The Pennsylvania State University

The Graduate School

Graduate Degree Program in Integrative Biosciences

GENETIC ENGINEERING OF *THEOBROMA CACAO* AND MOLECULAR STUDIES ON CACAO DEFENSE RESPONSES

A Thesis in

Integrative Biosciences

by

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Submitted in Partial Fulfillment
of the Requirements for the Degree of

Doctor of Philosophy

May 2003

ABSTRACT

Theobroma cacao, a tropical evergreen tree, holds great economic importance for many small crop farmers in developing countries. Cultivated on over five million hectares worldwide, cocoa production is limited by three major fungal diseases. Disease has resulted in average annual losses estimated at forty percent worldwide. In Brazil alone, production dropped from 400,000 to 100,000 metric tons over the past ten years due to single fungal pathogen

Research in plant molecular biology has developed an array of new techniques for crop improvement, including tissue culture, quantitative trait loci mapping, marker assisted selection, introduction of novel genes through genetic engineering and the use molecular techniques to investigate basic physiological mechanisms. Through my research I reported use of some of these techniques to improve the efficiency of *Agrobacterium* mediated transformation of cacao and further used this technique to generate transgenic cacao lines expressing a cacao class I chitinase gene. Furthermore, molecular biology techniques were utilized to initiate studies of defense responses in cacao, and investigate the expression patterns of five cloned cacao ESTs in response to infection and chemical elicitation. These studies will contribute to the development of new disease control strategies with the ultimate goal of providing alternative solutions to cocoa farmers.

ACKNOWLEDGEMENTS

My sincere thanks are extended to my thesis advisor Mark Guiltinan and all my committee members. I am appreciative of their guidance, advice, patience, time and support.

In addition I would like to thank all the members of the Guiltinan Laboratory 1998-2003, specifically Dr. Siela Maximova, Dr. Joe Verica, Dr. Yuan Yao, Carter Miller, JD Swanson, Sharon Pishak, Ann Young and Nianying Wang, Thanks for all the fun times and your constant help.

I am deeply indebted to my family. To my parents for teaching me that I could do anything I set my mind to. To my sister Pamela for assuring me that finishing a Ph.D. degree requires more patience and perseverance than anything else. To my sister Adriana, for teaching me how to make dilutions, solutions and figuring out molarity, without which my career in science would be a total chaos. To my brother Erik who is a constant source of joy, and to my sister Carolina my own little angel.

I am also extremely grateful to my husband Martin, who assured me that one day I would finish.

Thanks to my graduate school friends. To my friend Maggie and her husband Kevin who always kept their door and kitchen open. And to my Latin friends Gabi Mendez, and Carolina Bonilla.

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CHAPTER 1

CACAO FUNGAL PATHOGENS: A LITTERATURE REVIEW

1.1. INTRODUCTION

Theobroma cacao, a tropical tree belonging to the Malvaceae family, originated in the Amazon basin (Whitlock *et al.*, 2001). Archaeological records have revealed evidence of cacao plantations in Central America dated to 2000-4000 years before Spanish contact (Bergman, 1969). These plantations extended from Mexico to Costa Rica, and over time cacao cultivation spread to other locations in South America and the Caribbean (Wood and Lass, 1985). Currently cacao is cultivated on over five million hectares of tropical lowlands worldwide (Soberanis *et al.*, 1999; Duguma *et al.*, 2001; Ramirez *et al.*, 2001; Kraus and Soberanis, 2001). *T. cacao* is often cultivated as one component of complex agroecosystems, providing both economic and ecological benefits to both farmers and the producing countries (Wood and Lass, 1985).

T. cacao produces fruit (pods) on the trunk and branches of its tree. Seeds develop within the pods and are later removed for fermentation, which destroys seed viability and stimulates biochemical activities required for flavor development. Trade from dried cacao beans has an annual estimated value of 2.9 billion US dollars per year (Gray, 2000). Cacao beans are roasted and used to produce cocoa powder and butter, commodities of economic importance to the confectionery and cosmetic industries.

As with all crops, cacao farming is not without challenge. Its growing environment is often confining, shaded, and humid, conditions strongly favorable for pathogen development (Fulton, 1989). Because cacao plants are grown in many non-

native tropical environments around the world, its yield is affected by a number of opportunistic pathogens. Plantations in many countries are periodically subjected at repeated intervals to infection by three major fungal diseases: black pod (*Phytophthora sp*. (Butler) Butler), witches broom (*Crinipellis perniciosa* (Stahel) Singer), and monilia pod rot (*Moniliophthora roreri* (Ciferri) Evans). There are several other important pests and diseases of cacao. One of these is caused by cacao swollen shoot virus (CSSV), which is transmitted by mealybugs and is particularly destructive in West African producing areas. CSSV has also been reported in Trinidad, Sri Lanka (Orellana and Peiris, 1957) and Indonesia (Kenten and Woods, 1976). Another important disease is *Oncobasidium theobromae*, the causal agent of vascular-streak dieback, which is a problem in Southeast Asia. Additionally verticillium wilt, or sudden death caused by *Verticillium dahliae*, is found in several locations around the world including Brazil were it has become a disease of economic importance (Lass, 1985).

This chapter will review what is known about the three major cacao fungal diseases, the approaches taken towards their control, and possible future directions for research.

1.2. *PHYTOPHTHORA SP.*

The genus *Phytophthora* is one of the most widespread pathogenic fungi, affecting a wide range of host plants including *Theobroma cacao* (Prendergast and Spence, 1967). Classified as an Oomycete, *Phytophthora sp.* are capable of spreading disease through asexual sporangia to all parts of the cacao plant, though the severity of the attack depends on the *Phytophthora* species and the cacao variety.

For many years it was thought that black pod was caused by *Phytophthora palmivora*. However, it is now recognized that several species are pathogenic on cacao and that different species are dominant in different regions (Keane,1992). Estimated losses of cacao from black pod disease have varied between 10 percent in the 1980's (Wood and Lass, 1985) to 30 percent in the 1990's. However, losses have been reported as high as 90 to 100 percent, depending on location, cultivar, pathogenic strain, and present environmental conditions (Gregory, 1985; Iwaro and Screenivasan, 1997).

Pod rot is transferred from infected pods by wind or rain, contaminating the surfaces of nearby pods and trees. Under suitable weather conditions, germ tubes produced directly from sporangia or zoospores can penetrate pods through stomata or directly through the cuticle (Roreri, 1926). The fungus causes brown lesions on the pod husk and begins to sporulate two days after pod infection is initiated, covering the entire pod within a week. Beans may remain undamaged for several days after infection, thus partial yield loss can be prevented by frequent pod harvesting (Evans and Priori, 1987).

Of the seven *Phytophthora* species identified in the field (*P. palmivora, P. megakarya, P. capsici, P. citrophthora, P. megasperma, P. nicotianae, P. arecae*) (Iwaro *et al.*, 1997a), mycologists have now characterized four species which infect cacao (*P. palmivora, P. megakarya, P. capsici, P. citrophthora*) (Evans and Priori, 1987), (Table 1.1).

Table 1.1 Distribution of *Phytophthora* sp. Reported in cacao

Phytophthora sp.	Geographical distribution
P. palmivora	world wide
P. megakarya	Cameroon, Gabon, Ghana, Nigeria
P. capsici	Brazil, El Salvador, Guatemala, India, Jamaica, Mexico, Trinidad, Venezuela
P. citrophthora	Brazil, India, Mexico

Adapted from FAO/IPGRI Technical Guidelines for the Safe Movement of Germplasm (2000).

Of the four *Phytophthora* species affecting cacao, *P. palmivora* is regarded as the most widespread and devastating cacao pathogen. It is present in most cacao-producing countries, with important economic effects in many of them. It causes black pod disease, leaf blight and stem canker diseases (Iwaro *et al.*, 1997). The incidence of stem canker disease increased in West Africa following changes in stock trees from Amelonado to hybrid material, which appears to be more susceptible (Evans and Priori, 1987). In some cases, the stem canker encloses the trunk, resulting in sudden death of the tree (Keane, 1992).

P. megakarya, was first recognized during the work of the International Cocoa Black Pod Project in 1970. It is confined to parts of the West African continent, being the dominant *Phytophthora* species in Nigeria (Evans and Prior, 1987) and Ghana (Opoku *et al.*, 2002). It is the major cause of black pod in Nigeria and Cameroon, causing significant losses to black pod because of high rainfall and the absence of a dry season. *P. megakarya* is considered more aggressive than *P. palmivora*, because it has a different life cycle and relies more on root infection, rather than on flower cushions as does *P. palmivora* (Gregory *et al.*, 1984).

P. capsici is the most important *Phytophthora* specie present in Brazil, where it shares its niche with *P. palmivora* and *P. citrophthora*. Although *P. capsici* is not more virulent than *P. palmivora*, this *Phytophthora* species is favored by the climatic conditions and causes greater losses of cacao in Brazil (Kellen and Zentmyer, 1986; Keane, 1992). *P citrophthora* is the least wide spread *Phytophthora* specie, present only in the cacao growing region of Brazil. It is also considered the most virulent *Phytophthora* specie in the world for cacao black pod (Lawrence, 1998).

Researchers have been searching for host resistance to cacao fungal pathogens for the past 100 years. In 1902, an excursion to the Amazon to collect resistant cacao varieties failed because no immune varieties were found (Pound, 1943). The need for resistance prompted germplasm-collecting expeditions carried out by FJ Pound in 1942. He collected cacao clones *Scavina* 1 - *Scavina* 12, in the Upper Amazon of Perú, near

Iquitos (Pound, 1943). *Scavina* 6 and *Scavina* 12 are still considered highly resistant (Frias *et al.*, 1991; Ahnert, 2000).

The cacao variety *Scavina* 6 has consistently been more resistant than other cacao varieties such as Catongo and ICS1, as observed in various tests performed (Holliday, 1955; Spense, 1961; Iwaro *et al.*, 1997; Tahi *et al.*, 2000; Srujdeo *et al.*, 2001). Studies in 1997 by Iwaro *et al*. tested 12 common cacao clones, representing Forastero and Trinitario cacao-types. In their studies pods were infected with a *Phytophthora* sp. isolated from naturally infected pods in Trinidad. They found that *Scavina* 6 had the lowest lesion frequency and the smallest lesion size, while ICS1, which had the second lowest lesion number in leaves, made very large lesions on both leaves and pods. From these studies, *Scavina* 6 proved the most promising clone tested for resistance. Similarly, a study conducted by Srujdeo *et al.*, (2001) showed that of the five clones tested, *Scavina* 6 had both the fewest and smallest lesions, as well as the smallest ones when infected with *Phytophthora*. In addition, Tahi *et al.*, (2000) showed that regardless of the tissue tested (leaves, twigs or roots), the *Scavina* 6 clone continues to have high resistance to *Phytophthora* species. In this study the resistance of *Scavina* 6 was surpassed only by that of another Upper Amazon genotype, PA150 (Tahi *et al.*, 2000).

Variation in cacao host-pathogen interactions has been reported (Iwaro *et al.*, 1997; Tahi *et al.*, 2000). Early investigations of host pathogen interactions were initiated in 1929, by Ashby who characterized *P. palmivora* cacao isolates. The studies were extended by Turner 1960, and Spence in 1961, who obtained *P. palmivora* isolates from

the main cacao growing areas. In the Spence study, the *Phytophthora* strains were inoculated on cacao pods from Ghana (West Africa Amelonado) and Trinidad (ICS1 and *Scavina* 6). Spence concluded that when particular isolates of *Phytophthora* are inoculated onto a range of cacao pod varieties, the sporangia subsequently produced on these hosts exhibited phenotypic variations comparable with that between sporangia produced by different isolates on the same host (Spense, 1961). Most *Phytophthora* isolates showed distinct rates of spread on the different cacao varieties (Spense, 1961).

1.3. *CRINIPELLIS PERNICIOSA*

Crinipellis perniciosa (Stahel) Singer, the causal agent of witches' broom disease, is endemic to the Amazon basin region of South America (Purdy and Schmidt, 1996). This cacao fungal pathogen is thought to have developed concomitantly with *T. cacao* and causes one of the most feared cacao diseases. Witches' broom is associated with the decline of once-thriving cacao industries in a number of countries such as Ecuador, Surinam, Trinidad, (Evans and Prior, 1987) and currently Brazil (Purdy and Schmidt, 1996). The disease was first identified by Ferreira in 1785. Its causal agent, *Crinipellis perniciosa,* was identified by Pound in 1915 (Pound, 1943). *C. perniciosa* is found only in the New World and is present in all cacao-growing countries of South America, including important geographical regions (Purdy and Schmidt, 1996) that produce the bulk of the cocoa production (Evans and Bastos, 1980). Witches' broom of cacao was detected in Bolivia, Brazil, Peru and Venezuela (1785); Surinam (1895); Guyana (1906); Colombia (1917); Ecuador (1921); Trinidad (1928); Tobago (1939); Grenada (1948); St. Vincent (1988); Panama (1989) and the state of Bahia Brazil (1989).

The invading fungus has a two-phase life cycle. Its initial phase is monokaryotic, behaving as a biotrophic fungus. This stage is followed by a second dikaryotic-necotrophic phase (Griffith and Hedger, 1994). The fungus infects actively growing meristem tissue, causing growth hypertrophy and the distortion of both vegetative and reproductive tissue. Infection by biotrophic mycelia occurs through terminal axillary buds (Pound, 1943). Following penetration of the mycelia, the fungus ramifies in the host tissue intercellularly. After 3-9 weeks, dikaryotization begins and the biotrophic mycelia within the host tissue are replaced by the saprotrophic phase hyphae. During the transition, both forms of mycelia are present within the host (Frias *et al*., 1991). 4-8 weeks after the onset of rain, dikaryotic basidiocarps develop to form the necrotic brooms.

Crinipellis perniciosa causes major damage to the tree canopy and can cause a high incidence of pod rot, with potential serious socio-economic effects (Wood and Lass, 1985). The common massive vegetative infection reduces the plant photosynthetic efficiency, affecting pod setting and development indirectly. The pathogen can also infect flower cushions, affecting the plants' capacity to develop mature pods (Purdy and Schmidt, 1996).

Every season, the main sources of inoculum are the necrotic brooms left in the tree canopy from the previous season. After prolonged periods of wetting and drying, the brooms produce pink basidiocarp (Purdy and Schmidt, 1996). Basidiospores, the only infective propagule of the pathogen, are typically released at night and are dispersed by

wind and rain. Capable of germination once on the plant surfaces, basidiospores produce monokaryotic germtubes. Germtubes are attracted to stomatal openings for host penetration (Isaac *et al.*, 1992). Airborne spores landing on young pods germinate and grow within the tissue without cell invasion, necrosis or tissue death (Isaac *et al.*, 1993). After five to six months, as the pods approach maturity, brown or black lesions develop on the surface. Internally, the pod contents become completely colonized by the fungus, with symptoms ranging from destruction, compaction, and malformation of the beans and endosperm, to watery necrosis (Purdy and Schmidt, 1996).

The search for resistant cacao trees in Bahia, Brazil and Ecuador plantations has resulted in the selection of highly resistant trees, which have been evaluated under controlled conditions and in the field for their response to witches broom (Purdy and Schmidt, 1996; Ahnert, 2000). *Scavina* 6 selections are still the most important source of resistance and are widely used in the Brazilian cacao breeding programs (Ahnert and Pires, 2000). 15 selected resistant clones (mostly *Scavina* 6 derived) have been released recently by the Comissão Executiva do Plano da Lavoura Cacaueria (CEPLAC) to growers in Bahia and are being used to renew old plantations (Ahnert, 2000).

1.4. *MONILIOPHTHORA RORERI*

Monilia pod rot, also known as frosty pod rot, is caused by *Moniliophthora roreri* Cif (Evans and Benny, 1978). *Moniliophthora* was first described by Ciferri and Parodi (1933), from diseased cacao pods collected by J. B. Rorer in Ecuador. Infection occurs on all *Theobroma* and *Herrania* species. Originally restricted to the northwest region in

South America (Ecuador, Peru, Colombia) and Panama (Holliday, 1970), it now affects plantations in Nicaragua, Costa Rica and Venezuela (Wood and Lass, 1985).

The causal agent is the anamorphic state of a Basidiomycete that has been placed in a monotypic genus of the Fungi Imperfecti as *Moniliophthora roreri* (Evans, 1981 Evans and Priori, 1987). In the field, *M. roreri* attacks only pods, where it completes its entire life cycle. After inoculation, the fungus has a 45 – 90 day incubation period before necrosis of the tissue is evident. Dark brown lesions spread irregularly on the pod surface, followed by the development of white mycelia (Rocha, 1965). The extent of internal symptoms varies with pod age and variety. Tissue inside the pod may deteriorate into a disorganized gelatinous-watery substance. It can also cause the overproduction or compacting of tissue, producing a mass of dry rot (Evans, 1978).

The fungus survives in mummified pods, on which it may continue to sporulate for several months. The conidia are dry, powdery and thick walled, with some resistance to environmental stress, allowing them to spread over long distances (Keane, 1992). Air currents, rain and insects easily pick up spores on pod surfaces. Infected pods in the canopy retain viable conidia for up to nine months (Evans and Rodriguez, 1977). This constitutes the main inter-seasonal source for fungal infections (Evans and Priori, 1987).

The pods at a higher risk of Monilia infection are those that are young and actively growing (Evans, 1986). Susceptibility decreases with pod age during the first 90 days, after which the pods are immune. Therefore, field monitoring, 7 to 10-day

sanitation sweeps, and harvest at regular and frequent intervals have been strongly recommended to cacao farmers as an important management technique (Soberanis *et al.*, 1999; Rios-Ruiz, 2000; Kraus and Soberanis, 2001).

1.5. POD DISEASE MANAGEMENT

For the past years, five separate approaches to cacao pod disease management have been considered.

Breeding

Most cacao disease management has relied on sanitary measures, pruning of infected material, regular harvest of pods and rooting out trees infected by CSSV. However, breeding programs and nursery practices established throughout the last century, have also played important roles in disease control. The objectives of these programs are to develop genetically resistant cacao material, the most efficient approach for pathogen control (Ahnert, 2000). Genetic resistance has been used since the first outbreak of *Crinipellis* fungus in Ecuador in 1918 (Stell, 1934). Growers select clones from their plantations that show some degree of resistance, having survived the epidemic (Keane, 1992). This selection is possible in areas with heterogeneous plantations (Baker and Holliday, 1957); however, this has not solved the disease problem because the farmers' use of seed-derived material from out-crossed plants. Furthermore, in countries with more genetically uniform cacao plantations, resistance has not been possible (Ponsete, 1947). The *Scavina* 6 selections are still among the most important sources of

resistance, and they continue to be widely used in the Brazilian cacao breeding programs (Ahnert and Pires, 2000).

Quarantine

Evans and Priori (1987) observed that no other crop demonstrates the importance of quarantine better than cacao. As noted earlier, with the exception of *Phytophthora palmivora*, most fungal diseases are confined to specific geographical locations, and represent a terrible threat to other cacao growing regions. In a similar way the spread of the more virulent pathotypes, like *P. megakarya*, out of its limited region of Africa, or *P. citrophthora* from Brazil could increase cacao bean losses in any new territory infected. One recent example of this is the spread of witches' broom, thought to have been transported from sources in the upper Amazon to Bahia Brazil, with devastating consequences (Pereira *et al.*, 1996).

Material transfer between plantations and countries should be controlled both for the good of the farmers and the industry. The risk of spread of all these pathogens can be minimized by ensuring that only chemically treated seeds or budwood are transferred, and prohibiting the movement of soil and pods (Evans and Bastos, 1980). International guidelines for quarantine procedures for crop protection were released by the food and administration organization (FAO) in 1981, and while each country defines its quarantine regulations, agreements have been made and countries that share economic and political interests have formed committees to care for mutual interests. (Pereira and Menezes dos Santos, 2000). Analysis of risk involves a detailed review of the import material form of

entry, as well as the identification of potential noxious or quarantine pests that occur in the exporting country. The next phase in the evaluation of risk is determining the geographical distribution of the pest, the significant economic importance, and the potential establishment and dispersal of the pest. Following each of the first two phases, the regulation committee may or may not allow the transfer of the material. If the import material is allowed, the last phase is to administer, evaluate and compare the options for disease management.

Crop sanitation

Crop sanitation is a central aspect of crop management. Removal of the infected and sporulating pods can reduce or delay the dispersal of spores throughout a plantation. For some farmers this practice is the only cost-effective way to eliminate the primary inoculum sources, the mummified hanging pods produced by both *Phytophthora* pod rot and *Monilia* pod rot (Fulton, 1989), as well as the basidiocarps from dead witches brooms (Purdy and Schmidt, 1996). Crop sanitation through pod removal can also play an important role in preventing establishment of the diseases in new areas, by reducing the availability of the spores for distribution by air currents. For example, Soberanis *et al.* (1999) found that weekly removal of diseased pods significantly reduced the incidence of all fungal diseases. This practice produced yield increases of 26-36% with an increase in gross return that more than compensated for the increased expenditure in labor. Successful phytosanitation programs have now been implemented in other Peruvian cacao growing regions (Rios-Ruiz, 2000). However, for *Phytophthora* species it is important to remember that a major source of infection is not only the pod, but also the

soil surrounding the infected site. This is because the fungus is able to sporulate on multiple tissues of an infected tree, including the roots, making it impossible to eliminate (Prior, 1979).

Fungicides

Chemical application of copper-based fungicides, although very expensive for small crop farmers, and only economically viable when disease levels are extremely high, can be an effective way of controlling and delaying the development of these fungal disease (Evans and Bastos, 1980). However, spraying fungicides is only possible when the increased yield from spraying is greater than the cost of treatment. When discussing disease management, it is also important to consider the fluctuations of the cacao world market and the prices of dried beans. Chemical applications are markedly lower during periods of low bean prices. The reduced concentrations of fungicides in the field lead to a build up of fungal inoculum for next season and to the debilitation of cacao trees. This results in a series of drawbacks for the farmers in the following season, through a series of hidden costs aside from pod loss, including reduced tree vigor, and reduced capacity to develop mature fruit (Evans and Priori, 1987). Similarly fungicidal control should be considered only for producing areas or seasons when heavy rainfall will not make the efforts futile.

Biocontrol

Recent studies by Krauss and Soberanis, (2001) have reported the use of biocontrol mycoparasite mixtures to control cacao pod disease. They reported the use of

five native mycoparasitic strains of *Clonostachys rosea* and three of *Trichoderma spp.* isolated from healthy cacao tissue or from *Crinipellis perniciosa* basidiocarps. A host-range study was employed to select mycoparasites which could simultaneously attack *Moniliophthora roreri*, *Crinipellis perniciosa* and *Phytophthora palmivora*, and although none of the antagonists expressed all desirable characteristics on its own, and neither did the strain mixtures prepared to complement traits, the study did provide some positive results. Almost all selected treatment combinations reduced moniliasis disease by 15-25%. A combination of all five *C. rosea* strains, performed best against all three diseases, with increase of 17% in yield, and 24% net returns (Krauss and Soberanis, 2001).

1.6. CONCLUSIONS

Theobroma cacao is constantly challenged by a number of pathogens present in the tropical environment. Crop losses range up to 100% in different geographical regions. Each region is defined by the array of potential pathogens currently present within its boundaries. After years of research in disease control practices, the understanding is that control of pod diseases is only successful when it is part of an overall integrated crop management effort (Evans and Bastos, 1980). For example, controlling fungal pathogens on a tree incapable of bearing the extra fruit after pathogen removal would be both cost-and time-ineffective. Thus disease control is only one aspect of crop management, and its efficacy is increased by adequate nutrition, shade maintenance and the control of insect pests (Evans and Priori, 1987). Research is

focusing in the identification of disease resistant varieties, specific for the array of pathogens to each region. In addition, it is important to consider the few cacao varieties, which, in the past have shown levels of resistance and include other criteria, which should be selected in breeding programs, such as high yields.

Most of the cacao grown worldwide is cultivated by small crop farmers whose most distinctive feature is their labor, the main input component for their plantations. Thus, it is important to remember that any increased work load for disease control affects the grower both financially and in terms of personal effort (Priori, 1979).

The objectives of this thesis were to develop a system for crop defense upon which I could use current molecular biology techniques to study cacao's defense genes and initiate studies for the understanding of cacao defense signaling pathways. My studies contributed to the development of a tissue culture-based transformation system as well as to the development of cacao plants constitutively expressing a defense related gene, a cacao class I chitinase gene. Furthermore, to better understand the cacao defense signaling pathways, studies were performed with cacao seedlings treated with defense inducing elicitors. These studies were carried out in the belief that an understanding of the molecular interactions between cacao and its major pathogens could result in an increased capacity to produce disease-free planting material.

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CHAPTER 2

MOXALACTAM AS A COUNTER-SELECTION ANTIBIOTIC FOR *AGROBACTERIUM*-MEDIATED TRANSFORMATION AND ITS POSITIVE EFFECTS ON *THEOBROMA CACAO* SOMATIC EMBRYOGENESIS

2.1. ABSTRACT

Previous work in a number of laboratories has led to the development of a highly efficient cacao somatic embryogenesis protocol, establishing the basis for an *Agrobacterium tumefaciens*-mediated genetic transformation system. Such a system will be valuable as a basic research tool, and possibly in the future as a mean to introduce specific disease resistance or other genes into cacao. However, during our initial attempts at transformation, cacao tissue was frequently destroyed due to *Agrobacterium* overgrowth following co-cultivation. Furthermore, the addition of the antibiotic cefotaxime, commonly applied to tissue culture media to eliminate *Agrobacterium* post infection, decreased cacao somatic embryo production by 86%. To define antibiotic conditions sufficient to suppress *Agrobacterium* while not interfering with cacao somatic embryogenesis, we evaluated the efficacy of four antibiotics. Two cephalosporins, cefotaxime and moxalactam; and two penicillins, amoxicillin and carbenicillin. Moxalactam, a □-Lactam antibiotic, was proven to effectively suppress *Agrobacterium* growth. Furthermore, at certain concentrations, moxalactam significantly enhanced the efficiency of cacao somatic embryogenesis. A possible mechanism for this enhancement is proposed.

2.2. INTRODUCTION

Theobroma cacao, a perennial tree belonging to the Malvaceae family (Whitlock *et al.*, 2001) is native to the American tropics (Brooks and Guard 1952). Production of cacao is mainly the labor of small crop farmers, who cultivate close to five million hectares worldwide. Cacao seeds, fermented and dried, are a major cash crop for a number of developing countries with an annual value estimated at $2.9 billion US (Gray, 2000). Additionally, cacao farming provides ecological benefits such as habitat and watershed conservation, enhancement of the connectivity between ecosystem fragments, soil stabilization and carbon sequestration (Alves, 1990).

Due to increasing disease pressures, cacao farmers are in urgent need of pest and pathogen resistant varieties. For example, over the past decade Brazil lost 68% of its cacao production to "witches broom", a fungal disease caused by infection of *Crinipellis pernicosa*. Ghana has experienced 29.4% crop loss due to capsids insects, cacao swollen shoot virus and the fungal disease pod rot (Gray, 2000). Future predictions for crop loss are equally dim. The rapid spread of the lepidopteran pest cocoa pod borer, if left uncontrolled is predicted to reduce Indonesia's production by half, while Nigeria is predicted to incur production losses as much as 70% over the next few years if black pod continues to spread at the same rates (Gray, 2000). Recent efforts in plant breeding, disease management, and agro-forestry have begun to make a positive impact on these problems.

Research in plant biology has developed an array of new techniques for crop improvement including: tissue culture, quantitative trait loci mapping, marker assisted selection, genetic engineering and the use of molecular techniques to investigate basic physiological mechanisms. Our group, along with colleagues worldwide, is committed to applying these technologies to cacao tree improvement with the ultimate goal of enhancing the income of small-holder farmers. One of our objectives has been the development of a genetic transformation system for cacao for use as a basic research tool, and to enable the method for potential future use in crop improvement. Following on prior work (Lopez-Baez *et al*., 1993, Alemano *et al*., 1997a, Alemano *et al*., 1997b), we first developed an efficient *in vitro* primary somatic embryogenesis system for cacao using floral parts (Li *et al*., 1998), and an even more efficient secondary somatic embryogenesis protocol using cotyledon slices from mature primary somatic embryos (Maximova *et al*., 2002). These systems have established the regenerative basis for the development of a cacao genetic transformation protocol via *Agrobacterium tumefaciens* co-cultivation (Maximova *et al*., 2003). However, developing a transformation system requires not only a reliable regeneration system but also an efficient cell transformation procedure, a mechanism for selection of the transformed cells, and antibiotic conditions for the inhibition of *Agrobacterium* growth in the *in vitro* culture post-infection (Ellis *et al*., 1989). It is essential that the conditions used for each of these steps do not interfere with tissue regeneration.

Our initial attempts at developing an *Agrobacterium*-mediated transformation system were hampered by bacterial overgrowth, which often destroyed the infected tissue

weeks after infection. In our laboratory, *Agrobacterium* overgrowth during tobacco, *Arabidopsis* and apple transformations has been routinely controlled utilizing cefotaxime antibiotic at a concentration 400 mg L^{-1} (Fisher and Guiltinan 1995, Maximova *et al.*, 1998). However, using these conditions during cacao transformation resulted in *Agrobacterium* overgrowth, requiring frequent transfers of explants to media with fresh antibiotic and often, tissue necrosis. In addition, preliminary studies indicated that these conditions decreased somatic embryo regeneration by as much as 86% (Traore *et al.*, 2000).

In this study, we evaluated the effects of different antibiotics on somatic embryogenesis and their potential as *Agrobacterium* counter selection agents during cacao genetic transformation. A dilution series of two major antibiotic classes: cephalosporins (cefotaxime and moxalactam) and penicillin (amoxicillin and carbenicillin) were investigated. Using an *in vitro* disk diffusion assay, (Hackelford, 1996), we first established the efficacy of the antibiotics in controlling *Agrobacterium* growth. Each of the antibiotics were also added to embryo regeneration media at four different concentrations to evaluate their effects on callus and embryo formation during cacao secondary embryogenesis and to assess their efficacy in controlling *Agrobacterium* growth after co-cultivation with cacao somatic embryo cotyledon explants. My results indicated that moxalactam, a □-Lactam antibiotic, efficiently suppressed the growth of *Agrobacterium* strain AGL1 during the *in vitro* disk assays and tissue transformation. Surprisingly, the results also indicated that moxalactam significantly enhances the efficiency of somatic embryo formation. The results demonstrate that in addition to

controlling *Agrobacterium* overgrowth, moxalactam enhanced embryo production by approximately 2.5 fold. Using these conditions, the successful production of transgenic cacao embryos and plants has been achieved (Maximova *et al*., 2003).

2.3. MATERIALS AND METHODS

Antibiotics

All antibiotics were purchased from Sigma (St. Louis, MO, USA). Stock solutions of cefotaxime (Sigma #C7039), carbenicillin (Sigma #C3416) and moxalactam (Sigma #M8158) were dissolved in water (100mg L^{-1}), and filter sterilized. Amoxicillin (Sigma #A8523) was dissolved to the same concentration in 95% ethanol. Stock antibiotics were stored at –20ºC for up to 2 months. Antibiotics were added to the tissue culture media after media was autoclaved and cooled to 55°C, before plates were poured.

Bacterial cultures

25 ml of 523 media (Dandekar *et al*., 1989) containing 20 mg L^{-1} rifampicin (Sigma #R7382) and 50mg L^{-1} kanamycin (Sigma #K1377) were inoculated with *A. tumefaciens* strain AGL1 (Hood *et al*., 1986), containing plasmid pCP108. Binary vector pCP108 was constructed by the ligation of EGFP gene (Clontech) expression cassette as a *Hind*III-*Hind*III fragment into the *Hind*III cloning site of binary transformation vector pBI121 Clontech (Palo Alto, CA, USA) (Bevan *et al*., 1981). An EGFP gene was driven

by a CaMV 35s derivative, the E12Ω5' promoter (Bevan 1984). Additionally, pBI121 contains a neomycin phosphotransferase (*nptII*) plant selectable marker gene and a □-*glucuronidase* (GUS) reporter gene. *Agrobacterium* cultures were grown overnight on a rotary shaker at 200 rpm, at 25ºC. The optical density of the cultures was adjusted to 0.5 OD at 420nm by dilution with fresh 523 bacteria media just before use.

Disk Diffusion Assay

Filter paper discs, 5 mm in diameter, (Whatman 3-mm chromatography paper), were autoclaved and saturated with 15μl of antibiotic solutions, each with one of four different concentrations: 100, 150, 200, and 300 mg L^{-1}. Bacterial solution (50 μl, 0.5 OD at 420 nm) was evenly spread on the surface of solid 523 media, the four discs were placed on top of two replicate 523 agar plates. Plates were wrapped in parafilm and incubated for 48 hours at 25ºC. The zone of bacterial inhibition was defined as the distance in mm from the edge of the paper disc to the edge of the bacterial growth area.

Cacao tissue culture

Immature flowers were collected from *Scavina* 6 cacao plants grown in a greenhouse at The Pennsylvania State University. Primary somatic embryos were produced as described by Li *et al.*, 1998 by culturing staminode explants on primary callus growth (PCG) medium, followed by transfers to secondary callus growth (SCG) medium and were maintained on plant growth regulator free embryo development (ED)

medium (Li *et al.*, 1998). Secondary somatic embryos were initiated on SCG medium, followed by transfer to ED (Maximova *et al.*, 2002). Cotyledon pieces, 4 x 4 mm in size, were excised from mature primary somatic embryos at different times after primary culture initiation depending on the experimental objectives (see below). All moxalactam treatments contained 3 replicate plates with 25 explants each and the experiments were repeated three times, except for the initial experiment containing all four antibiotics, in which case only 25 explants per plate per treatment were initiated and recorded. Primary and secondary cultures were maintained in the dark at 25°C. In all experiments, embryo production and morphology was observed and recorded for the duration of the experiments. The total numbers of embryos present in the cultures were counted every other week for 22 weeks after culture initiation. Mature embryos were also classified as either normal (containing an axis, healthy hypocotyl and two cotyledons) or abnormal (multiple embryos fused at the axis, embryos lacking cotyledons or those with multiple cotyledons) as previously described (Maximova *et al.*, 2002).

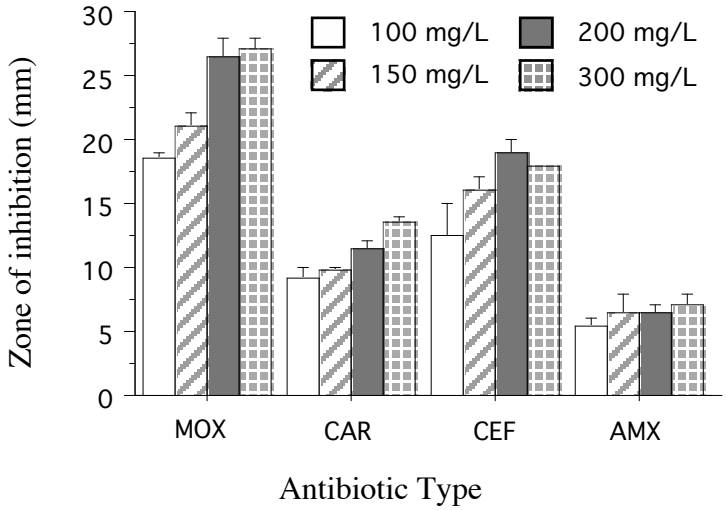
2.4. RESULTS

Effect of various antibiotics on the growth of *A. tumefaciens* strain AGL1

To determine the most effective antibiotic for controlling the growth of *A. tumefaciens*, a disk diffusion bioassay was performed using 4 antibiotics at concentrations of 100, 150, 200 and 300mg L^{-1}. From the antibiotics tested, moxalactam, at all concentrations demonstrated effective control over bacterial growth and produced the largest bacterial inhibition areas, up to 27 mm at 200mg L^{-1} (Fig. 2.1). The second

largest inhibition area from another antibiotic were observed for cefotaxime (18 mm) at the concentration of 200mg L^{-1}, same inhibition zone as that observed for the lowest moxalactam concentration. Carbenicillin and amoxicillin were somewhat effective in controlling bacterial growth in this assay, but the zones of inhibition were smaller than those observed for the other two antibiotics.

Figure 2.1. Antibiotic disk diffusion assay. *Agrobacterium* strain AGL1 was challenged with four antibiotics in a disk diffusion assay. The effect of the antibiotic on bacterial growth is represented as the zone of inhibition in millimeters (mm) from the paper disk to the edge of bacteria growth after 48 hours of co-cultivation at 25° C. Abbreviations are CEF-cefotaxime, CAR-carbenicillin, AMX-amoxicillin, and MOX-moxalactam.


30
25
20
15
10
5
0
Zone of inhibition (mm)
100 mg/L
150 mg/L
200 mg/L
300 mg/L
MOX
CAR
CEF
AMX
Antibiotic Type

Agrobacterium tumefaciens infection and counter selection

Cotyledon explants from 20 weeks-old primary somatic embryo cultures were inoculated and co-cultivated with *Agrobacterium tumefaciens* (AGL1::pCP108) as described for apple (Maximova *et al.*, 1998), with the addition of a 30 second sonication treatment in a Bransonic water bath (Model 1510,VWR, Pittsburgh, PA), at 25°C. After 48h co-cultivation in the dark at 25°C, the explants were transferred to SCG medium containing various antibiotics. The types and the concentrations of the antibiotics used for *Agrobacterium* counter selection were as described in the disk diffusion assay. The treatments included *Agrobacterium* infection and co-cultivation followed by counter selection with all four antibiotics at four different concentrations.

Moxalactam, at all concentrations tested, effectively controlled *Agrobacterium* growth with no explants lost in any of the treatments (Table 2.1). Explants grown on media with the other antibiotics needed more frequent transfer due to bacterial overgrowth, especially during the first 4 weeks following co-cultivation. Unlike the results from the disk assay, carbenicillin was more effective than cefotaxime at suppressing *Agrobacterium* growth after co-cultivation. This was demonstrated by the fewer explants lost on the carbenicillin medium compared to those on cefotaxime (Table 2.1), especially at the lower concentrations (100 mg L^{-1}, 150 mg L^{-1}) where considerably fewer explants were lost on the carbenicillin plates than on cefotaxime. Amoxicillin, provided little control over *Agrobacterium*, resulting in a high percentage of explants lost at all 4 concentrations tested.

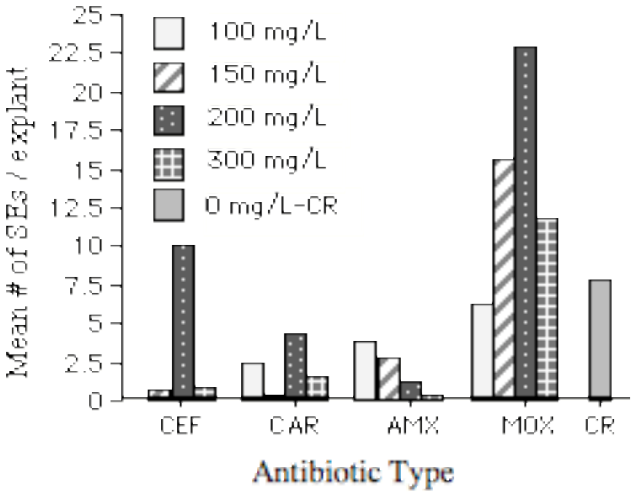
Table 2.1. Effect of antibiotics on the percent of explants lost to *Agrobacterium*-AGL1 overgrowth 10 weeks after co-cultivation. The percentages were calculated from the total numbers of explants lost per treatment plate. One plate containing 25 total number of explants was initiated per concentration and antibiotic treatment. Abbreviations are CEF-cefotaxime, CAR-carbenicillin, AMX -amoxicillin, and MOX moxalactam.

Concentration	CEF	CAR	AMX	MOX
100ug/mL	15	5	30	0
150ug/mL	14	2	24	0
200ug/mL	10	0	20	0
300ug/mL	8	0	15	0

To investigate the effects of the antibiotics on somatic embryogenesis (SE), cotyledon explants were cultured with the various antibiotics at varying concentrations. Tissues were transferred to fresh media every other week. Twenty-five explants were cultured per treatment. Ten weeks after culture initiation, the numbers of explants lost to *Agrobacterium* overgrowth were recorded. Additionally, the total numbers of SEs produced per treatment and the numbers of explants producing SEs were recorded.

At the concentrations tested, all antibiotics had some effect on the regeneration potential of cacao secondary somatic embryogenesis compared to the control cultures without antibiotics (Fig. 2.2). Cotyledons exposed to 200 mg L^{-1} moxalactam responded positively, with the highest increase in the total number of embryos produced per cotyledon explant. Moxalactam at concentrations above 100 mg L^{-1} showed a strong positive effect on embryo regeneration. The highest production was achieved at 200 mg L^{-1} with an average number of embryos per explant three fold higher than the control plates without antibiotic.

Figure 2.2. Effect of antibiotic type and concentration on the average number of embryos produced per cotyledon explant in the absence of *Agrobacterium*. 25 explants per treatment were plated on media containing various antibiotics. The total number of embryos produced, and the total numbers of explants initiated per treatment were recorded. The average number of embryo per total explant was calculated. Abbreviations: CEF-cefotaxime, CAR-carbenicillin, AMX-amoxicillin, and MOX-moxalactam.


Mean # of SEs / explant
25
22.5
20
17.5
15
12.5
10
7.5
5
2.5
0
100 mg/L
150 mg/L
200 mg/L
300 mg/L
0 mg/L-CR
CEF
CAR
AMX
MOX
CR
Antibiotic Type

At 300 mg L^{-1}, the effect of moxalactam on embryogenesis was reduced to an average of 11 embryos per explant compared to 200 mg L^{-1}, with an average of 17 embryos per explant. At 300 mg L^{-1} we observed an average increase in the total number of embryos compared to the control regeneration plates, which only produced 7 embryos per explant. Cefotaxime enhanced regeneration only at 150 mg L^{-1}. Negative effects were observed at all of other cefotaxime concentrations tested, including 300 mg L^{-1}, the minimum concentration required for moderate control of the *Agrobacterium* during transformation. Carbenicillin and amoxicillin had negative effects at all concentrations tested, decreasing cacao secondary somatic embryogenesis.

Detailed analysis of the effects of moxalactam on cacao primary and secondary embryogenesis

To determine the optimal moxalactam concentration for primary embryogenesis, and the moxalactam concentration in combination with explant age for secondary embryogenesis, primary embryogenesis was initiated immediately after flower harvest, and cotyledon explants were excised from mature SEs at 20, 21 and 24 weeks after primary culture initiation from primary SE cultures. Moxalactam concentrations applied were 100 mg L^{-1}, 150 mg L^{-1}, 200 mg L^{-1} and 300 mg L^{-1} (Table 2.2).

Table 2.2. Average number of normal embryos produced per treatment after 20 weeks culture initiation. Data represents the average number of normal embryo per explant ± standard error. Averages were generated from 3 replicate plates containing 25 explants each. Abbreviations: MOX – moxalactam. Concentrations are expressed in mg/L.

Explant	Age of tissue	Control	MOX 100	MOX 150	MOX 200	MOX 300
Staminode	0 weeks	11 ± 2.1	35 ± 2.8		35 ± 3.7	22 ± 3.0
Cotyledon	20 weeks	141 ± 3.6	153 ± 7.3	163 ± 6.4	218 ± 4.0	158 ± 3.4
Cotyledon	21 weeks	85 ± 5.9	135 ± 3.0	146 ± 6.2	249 ± 6.2	147 ± 5.3
Cotyledon	24 weeks	97 ± 4.3	140 ± 2.4		361 ± 4.7	124 ± 3.9

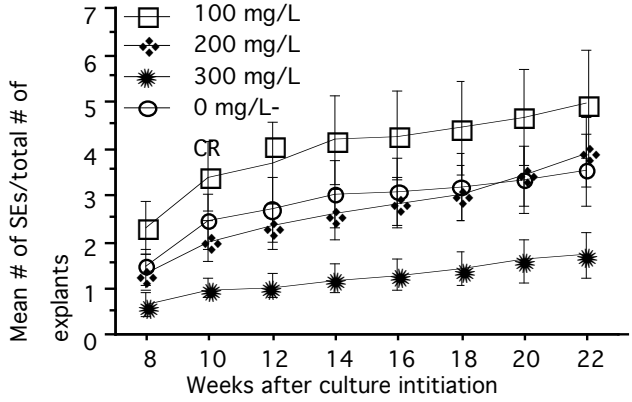
Effect of moxalactam on primary somatic embryogenesis

Staminode cultures were monitored for a 22-week period. The total number of embryos produced and the number of explants producing embryos were recorded. As seen in the previously described experiment, we observed that moxalactam had a significant positive effect on regeneration, increasing primary embryo production by 42 % at 100 mg L^{-1}. No significant effect was observed for explants plated on 200 mg L^{-1} compared to the control, while at 300 mg L^{-1}, moxalactam had negative effects on the tissue regeneration (Table 2.2), producing white hard calli, forming numerous roots and developing very few embryos.

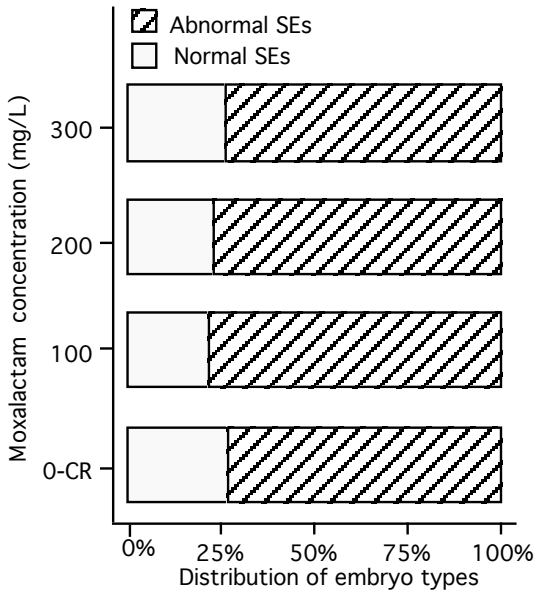
In previous work, we have observed the appearance of both normal and abnormal embryos in culture (Li *et al.*, 1998, Maximova *et al.*, 2002). Thus, we counted the numbers of normal and abnormal embryos in these experiments to determine possible antibiotic effect on the embryo quality (Fig. 2.3b). Although moxalactam at concentrations higher than 100 mg L^{-1} had a negative effect on the number of embryos produced, the higher concentrations did not affect the percentage of normal and abnormal embryos produced, 25% to 75% respectively. A one-way ANOVA analysis at the 95% confidence interval showed no significant difference ($p=0.772$) in embryo quality among all antibiotic treatments.

Figure 2.3. Effects of moxalactam antibiotic and concentration on primary embryogenesis. Staminode explants were cultured on media containing various concentrations of moxalactam. Quantitative and qualitative data of the embryo production were collected every 2 week intervals for the entire culture period. A. Average numbers of somatic embryos produced per cotyledon explant. Data points represent the means of 3 replicate plates. B. Percentages of normal versus abnormal embryos produced on moxalactam compared to control regeneration without antibiotic. Stack columns represent cumulative data at 20 weeks of mean percentages from 3 replicate plates. Standard errors vary from 1.2 - 2.5% for all data sets collected. C. Percentages of explants producing somatic embryos overtime. Data points represent mean percentages of 3 replicate plates. Mean separations were performed using Fisher's PLSD test at 5% level of significance.

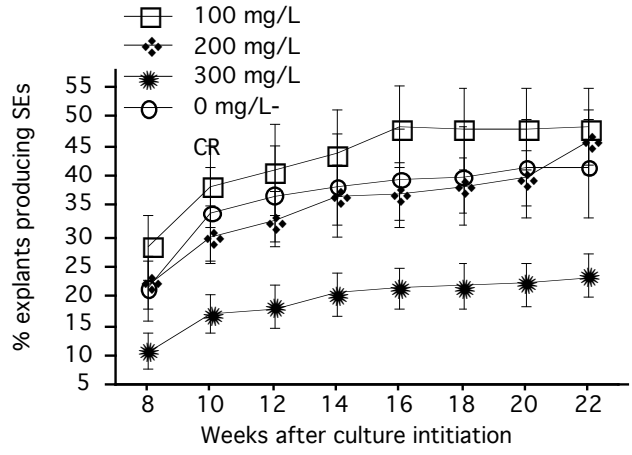
(A)


100 mg/L
200 mg/L
300 mg/L
0 mg/L-CR
Mean # of SEs/total # of explants
0
1
2
3
4
5
6
7
8
10
12
14
16
18
20
22
Weeks after culture intitiation

(B)


Abnormal SEs
Normal SEs
Moxalactam concentration (mg/L)
300
200
100
0-CR
0%
25%
50%
75%
100%
Distribution of embryo types

(C)


100 mg/L
200 mg/L
300 mg/L
0 mg/L-CR
% explants producing SEs
5
10
15
20
25
30
35
40
45
50
55
8
10
12
14
16
18
20
22
Weeks after culture intitiation

Another important parameter associated with the efficiency of cacao somatic embryogenesis is the percentage of explants, which regenerate and produce at least one embryo (Fig 2.3c). Moxalactam at 100 mg L^{-1} had a positive effect on the percentage of staminodes producing embryos. However, as reported in Table 2.2, antibiotic concentrations higher than 100mg L^{-1} had negative effects on the number of SEs produced from staminodes, an effect that became accentuated with increasing antibiotic concentrations.

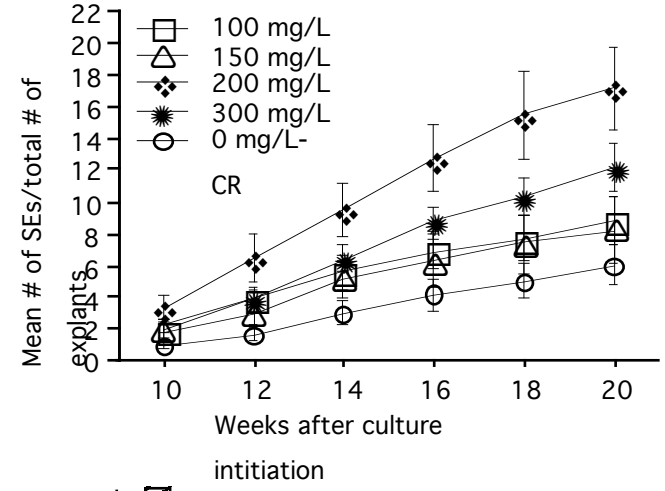
Effect of moxalactam on secondary somatic embryogenesis

An increase in embryo production was also observed for secondary somatic embryogenesis (2-SE) cultures at all concentrations of moxalactam tested. The highest numbers observed were at the antibiotic concentration of 200 mg L^{-1} (Table 2.2). The age of the primary culture at the time of 2-SE initiation was also important, even in the presence of moxalactam. Moxalactam treatment resulted in increases of 36, 91 and 264 percent embryo production for the cultures initiated at age 20, 21 and 24-weeks respectively after primary culture initiation. The effects of moxalactam on 2-SE (Fig. 2.4) were similar to those observed with primary embryogenesis, (Fig 2.3). At all concentrations tested, moxalactam increased embryo production compared to the control treatments. Similar to the result with primary embryogenesis, a significant increase in the number of embryos was recorded at all treatments, with optimal moxalactam concentration at 200 mg L^{-1} (Figs. 2.4a and 2.4c).

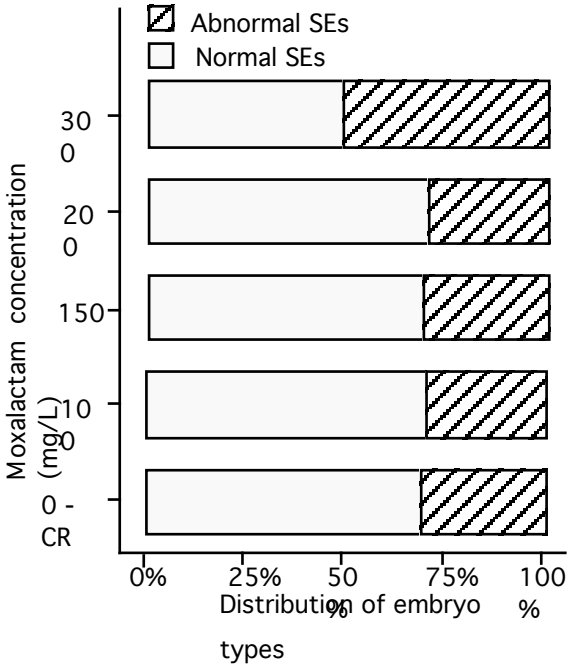
In my experiments, the control treatments exhibited the lowest average number of embryos per explant, followed by 100mg L^{-1}, and 150mg L^{-1} moxalactam. At moxalactam concentrations of 200mg L^{-1} we observed the best response from the tissue, and at 300mg L^{-1} embryo production started to decline compared to 200 mg L^{-1} (Fig 2.4a). At the lower 3 concentrations, no difference in the percentage of normal versus abnormal embryos was recorded ($p=0.954$). However, at 300mgL^{-1} a statistically significant decrease ($p<0.001$) in the production of normal embryos was observed (Fig. 2.4b) when compared with all other treatments (two-sample T-tests at the 95 percent confidence interval).

Figure 2.4. Effects of moxalactam antibiotic concentration on secondary embryogenesis. Cotyledon explants were cultured for 20 weeks on media containing various concentrations of moxalactam. Quantitative and qualitative data of the embryo production were collected every 2 week intervals for the entire culture period. A. Average numbers of somatic embryos produced per cotyledon explant. Data points represent the means of 3 replicate plates. B. Percentages of normal versus abnormal embryos produced on moxalactam compared to control regeneration without antibiotic. Stack columns represent cumulative data at 20 weeks of mean percentages from 3 replicate plates. Standard errors vary from 1.3 to 2.29% for all data sets collected. C. Percentages of explants producing somatic embryos overtime. Data points represent mean percentages of 3 replicate plates. Mean separations were performed using Fisher's PLSD test at 5% level of significance.

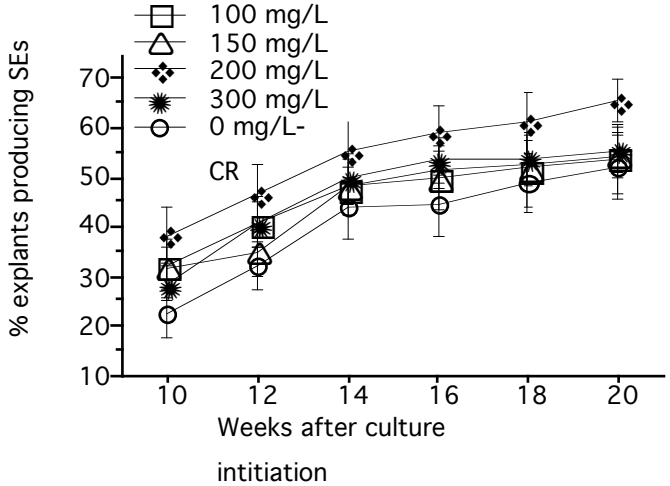
(A)


100 mg/L
150 mg/L
200 mg/L
300 mg/L
0 mg/L-
CR
Mean # of SEs/total # of explants
Weeks after culture intitiation

(B)


Abnormal SEs
Normal SEs
Moxalactam concentration (mg/L)
CR
Distribution of embryo types

(C)


100 mg/L
150 mg/L
200 mg/L
300 mg/L
0 mg/L-
CR
% explants producing SEs
Weeks after culture intitiation

Comparison of cefotaxime and moxalactam effects on cacao secondary embryogenesis

In an effort to further investigate the effects of cefotaxime and moxalactam on cacao SE, an experiment was designed to evaluate the antibiotics effects on embryo production and quality. For this experiment cotyledons from 24-week old primary culture were exposed to cefotaxime and moxalactam at the concentrations of 0, 100, 200 and 300 mg L^{-1} (2X4=8 treatments). At the concentrations tested, both antibiotics altered secondary embryo production (Fig. 2.5a,b). The highest numbers of embryos produced by the cefotaxime treatment was at 100 mg L^{-1}, with the average number of embryos decreasing at the higher concentrations of 200 and 300 mg L^{-1}. In contrast, moxalactam averages peaked at 200 mg L^{-1}, decreasing at 300 mg L^{-1} with averages well above the control regeneration plates. When comparing the numbers of embryos produced at 300 mg L^{-1} cefotaxime, the values are not significantly different from those of the control regeneration ($p=0.412$). Nonetheless, at 300mg L^{-1} we observed a slight increase ($p=0.044$) in embryo abnormality compared to the control (Fig. 2.5b). Similar increases in embryo abnormality were also observed at 300 mg L^{-1} moxalactam (Fig. 2.4b). In addition, we compared the cefotaxime normality rates using a two-sample T-test. In this analysis, we observed no significant difference between abnormal embryo production in the controls 47% ($p=0.412$), the 100 mg L^{-1} treatments 34% ($p=0.44$), or in the 200 mg L^{-1} treatment 45% ($p=0.135$) assuming equal variance. A significant difference was identified at the 300mg L^{-1} concentration when the treatment produced a total of 53% abnormal embryos ($p=0.002$) compared to all the other treatments.

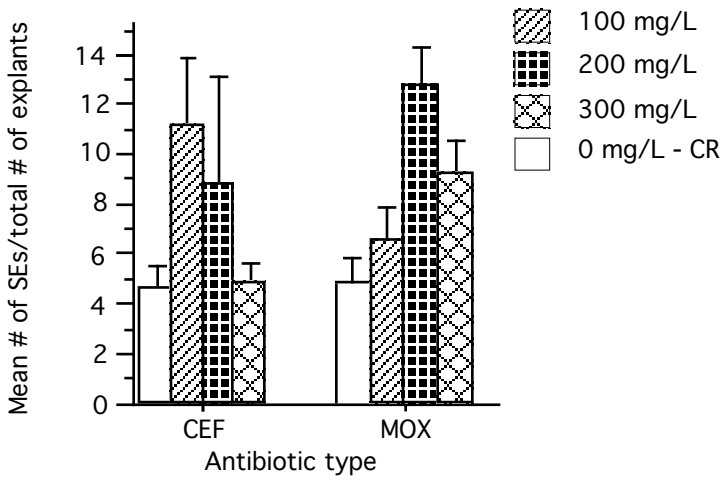
Effects of moxalactam time exposure on cacao secondary embryogenesis

In the previous experiments, the explants were cultured continuously on antibiotics; therefore, we also investigated the effect of moxalactam time exposure on secondary embryogenesis. 24 weeks-old primary cotyledons were placed on SCG for secondary embryogenesis and exposed to 200 mg L^{-1} moxalactam for 0, 4, 6, or 20 weeks, followed by growth on antibiotic free media. Starting 8 weeks after culture initiation, the number of explants producing embryos and the total number of embryos produced per treatment were recorded every other week for 20 weeks.

Our data indicated that 4 weeks of exposure to moxalactam had a positive effect on embryogenesis compared to untreated controls (Fig. 2.6). The numbers of embryos produced increased further after 6 weeks exposure to moxalactam and were slightly higher for those explants that remained in the 200 mg L^{-1} antibiotic concentration throughout the experiment. Thus we can conclude that for the first 20 weeks after culture initiation, moxalactam had no negative effect on the regeneration potential of cacao cotyledon tissue; conversely, extended exposure to the antibiotic results in increased regeneration.

Figure 2.5. Effects of cefotaxime antibiotic and concentration on secondary embryogenesis. A. Direct comparison between the effects of cefotaxime and moxalactam antibiotic after a 20 week cultivation period of tissue on media containing antibiotics at the indicated concentrations. B. Percentages of normal versus abnormal embryos produced on moxalactam compared to control regeneration without antibiotic. Stack columns represent cumulative data at 20 weeks of mean percentages from 3 replicate plates. Standard errors vary from 2.37-3.83% for all data sets collected.

(A)


Mean # of SEs/total # of explants
14
12
10
8
6
4
2
0
CEF
MOX
Antibiotic type
100 mg/L
200 mg/L
300 mg/L
0 mg/L - CR

(B)

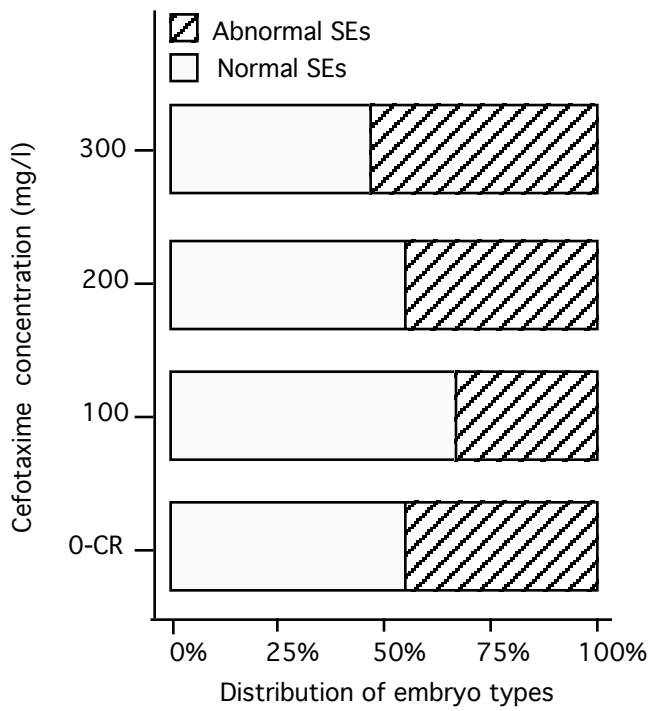
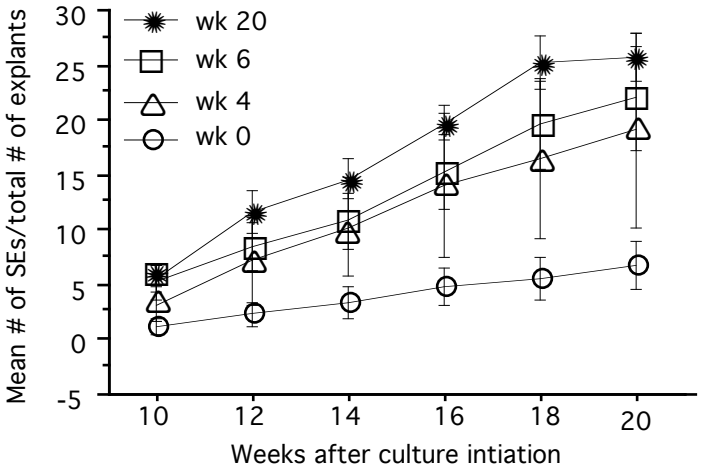

Abnormal SEs
Normal SEs
Cefotaxime concentration (mg/l)
300
200
100
0-CR
0%
25%
50%
75%
100%
Distribution of embryo types

Figure 2.6. Effect of time exposure of the antibiotic moxalactam on secondary embryogenesis. 25 cotyledon explants per plate in triplicate were plated on media containing 200mg L^{-1} moxalactam antibiotic for the four specific time treatments of zero, 4, 6 or 20 weeks. After treatments, explants were grown on antibiotic free media for a total of 20 weeks. The total number of embryos produced, and the total numbers of explants initiated per treatment were recorded. The average numbers of embryos produced per explant were calculated. Abbreviations: CEF-cefotaxime, CAR-carbenicillin, AMX-amoxicillin, and MOX-moxalactam.


wk 20
wk 6
wk 4
wk 0
Mean # of SEs/total # of explants
30
25
20
15
10
5
0
-5
10
12
14
16
18
20
Weeks after culture intiation

2.5. DISCUSSION

Development of an effective transformation system for cacao depends on the availability of tissue culture techniques that permit efficient DNA delivery, selection of transformed cells and the regeneration and recovery of whole transgenic plants. *Agrobacterium* mediated transformation is a method of choice for the introduction of foreign genes into plants. During the development of a transformation protocol for cacao, it was noted at an early stage that controlling *Agrobacterium tumefaciens* growth after gene delivery and increasing the efficiency of the somatic embryogenesis system would be critical for success. The research described here contributed to the development of a working transformation system (Maximova *et al.*, 2003), which is very useful as a basic research tool for cacao. Using moxalactam antibiotic in the transformation protocol, we have been able to generate 17 independent transgenic secondary somatic embryogenesis lines from the first 10 *Agrobacterium*-mediated transformation experiments (Maximova *et al.*, 2003). Transgenic lines have been generated using various transformation vectors, including a vector containing a *Theobroma cacao* class I chitinase and several other marker genes. Further analysis of these lines awaits embryo development into plants containing sufficient tissue for molecular examination.

The antibiotics cefotaxime and carbenicillin are commonly used for counter-selection of *Agrobacterium* (Ellis *et al.*, 1989, Hackelford, 1996). It is generally assumed that these antibiotics, at concentrations required for bacterial control, are not detrimental to the plant tissue *in vitro*. The effect of these antibiotics on morphogenesis

of plants in highly regenerative microculture systems like tobacco leaf discs may be negligible (Ellis *et al*., 1989). However, that is not the case for the *T. cacao* somatic embryogenesis system, which in the past has proven relatively recalcitrant to tissue culture practices.

The antibiotics tested in this study belong to two major classes, cephalosporins and penicillins. These drugs are known as β-Lactams, which despite their different structural compositions inhibit the synthesis of bacterial cell walls (Bycroft, 1987). Both classes of antibiotics become covalently linked to the cell's penicillin-binding proteins, enzymes responsible for constructing or modifying the bacterial cell wall. Antibiotic binding prevents cell wall synthesis, causing leakage and/or triggering of active enzymatic processes, leading to the degradation of the cell wall (Bycroft, 1987). The cephalosporin antibiotics tested in this study were moxalactam and cefotaxime, and we observed that both cephalosporin type antibiotics showed good control of *Agrobacterium*, while also increasing embryo regeneration, compared to control regeneration.

In an effort to determine the optimal counter-selection conditions for *Agrobacterium*-mediated transformation of cacao, we tested various antibiotics and their individual effects on *Agrobacterium tumefaciens* growth and on the somatic embryogenesis regeneration system. The results from the disk diffusion assay demonstrated that moxalactam is most effective for growth inhibition of strain AGL1. Similar results were observed when testing the same four antibiotics with *Agrobacterium* strains EHA 101 and LBA4404, where moxalactam proved to be the antibiotic with the

largest bacterial zone of inhibition, even at the low concentration of 100mg L^{-1} (Hackelford, 1996). Similarly, for strain AGL1::pCP108, we observed that the application of moxalactam at 100 mg L^{-1} concentration resulted in an inhibition zone of 18 mm, equivalent to that of the next most effective antibiotic, cefotaxime at 200 mg L^{-1}. The addition of 200 mg L^{-1} moxalactam to the regeneration media not only effectively eliminated bacterial growth, but also enhanced secondary embryo production even in the absence of *Agrobacterium*. Moxalactam also contributed to an increased number of cotyledon explants capable of embryo regeneration.

The effects of moxalactam antibiotic were evaluated in combination with the other factors required for a successful transformation protocol, including explant type, tissue age, and *Agrobacterium* strain. Here, we demonstrated that the use of moxalactam in cacao primary and secondary somatic embryogenesis not only controlled *Agrobacterium*, but also enhanced embryogenesis frequencies. Consistent with previous observations (Maximova *et al*., 2002), my results showed that secondary embryogenesis is more efficient than primary embryogenesis (3.5 fold higher) in terms of average numbers of embryos produced per explant cultured. Similarly, secondary embryogenesis produced a higher percentage of normal embryos per explant. This indicates the distinct advantages of utilizing secondary embryogenesis for development of a transformation protocol.

A clue to a possible mechanism of action explaining the enhancement of cacao somatic embryogenesis by moxalactam is found in the structural composition of this

antibiotic. After its breakdown, moxalactam is thought to degenerate into several key functional groups (Webber and Yoshida, 1979), one of which resembles phenylacetic acid, a naturally occurring auxin. This suggests that increased auxin concentration during somatic embryogenesis may promote higher regeneration frequencies. However, for our transformation system, moxalactam function is doubled by not only serving potentially as an additional auxin source, but also by exhibiting β-Lactam activity against gram negative bacteria, including *Agrobacterium* (Bycroft, 1987, Neu *et al*., 1979), thus enhancing embryogenesis, preventing bacterial overgrowth and tissue loss.

It is important to note that significant genotypic effects on cacao SE efficiencies has been observed (Maximova *et al*., 2002). The results presented here were obtained with the genotype *Scavina* 6 and it is likely that the effects of antibiotics on other genotypes will vary significantly. Additionally there is a need to test the seasonal effects on cacao tissue culture initiation, since a difference in the frequency of somatic embryos produced from tissue initiated at different times of the year and at different years (Li *et al*., 1998, Maximova *et al*., 2002) generated different fold induction results in the *Theobroma cacao* somatic embryogenesis protocol.

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CHAPTER 3

OVEREXPRESSION OF A CACAO CLASS I CHITINASE GENE IN TRANSGENIC *THEOBROMA CACAO* L.

3.1. ABSTRACT

Transgenic *Theobroma cacao* plants over-expressing a cacao class I chitinase gene (Chi A) were obtained by *Agrobacterium*-mediated transformation. PCR analysis confirmed the presence of the transgene in three independent transgenic lines, SAG31, SA39 and SA44, initially selected by GFP expression. Northern blot analysis verified expression of the endochitinase gene in two of the three lines, while transcript levels of the chitinase gene in SAG31 were not detected.

Transformed lines were also tested for expression of endochitinase activity using an *in vitro* fluorometric assay. Leaves of mature SA39 and SA44, but not SA31, transgenic plants had up to a 2.5-fold increase in endochitinase activity compared to controls. In addition, plants from the SAG31 and SA39 lines were inoculated with *Crinipellis perniciosa*, the causal agents of witches broom disease of cacao to assess resistance.

3.2. INTRODUCTION

The life cycle of plant pathogenic fungi range from the opportunistic colonist, which invades plant wound sites, to specialized biotrophs and necrotrophs capable of colonizing intact living tissue (see Chapter 1). *Theobroma cacao* fungal pathogens exhibit both lifestyles. Of particular importance to South American cacao-producing countries is the specialized biotroph *Crinipellis perniciosa* (Stahel) Singer, the causal agent of witches' broom disease of cacao. *Crinipellis perniciosa*, endemic to the Amazon basin region, (Purdy and Schmidt, 1996), affects cacao production in all South American cacao-producing countries, including the largest production area, Bahia, Brazil.

Plants respond to pathogen recognition by the induction and expression of a number of genes encoding diverse proteins, many of which are believed to play a role in defense (Collinge *et al.*, 1993). A group of these proteins, known as pathogenesis-related (PR) proteins, accumulate locally and often systemically in plant tissue in response to infection. PR proteins are classified into several groups. One widely studied group comprises plant chitinases (group 3 PR proteins). These enzymes are thought to play a role in plant defenses by cleaving a bond between the C1 and C4 of two consecutive N-acetylglucosamine links of chitin polymers, a key component of fungal cell walls (Collinge *et al.*, 1993), releasing internal β-1,4 linkages (Boller *et al.*, 1988).

Chitinase genes, cloned from plants and microorganisms, have been inserted in the genome of a number of plant species in order to achieve resistance against a broad range of fungal pathogens (Mora and Earle, 2001). Crops transformed with chitinases

include tobacco (Broglie, 1991, Lorito *et al*., 1998), canola (Broglie, 1991; Benhamou *et al*., 1993), tomato (Tabaeizadeh, 1997), potato (Lorito *et al*., 1998), broccoli (Mora and Earl, 2001), apple (Bolar *et al*., 2000), carrot and cucumber (Punja and Raharjo, 1996) to mention a few. Studies performed by Broglie (1991) reported that transgenic tobacco and canola plants over-expressing a bean endochitinase gene, showed increased resistance to the soil-borne pathogen *Rhizoctonia solani*. Furthermore, in 1993, Benhamou *et al*. (1993) observed that *Rhizoctonia solani* colonization differed in transgenic canola plants expressing chitinase transgene compared to non-transgenic controls. In the transgenic plants, infection was restricted to the cortex, regardless of penetration of the host cuticle and epidermis by the fungus. In a separate study, Lorito *et al*. (1998) described transgenic tobacco and potato plants transformed with an endochitinase derived from the mycoparasitic fungus *Trichoderma harzianum*. Similarly, transgenic plants ranged from highly tolerant to completely resistant to the foliar pathogens *Alternaria alternata, Alternaria solani*, *Botrytis cinera* and *Rhizoctonia solani*. Transgenic broccoli (Mora and Earl, 2001) expressing *Trichoderma harzianum* endochitinase showed a decrease in disease symptoms compared to the control plants when treated with *Alternaria brassicola*. This reduction in disease severity was comparable to protection by fungicide on non-transgenics (Mora and Earl, 2001).

Expression of chitinase transgenes does not always result in resistance. For example disease resistance in transgenic apple lines expressing the same chitinase gene as the one described by Lorito *et al*. (1998), and Mora and Earl (2001) resulted in transgenic lines with a wide range of resistance. Resistance ranged from 0% to 99.7% in the number

of lesions after infection of transgenic apple lines compared to controls. Similarly, transformation of carrot and cucumber with a chitinase gene resulted in elevated disease resistance only in the former (Punja and Raharjo, 1996). Reports from transgenic lines having a limited resistance or no resistance when compared to non-transgenics indicate the limitations of incorporating a gene encoding the end product of a defense pathway. In many cases this approach will be highly specific for few pathogens, providing resistance to a limited range pathogen, or in some cases no protection at all.

It is for this reason that in addition to chitinases, genes encoding other hydrolyses, such as □-glucanases, have been used in combinations with chitinases to generate transgenics with added disease resistance (Sela-Bourlage *et al.*, 1993; Jach *et al.*, 1995; Anand *et al.*, 2003). In addition it is important to recognize that the value of transgene in the transformed plant will depend on the endogenous defense mechanisms already present in the non-transformed plant (Stuiver and Custer, 2001).

In 1995, a study by Snyder and Furtek at The Pennsylvania State University resulted in the isolation and characterization of a genomic chitinase clone from *Theobroma cacao* L (Snyder and Furtek, 1995). Here, we report the insertion of this cacao endochitinase gene, under the control of a constitutive promoter, into the cacao genome. *Agrobacterium* mediated transformation was used to generate transgenic cacao plants which were characterized for the presence of the T-DNA insertion, the expression of the *T. cacao* endochitinase gene, and endochitinase activity measured using an *in vitro*

assay. Studies were initiated to evaluate the lines for resistance to the fungal pathogen *Crinipellis perniciosa*, using an *in vivo* bioassay.

3.3. MATERIALS AND METHODS

Development of transgenic lines

Secondary somatic embryogenesis cultures were maintained as previously described (Maximova *et al*., 2002) and used for transformation as described by (Antunez de Mayolo *et al*., 2003, Maximova *et al*., 2003). Thirteen independent transgenic cacao lines expressing pGAM00.0511 T-DNA construct have been recovered. Three of these transgenic lines were used for the present study.

Vegetative propagation of *T. cacao* plants

Transgenic plant acclimation to greenhouse condition followed the procedures of Traore *et al*., 2003. *Theobroma cacao* rooted cuttings were generated from transgenic lines and from non-transgenic *Sca*- 6 and ICS1 plants as controls. Cuttings, approximately 10 cm in length with at least 2 leaves each of 5-8 cm, were dipped for 5 seconds in 4 gm L^{-1} indole butyric acid (IBA), 4gm L^{-1} naphthalene acetic acid (NAA) dissolved in 50% ethanol, to induce root formation. Cuttings were placed on soaked silica sand on a misting bench for 2-3 months in the greenhouse under previously described greenhouse conditions (see laboratory protocol for recent updates: http://guiltinanlab.cas.psu.edu/Research/Cocoa/protocols.htm).

Fluorescent imaging of GFP

Fluorescent GFP images of transgenic leaves were obtained as previously described (Maximova *et al.*, 2003). Fluorescence leaf-GFP intensity from four independent transgenic lines: SA25, SAG31, SA39, SA44 was measured and compared to non-transgenic embryos, *Scavina* 6 (*Sca*-6) variety. The measurements were taken using a 3 CCD video camera and 35nm Nikon lens, magnification was 1.5X with a 1/2-sec. exposure time. Mean intensities (integrated pixel values) were measured using NIH Image 1.6 image processing and analysis software.

Genomic PCR analysis.

Genomic PCR was performed as described by Maximova *et al.*, (2003). PCR primers OmegaXba+ and 35tXba- were used for analysis of transgene insertion into the genome. Primer sequences were OmegaXba+ (Forward) 5'-CAA CAA CAT TAC ATT TTA CAT TCT -3' and 35tXba- (Reverse) 5'-AAG AAC CCT AAT TCC CTT ATC T -3'. The primer set was designed to simultaneously amplify genes flanked by an E12 promoter and 35S terminator present in our transformation vectors. PCR products were a 757 bp GFP fragment and 993 bp NPTII selectable marker fragment present in both pGH00.0126 and pGAM00.0511 constructs. In addition to these two fragments, the pGAM00.0511 plasmid contained an additional 1.2 kb fragment of a cacao class I chitinase gene. A second set of primers were used as a control to assess *Agrobacterium tumefaciens* contamination in the transgenic lines. This set of primers was designed by

Maximova *et al.*, (2003) to amplify a 411 bp fragment of the backbone sequence of the pBin19A vector, which should not be present in *Agrobacterium*-free transgenic plants.

For PCR analysis, genomic DNA was extracted from fully developed leaves using Qiagen DNAeasy plant mini kit (Qiagen, Valencia CA). Samples from a non-transgenic *Sca*- 6 leaf was used as a negative control. Reactions contained 10 ng of DNA, 10 µl REDExtract-N-Amp PCR jumpstart reaction mix (Sigma-Aldrich, Austin, TX) and both forward and reverse primers at final concentrations of 0.25 µM in a final volume of 20 µl. Control PCR reactions were performed with DNA from vector pGH00.0126 and backbone vector pBin19 containing only left and right Ti borders (pBin19A), and a PCR reaction without DNA was used for a negative control. For the plasmid reactions, DNA was isolated using QIAGEN plasmid midi purification kit (QIAGEN Inc., Valencia, CA). PCR conditions for all reactions were: 94ºC for 4 min, followed by 35 cycles at 94ºC for 1 min, 54ºC for 1 min, and 72ºC for 1 min. A final extension cycle at 72ºC for 5 min was followed by incubation at 4ºC. Ten µl of each PCR reaction with 1 µl 6X loading dye were loaded onto 2% high-resolution agarose gels (Sigma-Aldrich Co., St. Louis, MO, #A-4718) for electrophoresis.

Northern analysis

Total RNA was extracted from SAG31, SA39 and SA44 transgenic plants containing T-DNA insert from pGAM00.0511, as well as from control leaves from non-transgenic *Sca*- 6 plants. RNA extractions initially followed Chang *et al.*, (1993). Frozen

leaf samples were ground with a mortar and pestle in liquid nitrogen to a fine powder. Approximately 1 gm tissue was re-suspended in 15 ml extraction buffer containing 2% CTAB (hexadecyltrimethyl-ammonium bromide), 2% PVP (polyvinylpyrrolidone), 100 mM Tris base, 25 mM diNaEDTA, 2 M NaCl, 0.5 gm L^{-1} spermidine and 2% β-mercaptoethanol, then incubated at 65 °C for 10-30 min. 15 ml chloroform was added to each sample which were then mixed and centrifuged for 20 min at 8000 x *g*. The top aqueous layer was transferred to tubes containing 5 ml 8 M LiCl (DEPC treated), and stored overnight at 4°C, followed by centrifugation for 30 min at 8000 x *g*. Following LiCl RNA precipitation, RNA was recovered following the Qiagen RNeasy based method (QIAGEN Valencia, CA) as follows: pellets were re-suspended in 500μl RLC Qiagen buffer, samples in RLC buffer were added 0.5 volumes ethanol (100%) and transferred to RNeasy mini column, and centrifuged for 15 sec at 8000 x *g*. Samples were washed with 700μl RW1 buffer, then again using RPE buffer; after each step samples were centrifuged (15 sec at 8000 x *g*). RNA samples were eluted with two 70μl RNase-free water aliquots.

Northern blotting

Total RNA (20 μg) was denatured for 30 minute at 55º C with 1:1 (v/v) sample and Glyoxal/DMSO loading dye (Amersham Bioscience, Piscataway, NJ). Samples were run on 1% agarose gels, capillary blotted with 20X SSC buffer (NaCl, $C_6H_5Na_3O_7$) onto positively charged nylon membranes (Hybond, Amersham, Arlington Heights, IL). RNA was crosslinked to membranes by UV crosslinking [1200 J m^{-2}] (UV Crosslinker 1800,

Stratagene, La Joya, CA). DNA probes (20ng) were labeled with 0.01 mCi [P^{32}] dCTP using Megaprime DNA labeling system (Amersham Bioscience, Piscataway, NJ). Northern blots were hybridized three times. The first probe was the *Theobroma cacao* class I chitinase gene, Genebank accession number TCU30324 (Snyder, 1995), used in the transformation vector. The second probe was the cacao class I chitinase gene, basic isoform, provided by Dr. J. Verica at the Pennsylvania State University. The third probe was a soybean 26 S ribosomal probe (26S) used to standardize gel loading, provided by Dr. B. A. Bailey at the United States Department of Agriculture, Beltsville.

RNA blots were prehybridized for 2 hours with 15 ml ExpressHyb buffer (Clonetech, Palo Alto, CA) and then hybridized overnight at 65 °C in ExpressHyb buffer and 20ng labeled probe. Hybridized blots were washed with 2X SSC/0.1% (v/v) SDS (2 x 5 min) and 0.2x SSC/0.1 % (v/v) SDS (15 min). Blots were covered in plastic wrap and exposed to a phosphor screen for 15 hours (Molecular Dynamics, Amersham Bioscience). Sizes of hybridizing fragments were determined by comparison with mobility of known RNA markers (RNA ladder, Ambion Inc., Austin, TX). Hybridizing band intensity was quantified by Phosphorimager scan (Molecular Dynamics, Amersham Bioscience) and ImageQuant software (Molecular Dynamics, Amersham Bioscience). All measurements were within the linear range of phosphorimager.

In vitro endochitinase activity assay

Total protein extracts from mature leaf samples were obtained from non-transformed *Sca-* 6 and transgenic lines SA 25, SAG31, SA39, SA44. Fresh leaves were placed in liquid nitrogen and ground to a fine powder. 5 ml of extraction buffer (50 mM sodium acetate, pH 5.0, 2 mM EDTA, 3 mM sodium metabisulphite, 1% polyvinyl polypyrrolidone, 5 mM dithiothreitol (DTT) and 250 mM sodium chloride), was added per gm of fresh leaf tissue. Samples were centrifuged at 6000 x *g* for 15 min to remove leaf debris. Total protein content was quantified using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond CA).

Endochitinase activity was measured by the fluorometric assay method (Mora and Earl, 2001). The activity assay used 4- 4-methylumbelliferyl □-D-N, N', N" –triacetylchitotrioside substrate (Sigma M5639), which becomes fluorescent upon cleavage by endochitinase (Brants, 1999; Brants and Earl, 2001; Mora and Earl, 2001). Plates were read on fluorescence plate reader (FluoroCount Packard BioScience, Meriden CT) at 360/480 nm ex/em. Data was collected using the FluoroCount software package. Endochitinase activity units are expressed as the absolute fluorescence value in nmol of substrate per minute per □g total protein.

Crinipellis perniciosa in vivo bioassay

Cacao plants generated under greenhouse conditions were transferred from silica sand to potting soil 3-4 weeks before being moved to a growth chamber. Plants used for experiments were chosen to be of similar height and size, with the presence of an actively growing apical meristem with very young immature leaves. Cacao *comun* seedlings; considered susceptible to witches broom infection, were kindly provided by the United States Department of Agriculture (USDA), Beltsville Maryland

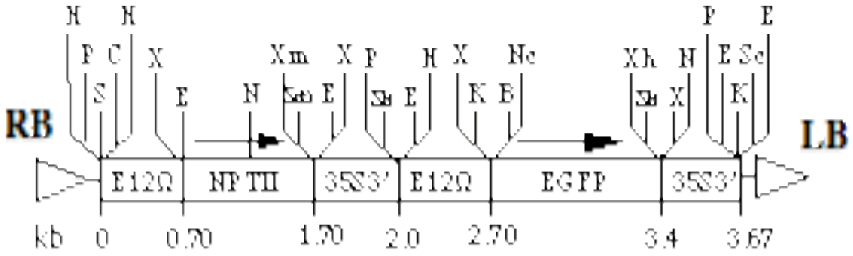
For infection with *Crinipellis perniciosa*, basidiospores were harvested and provided by Dr. Bryan Bailey at the USDA facility Beltsville (APHIS permit # 53959), at a final spore count of $2x10^4$. Spores were suspended in 0.4% warm agarose in water not exceeding 45°C. A 25 □l drop containing approximately 500 spores was applied to the tip of the growing apical meristem of each plant. The agar was allowed to gel for 15 minutes after which the plants were transferred to dew chambers set to 25°C wall and 30°C water to allow dew formation on the leaves without causing runoff drops that might wash the spores from the meristem surface. Plants remained in dew chambers at 100% humidity for 48 hours in the dark. Plants were then transferred to a growth chamber with 12/12 hours light/dark photoperiod with 450 mol* m^{-2} sec^{-1} light. Temperatures were set at 28/25 °C light/dark period, with 75% humidity. Irrigation was applied once a day with 1/10 Hoagland's solution (160 ppm N) during the first month and as often as required for the remaining of the experiment.

3.4 RESULTS

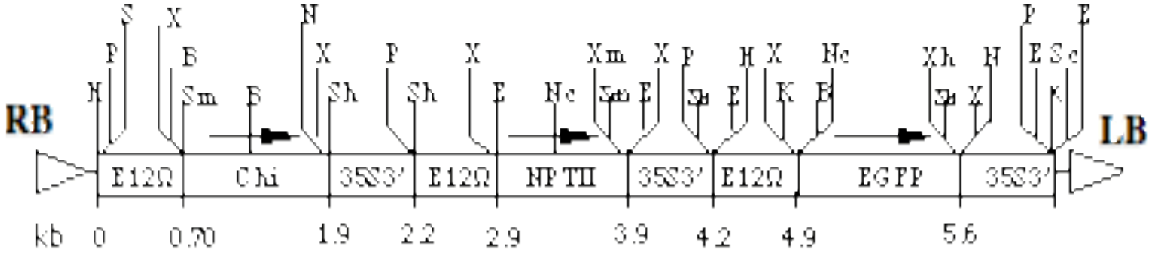
Transformation and transgenic embryo formation

Standard recombinant DNA techniques were used to construct plasmid pGH00.0126 and pGAM00.0511 (Fig. 3.1) which were introduced into *Agrobacterium tumefaciens* strain AGL1 as described by Maximova *et al*. (2003). Both vectors contained the pBin19A backbone and left and right T-DNA borders. The T-DNA region included reporter gene EGFP (Clonetech, Palo Alto, CA) and marker gene NPTII (de-Block *et al*., 1984), both driven by E12- promoter (Mitsuhara *et al*., 1996). In addition, plasmid pGAM00.0511 contains a gene cassette with the genomic copy of the class I chitinase gene isolated from *Theobroma cacao* L., genebank accession number TCU30324 (Snyder and Furtek, 1995). For construction of the pGAM00.0511, the class I chitinase gene was cloned by PCR using Forward 5'-GGCCATGGGTTTACCAAAG TCATTCGAC-3' and Reverse 3'-CAGCTTACTGAAACCATTTGGGTACCGG-5' primers, to include a fragment extending from the TATA box to the polyadenylation site (1.2Kb). The genomic copy of the chitinase gene includes two introns at position 418 and 660 of relative size 89 and 105 bp, respectively. This gene was introduced by blunt end ligation into a gene cassette driven by the E12- promoter (Mitsuhara *et al*., 1996). Gene cassette and pGH00.0126 plasmid were digested with ClaI and ligated next to the right T-DNA border.

Figure 3.1. Transformation vectors pGH00.0126 and pGAM00.0511. (A) Vector pGH00.0126 contains a 3.7 kb T-DNA containing an NPTII selectable marker gene and GFP gene encoding green fluorescent protein. (B) Vector pGAM00.0511 contains a 5.9 kb T-DNA vector containing an NPTII selectable marker gene, and a GFP reporter gene and a cacao class I endochitinase gene. E12 promoter (a CaMV 35S derivative) drives all transgenes in both vectors. Restriction enzymes recognition sites are shown at the top of each map and are abbreviated as follows: B, BamHI; C, ClaI; E, EcoRI; K, KpnI; N, NotI; Nc, NcoI; P, PstI; S, SalI; SacI; Sh, SphI; Sm, SmaI; Sp, SpeI; X, XbaI; Xh, XhoI; Xm, XmaI. The distance from the left most site to each restriction enzyme site is indicated below each map in kilobase pairs (kb). Figure 3.1 was reproduced with permission from Maximova *et al.*, 2003.



pGH00.0126



pGAM00.0511

In order to develop transgenic cacao plants containing the chitinase transgene, primary somatic embryo cotyledon explants were co-cultivated with *Agrobacterium* Cotyledon explants were examined for GFP expression using fluorescent microscopy. Transgenic lines identified through GFP expression were cultured for several months until somatic embryos developed more than one cotyledon. All transgenic lines showed fluorescence levels higher than the non-transgenic *Scavina* 6. A total of 13 independent transgenic lines were established from the *Scavina* 6 cacao variety using pGAM00.0511 vector. To propagate the independent transgenic embryos, cotyledon explants from mature transgenic embryos were plated on embryo induction medium. Clonal propagation of transgenic embryos provided more tertiary transgenic embryos derived from each independent transformation event. Only three independent lines produced sufficient leaf material in the time frame of the present analysis. Transgenic lines grown in greenhouse for transgene analysis were also quantified for leaf GFP expression (Table 3.1).

Characterization of transgenic lines

To examine the insertion of the T-DNA region into the genome of the transgenic lines, PCR analysis was performed on transgenic lines SA25, SAG31, SA39 and SA44. PCR analysis confirmed the insertion of T-DNA into the cacao genome and at the same time tested for the possibility of *Agrobacterium* contamination in the samples, which might occur if insufficient antibiotic selection were applied.

Table 3.1. Mean values of GFP fluorescence intensity ± standard deviation.

Fluorescence pixel intensity value, from 3 independent green pliable mature leaf samples, approximately 25 cm in length, was measured using a Nikon SMZ-4 dissecting scope and a 3CCD video camera system (Optronic Engineering, Goleta, CA) with 1/2 sec. exposure time. Mean intensities (integrated pixel value) were recorded for non transgenic *Scavina* 6 line as well as for SA25, SAG31, SA39 and SA44 transgenic lines, containing GFP gene driven by 12E promoter.

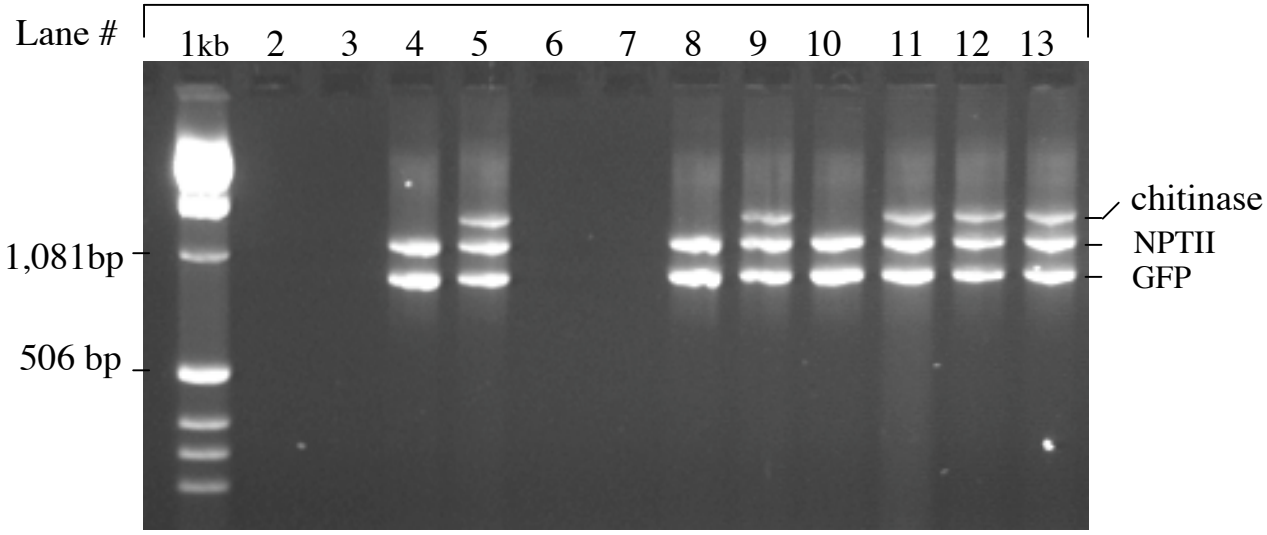
	Sca 6	SA25	SAG31	SA39	SA44
Mean Intensity	84.46	204.34	223.57	214.33	206.93
StDev	± 6.96	± 3.42	± 5.22	± 4.23	± 7.01

In order to determine the sensitivity and specificity of the PCR assay, a series of controls were included. Control plasmid DNAs, were diluted to 15 pg, equivalent to 10 ng of cacao genomic DNA. The OmegaXba+ and 35StXba – primer set was used to detect the presence of T-DNA in transgenic genomic DNA samples. Figure 3.2a shows that as expected, there was no amplification with samples containing only the salmon sperm DNA (Fig. 3.2, lane 2), or vector pBin19A (Fig. 3.2a, lane 3), showing the specificity of the primers to the T-DNA region of the construct. As expected, plasmid pGH00.0126 produced two fragments: a 757 bp EGFP fragment and a 993bp NPTII (Fig. 3.2a, lane 4), and plasmid pGAM00.0511 produced three fragments EGFP, NPTII and the 1.2 kb chitinase fragments (Fig. 3.2a, lane 5). Negative controls of non-transgenic *Sca-* 6 DNA and SSDNA generated no PCR band (Fig. 3.2a, lane 6), nor did the combination of pBin19A with non-transgenic *Sca-* 6 (Fig. 3.2a, lane 7). For positive controls, the combination of non-transgenic *Sca-* 6 and SSDNA with a genomic equivalent of pGH00.0126 produced the two expected fragments (Fig. 3.2a, lane 8). Similarly, non-transgenic *Sca-* 6 DNA mixed with pGAM00.511 produced all three expected PCR fragments (Fig. 3.2a, lane 9). DNA from transgenic line SA25 resulted in both EGFP and NPTII fragments (Fig. 3.2a, lane 10), while the three transgenic lines SAG31, SA39 and SA44 containing T-DNA from pGAM00.0511, produced all three fragments (Fig. 3.2a, lane 11, 12 and 13 respectively).

Figure 3.2. Genomic PCR analysis of transgenic cacao lines. (A). PCR analysis was performed using OmegaXba+ and 35StXba- primer set. Control PCR amplification used plasmid DNA of pBin19A, transgenic vector backbone, pGH00.0126 and pGAM00.0511, as well as with non-transgenic *Scavina* 6 DNA. All plasmid samples were diluted to 15 pg per reaction or 10 ng genomic DNA using salmon sperm (SS) DNA as a carrier with a final concentration of 10 ng per reaction. The template for each reaction is indicated in the panel above the figure as follows: 1kb ladder, lane 1; salmon sperm (SS) DNA, lane 2; pBin19A, lane 3; pGH00.0126, lane 4; pGAM00.0511, lane 5; *Sca*-6, lane 6; *Sca*-6 plus pBin19A, lane 7; *Sca*-6 plus pGH00.0126, lane 8; *Sca*-6 plus pGAM00.0511, lane 9; and DNA of transgenic (TR) lines SA25, SAG31, SA39 and SA 44 in lanes 10, 11, 12 and 13 respectively. (B). PCR analysis was performed using pBin19A Forward and Reverse primer set. Control PCR sample contained plasmid DNA pBin19A. Template for each lane was as follows: 1kb ladder, lane 1; pBin19A, lane 2; transgenic (TR) lines SA25, SAG31, SA39 and SA 44 in lanes 3, 4, 5, 6 respectively.

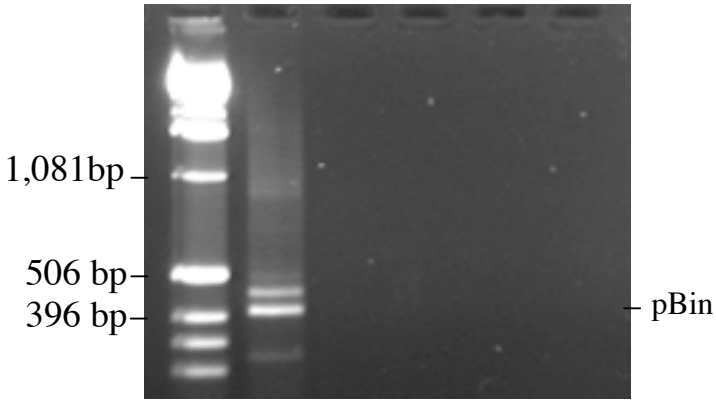
(A)

SS	+	+	+	+		+	+	+				
pBin		+				+						
p126			+				+					
p0511				+				+				
*Sca*6					+	+	+	+				
TR									+	+	+	+



(B)

SS	+				
pBin	+				
TR		+	+	+	+

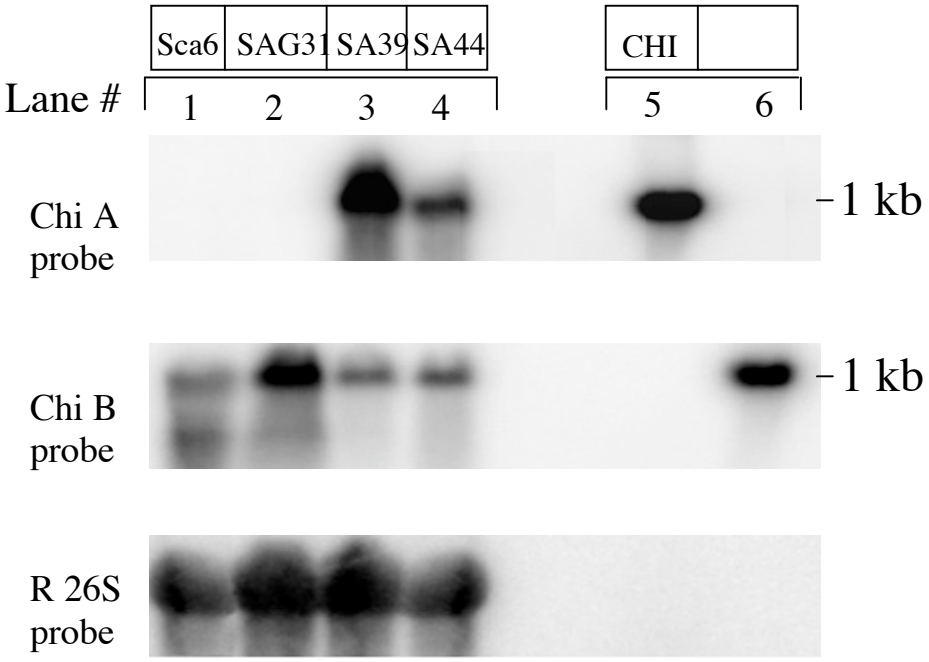


As expected pBin19A backbone fragment amplifications were only observed when using pBin19A plasmid DNA (Fig 3.2b, lane 2). Genomic DNA from the transgenic plants SA25, SAG31, SA39 and SA44 with the pBin19A primer set (Fig 3.2b, lane 3, 4, 5, and 6) resulted in no amplification with these primers, indicating that these samples did not contain the sequences flanking the Ti borders. This demonstrates that *Agrobacterium* was effectively eliminated from the tissue below detectable levels during the selection and culture process.

Northern blot analysis

Northern blot analysis was used to determine the transcription levels of the Chi A transgene in lines SAG31, SA39 and SA44 (Fig. 3.3). Northern blots containing total RNA from leaf tissue, were stained with methylene blue revealing discrete ribosomal RNA bands, indicating the integrity of the RNA, as well as the uniformity of loading between different samples (data not shown). Ten ng of DNA fragments representing portions of the Chi A and Chi B cDNA were loaded in adjacent lanes as hybridization controls, as seen in Fig. 3.3, lane 5 and 6. The results demonstrate that in the conditions used here, the Chi A and Chi B probes were gene-specific, showing no detectable hybridization to each other. Northern blots were probed first with ChiA, corresponding to the transgene, also expected to hybridize to the endogenous transcript if present. No hybridization between the Chi A probe and the *Sca-* 6 RNA was detected (Fig. 3.3, lane 1). Surprisingly, no hybridization was observed between the Chi A probe and the RNA

Figure 3.3. Northern blot analysis of chitinase gene expression in transgenic and non-transgenic cacao plants. Total RNA (20 □g) isolated from non-transgenic *Sca*-6 (lane 1) and transgenic lines SAG31, SA39 and SA44 (lanes 2, 3 and 4). Blots were hybridized to a chitinase A (Chi A) probe, and then with a chitinase B (Chi B) probe, as indicated on the left side of the panel. Ribosomal 26S RNA probe was used to standardize gel loading.


Sca6
SAG31
SA39
SA44
CHI
Lane #
1
2
3
4
5
6
Chi A probe
1 kb
Chi B probe
1 kb
R 26S probe

from the transgenic line SAG31 (Fig. 3.3, lane 2). However, hybridization to SA 39 and SA 44 RNA at the expected 1 kb size was easily detected (Fig. 3.3, lanes 3 and 4 respectively). Hybridization was 3.2-fold higher to SA 39 RNA than to SA 44 RNA. The blot was stripped of probe and hybridized to Chi B probe. Unlike Chi A, the Chi B probe showed hybridization to all of the cacao leaf RNA samples (Fig. 3.3), indicating that the two class I chitinase genes are differentially expressed in cacao leaves. The probe was rehybridized to a 26 S ribosomal probe to assess loading uniformity (Fig. 3.3).

In vitro endochitinase activity assay

A *in vitro* endochitinase activity assay was used to assess functional expression of the transgene product. Since all transgenic lines were derived from the *Sca*- 6 cacao variety, *Sca*-6 sample leaves were used to measure the endogenous endochitinase activity levels. Transgenic line SA25 with the GFP transgene alone was also used as a control to assess the contributions of GFP to the fluorometric reading. Transgenic lines SA39 and SA44 exhibited increased chitinase activity compared to controls (Fig. 3.4). Transgenic line SAG31, which showed no detectable Chi A mRNA in leaf samples, showed chitinase activity comparable to line SA25, containing only the GFP gene. *In vitro*, endochitinase levels compared to the non-transgenic *Sca*-6 samples and boiled samples are summarized in Table 3.2.

Figure 3.4. *In vitro* endochitinase activity of transgenic and control leaf protein extracts. 50 μg total protein samples, including non-transgenic *Scavina* 6; transgenic SA25, containing only the GFP and NPTII transgene; and SAG 31, SA39 and SA44, transformed with pGAM00.0511 were analyzed to assess the functional expression of the transgenic chitinase protein. Endochitinase activity was measured by a fluorometric assay using 4-4 methylumbelliferyl □-D-N, N', N"-triacetylchitotrioside substrate, which becomes fluorescent upon cleavage by endochitinases. Boiled samples were obtained by boiling a 50 μg total protein extracts from all samples and averaging the fluorescence emission. All protein extractions were performed in triplicates at pH 5.0.

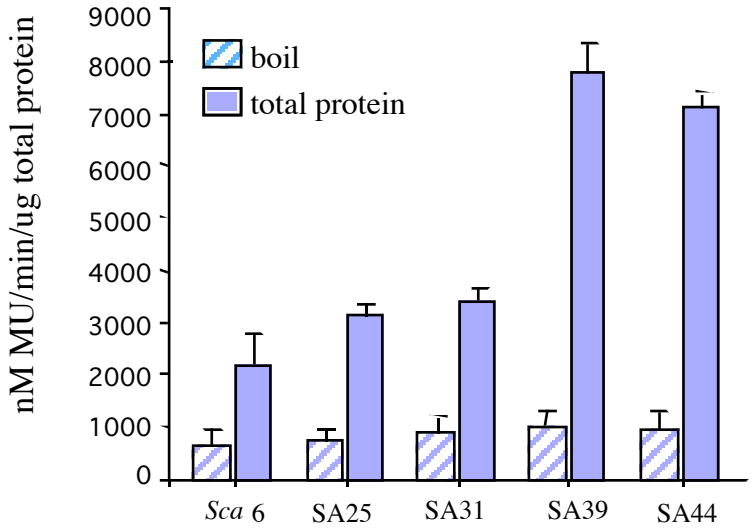

nM MU/min/ug total protein
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
boil
total protein
Sca 6
SA25
SA31
SA39
SA44

Table 3.2. Fold increase of endochitinase activity in leaves of transgenic plants. Endochitinase activity was measured by fluorometric assay using 4-4 methylumbelliferyl β-D-N, N', N"-triacetylchitotrioside substrate, which becomes fluorescent upon cleavage by endochitinases. Endochitinase activity was calculated from average readings of the total protein samples from transgenic lines compared to SA 25 transgenic line, containing only the GFP and NPTII transgene as well as from *Scavina* 6 non-transgenic controls. In addition, activity was also compared between the average activity of each sample over the average activity of the same sample after 5 minutes boiling. Values are followed by ± standard error of the means.

	Sca6	SA25	SA31	SA39	SA44
Sample/SA25 avg.	0.62 ± .12	1.00 ± .00	1.12 ± .16	2.50 ± .45	2.29 ± .56
Sample/*Sca* 6 avg.	1.00 ± .00	1.60 ± .20	1.77 ± .23	3.98 ± .32	3.67 ± .32
Sample/boil avg.	2.87 ± .18	4.59 ± .16	5.09 ± .21	11.40 ± .56	10.51 ± .28

In vivo infection with *Crinipellis perniciosa*

An *in vivo* bioassay was performed to assess whether or not the increased chitinase activity enhanced the capacity of the transformed plants to resist infection by fungal pathogen *Crinipellis perniciosa*. Clonaly propagated rooted cuttings from non-transgenic *Sca*-6, and transgenic SAG31, and SA39 were tested. Since *Sca*-6 is considered to be a cacao variety with high levels of resistance to disease, two additional cacao varieties were tested, cacao comun seedlings and ICS1 rooted cuttings. Furthermore, SA25, a line containing only the GFP and NPTII transgenes was also tested. For infection, a 25 □l spore suspension was placed on the tip of an actively growing apical meristem. The treated plants were placed in the dark for 2 days at 100% humidity and then transferred to a growth chamber.

The initial average height for all rooted cuttings, both treatment and control, was 11.67 $\pm$ 3.2 cm, with stem diameter of 2.54 $\pm$ 0.67 mm. One month after infection, the heights increased an average of 9.56 $\pm$ 2.3 cm and an average of 1.98 + 0.23 mm in stem diameter. In addition, an increase was observed in the total number of meristems found on the cacao *comun* seedlings; with 3 out of the 5 plants exhibiting additional meristems from the leaf axis directly below the point of infection. Furthermore, one of the ICS1 cuttings clearly showed signs of infection, having formed a terminal broom out of which we were able of isolate *Crinipellis perniciosa*. Further studies are needed to complete this analysis.

3.5. DISCUSSION

Chitin is the second most abundant polymer in nature (Cohen-Kupiec and Chet 1998). It is present in insects, crustaceans and in most fungal cell walls. Although chitin is not synthesized by plants they do synthesize chitinases, a chitin-degrading enzyme believed to play a role in defense against chitin-containing organisms, including fungi, insects and nematodes. Based on amino acid sequence similarities, five classes of chitinases have been described, all of which are found in plants (Sahai and Manocha, 1993, Hamel *et al.*, 1997, Melchera *et al.*, 1994). Of particular interest for my studies are the class I chitinases. Many genes of this family have been used to generate transgenic plants, (Broglie, 1991, Benhamou *et al.* 1993, Punja and Raharjo, 1996, Lorito *et al.*, 1998, Bolar *et al.*, 2000, Mora and Earl, 2001), some of which have showed promising levels of resistance to various fungal pathogens (Broglie, 1991, Lorito *et al.*, 1998) (see chapter I).

Only two genes belonging to the chitinase class I family have been identified and studied so far in *Theobroma cacao*. An acidic form of the class I chitinase (Chi A) was cloned and characterized by Snyder and Furtek (1995), and a basic class I chitinase (Chi B) was recently identified by Dr. J. Verica (personal communication). In leaves, endogenous Chi A RNA is only detectable reverse transcriptase PCR, as the fragment was amplified using a *Sca*-6 cDNA template. For my studies, I cloned a genomic copy of the acidic chitinase gene into a transformation vector under the control of a E12□ promoter and used it to generate transgenic cacao plants constitutively over-expressing

this gene. The genomic copy of the gene was used because previous authors (Abler and Green, 1996; Koziel *et al.*, 1996; and Lorito *et al.*, 1998) had reported that the level of transcript obtained with the genomic copy of the chitinase gene was higher than with the cDNA copy. In these studies, it was reported that the presence of introns not only improved gene expression levels in plants but also improved mRNA stability. Incorporation of the chitinase transgene into the plant genome of transgenic lines was verified by PCR. In addition, GFP expression in the detached leaves of the four transgenic lines, indicated the transcription and translation the GFP transgene.

An increase in the level of Chi A mRNA in two out of the three transgenic lines was detected by northern blot analysis. Two lines showed a significant increase in gene expression levels compared to the controls. Line SA39 showed a chitinase transcript level 3.2-fold higher than SA44, while the Chi A probe did not hybridize to SAG31 RNA. Differences in transcript levels between different transgenic lines containing the same construct have been reported many times (Lorito *et al.*, 1998, Mora and Earl 2001). Variation in expression levels of transgenes have been attributed to differences in the number of T-DNA copies incorporated into the genomes, and/or positional effects upon insertion at different sites within the genome.

Consistent with the northern blot results, the *in vitro* endochitinase assay showed increased chitinase activities in transgenic lines SA39 and SA44 compared to controls but not in SAG31. The SA39 and SA44 lines showed 2.50-and 2.29-fold higher chitinase activity respectively compared to control transgenic line SA25.

Lastly, an *in vivo* bioassay using cacao fungal pathogen *Crinipellis perniciosa* was used to infect both SAG31 and SA39 transgenic lines and the appropriate controls. One month after infection, a doubling of stem length and width was observed for all treated plants. In addition, cacao plants from *ICS1* and cacao *comun* varieties are beginning to show abnormal growth, with at least one ICS1 cutting showing clear signs of *Crinipellis perniciosa* infection. However, I did not see any difference between the transformed and non-transgenic *Scavina* 6 plants. Because *Scavina* 6 derived plants are believed to have resistance against *Crinipellis perniciosa* it is likely that a higher spore inoculum will be needed to infect *Scavina 6* plants and to determine the value of the transgene insertion for resistance to witches' broom.

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CHAPTER 4

GENE INDUCTION IN *THEOBROMA CACAO* IN RESPONSE TO BENZOTHIADIAZOLE, NEP 1 AND *XANTHOMONAS CAMPESTRIS MALVECEARUM*

4.1. ABSTRACT

An understanding of plant defenses is rapidly growing, yet little is known about the molecular or physiological responses of *Theobroma cacao* during pathogen infection. The objectives of the studies presented in this chapter were to better understand some aspects of cacao defense responses at a molecular level and to provide insight into the defense pathways used by cacao. Here, I report the use of three elicitors of defense responses on *Theobroma cacao* and the molecular responses at the RNA level of several selected marker genes. The treatments used included: 1) *Xanthomonas campestris malvecearum* (*Xcm*), a bacterial plant pathogen, which is non-pathogenic to *Theobroma cacao*, 2) benzothiadiazole (BTH), a salicylic acid analogue and 3) Nep 1 protein of *Fusarium oxysporum*. A set of five cacao homologues of genes that contribute to plant defenses in other plant species were selected as marker genes to detect the activation of defense pathways, two chitinases, a □-glucanase gene, a phosphatase and a trypsin inhibitor gene.

The results show that cacao is able to respond to *Xanthomonas*, BTH and Nep 1 protein, demonstrating cacao's capacity to activate a defense response in treated leaves. Treated leaves showed a significant increase in expression levels of two different members of the PR gene family, the cacao □-glucanase and the basic form of the class I chitinase gene (*Chi B*). Furthermore, response patterns for *Xcm*, BTH and Nep1 treatments differed, indicating distinct responses to each of the treatments.

4.2. INTRODUCTION

Theobroma cacao is a small tropical tree belonging to the *Malvaceae* family (Whitlock *et al.*, 2001). Cacao is grown in more than fifty countries usually within twenty degrees latitude of the equator. Plantations world-wide face many pathological challenges including fungal, viral and insect infestation, that result in crop losses and occasionally, in field devastation and abandonment (Chapter 1). Yet, there is little understanding of the unique host-pathogen interactions, the defense pathways or of the mechanisms of resistance within the cacao tree.

It is believed that most plants are resistant to most potential plant pathogens. This resistance arises from complex defense mechanisms that plants have evolved to withstand penetration, invasion and/or replication of their pathogens (Dixon, 2001). In addition, plants have evolved the ability to perceive certain pathogens and defend themselves against their attack. Recognition can be immediate and triggered by a race-host specific recognition, as in the 'gene for gene' type interaction where a protein encoded by the resistance (*R*) gene of the host recognizes a protein elicitor encoded by the avirulence (*avr*) gene of the pathogen (Flor, 1971; Staskawicz *et al.*, 1995). For recognition, both elicitor and receptor must be present. In the absence of either elicitor or receptor, the pathogen invades the tissue unrecognized by the host, resulting in disease.

Plants also possess inducible defense mechanisms to protect themselves against pathogen attack (Stuiver and Custer, 2001). One of these mechanisms is the

hypersensitive response (HR) (Morel and Dangl, 1997). HR is defined as the death of host cells within a few hours of pathogen contact (Agrios, 1988). It occurs at the site of pathogen entry and involves the activation of programmed cell death to arrest pathogen growth. HR is accompanied by the induction of other plant defense response pathways that serve to confine the pathogen and protect the plant (Lam *et al.*, 2001).

Another defense response pathway is the salicylic acid (SA)– dependent systemic acquired resistance (SAR) pathway. SAR is activated upon infection by pathogens (Ryals *et al.*, 1996) including *Xanthomonas campestris* bacteria. SAR refers to a distinct signal response that plays an important role in the ability of plants to defend themselves against pathogens (Ryals *et al.*, 1996). It can be distinguished from other disease resistance responses by both the wide range of pathogen protection as well as by the associated changes in gene expression (Ward *et al.*, 1991, Uknes *et al.*, 1993, Ryals *et al.*, 1996). Associated with SAR is the increased accumulation of the plant-signaling molecule SA and the expression of a set of genes called SAR genes (van Loon, 1985; Ward, 1991). Analysis of SAR proteins has shown that most proteins belong to the class of pathogenesis-related (PR) proteins (van Loon and van Kammen, 1970; van Loon, 1985) including PR-1 as well as antifungal proteins, PR-2 group, □-glucanases and PR-3 group, plant chitinases.

A third widely studied defense response is that induced by jasmonic acid (JA) and ethylene. Although it is known that JA and ethylene accumulate naturally in plants, it has been shown that accumulation can also be associated with a response to a variety of stress

stimuli, including plant pathogens. JA-dependent resistance can be induced in several plant species by necrotrophic fungi, including *Fusarium oxysporum* (Pieterse *et al.*, 1996; van Wess *et al.*, 1997). Gene products associated with JA-defense response include various key enzymes of the phenylpropanoid phytoalexin biosynthetic pathway. These include phenylalanine ammonia lyase and chalcone synthase, the antifungal proteins thionin, osmotin and plant defensins, as well as proteinase inhibitors among others (Shewry and Lucas, 1997).

Various substances, including the signaling molecules SA, JA and ethylene can activate defense responses (Boller, 1991; Ryal *et al.*, 1996). Because external application of SA has been shown to be toxic to most cultivated plants it has limited commercial use (Resende *et al.*, 2002). Therefore, research in plant defense responses have also focused on identifying and developing elicitor molecules that can specifically induce defense responses without the toxic effect on crops. One such molecule is benzothiadiazole (BTH), an SA analogue, which behaves as a potent activator of SAR (Görlach *et al.*, 1996). By treating plants with a molecule capable of activating SAR, such as BTH, plants can induce an array of defense-related proteins prior to encounter with a pathogen, thus facilitating protection of the crop under field conditions. Protection by BTH has been observed in many crops (Görlach *et al.*, 1996), including *Theobroma cacao* (Resende *et al.*, 2002). Recently Resende *et al.* (2002) reported the use of BTH on cacao seedlings of *Catongo* variety, susceptible to *Crinipellis perniciosa*, the pathogenic agent of witches broom disease of cacao. In addition BTH was also used to treat cacao seedlings of *Theobahia* variety, susceptible to *Verticillium dahliae*, which causes vascular

wilt (Chapter 1). In these studies, the use of BTH prior to pathogen inoculation resulted in the reduction of witches broom incidence by up to 85%, and in the reduction of the severity of vascular wilt by 54%.

The objectives of these studies were to use the knowledge and techniques developed in other plant systems to identify defense-related genes in cacao and to use a few key marker genes to initiate research into the molecular events occurring during defense responses. The five genes selected to detect the activation of the defense signaling pathways are known to play a role in plant defenses in other plant species. Three of these genes encode pathogenesis-related (PR) proteins, including two secreted isoforms of the class I chitinase gene, one acidic and one basic, as well as a □-glucanase gene. Since □-glucans and chitin polymers are present in the cell walls of many pathogenic fungi, it is not surprising that both groups of endohydrolases have been shown to have antifungal properties (Shewry and Lucas, 1997), and have been associated with the induction of SAR (van Loon, 1985). The other two genes used were a cacao phosphatase and a trypsin inhibitor gene. The cacao phosphatase and trypsin inhibitor genes were identified by Dr. J. Verica at The Pennsylvania State University, as being up-regulated in cacao leaves 30 min after treatment with BTH. In addition, the trypsin inhibitor gene is highly expressed in cacao seeds and thought to regulate and inhibit endogenous and exogenous proteases, functioning as a protective compound against herbivory (Dodo *et al.*, 1992). Moreover, because the trypsin inhibitor has a high cystein content, it could also function as a source of sulfur (Spence and Hodge, 1991). Sulfur is required to produce a broad range of antimicrobial compounds (Dixon, 2001), including

elemental sulfur. Elemental sulfur was first identified in *Theobroma cacao* as the only known inorganic antimicrobial agent produced by plants, accumulating in cacao leaves when infected with vascular wilt fungi (Cooper, 1996).

In this study, I demonstrated induction of defense genes in cacao by three different elicitors. The first treatment included treatment with a plant pathogenic bacteria, *Xanthomonas campestris malvecearum* (*Xcm*). *Xcm*, known to induce SAR (Jetiyanon, 1994). It is the causal agent of angular leaf spot disease in some members of the *Malvaceae* family, such as cotton, although it is not pathogenic to *T. cacao*. The lack of pathogenicity to cacao may be attributable to the inability of the pathogen to penetrate the host, or to the capacity of the cacao plant to recognize the pathogen and prevent its spread. To eliminate the first possibility, Silwet L-77 was incorporated in the treatment, to provide a means for the bacteria to enter cacao leaves through the leaf stomata and hydathodes.

The second treatment was benzothiadiazole (BTH), an inducer of plant defense responses (see above). This compound is of particular importance for my research because of its current use and experimentation in Brazilian cacao plantations (Resende *et al.*, 2002). The third elicitor was Nep 1, a 24 kD protein first isolated from *Fusarium oxysporum* f. sp. erythroxyli, a plant fungal pathogen (Bailey *et al.*, 2002). This protein has been shown to induce necrosis and stimulate ethylene production in dicots (Jennings *et al.*, 2000), as well as having the capacity to induce HR in *Centaurea maculosa* (Bailey *et al.*, 2000).

4.3. MATERIALS AND METHODS

Plant material

Theobroma cacao pods, ecotype *comun*, were obtained from Alan Pomella at the Almirante cacao farm in Bahia, Brazil. Flowers were open-pollinated and mature pods were transferred to the USDA facility in Beltsville, Maryland. Once at the USDA, the pods were opened, seeds emptied, washed to remove mucilage, and planted. Seeds were germinated in potting soil, and grown under greenhouse conditions with minimum temperatures of 24º C.

Xanthomonas cultures and growth curve studies

Xanthomonas campestris pv. *malvacearum* pathogen strain 89-15 (*Xcm*) was isolated by Dr. C. J. Chang at University of Georgia and kindly provided to Dr. P. Backman at The Pennsylvania State University. Cultures were streaked on potato dextrose agar (PDA) solid media three days prior to infection. The bacterium were then scraped and resuspended in 0.1M $KHP0_4$ buffer (pH 7.0) solution. Liquid cultures were adjusted to an OD of 0.1. Before spraying, Silwet was added to the liquid culture to a final concentration of 0.2% (v/v). Control plants were sprayed with buffer at the same concentrations as treatment.

Four-month-old greenhouse-grown *Theobroma cacao* plants, producing at least three to four true leaves, were sprayed using a fine mist hand-held aerosol sprayer (Crown power pack, Fisher Scientific, Pittsburgh, PA). The plants were sprayed at a distance of 30 cm from the leaves until leaves were wetted. Plants were treated before eight a.m. with: 1) 0.2% Silwet containing $1x10^7$ cfu ml^{-1} of *Xcm* in 0.1 M buffer; or 2) 0.2% Silwet in 0.1 M buffer.

Measurement of the *Xanthomonas* population in cacao leaves

To assess the number of *Xcm* cells viable within *Theobroma cacao* leaves after treatment one-gram leaf samples were weighed and processed with a stomacher blender (Tekmar, Cincinnati, OH) for approximately 2-3 min. A 1:10 leaf to buffer ratio (w/v) was used to break the leaf tissue and release the bacteria into the solution. Samples were serially diluted with buffer (1:10, 1:100, 1:1000 v/v) and plated onto PDA solid agar plates with a spiral plater (Spiral Systems, Inc. Bethesda, MD). Plates were incubated at 25° C for 2 days. Quantification of viable bacteria was determined by counting *Xcm* colonies. Leaf samples were collected in triplicates at 1 hr, 1, 3, 7, 10, 14, 21, and 28 days after spraying. Two additional leaves were excised from the same set of plants freeze dried in liquid nitrogen, and stored at –80 °C for RNA extraction and northern blot analysis.

BTH and Nep1 treatment

Benzothiadiazole elicitor (Syngenta, North Carolina) was diluted in water at a concentration of 1000 mg L^{-1}. Nep 1 protein was used at 5000 mg L^{-1}, in water containing 0.2% of a non-ionic organosilicone surfactant, Silwet L-77. This surfactant allows for the delivery of the elicitor directly into natural openings of the plants. For each individual treatment and the control spray without elicitor, 20 individual plants were sprayed. Samples were collected at the following time points: 0, 1 h, 4 h, 24 h, and 9 days (216 h). Two mature leaves, light green and pliable, were collected per plant from each treatment and set of controls. Each leaf pair belonged to individually treated plants used for only one collection time, and each collection was replicated three times.

Analysis of gene induction

Total RNA was extracted from treated and control as described by Chang *et al.* (1993) with some modifications. Frozen leaf samples were ground with a mortar and pestle in liquid nitrogen to a fine powder. Approximately, 1 gm of tissue was resuspended in 15 ml extraction buffer containing 2% CTAB (hexadecyltrimethyl-ammonium bromide), 2% PVP (polyvinylpyrrolidone), 100 mM Tris base, 25 mM diNaEDTA, 2 M NaCl, 0.5 gm/L spermidine and 2% □-mercaptoethanol, then incubated at 65 °C for 10-30 minutes. A total of 15 ml chloroform was added, samples were mixed and then centrifuged for 20 min at 8000 x *g*. The top layer of each sample was then transferred to fresh tubes containing 5 ml 8 M LiCl (DEPC treated), and stored overnight at 4°C. RNA

was centrifuged for 30 min at 8000 x *g*. RNA pellets were re-suspended in 500 μl RLC Qiagen RNeasy buffer (QIAGEN Valencia, CA) and purified using Qiagen RNeasy mini based protocol. RNA samples were recovered as follows: 250 μl ethanol (100%) was added to sample and the mixture transferred to RNeasy mini columns. Samples were centrifuged for 15 sec. to one minute at 8000 x *g*. Samples in the mini column were washed with 700 μl RW1 buffer, followed by another rinsing step using RPE buffer; after each step samples were centrifuged (15 sec at 8000 x *g*). RNA samples were eluted with two 70 μl RNase-free water aliquots.

Northern blotting

Total RNA (20 μg) was denatured for 30 min at 55° C by incubating RNA samples in a 1:1 (v/v) with Glyoxal/DMSO loading dye (Amersham Bioscience, Piscataway, NJ). Samples were run on 1% agarose gels, capillary blotted with 20X SSC buffer (NaCl, $C_6H_5Na_3O_7$) onto positively charged nylon membranes (Hybond, Amersham, Arlington Heights, IL). RNA was UV cross linked to the membrane with 1200 J m^{-2} (UV Crosslinker 1800, Stratagene, La Joya, CA). Variation in total RNA loading was evaluated by staining the membrane with 0.02% methylene blue. Digital images of all membranes were recorded using a Sony digital camera.

Hybridization probes

Short cDNA fragments or expressed sequence tags (ESTs) were isolated by the following methods. A glucanase (Gluc) EST was cloned by reverse transcriptase polymerase chain reaction (RT-PCR). For this purpose, a sequence alignment was generated using *Arabidopsis thailiana* □-*glucanase* [Genebank accession #AF372940, bp770-1028] and *Gossypium hirstum* □-*glucanase* [accession # D88417, bp129-387] sequences using Vector NTI suite 7.0 software package (Invitrogen Inc., Carlsbad CA) to determine a consensus sequences. This consensus was used to design degenerate oligonucleotide primers for polymerase chain reaction (PCR), using Primer3 software (MIT Whitehead Institute); (http://www.basic.nwu.edu/biotools/primer3.html). Primers were chosen from highly conserved sequences to amplify a DNA fragment of approximately 300 base pairs in length. Glucanase-F and Glucanase-R primers used were 5'-CAG AGG MAA ATA CGA CAG CA- 3' (M= A or C) and 5'-AKW CCA GCT TTG CCT TGC AT-3' (K= G or T; W= A or T) respectively. cDNA was amplified through reverse transcriptase polymerase chain reaction, (RT-PCR), from untreated *Scavina-6* leaf cDNA. PCR conditions for all reactions were: 94ºC for 4 min, then 30 cycles of 94ºC for 1min., 50ºC for 1min., 72ºC for 1 min, a final extension cycle at 72ºC for 5 min followed by incubation at 4ºC. PCR products were separated by gel electrophoresis and fragments of the expected size (258 bp) were excised and gel purified, cloned into plasmid, pCR®4-TOPO® (Stratagene), and transformed into *E. coli* by heat shock. Ten colonies from each primer set were sequenced and compared to the available databases. chitinase (*Chi* B), phosphatase (*Phos*) and trypsin inhibitor (*Tryp*)

ESTs were cloned by suppression subtractive hybridization (SSH) library, and kindly provided by Dr. J. Verica, The Pennsylvania State University. cDNA was generated from poly-A mRNA isolated from cacao leaves after treatment with BTH (1000 mg L^{-1}) or with control spray (water). The fifth probe was a second chitinase gene, a cacao class I chitinase gene, (*ChiA*) [Genebank accession # AAA80656] (Snyder, 1995). Templates were gel purified following PCR amplification. DNA probes (20 ng) were labeled with dCTP [P^{32}] using Megaprime DNA labeling system (Amersham, Bioscience, catalogue # RPN 1604 Piscataway, NJ). Table 4.1 shows the cDNA templates used to make probes for northern blots.

Blotted RNA membranes were prehybridized for 2 hours and hybridized overnight at 65 ° C in ExpressHyb buffer (Clonetech, Palo Alto, CA). Hybridized blots were washed with 2X SSC, 0.1% (v/v) SDS (2 x 5 min) and 0.2x SSC, 0.1 % (v/v) SDS (15 min). Blots were covered in plastic wrap and exposed to a phosphor screen (Molecular Dynamics, Amersham Bioscience). The sizes of hybridizing bands were determined with respect to an RNA ladder (Ambion Inc., Austin, TX). Hybridizing band intensity was quantified using ImageQuant software (Molecular Dynamics, Amersham Bioscience). After six cycles of probing and stripping in boiling 0.5% (v/v) SDS, RNA loading was evaluated by hybridization to a soybean 26S ribosomal gene.

Table 4.1. *Theobroma cacao* cDNA sequences used as hybridization probes to examine defense response induction by *Xcm*, *Xanthomonas campestris malvecearum*, BTH, benzothiadiazole and Nep 1 protein. Cacao chitinase A (ChiA) and cacao trypsin inhibitor (Tryp) complete sequences are in genebank. The chitinase B (ChiB), glucanase (Gluc) and phosphatase (Phos) cacao genes, were identified at The Pennsylvania State University and the table indicates the most significant homologue in the database and the e value s determined by BLAST analysis. cDNA fragments used for probes varied in length from Chi A, 453bp; Chi B, 360 bp; Gluc, 258 bp; Phos, 300 bp; and Tryp, 220 bp.

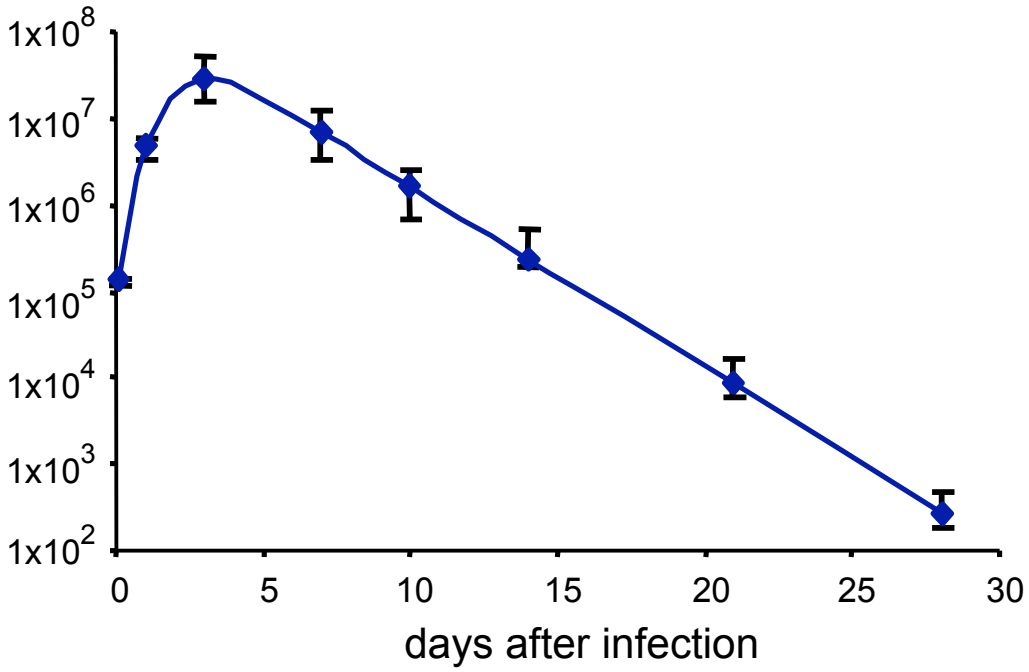
cDNA clone	BLAST NT Sequence homology	[e]
Chi A	Acidic endochitinase, *Theobroma cacao*, TCU30324	0.00
Chi B	Basic endochitinase, *Arabidopsis*, AAF29391	2e-64
Gluc	endo glucanase, *Arabidopsis*, AF372940	7e-34
Phos	Phosphatase subunit, *Arabidopsis* AAL24096	7e-61
Tryp	Trypsin Inhibitor, *Theobroma cacao*, X56509	0.00

4.4. RESULTS

Xanthomonas campestris malvecearum population dynamics in infiltrated leaves

To study *T. cacao* defense responses, *Xanthomonas campestris malvecearum* bacteria, and cacao *comun* seedlings were used as a cacao defense model system. For this, cacao seedlings were sprayed with a buffer solution containing 10^7 cfu ml^{-1} *Xcm* bacteria. Bacterial populations were monitored for a month after treatment (Fig. 4.1). After treatment, a logarithmic increase in leaf associated bacteria was observed from 10^5 cfu ml^{-1} 1 hour after treatment, to a peak of 10^7 cfu ml^{-1} 3 days after treatment, indicating the capacity of the bacteria to reproduce in the intercellular spaces of cacao leaves. Twenty-eight days after treatment, however; *Xcm* bacteria was nearly eliminated from the leaves. There were no visible signs of disease on the leaf surfaces, indicating that the bacteria was unable to cause disease even after penetration in *Theobroma cacao* leaf intercellular spaces.

Figure 4.1. Mean number of *Xanthomonas campestris* pv. *malvecearum* colonies in *Theobroma cacao* leaves, genotype *comun*. Cacao seedlings were sprayed to run off with *Xanthomonas* bacteria at 1 x 10^{7} cfu ml^{-1} concentration. Following treatment, leaf samples were collected at 1hr, 1, 3, 7, 10, 14, 21 and 28 days. At each time point, leaves from three replicate treated plants were analyzed to assess the number of *Xcm* bacteria (cfu per gram of leaf tissue).


1x10^8
1x10^7
1x10^6
1x10^5
1x10^4
1x10^3
1x10^2
0
5
10
15
20
25
30
days after infection

Xanthomonas campestris malvecearum-induced responses in *Theobroma cacao* seedlings

In order to determine whether defense responses had been activated in the treated seedlings, northern blot analysis was performed to measure the expression levels of five-selected cacao genes (Table 4.1). RNA samples were extracted from leaves at the same times as for the bacterial population study and both studies contained triplicate samples. To examine the possibility of a systemic defense response, non-treated leaves from above the infection site were also collected 7 days after treatment and RNA was extracted. Figure 4.2 shows a representative sample of the hybridization results indicating the magnitude of gene induction in infested leaves at different times after exposure to bacteria.

To measure induction of the selected marker genes for each of the treatments, fold induction was calculated by dividing all treatment phosphorimager integrated pixel values, by the value observed for the control samples at each time point (Table 4.2).

Figure 4.2. Accumulation of defense response gene transcripts in controls and *Theobroma cacao* leaves treated with *Xanthomonas campestris* pv. *malvecearum*. Five month old cacao *comun* seedlings, were sprayed with 0.1 M phosphate buffer containing 0.2% Silwet (control). A separate set of plants was sprayed with the same solution containing *Xanthomonas* bacteria at a concentration of 10^{7} cfu ml^{-1}. Two leaves per plant were collected from each *Xcm* treated and buffer control for RNA extraction. Leaves of three individually treated plants were collected per time point at 1hr, 1, 3, 7, 10, 14, 21 and 28 days. 20 μg total RNA isolated from each treated and control sample were analyzed. In addition, 7 days after treatment with bacteria, leaves from apical non-sprayed (systemic) leaves were collected for each of the remaining collection points and labeled systemic. Gene abbreviations: *Chi A*, acidic endochitinase (genebank accession # TCU30324); *Chi B*, basic endochitinase; *Gluc*, □ 1,4-*glucanase*; Phos, *phosphatase*; Tryp, *trypsin inhibitor* protein (genebank accession # X56509); 26S, ribosomal 26S RNA (RNA loading control).

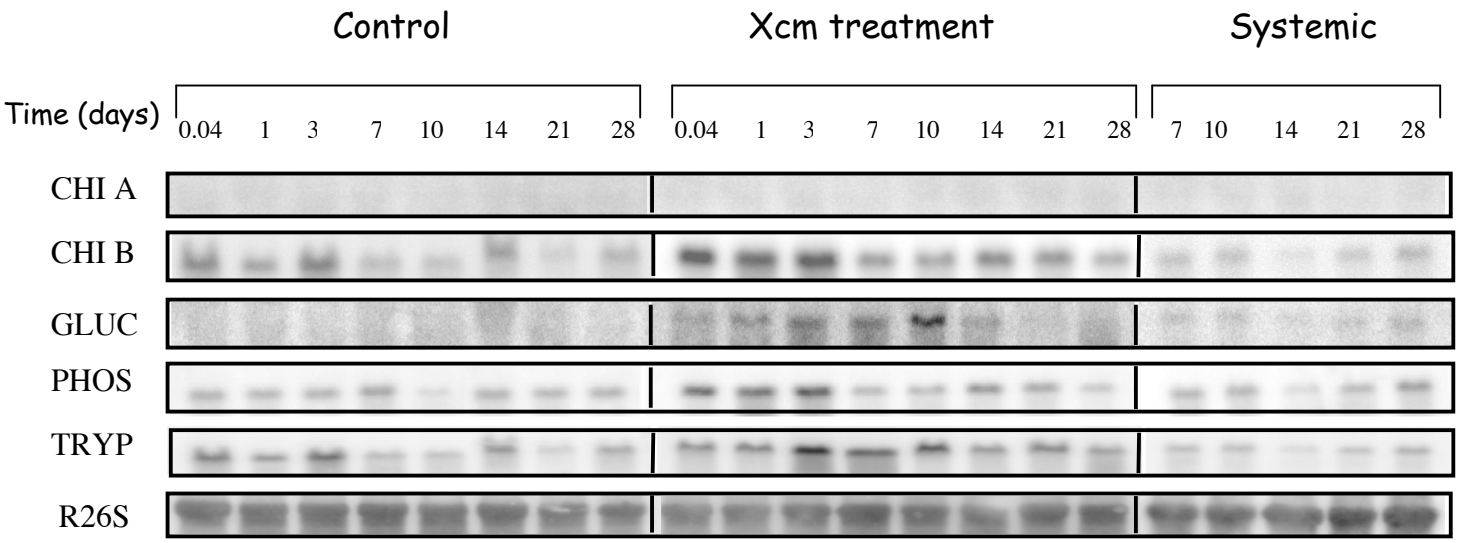

Control
Xcm treatment
Systemic
Time (days)
0.04 1 3 7 10 14 21 28
0.04 1 3 7 10 14 21 28
7 10 14 21 28
CHI A
CHI B
GLUC
PHOS
TRYP
R26S

Table 4.2. Effects of elicitor treatments on gene expression compared to untreated control at same collection time. Treatments were as follows A. *Xanthomonas campestris malvecearum*; B. Benzothiadiazole; C. Nep1 protein. All samples at each time point were analyzed in triplicate. Induction of marker genes was measured by northern blot analysis and quantified with ImageQuant software package. Phosphorimager counts were normalized among replicate membranes. Treatment and Control columns indicate the average mean counts for each signal ± standard error. Fold induction was calculated by dividing the treatment values by their control value. Values followed with an asterix (*) indicate statistical significant differences.

A. *Xanthomonas campestris malvecearum*

Glucanase

Time day	Treatment	Control	Fold Induction
0.04	2566.64 ± 9.41	2398.34 ± 9.41	1.1
1	5685.22 ±10.14	2659.71 ±10.14	2.1*
3	7312.65 ± 9.85	2606.37 ± 9.85	2.8*
7	6839.23 ±12.94	2818.80 ±12.94	2.4*
10	8313.49 ±14.53	3490.26 ±14.53	2.4*
14	3770.55 ± 8.80	2553.94 ± 8.80	1.5
21	3120.16 ± 5.57	2471.72 ± 5.57	1.3*
28	2671.34 ± 4.26	2669.01 ± 4.26	1.0

Chitinase B

Time day	Treatment	Control	Fold Induction
0.04	592014 ± 91.49	193793 ± 91.49	3.1*
1	413035 ±136.25	384419 ±136.25	1.1
3	824244 ±141.30	496422 ±141.30	1.7
7	310810 ±129.44	281870 ±129.44	1.1
10	466925 ± 92.72	158285 ± 92.72	2.9*
14	271277 ± 90.01	226691 ± 90.01	1.2
21	279252 ±128.09	174321 ±128.09	1.6
28	211410 ±128.35	179552 ±128.35	1.2

Phosphatase

Time day	Treatment	Control	Fold Induction
0.04	543719 ± 410.47	403612 ± 410.47	1.3
1	416280 ± 472.60	368516 ± 472.60	1.1
3	881496 ± 476. 77	508541 ± 476. 77	1.7
7	883469 ± 452.56	384117 ± 452.56	2.3
10	501103 ± 224.41	113586 ± 224.41	4.4*
14	265803 ± 238.02	258515 ± 238.02	1.0
21	213317 ± 402.98	172583 ± 402.98	1.2
28	216447 ± 371.90	330329 ± 371.90	0.7

Trypsin Inhibitor

Time day	Treatment	Control	Fold Induction
0.04	33198.1 ± 40.24	33350.9 ± 40.24	1.0
1	27426.5 ± 29.45	25828.9 ± 29.45	1.1
3	42003.4 ± 28.80	31841.9 ± 28.80	1.3
7	28117.6 ± 27.98	30696.4 ± 27.98	0.9
10	19469.5 ±15.99	12976.5 ±15.99	1.5
14	22621.9 ±25.50	13314.8 ±25.50	1.7
21	22540.1 ±35.39	18856.6 ±35.39	1.2
28	11904.1 ±24.05	22314.3 ±24.05	0.5

B. Benzothidiazole

Glucanase

Hours	Treatment	Control	Fold Induction
0	13980.23 ±17.35	13980.23 ±17.35	1.0
1	11879.29 ±10.27	9744.34 ±10.27	1.2
4	11656.79 ± 3.82	9844.21 ± 3.82	1.2
24	10518.35 ± 6.73	9739.19 ± 6.73	1.1
216	8973.91 ± 4.84	9358.53 ± 4.84	1.0

Chitinase B

Hours	Treatment	Control	Fold Induction
0	407985 ± 17.35	407985 ± 17.35	1.0
1	1621093 ± 10.27	386607 ± 10.27	4.2*
4	1491792 ± 3.82	341492 ± 3.82	4.4*
24	1297747 ± 6.73	404926 ± 6.73	3.2*
216	655640 ± 4.84	369783 ± 4.84	1.8*

Phosphatase

Hours	Treatment	Control	Fold Induction
0	876314 ± 444.24	876314 ± 444.24	1.0
1	858775 ± 324.66	715831 ± 324.66	1.2
4	818227 ± 266.15	809422 ± 266.15	1.0
24	884935 ± 408.08	574355 ± 408.08	1.5
216	770907 ± 211.74	837177 ± 211.74	0.9

Trypsin Inhibitor

Hours	Treatment	Control	Fold Induction
0	37256.4 ± 19.82	37256.4 ± 19.82	1.0
1	59567.6 ±17.76	42066.1 ±17.76	1.4*
4	49927.1 ± 23.13	34256.6 ± 23.13	1.5*
24	44439.6 ±11.09	60574.4 ±11.09	0.7*
216	25545.9 ±19.82	31390.8 ±19.82	0.8

C. Nep 1 protein

Glucanase

Hours	Treatment	Control	Fold Induction
0	1547.57 ± 3.95	1547.57 ± 3.95	1.0
1	1518.38 ± 5.27	1324.38 ± 5.27	1.2
4	1438.81 ± 3.89	1634.81 ± 3.89	0.9
24	1413.89 ± 5.42	1209.00 ± 5.42	1.2
216	1617.38 ± 6.22	1668.97 ± 6.22	1.0

Chitinase B

Hours	Treatment	Control	Fold Induction
0	13945.8 ± 26.08	13945.8 ± 26.08	1.0
1	7589.78 ± 28.23	13223.6 ± 28.23	0.6
4	25543.8 ± 38.97	20205.3 ± 38.97	1.3
24	44077.1 ± 20.36	21135.3 ± 20.36	2.1*
216	10211.1 ± 22.00	8564.39 ± 22.00	1.2

Phosphatase

Hours	Treatment	Control	Fold Induction
0	643218.5 ± 13.58	643218.5 ± 13.58	1.0
1	631960.3 ± 20.93	500421.6 ± 20.93	1.3
4	1359286 ± 18.12	1151157 ± 18.12	1.2
24	1243010 ± 10.35	1178554 ± 10.35	1.1
216	352879.3 ± 11.76	352879.3 ± 11.76	1.0

Trypsin Inhibitor

Hours	Treatment	Control	Fold Induction
0	4313.70 ± 7.53	4313.70 ± 7.53	1.0
1	5195.64 ± 8.35	3208.11 ± 8.35	1.6
4	2606.05 ± 12.98	6583.45 ± 12.98	0.4
24	7176.22 ± 17.89	5725.53 ± 17.89	1.3
216	2381.96 ± 16.26	4152.71 ± 16.26	0.6

Table 4.3. Statistical analysis of *Xcm*, BTH and Nep 1 treated plants and their control samples. Two independent statistical analyses were performed with all three treatments data sets. This table shows the results for the marker genes that showed a statistically significant difference between the treatment and controls. First, a balanced one way ANOVA test was conducted to report significant difference between the treatment and the control samples regardless of the various time collections. If a significant difference was reported by the one way ANOVA, a two sample t-test was performed to compare the treatment and the control sample at each individual time collection. Both tests were performed at the 95% confidence level. p value followed with an asterix (*) indicate significant difference. Time collections are in days for the *Xanthomonas* treatment and in hours for benzothiadiazole and Nep 1 protein. A. *Xanthomonas campestris malvecearum*, (DF=4). B. benzothiadiazole (DF=4) and C. Nep1 protein (DF=4).

A. *Xanthomonas campestris malvecearum*

	Glucanase	Chitinase B	Phosphatase
ANOVA	p<0.001	p<0.001	p=0.001
Day	**t-test**	**t-test**	**t-test**
0.04 day	p=0.989	p=0.003*	p=0.321
1	p=0.016*	p=0.844	p=0.786
3	p=0.003*	p=0.089	p=0.089
7	p=0.031*	p=0.826	p=0.167
10	p=0.040*	p=0.008*	p<0.001*
14	p=0.090	p=0.496	p=0.866
21	p=0.047*	p=0.433	p=0.713
28	p=0.987	p=0.805	p=0.925

B. Benzothiadiazole

	Glucanase	Chitinase B	Trypsin Inhibitor
ANOVA	p=0.011;	p<0.001;	p=0.002
Hours	**t-test**	**t-test**	**t-test**
0.0	p=0.327	p=0.327	p=0.327
1	p=0.443	p<0.001*	p=0.002*
4	p<0.001*	p<0.001*	p=0.016*
24	p=0.079	p=0.001*	p=0.002*
216	p=0.089	p=0.002*	p=0.113

C. Nep 1 protein

	Chitinase B
ANOVA	p=0.012
Hours	**t-test**
0.0	p=0.560
1	p=0.390
4	p=0.341
24	p=0.002*
216	p=0.648

-Glucanase mRNA levels in *Xanthomonas*-treated leaves increased after infection. The highest transcript level was 3.5-fold greater than the untreated control 10 days after treatment (Fig 4.2 and Fig 4.4a). Figure 4 presents both a representative sample of the changes in transcript level observed in the northern analysis (Fig 4.2), as well as the average pixel intensity value calculated from the three replicate samples for the 10 day time samples (Fig 4.4a). The cacao glucanase gene showed an increase in transcript levels after 1 day of treatment, and the transcript level continued to increased for the first 10 days following treatment. This change was shown to be significant after a balance analysis of variance (ANOVA) test ($p<0.001$) (Table 4.3). Additionally a two sample t-test showed significant difference between the treatment and control at the 95% confidence level. The same set of analyses was performed for all other probes (Table 4.3). The chitinase B gene (Chi B), another member of the class I chitinase family, was induced in response to *Xcm* (Table 4.2). Analysis of the phosphatase gene indicated activation of the gene 10 days after treatment, the same time point as the peak of glucanase gene transcription. No significant changes in transcript levels were observed for the Chi A and trypsin inhibitor genes. Systemic induction of the selected cacao *PR* genes by *Xanthomonas campestris* was not detected (Fig 4.2).

Gene Induction Following BTH Treatment

The second elicitor analyzed was the salicylic acid analogue BTH. In order to test whether BTH is capable of inducing an SA-dependent response in cacao, cacao seedlings were treated with BTH and samples were collected at 1, 4 and 24 hours, and 9 days (216

h) following treatment. Figure 4.3a shows hybridization results for RNA blots probed with the selected cacao ESTs (Table 4.1). Figure 4.4b shows the average Chi B gene induction in BTH treated leaves for three replicates from treated seedlings. Chi B transcript level was 3.7-fold higher in treated plants compared with control plants after 1 hr (Fig 4.4b and Table 4.2).

The Chi B probe gave the strongest signal among all the tested probes for the BTH treatment compared to the control samples. Statistically significant differences were observed between the treated and the control samples. This difference lasted throughout the four collection time points ($p=0.001$). In addition, statistically significant differences in the expression levels of glucanase transcript ($p=0.011$) and the trypsin inhibitor ($p=0.002$) gene were also observed. No difference was found between treated and control samples for the phosphatase gene (Table 4.2 and 4.3).

Figure 4.3. Accumulation of defense response gene transcripts in *Theobroma cacao* leaves sprayed with BTH, a chemical elicitor of SAR and Nep 1, a protein exudate of *Phytophthora* sp. and *Fusarium oxysporum*. For both treatments, 3 month-old cacao *comun* seedlings were sprayed with (A) BTH (1000 mg L^{-1}) or with water (control); or (B) Nep1 (5000 mg L^{-1}) or with 0.1M Phosphate buffer and 0.2% Silwet (control). Leaves of three treated plants were collected for each time point for both treated and control plants at 0, 1, 4, 24 hours and 9 days (216 hrs) after spraying. Two leaves were collected for total RNA extraction from each plant. Blots of total RNA (20 μg) isolated from treated leaves collected from sprayed control plants and treated plants. Gene abbreviations: Chi B, basic endochitinase; Chi A, acidic endochitinase; Gluc, β 1,4-glucanase; Phos, phosphatase; Tryp, trypsin inhibitor protein; 26S, ribosomal 26S RNA (RNA loading control).

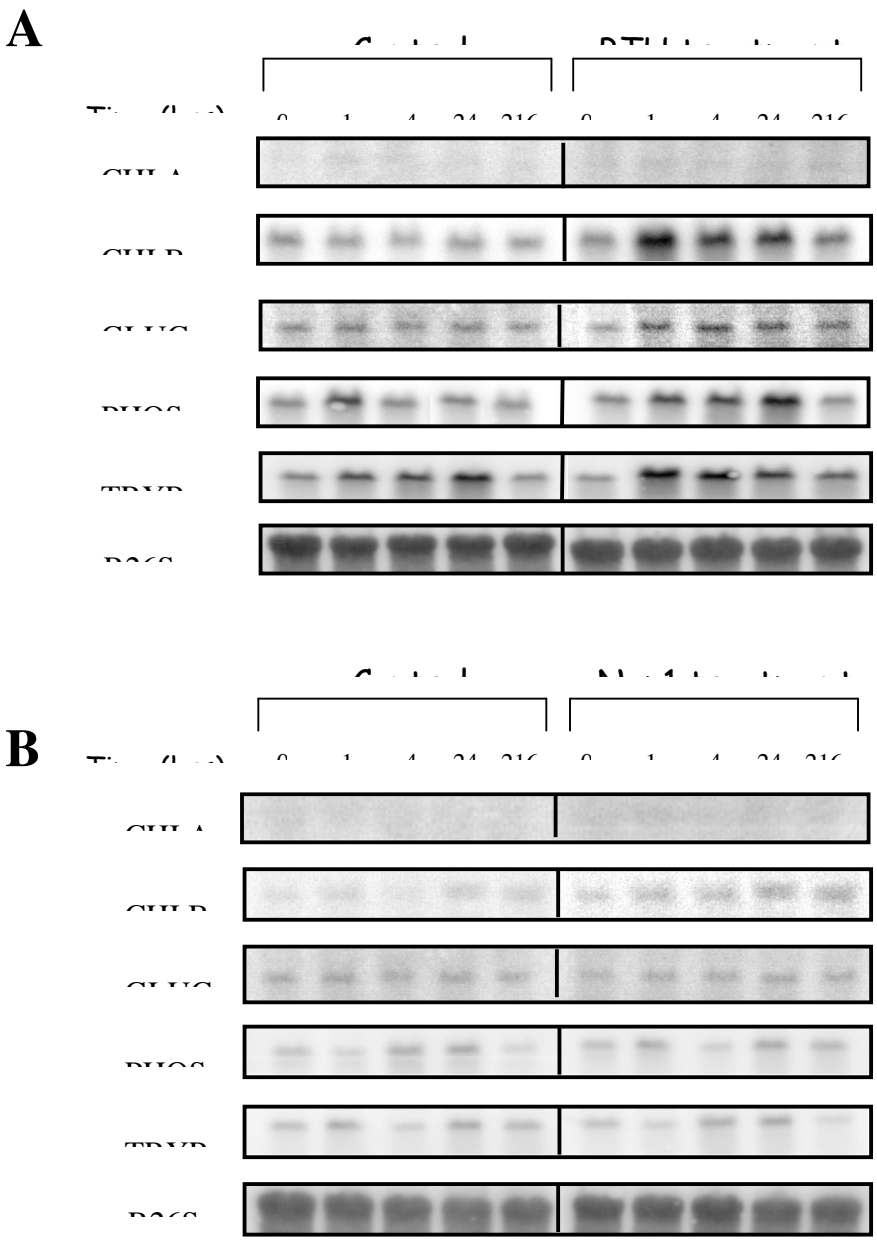
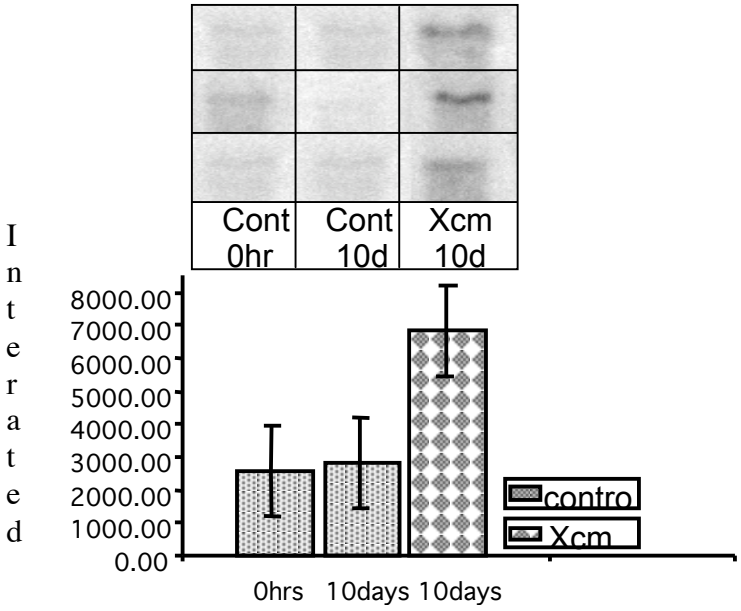

A
B

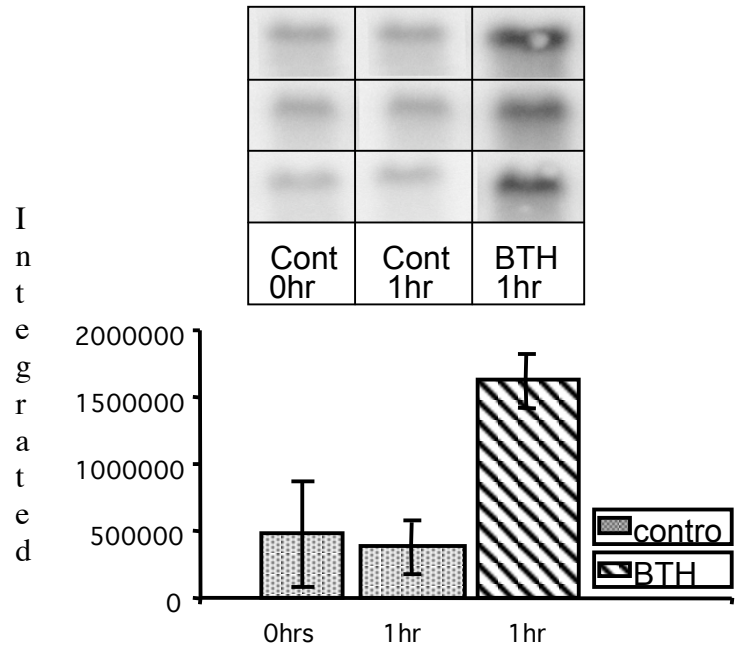
Figure 4.4. Transcript levels for selected defense response genes in *Theobroma cacao* leaves after BTH treatment and exposure to *Xanthomonas campestris malvecearum*. Blots of total RNA isolated from three replicate plants were hybridized to P^{32}-labeled *ChiA* and *Gluc* cDNA probes. Bands detected with a phosphorimager were quantified to assess the expression level of each genes in treated and control leaves. Integrated pixel values were normalized between the three replicate blots for mean relative mRNA level. Images obtained from each individual replicate blot are shown together, containing control sample at time 0 h, treated sample at time of peak expression level, and control sample at same time as treated sample. The graph below indicates the mean normalized pixel intensity value obtained from blots, indicating the maximum induction levels in treated versus the control plants. (A) Response of *ChiA* mRNA after 1h treatment with BTH (1000 mg L^{-1}) on cacao *comun* leaves. Control leaves were sprayed with water. (B) Response of *Gluc* mRNA after 10 days treatment with *Xcm*. Control leaves were sprayed with buffer.

A.



Glucanse probe

B.



Chitinase B probe

Gene Induction following Nep 1 treatment

The Nep1 protein of *Fusarium oxysporum* is known to play a role in the induction of defense responses and hypersensitive response in other plant species (Bailey *et al.*, 2000). However, when the fungal protein was applied to cacao seedlings, despite the signs of necrosis that appeared in the leaf tissue 3 to 5 days after treatment (data not shown), we found very little variation in gene expression levels. The only statistically significant difference was for the *Chi B* gene, which showed a difference between the treated and control samples, 24 hours after treatment ($p=0.002$) (Table 4.2 and 4.3). ANOVA tests at the 95% confidence level for glucanase, phosphatase and trypsin inhibitor probes showed no significant difference.

4.5. DISCUSSION

Plants have inducible defense mechanisms to protect them against pathogen attack (van Wess *et al.*, 1999, 2000). A range of treatments, including those used in the current study, can activate these responses. In an effort to investigate *Theobroma cacao* defense response mechanisms and provide evidence that cacao activates defense genes in a fashion similar to other plants, we tested three elicitors of defense (*Xcm*, BTH and Nep1) on cacao. Following each treatment, we monitored their effects on the transcriptional level of five cacao genes (ChiA, Chi B, □-glucanase, phosphatase and trypsin inhibitor). The results demonstrate that cacao leaves responded to all of the treatments by the activation of genes previously associated with the defense response. Some of the

responses were detected at the level of gene expression, such as the activation of PR genes, while others were more generally observed, such as physical changes observed on the leaf surface of leaves treated with Nep 1, including the formation of necrotic lesions.

Although an association between the bacterium *Xanthomonas campestris malvecearum* and *Theobroma cacao* has not been reported in nature, this pathogen is capable of causing disease to a member of the *Malvaceae* family (*Gossypium herbaceum*). In our experiments, this pathogen was introduced into cacao leaves with the aid of Silwet L-77 surfactant. Leaves receiving Silwet treatments showed darkened water-soaked tissue areas following the solutions infiltration through the stomates and hydathodes. The effect was only visible during the first 10 min following treatment. Populations of *Xcm* detected in cacao leaves after spraying with 0.2% Silwet (v/v) indicated that the bacteria were capable of penetrating through the natural openings of leaves. This study also indicated that Silwet L-77 could be used to deliver biocontrol organisms into cacao plants very rapidly and more conveniently than by hand-infiltration techniques. After treatment with *Xcm*, collection of leaf samples showed that bacterial populations increased during the first three days after treatment. The *Xcm* population decreased afterwards throughout the experiment until undetectable 28 days after treatment (Fig 4**.**1).

Following treatment with bacterial suspension, a diverse set of genes with known roles in defense, were induced in *T. cacao* plants inoculated with *Xanthomonas campestris malvecearum*. Furthermore, the observed transcriptional levels of the *T.*

cacao PR-2 and *PR-3* genes (Table 2) varied in response to the *Xcm* treatment with *PR-2* genes levels increasing throughout the first 10 days following treatment. This increase in gene transcript level could have resulted in the decline of the bacterial population. The coordination of the two events may signify the activation of the SA-dependent response pathway, known to result in the induction of *PR* genes in leaves (Staskawicz, 1985). Possible signals from the bacterium that resulted in the bacteria recognition by the plant could include the perception of *Xcm* bacteria cell wall fragments, wall polysaccharides, or bacteria exudates (Stadnik *et al.*, 2001).

Although we were unable to detect a systemic induction of cacao marker genes in the *Xcm*-treated plants, studies in other plant species have reported the induction of systemic resistance in both greenhouse and field trials in response to *Xcm* (Jetiyanon, 1994). Therefore, to study the systemic induction of defense responses in cacao, future studies should test if *Xcm* treated cacao plants acquired resistance against cacao pathogens following treatment with *Xcm*, and monitor the activation of other *PR* genes that could be induced on treated as well as on untreated leaves. Moreover, studies with other systems have demonstrated that induced resistance can dependent on the concentration of the inducing inoculum, the age of the plant at induction, temperature, and the length of time between induction and challenge (Jetiyanon, 1994), variables which should be taken into consideration when working with *Xcm*.

Cacao responded differently to the three individual treatments. As predicted, treatment with BTH resulted in the activation of SA-dependent responses. Following

treatment, a 3-fold increase of basic chitinase, *Chi B*, (*PR-3* group) was detected in the BTH treated samples. The response of *Chi B* differed both in time and extent, compared to the response of *Chi B* after the *Xcm* treatment. In response to BTH treatment, the *Chi B* transcript level differences were observed at 1 hour, 1 day and 9 days, with transcript levels as high as 4.2-, 3.2- and 1.8-fold increase compared to the control at same time points respectively. In addition, the □-*glucanase* gene expression pattern also varied in response to BTH treatment compared to *Xcm* treatment, with a distinct increase in transcript levels of the □-*glucanase* gene in *Xcm* treated plants. Significant difference was first observed at 1 hour after treatment (1.1-fold increase) and gradually increasing in all of the time points until it reached a peak at 10 days after treatment (2.4-fold increase). This difference in the transcript levels of cacao □-*glucanase* gene was not observed on the samples from the BTH treatment. Despite the differences in temporal activation of defense response genes in *Xcm* and BTH treatment, both treatments not only activated the cacao *PR* genes, but also elicited similar levels of gene induction, suggesting that both treatments could activate genes involved in a SA-response. Future studies would be necessary to test whether both treatments induce the same SAR response in cacao and to investigate if treatment with both elicitors can generate systemic resistance to the same range of pathogens.

Unlike what was observed with BTH and *Xcm* treatment, and despite the noticeable necrotic leaf formation caused on the cacao leaves in response to Nep 1 (Dr. Bryan Bailey, personal communication), no major transcript level changes were observed in response to Nep 1 application for the genes tested in this study. The observed

formation of necrotic lesions on treated leaves a few days after treatment were observed at the USDA facility, results similar to those observed on other plant species (Bailey, 2000). Reports on Nep 1 treatments indicate the protein has capacity to induce ethylene production and a SA-independent defense responses (Bailey *et al.*, 2000, Jennings *et al.*, 2001), results which are comparable to those found on the cacao leaves after Nep 1 treatment (Bryan Bailey, personal communication). It is, therefore, very likely that a defense response did occur. A different set of marker genes responsive to the JA-dependent pathway should be used to detect changes in gene expression levels in response to Nep 1.

Studies on plant defense responses provide a growing understanding of the mechanisms involved in plant-pathogen recognition, gene activation and function, and the defense response that results in disease arrest. Through these studies, I have been able to establish a system to study cacao defense responses. In addition, I have demonstrated that the cacao defense genes tested in my studies follow responses similar to those observed in other plant species. These responses included the induction of *PR* genes, and reduction in *Xcm* bacterial pathogen, suggesting the activation of an SA-dependent response. Ongoing studies in cacao defense induction are being carried out to provide candidate defense genes for future research.

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CHAPTER 5

CONCLUSIONS AND FUTURE RESEARCH

5.1. CONCLUSIONS AND FUTURE RESEARCH

The main objectives of my research were to optimize *Theobroma cacao* tissue culture techniques, specifically optimizing the type and concentration of antibiotic used to select against *Agrobacterium tumefaciens* (Antúnez de Mayolo *et al*., 2003). My results indicated that the use of moxalactam, a β-lactam antibiotic, not only controlled the growth of *Agrobacterium tumefaciens* after transformation of cacao tissues, but also significantly enhanced the frequency of secondary somatic embryogenesis. In doing so, I contributed to the development of the current *Theobroma cacao* transformation protocol (Maximova *et al*., 2003).

Most of the cacao tissue culture studies presented so far have focused on a limited number of cacao genotypes. Among the genotypes tested, great variability of the frequency of somatic embryogenesis has been observed (Maximova *et al*., 2002). The genotype *Scavina* 6 is the only one from which transgenic plants have been attempted to date. Therefore, future research in cacao tissue culture will need to focus on the optimization of tissue culture conditions for other genotypes of interest. Additionally, there is a need to test the seasonal effects on cacao somatic embryogenesis, since the observations in our laboratory over time have indicated a difference in the frequency of somatic embryos produced from tissue initiated at different times of the year. Long term experiments to test these effects have been initiated.

The second main objective was to use the tissue culture and transformation techniques to develop transgenic cacao lines, that over-express a cacao pathogenesis

related (PR) defense protein. A class I chitinase gene thought to play a role in defense against *Crinipellis perniciosa* in cacao pods (Snyder and Furtek, 1995) was used. The genomic copy of this chitinase gene was introduced into a T-DNA cassette containing two marker genes used for the selection of transgenic lines.

Molecular techniques were used to detect stable gene insertion into the cacao genome and verify transcription of the chitinase transgene in the transgenic lines. Northern blot analysis indicated increases in the transcript levels of Chi A in both SA39 and SA44 transgenic line compared to the non-transgenic controls. This change in the transgene expression was positively correlated with an increase in endochitinase activity in the same transgenic lines. Ongoing studies with transgenic lines will continue to evaluate the chitinase transgene both *in vitro* and *in vivo*. It is important that as soon as more lines are available, and rooted cuttings are prepared from all the lines, large-scale bioassay experiments are initiated not only with *Crinipellis perniciosa* cacao pathogen, but possibly with other cacao pathogens, such as *Moniliopthora roreri*.

Furthermore, future experiments should focus on the combination of more than one PR transgene insertion. For example, a combination of the chitinase and □-glucanase genes has in other plant species been shown to increase resistance and in some instances resulted in resistance to a broader range of pathogens (Sela-Bourlage *et al.*, 1993; Jach *et al.*, 1995; Anand *et al.*, 2003). It is also very important to recognize that consumers vary in their understanding of transgenics crops and that ultimately consumers' acceptance of

transgenic crops over the next years will very likely drive the use of this technology in agriculture.

The last component of my thesis was designed to develop a working model system to explore cacao defense responses. Despite the rapidly growing understanding of plant defenses, little is known about the molecular aspect of *Theobroma cacao* defense signaling pathways. My studies characterized the expression patterns of five cacao genes in response to three elicitors: *Xanthomonas campestris malvecearum*, benzothiadiazole (BTH), and Nep 1 protein. Treated leaves showed a significant increase in transcript levels of two different members of the PR family, the cacao □-glucanase and the basic form of the class I chitinase gene (Chi B). Similarly, treatment with BTH resulted in significant increase of the Chi B mRNA. However, transcript levels for five marker genes in response to three different treatments showed that cacao plants are capable of differentially activating distinct genes in response to each elicitor.

Due to the limited number of *Theobroma cacao* gene sequences currently available in the NCBI database and the even shorter list of genes associated with defense responses in cacao, ongoing work in our laboratory has focused on the development of subtractive hybridization cDNA libraries (Dr. Joe Verica, personal communication). These libraries have been design to identify and clone genes differentially expressed in treated and untreated cacao samples. Libraries have been generated from benzothiadiazole (BTH), Nep 1 protein of *Fusarium oxysporum*, ethylene- and jasmonic-acid treated plants are being screened for candidate genes. This research will provide a

much larger selection of defense marker genes for each of the different signaling pathways, allowing each pathway to be studied in detail. The use of cacao cDNA libraries in combination with microarray techniques and proteomic studies could provide a better understanding of the cacao defense response. Understanding cacao defense responses could result in a better list of candidate genes for transgenics and marker genes for a breeding program. It could also lead to the discovery of plant protection chemicals capable of stimulating disease resistance in the cacao plants.

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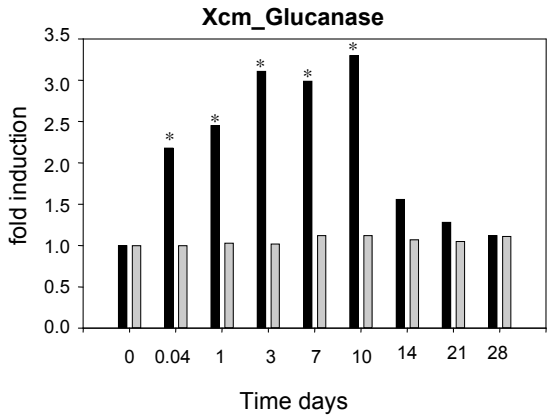
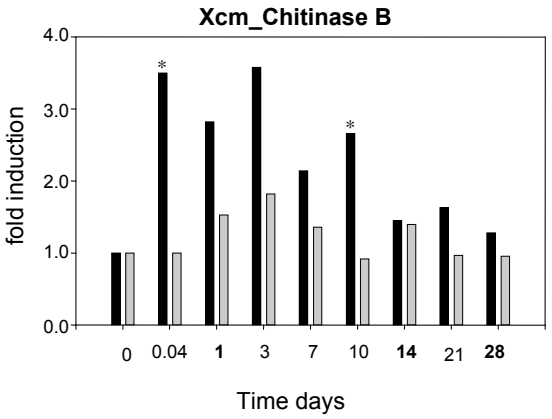
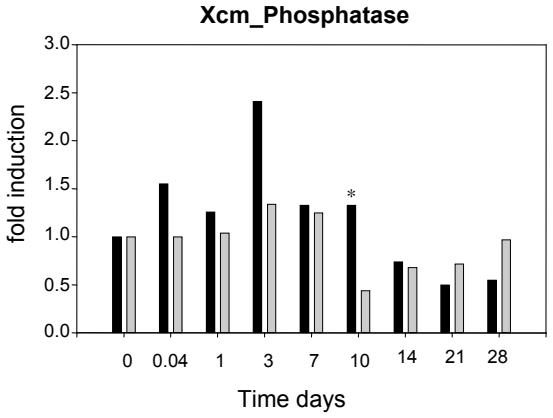
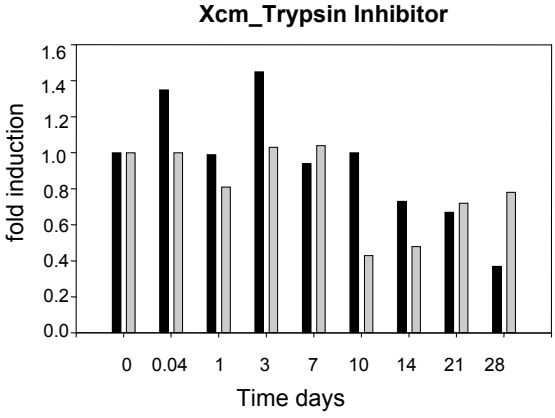
Appendix A

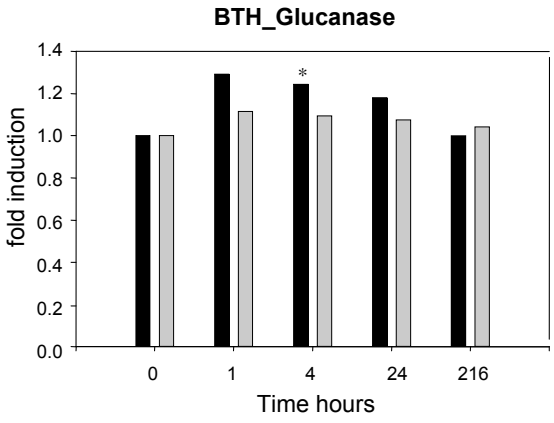
Effect of elicitor treatment on gene induction compared to untreated control at time 0 hours. Abbreviations are Xcm, *Xanthomonas campestris malvecearum*; BTH, benzothiadiazole; and Nep, Nep1 protein. Marker genes were Glucanase, Chitinase B, Phosphatase, and Trypsin Inhibitor. Graphs were generated by calculating the average fold induction of the signals generated by maker gene for both treatment and control samples. Fold induction values were determined by dividing values at each time point by values of untreated control at time 0 hours. This analysis is another way to look at the results from Chapter 4 and at the values presented in Table 4.2. Column bars on graphs are treatment, black, followed by control, grey. Standard error and statistical analysis are the same as for values shown in Table 4.2, and Table 4.3.

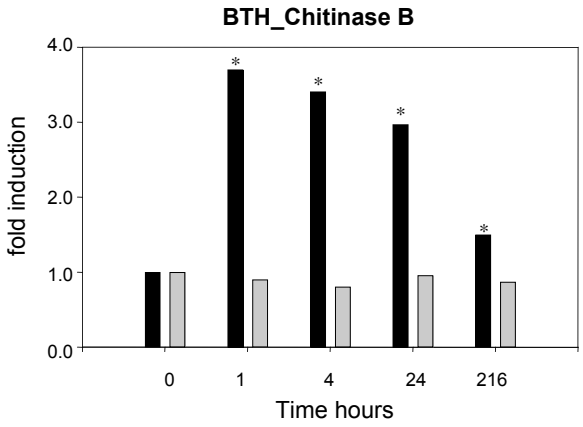

Xcm_Glucanase
fold induction
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0 0.04 1 3 7 10 14 21 28
Time days

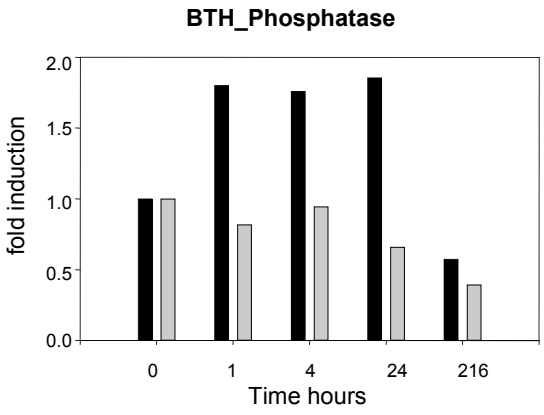

Xcm_Chitinase B
fold induction
4.0
3.0
2.0
1.0
0.0
0 0.04 1 3 7 10 14 21 28
Time days

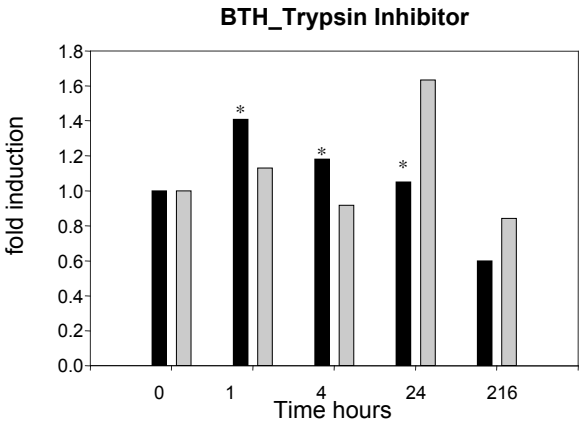

Xcm_Phosphatase
fold induction
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0 0.04 1 3 7 10 14 21 28
Time days

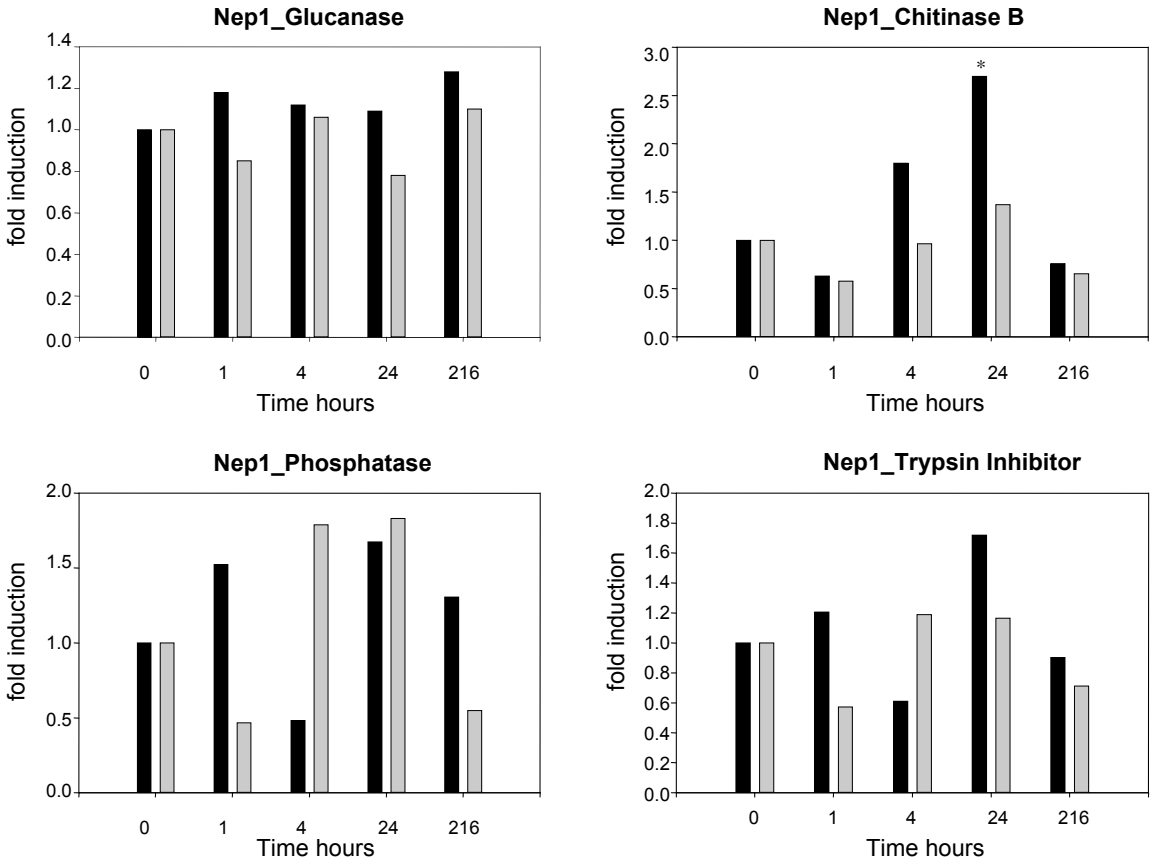

Xcm_Trypsin Inhibitor
fold induction
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 0.04 1 3 7 10 14 21 28
Time days


BTH_Glucanase
fold induction
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
0
1
4
24
216
Time hours


BTH_Chitinase B
fold induction
4.0
3.0
2.0
1.0
0.0
*
*
*
*
0
1
4
24
216
Time hours


BTH_Phosphatase
fold induction
2.0
1.5
1.0
0.5
0.0
0
1
4
24
216
Time hours


BTH_Trypsin Inhibitor
fold induction
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
0
1
4
24
216
Time hours


Nep1_Glucanase
fold induction
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
1
4
24
216
Time hours
Nep1_Chitinase B
fold induction
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
0
1
4
24
216
Time hours
Nep1_Phosphatase
fold induction
2.0
1.5
1.0
0.5
0.0
0
1
4
24
216
Time hours
Nep1_Trypsin Inhibitor
fold induction
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
1
4
24
216
Time hours