-old *C. odorata* and *S. macrophylla*, respectively, was reduced by combining the use of NaOCl or PPM^{TM} for surface sterilization or added to the culture media with a predisinfection treatment containing fungicides Benomyl and Manzate and the bactericide Agrimycin. Agrimycin and Benomyl combination as pretreatment is commonly used for explants that show high contamination *in vitro* (Dalsaso & Guevara, 1989).

Contamination was mainly due to bacteria in *C. odorata* and due to fungi in *S. macrophylla*, which could be explained by the fact that different plant species grown *in vitro* create distinct environments inside the culture vessels, which in turn allow or prevent the growth of different contaminants (Leifert & Waites, 1992).

The different contaminants between both species could be the reason why PPM^{TM} added to the medium worked better on *S. macrophylla* than on *C. odorata* reducing contamination and increasing bud break. Moreover PPM^{TM} at 2 ml l^{-1} also favored the growth of sprouted buds on *S. macrophylla*.

Specific objective 2.2. To improve the growth of *C. odorata* and *S. macrophylla* nodal explants in the *in vitro* establishment.

Cedrela odorata and *S. macrophylla* established *in vitro* by nodal explants responded positively to BAP, silver nitrate, activated charcoal, and sealant type and caps treatments. For nodal explants from 7-year-old plants from both species, BAP at 0.5 mg l^{-1} seemed to favor the bud break. For *S. macrophylla*, the highest BAP concentration resulted in higher percentages of bud break than in *C. odorata*, which could be explained because although both species were the same age, S*. macrophylla* tissues are woodier than *C. odorata*. Both species responded differently to BAP treatments, and in general the bud break was almost twofold higher for *S. macrophylla* compared to *C. odorata* (44.7% and 23.5%, respectively).

Neither activated charcoal nor $AgNO_3$ alone or combined prevented defoliation. $AgNO_3$ at 3 mg l^{-1} lacked a statistical significant effect it decreased contamination but also increased defoliation. The effect of $AgNO_3$ increasing defoliation could be explained by a phytotoxic effect of Ag^+ which is a heavy metal (Pua, 1999). Activated charcoal also helped to prevent contamination. Activated charcoal provided a dark environment, which could have prevented the photodegradation of NaOCl in the culture medium and favored the controlling of the contaminants. The interaction of both factors prevented contamination but also increased defoliation.

Bud break was twofold higher for nodal explants established in vessels using vented caps than for intact caps. Also, vessels covered with vented caps had fewer contaminated

nodal explants than those with normal caps. These results could be explained by a positive effect of ventilation in the vessel headspace, specifically the filter of vented caps might have allowed the interchange air without allowing pathogens or other microbial contaminants to enter the vessels.

Although both types of sealant had similar effects, fewer Micropore sealed vessels were contaminated yet these bud break percentages higher than those for vessels sealed with plastic film. Therefore, Micropore could be combined with vented caps in order to obtain better control of the *in vitro* atmosphere, which should result in a better response of *C. odorata* and *S. macrophylla* nodal explants.

Recommendations

- To confirm the resistance of grafted plants under field conditions, as well as their phenotypic response over time by establishing pilot plots of these trees in a wide range of environments.
- To examine, on ecological, silvicultural and economic grounds, the possibility of planting *T. ciliata* and *K. senegalensis* either in pure plantations or within agroforestry systems in neotropical countries.

Indicated additional research to improve *H. grandella* management

- In the short term (1 to 5 years)

- To improve the grafting method looking for *C. odorata* and *K. senegalensis* combination missing in the present research, and then test the grafted plants against *H. grandella*.

- To analyze chemically and separately alkaloids, limonoids, and phenolics looking for specific substances that provides the resistance of grafted plants against *H. grandella*.
- To complete the protocol for micropropagation of *S. macrophylla* and *C. odorata* by further reducing contamination rates of nodal explants taken from older plants.

- In the short and middle term (1 to 10 years)

- To evaluate the phenology of grafted plants in the field as during aging some grafted plants may become unsuitable for commercial purposes.
- To determine if the aging process of grafted plants affect the resistance to *H. grandella*.
- To start developing protocols for somatic embryogenesis for *C. odorata* and *S. macrophylla*.
- To start developing genetic transformation techniques for improving *C. odorata* and *S. macrophylla* resistance against *H. grandella*.

- In the long term (1 to 20 years)

- If grafted plants develop similar to intact plants, then study the characteristics (workability, finishing, color and durability) of their wood.
- To evaluate the progeny of grafted plants to determine the retention of resistance.

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APPENDICES

Appendix 1 Bioassay with fractions.- Mean comparison by fractions.

Fractions and controls	Leaf consumption (mm^2)	Weight gain (mg)	Days to pupa	Days to adult	Pupa weight (mg)	Pupa length (mm)	Survival (%) a	Survival (%) b	Survival (%) c
Alkaloids	**245.5** a	**52.5** a	**21.1** ab	**30.0** b	**158.1** a	15.1 a	88.7 a	76.0 ab	65.3 a
Limonoids	250.3 a	58.1 a	21.3 ab	30.4 b	170.6 a	15.6 a	**84.3** a	**71.0** ab	**63.7** a
Phenolics	247.1 a	57.9 a	20.7 ab	30.3 b	170.3 a	15.7 a	92.7 a	78.0 ab	72.0 a
Ether	**246.9** a	**53.3** a	19.8 b	**29.4** b	**161.6** a	15.5 a	89.6 a	82.6 a	69.3 a
DCM	208.6 a	45.8 a	21.1 ab	30.1 b	157.1 a	15.1 a	90.0 a	**70.0** ab	**66.7** a
Water	**274.2** a	**56.8** a	**22.2** ab	**30.8** b	**161.4** a	15.7 a	**90.0** a	**86.7** a	**76.3** a
Alone larva	-	-22.3 b	**24.0** a	**35.3** a	**162.8** a	15.4 a	**45.0** a	**30.0** b	**30.0** a

Each mean is the average of n = 150 data for fractions and n = 30 data for controls. Data are the means from three bioassays combined into one data set analysis. Aa, b, and c are mean for survival at 2, 10 and 25 days after starting bioassays. DCM = Dichloromethane.