STUDIES ON SECONDARY METABOLITES ASSOCIATED WITH WITCHES' BROOM DISEASE, FLORAL BIOLOGY, AND SEED FERMENTATION IN CACAO

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ABSTRACT OF THE DISSERTATION

STUDIES ON SECONDARY METABOLITES ASSOCIATED WITH WITCHES' BROOM DISEASE, FLORAL BIOLOGY AND SEED FERMENTATION IN CACAO

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Theobroma cacao L., a tree native to the Amazon, is cultivated in the tropics throughout the world for its seeds, used primarily for chocolate production. Cacao production is limited by several problems. Cocoa pod borer, an insect that burrows through pods, damages seeds, allowing contamination by toxigenic fungal species. Many fungal diseases infect cacao. Among them, *Moniliophthora perniciosa*, the causal agent of witches' broom disease, severely affects plantations throughout South America and the Caribbean. Cacao yields are further limited by the naturally low rates of fruit set. Moreover, disease tolerant varieties are usually self-incompatible low producers and do not give a superior chocolate flavor compared to some disease susceptible and self-compatible genotypes with highly valued aroma compounds.

During this project, problems associated with three main aspects of cacao were investigated: disease, production and processing. Studies on plant-endophyte-pathogen interactions allowed for the identification of new possible mechanisms of disease control; studies on cacao flower physiology indicated ways to improve pollination and therefore increase fruit set and crop yield; and investigations of the fermentation step of cacao processing permitted discovery of a method for maintaining higher levels of compounds valued by cacao manufacturers.

Flavan-3-ol monomers and oligomers, purine alkaloids and salicylic acid, volatile organic compounds, polyketides and other phenolic compounds were among the determined bioactive compounds found in cacao, pathogens and endophytes, with influence on disease, production, and processing.

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You may travel the world, meet a million people and still if you do not know what you are looking for, you will never be satisfied with what you find.

An experience may be very pleasant, but if you do not feel you are building something you won't be happy.

I may have heard them or may have read them; I sure did learn and believe them.

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1. INTRODUCTION

In 1720, Linnaeus named a tree *Theobroma cacao*, from the Greek "Theo" meaning God and "broma" meaning food, based on the Aztecs who cultivated cacao and believed it was of divine origin and possessed godly powers. The Aztecs made a stimulant drink, chocolatl, by combining ground roasted cocoa beans with maize meal, vanilla, and chili peppers, which was served in gold cups to emperor Montezuma (Wood and Lass, 1987; Young, 2007). Cacao has also been used as an antiseptic, diuretic, emmenagogue, and parasiticide, as well as a folk remedy for alopecia, burns, cough, dry lips, listlessness, pregnancy, rheumatism, snakebite, and wounds (Duke, 2007). In some countries, the beans served as currency; in 16th century Guatemala, one could buy a horse with 50 cocoa beans and a slave with 100 beans (Young, 2007). Currently, the tree native to the Amazon is cultivated in the tropics throughout the world for its beans, which serve as raw material for the manufacture of chocolate, cocoa butter, and several other food and cosmetic products.

Worldwide, 7,000,000 hectares of land are devoted to the cultivation of cacao. Cocoa bean yields vary from 200 $kg \cdot ha^{-1}$ to 1500 $kg \cdot ha^{-1}$. Each tree produces an average of 30 to 40 pods a year, with ten pods yielding one pound of dry cocoa beans. Major producing countries include Ivory Coast with 1,286,000 tons per year, followed by Indonesia (610,000 tons per year), Ghana (599,000 tons per year), Nigeria (441,000 tons per year), and Brazil (236,000 tons per year) (2005 data, FAO). In the United States, the main importer of cocoa beans, more than 1,900,000 jobs are dedicated to the manufacture, distribution and sale of chocolate products. The chocolate industry in the

US exports chocolate and chocolate products to more than 50 countries, and in the year 2003 generated $23.5 billion in sales (Young, 2007).

The word “cocoa” originated from a loss in translation to English and has been used to describe finished products, while “cacao”, allegedly the correct name, serves to describe the crop and raw materials prepared from it (Chatt, 1954). In order to avoid inaccuracies, many local terms used by indigenous people from cacao growing regions have been preserved, which brings more peculiarity to a very distinct plant.

Cacao can be cultivated under a system called “Cabruca”, where a forest is thinned and the original understory is replaced with cacao, allowing for the preservation of native species. When cacao, which has a canopy height of about four to ten meters, is planted in deforested areas, other taller species are introduced as shade trees. The choice of shade tree varies according to regional adaptability and local culture. Leguminous species such as *Inga* sp., *Gliricidia* sp. and *Erythrina* sp. are used preferentially due to their nitrogen fixation capacity, but other trees such as coconuts, rubber trees, and macadamia may be used and serve as secondary crops and an alternative source of income. During the establishment of a cacao plantation, banana trees in Latin America, and plantains in Africa, are used temporarily for shade to protect young trees. Cacao can also be cultivated under another system, full sun, in which there are no shade trees. Although more productive, this system demands higher fertilization and the life span of the trees is shortened.

Cacao requires about 1250 to 2800 mm of rain per year and temperatures around 26 ^{o}C. In Salvador, BA, where more than 90 % of Brazil’s cacao is cultivated, the rainy season starts in March, goes until August, and peaks in May with 350 mm of rain for the

month. The highest temperatures of about 30 ^{o}C occur in January and February, while the lowest temperatures of around 21 ^{o}C occur in July and August. The minimum number of daylight hours occurs in June (165 hours) and the maximum in January (245 hours) (2007 data, INMET).

Cacao is a dicotyledonous, C3 plant, with 2n = 2x = 20 chromosomes and an estimated genome size of 4.15 x 108 bp (Figueira et al., 1992). The tree has some unique morphological characteristics. After a brief period of monopodial growth, five horizontal fan branches develop simultaneously from buds just below the terminal growing point on the main stem in a structure known as a jorquette. At the base of the jorquette, a vertical shoot (known as a chupon) is formed, and from the chupon other fan branches eventually arise. Chupons also stem from the bottom of the tree and it is common practice to renovate old trees by cutting down the main trunk and allowing one chupon to take over. On fan branches, leaves are oppositely arranged, whereas on vertical stems, leaves are spirally arranged in a 3/8 pattern. When counting from the first leaf it takes three turns around the stem for the 9th leaf to be at the same position as the 1st (Chatt, 1954).

Cacao has alternate periods of rapid shoot growth and dormancy, a type of growth often referred to as flush growth. New flush leaves are thin and tender, and can be pale green or pink, and only when fully expanded become dark green, thicker and sturdier.

Cacao has a cauliflorous habit with flowers growing from the trunk in patches called flower cushions. Each flower can bear a single fruit called a pod. During development the pods are green, and after five to six months they ripen to a yellow or reddish purple color, depending on the variety. Pods do not drop off the trees even when

fully ripe. Each pod is approximately 30 cm long and 15 cm wide at the middle, and bears an average of about 40 beans.

Cacao trees begin to produce pods after three to five years, and at ten years are fully productive. There are usually two harvests a year, a small one prior to the rainy season and the major harvest at the end of the rainy season. Once harvested, the pods are cracked open with a machete and the beans, covered by a mucilaginous pulp, are placed in piles, covered with banana leaves, and allowed to ferment. During fermentation, yeasts and bacteria convert sugars to alcohols to acids, which allow for the development of aroma precursor compounds. After fermenting for a few days, beans are sun dried and then shipped to be roasted and manufactured.

Around the world, several problems threaten cacao production ranging from low productivity to insect pests to devastating diseases. Overall losses due to insects and disease are estimated at 30 % of the potential crop per year. In Indonesia, the main pest problem is cocoa pod borer (*Conopomorpha cramerella*), an insect that burrows through the pods and feeds on the pulp causing malformation of seeds. Moreover, the wounds serve as an entry point for opportunistic organisms such as toxigenic fungi that contaminate beans and pose a threat to human health. In Africa, *Phytophthora* species are the main threat and can attack all parts of the tree. Species from the *Phytophthora* genus are present in all cacao growing regions and cause black pod disease, 'mancha de agua' and Phytophthora canker. In Brazil, the most destructive pathogen is the fungus *Moniliophthora perniciosa*, causal agent of witches' broom disease, which under high disease incidence can cause total crop loss. The fungus is present in growing countries of the Americas and can also attack other species of Theobroma and the closely related

genus Herrania. In addition to *Phytophthora* and witches' broom disease, cacao plantations in northwestern South America, Ecuador, and Colombia also have to deal with *Moniliophthora roreri,* the causal agent of frosty pod disease, which is characterized by the frosty appearance of mycelia and spore covered pods. Cacao yields are further limited by the naturally low rates of fruit set, ranging from one to five percent. Moreover, disease tolerant varieties are usually self-incompatible low producers and do not give a superior chocolate flavor compared to some very disease susceptible and self-compatible genotypes with high valued aroma compounds.

Cacao is an interdisciplinary crop in that unlike many other food crops, it passes through complex processing before becoming a consumable food. Therefore, a complete understanding of agricultural, processing and manufacturing aspects of this crop is essential for determining its success.

During this project, problems associated with three main aspects of cacao were investigated: diseases, production and processing. The dissertation is divided into 3 sections: (1) Studies on plant-endophyte-pathogen interactions that allowed for the identification of new possible mechanisms of disease control; (2) Studies on cacao flower physiology that indicated ways to improve pollination and therefore increase fruit set and crop yield; and (3) Investigations on the fermentation step of cacao processing permitted discovery of a method for maintaining higher levels of compounds valued by cacao manufacturers for chocolate production.

2. PLANT-ENDOPHYTE-PATHOGEN INTERACTIONS

2.1. PLANT

2.1.1. INTRODUCTION

Cacao disease resistance is multigenic and several genes associated with resistance have been identified (Leal et al., 2007). However, there are no completely resistant cultivars to any of the three main diseases that attack cacao, and the array of pathogens and their high genetic variability make breeding for resistance complicated. Other forms of crop protection employed for cacao include cultural practices such as phytosanitary pruning and pod stripping, as well as the use of pesticides and biological control agents. None of these procedures is very effective against cacao pathogens alone, although when used in combination, depending on environmental conditions, satisfactory levels of disease control may sometimes be attained. Despite the relative success of current practices, crop production is significantly below capacity worldwide, *M. roreri* and *M. perniciosa* are spreading and new forms of control should be pursued in an effort to keep the pathogens and diseases manageable.

Activation or induction of plant defense responses, using chemicals considered plant activators, is an alternative approach to pesticides for disease management. Plants respond to chemical elicitors by activating metabolic pathways leading to systemic acquired resistance (SAR). SAR is an inducible plant defense response that results in a non-specific and long lasting systemic resistance to a variety of pathogens. Following

SAR there is an accumulation of signaling molecules, which trigger the activation of a series of defense pathways and results in production of a variety of phytoalexins in cacao and other plants. The phenylpropanoid pathway is one important source of phytoalexins. It starts (meaning it derives from primary metabolism at this point) from phenylalanine, which is deaminated by the action of the enzyme phenylalanine ammonia lyase (PAL; EC 4.3.1.5), to trans-cinnamic acid, a precursor of flavonoids and other metabolites.

Flavonoids, a class of compounds with a basic structure of 15 carbons arranged in two aromatic rings and a heterocycle containing oxygen is subdivided into several groups including the proanthocyanidins. Both induced and constitutive flavonoids have been associated with plant resistance to biotic and abiotic stresses (Harborne and Williams, 2000). In cacao, flavonoids have been implicated in the resistance against pathogens (Nojosa et al., 2003). Proanthocyanidins, also known as condensed tannins, possess a polyflavan-3-ol structure differing in the hydroxylation pattern. The most common are the di-hydroxylations (catechins) and tri-hydroxylations (gallocatechins) in the B ring of the flavonoid skeleton. Procyanidins are the largest class of proanthocyanidins, composed of polymers of flavan-3-ol subunits mostly linked by carbon-carbon bonds through C4 and C8 and to a lesser extent linked by C4 to C6 (Figure 1). These single linked molecules are referred to as B type procyanidins. The A type linkages have an additional bond between C2 and O7 and have not been reported in cacao (Kelm et al., 2006).

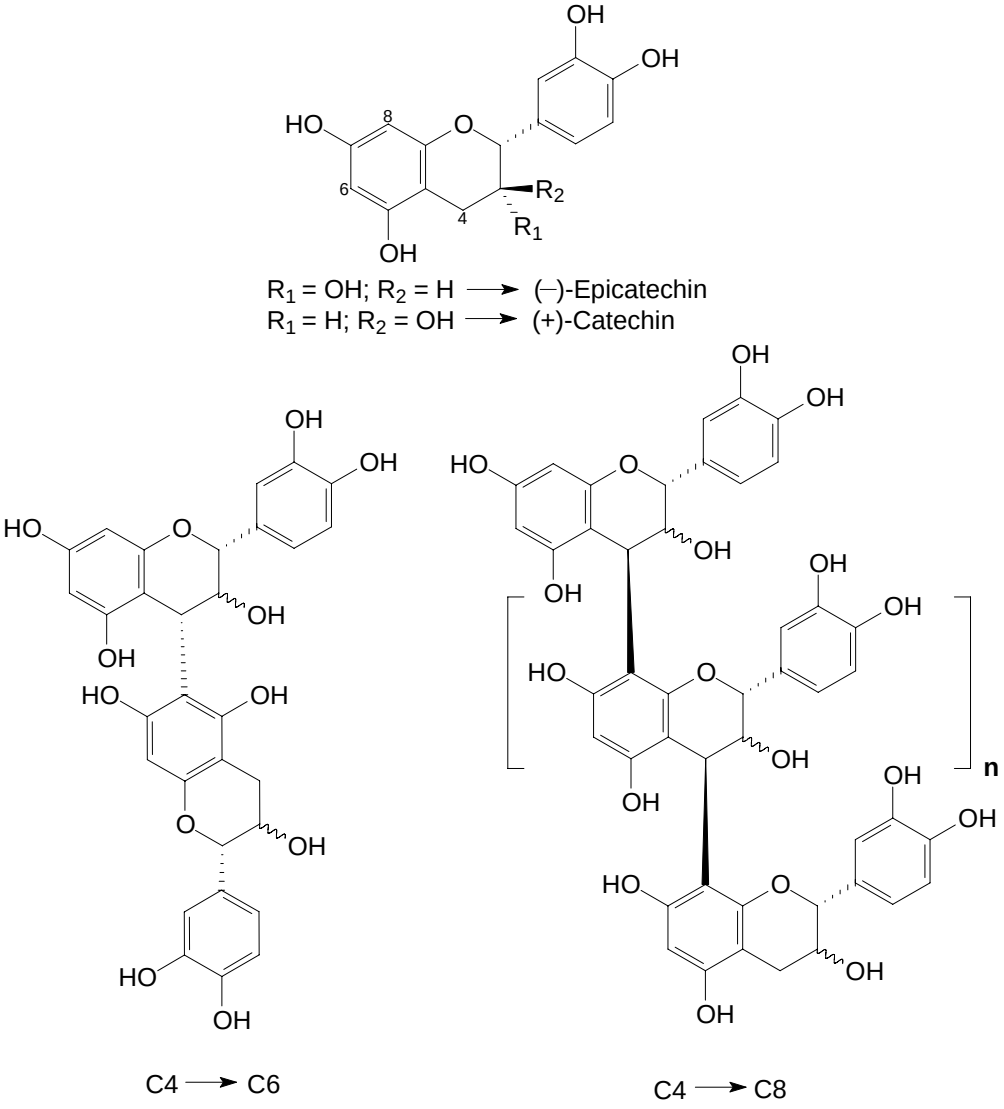
Purine alkaloids are another important group of compounds present in significant amounts in cacao. The main purine alkaloids found in cacao leaves are theobromine (3,7-dimethylxanthine) and caffeine (1,3,7-trimethylxanthine) (Zheng et al., 2004; Hammerstone et al., 1994). In the alkaloid pathway, xanthosine is converted to

theobromine after two methylation steps and the hydrolysis of a sugar group. Caffeine originates from theobromine after another methylation step. The conversion of theobromine to caffeine may be slower in cacao leaves when compared to coffee and tea, which contain higher caffeine levels. Both alkaloids have been linked to resistance against pathogens (Nathanson, 1984; Pearson and Marth, 1990; Aneja and Gianfagna, 2001).

Salicylic acid (SA) has been identified as a signaling molecule that triggers plant defense responses including activation of both the purine alkaloid and phenylpropanoid pathways. Moreover, tobacco plants inoculated with tobacco mosaic virus respond by accumulating SA and salicylic acid glucoside (SAG) (Hennig et al., 1993; Kawano et al., 2004). After biosynthesis, most free SA is removed by esterification to methylsalicylate (MeSA) or conjugation to SAG, which may work as a slow release storage form of SA that maintains SAR over time.

SA and S-methyl-benzo[1,2,3]thiadiazole-7-carbothioate (Acibenzolar-S-methyl, ASM; or BTH for benzothiadiazole), a synthetic analogue of SA (Kunz et al., 1997), induce caffeine accumulation when applied to young flush leaves of cacao (Aneja and Gianfagna, 2001). ASM application before inoculation of cacao seedlings with *Moniliophthora perniciosa* reduced disease incidence by 84.5 %. In addition, ASM application prior to infection protected cacao against verticilium wilt, an effect comparable to that found upon treatment with cuprous oxide or tebuconazole (Resende et al., 2002). With grapes, ASM enhanced anthocyanin levels and improved resistance against the fungal pathogen *Botrytis cinerea* (Iriti et al., 2004).

Figure 1. Chemical structure of B-type procyanidin monomers and oligomers



Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. Suspended cell cultures accumulate secondary metabolites upon application of methyl jasmonate (MeJA). MeJA initiates de novo transcription of PAL genes. The induction of anthocyanins in germinating soybean seedlings upon application of MeJA indicates that this defense mechanism is present not only on plant cell suspension cultures but in differentiated plants (Gundlach et al., 1992).

It has been reported that SA blocks JA biosynthesis in tomato leaves (Pena-Cortes et al., 1993), on the other hand, SA production is inhibited by JA in wounded transgenic tobacco plants (Sano et al., 1996). Wound-induced accumulation of transcripts and proteins for some PR genes (pathogenesis related) is enhanced in the presence of MeJA, and inhibited in the presence of SA, in contrast, the expression and transcription of other PR genes is induced by SA and inhibited by MeJA (Niki et al., 1998). Thus, there are many cases in which the production of SA and JA are mutually antagonistic.

To study wound-induced gene expression, chemicals such as MeJA and ethylene have been used due to their known involvement as signal molecules in the wound-induced expression of proteinase inhibitor genes (Niki et al., 1998; Pieterse and van Loon, 1999). Ethylene has also been reported to be an inducer of PR proteins. Wounded leaves treated with ethylene or MeJA accumulated PR transcripts faster than leaves that were only wounded, suggesting that ethylene and MeJA might mediate wound-induced transcriptional gene stimulation (Niki et al., 1998; Kim et al., 2006).

In addition to hormones, other compounds have been used to activate SAR experimentally. Aminoethoxyvinylglycine (AVG) reduces ethylene and protein biosynthesis in excised discs of mature-green tomato pericarp tissue. AVG is frequently

used as a specific inhibitor of ethylene biosynthesis to determine the effects of ethylene on plant growth, development, and response to stress. AVG has also been used to study fungal pathogenesis, nodulation in legumes and response to chilling stress (Saltveit, 2005). Glutathione, another chemical elicitor, causes rapid, marked but transient gene expression in soybean protoplasts. Both reduced glutathione and oxidized glutathione elicited the phytoalexin response in cell-suspension cultures of beans (*Phaseolus vulgaris*) (Edwards et al., 1991). *Agrobacterium rhizogenes* transformed root cultures of *Lotus corniculatus* treated with glutathione, accumulated isoflavan phytoalexins in both tissue and culture medium. This accumulation of phytoalexins was preceded by a transient increase in the activity of PAL (Robbins et al., 1991).

Small increases of different compounds by synergism may enhance defenses more effectively than greatly increasing the concentrations of one specific compound. Such interactions cannot be evaluated if only a single compound is analyzed. Since cacao produces both flavonoids and purine alkaloids that may be regulated by SA, the levels of procyanidins, salicylic acid, salicylic acid glucoside, caffeine and theobromine in leaves of different cacao varieties with varying disease tolerance levels at different stages of development, before and after elicitation, and after pathogen inoculation, were examined in order to better understand the chemical mechanisms of plant defense.

2.1.2. MATERIALS AND METHODS

2.1.2.1. Chemical elicitors

Young developing flush leaves and fully developed mature leaves of greenhouse grown cacao varieties used, differed in disease susceptibility. IMC 30 (very disease susceptible), TSH 565 (Trinitario Selected Hybrids, moderately disease susceptible), Scavina 6 (SCA 6, is one of the progenitors of TSH; the least disease susceptible) and Amelonado (which is very susceptible to diseases and the only self-compatible of the four). Fully developed leaves were cut into 1 cm disks with a cork borer and young leaves were cut in approximately 1 cm pieces. 0.5 g of leaves were placed in an erlenmeyer flask containing: 10 mL of a solution composed of 30 mM pH 5.6 buffer potassium phosphate, 10 mM of sucrose and one of the following chemical elicitors, 1 mM of glutathione, 1 mM of aminoethoxyvinylglycine (AVG), 1 mM of BTH (acti-guard, product containing BTH, from Novartis, USA), 1 mM of jasmonic acid. The flasks were kept in a shaker (100 rpm) at room temperature for 24 hours. Leaves were then rinsed with distilled water, blotted and kept at -80 ^{o}C until they were analyzed.

2.1.2.2. Pathogen inoculation

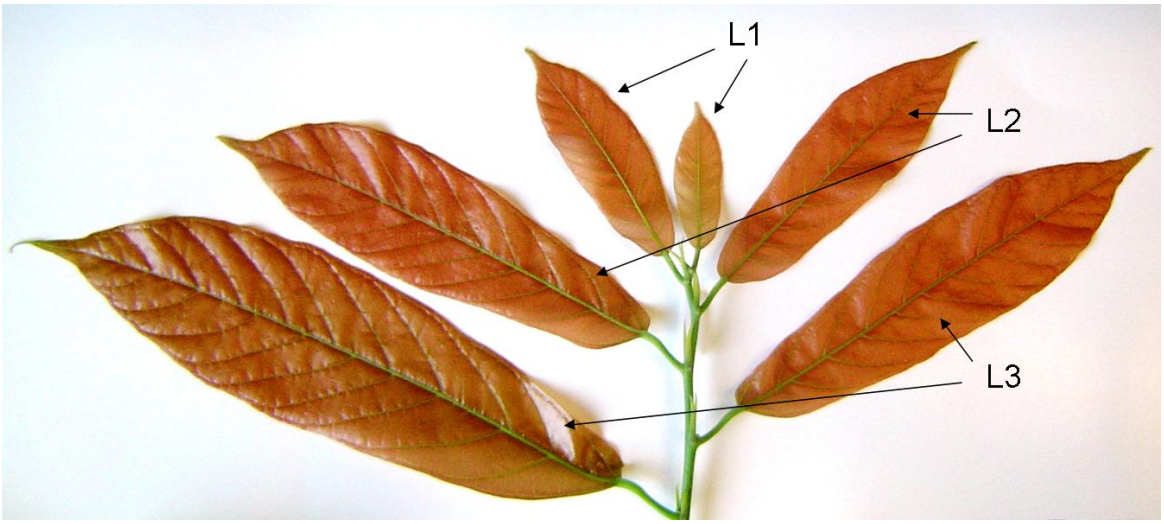
Field grown cacao trees from Bahia, Brazil, of two varieties, Cacao Comum (CCO), disease susceptible, and Scavina 6 (SCA), disease tolerant, were used for this study. The youngest pair of leaves in a flush (< 6 cm) was inoculated with basidiospores

of *M. perniciosa*. After 48 hours, the inoculated leaves (L1) and the two older proximal pairs in the same flush (L2 and L3) were collected (Figure 2). The respective control leaves (L1, L2 and L3) were collected from trees not inoculated with the pathogen. The leaves were freeze dried and stored at -80 ^{o}C until analysis.

2.1.2.3. Leaf size experiment

Cacao leaves from greenhouse grown trees from the varieties IMC 30, TSH 565, SCA 6 and Amelonado, were sampled and separated in nine categories according to leaf size, 1: <2 cm, 2: 2-4 cm, 3: 4-6 cm, 4: 6-8 cm, 5: 8-10 cm, 6: 10-15 cm, 7: 15-20 cm, 8: >20 cm young flush leaves, 9: >20 cm old fully expanded green leaves. Leaves were harvested, flash frozen in liquid nitrogen, freeze-dried, and kept at -80 ^{o}C until analysis.

Figure 2. Cacao leaves used in the pathogen inoculation study



L1 ≤ 6 cm

2.1.2.4. SA and SAG analysis

SA extraction and HPLC analyses followed the method of Verberne et al. (2000). The protocol was the following: grind 0.5 g of leaves in liquid nitrogen and transfer to an eppendorf tube; add 1 mL of methanol (90 %) and 2.5 µL of internal standard (3,4-DHBA, dihydroxy benzoic acid, 10 $\mu g \cdot \mu L^{-1}$); vortex for one minute and sonicate for five minutes; centrifuge for five minutes at 13,000 rpm and separate the supernatant; resuspend the pellet in 0.5 mL methanol; vortex for one minute and sonicate for five minutes; centrifuge for five minutes and combine the supernatants; centrifuge again for five minutes and discard the pellet. To the supernatant, add 10 µL of sodium hydroxide (0.2 M); concentrate in a speedvac; add 250 µL of tricholoroacetic acid (5 % in water) and vortex for 30 seconds; partition with 800 µL of ethylacetate:cyclohexane (1:1) twice; the upper phases contain SA and the lower phases (aqueous) contain SAG; add 60 µL of 0.2 M sodium acetate buffer pH 5.5 to the combined upper layers; concentrate in the speedvac. Add 600 µL of the HPLC mobile phase (0.2 M sodium acetate buffer pH 5.5 : methanol; 90 : 10) and vortex for 30 seconds; centrifuge and use the supernatant to inject in the HPLC; to the aqueous phase add 300 µL of 8 N HCl and heat the sample at 80 ^{o}C for one hour; partition acid fraction with 800 µL of ethylacetate:cyclohexane (1:1) twice; add 60 uL of sodium acetate buffer to the combined upper layers and concentrate in the speedvac; add 600 µL of the HPLC mobile phase and vortex for 30 seconds; centrifuge and inject the supernatant in the HPLC. A Shimadzu LC-10AT equipped with a RF-10A spectrofluorometric detector and a C18 column (140 mm x 4.6 mm x 0.5 µm particle size) was used. The fluorescence detector was set at an excitation wavelength of 305 nm

and emission of 407 nm; the flow rate was 0.8 $mL \cdot min^{-1}$ using a mobile phase of 90 % 0.2 M sodium acetate buffer pH 5.5 and 10 % methanol.

2.1.2.5. Purine alkaloids and procyanidin monomer analysis

For the extraction, flush leaves from greenhouse grown trees were ground in liquid nitrogen until powdered and transferred to an eppendorf tube. Then 1 mL of 90 % methanol was added to the tube, which was vortexed for one minute, sonicated for 12 minutes and centrifuged for five minutes at maximum speed in a bench top centrifuge. The supernatant was saved, and 0.5 mL of methanol was then added to the pellet. The sample was vortexed for one minute, sonicated for 12 minutes and centrifuged for five minutes. The supernantants were combined and centrifuged again. Then 0.25 mL of water and 0.25 mL of chloroform were added to the supernatant. The sample was vortexed for 30 seconds and centrifuged for one minute to break the emulsion. The chloroform layer was discarded and the remaining solution was concentrated in the speedvac. Methanol was added to complete the final volume to 1 mL. HPLC conditions: a reverse phase (C18) column (140 mm x 4.6 mm x 0.5 μm particle size) was used; the mobile phase composed of solvent A (0.1 % acetic acid in water) and solvent B (0.1 % acetic acid in methanol) ran at a flow rate of 1 $mL \cdot min^{-1}$. The gradient started with 15 % B in A, then at 15 minutes 30 % B in A, at 25 minutes 65 % B in A, and at 26 minutes back to 15 % B in A. The photo diode array detector was set at 250-290 nm.

2.1.2.6. Procyanidin oligomer analysis

Freeze-dried leaves were ground to powder in liquid nitrogen and defatted with hexane. The dried defatted sample was then extracted with an acetone:water:acetic acid solution (70:29.5:0.5). After filtering through a 0.22 μm nylon membrane filter, procyanidins were separated by HPLC (LC-10AT, Shimadzu) using a silica column (Lichrosphere 250 mm x 4.6 mm, Phenomenex) and analyzed by UV (SPD-M10A, Shimadzu) (200 nm - 400 nm) and fluorescence detectors (FLD) (RF-10A, Shimadzu) (ex. 276 nm; em. 316 nm). The mobile phase was composed of two solvent solutions: A – 82 % dichloromethane, 14 % methanol, 4 % water:acetic acid (1:1), and B – 96 % methanol, 4 % water:acetic acid (1:1). The gradient employed started with 5 % B in A and was raised to 20 % B in A at 30 minutes. It remained at 20 % until 45 minutes and increased to 90 % at 55 minutes, then returned to the starting conditions of 5 % B in A at 60 minutes and ran for an additional 10 minutes at 5 % B in A, at a flow rate of 1 mL $\cdot min^{-1}$. Cacao procyanidin standards were stored in a -80 ^{o}C freezer and solutions were prepared just prior to use.

2.1.2.7. Statistical analysis

Statistical analysis of the data was performed using SAS version 9.1 for Windows (SAS Institute, USA) and Metrixus, a Microsoft Office Excel XLL Add-In (Duxus, Brazil). Using SAS, the data was subjected to an analysis of variance for completely randomized samples and the least significant difference was determined at the level of 5

% of probability. Using metrixus, the data was subject to principal component analysis (PCA). PCA was used to reduce the dimensionality of the data by combining the data within a group of new variables. It was performed by assigning the data sample from the cacao varieties treated with the different elicitors and setting the measured compounds (SA, SAG, caffeine, theobromine, (-)-epicatechin and (+)-catechin) as variables.

2.1.3. RESULTS

2.1.3.1. Endogenous levels of purine alkaloids and procyanidin monomers in flush leaves of greenhouse grown trees

On average, independent of cultivar, cacao flush leaves had 1.19 $mg \cdot g^{-1}$ dw total purine alkaloids. Theobromine levels were higher than caffeine levels, 0.66 $mg \cdot g^{-1}$ dw and 0.42 $mg \cdot g^{-1}$ dw respectively, but there was no significant statistical difference. There was also no significant statistical difference when comparing the total purine alkaloid content (theobromine and caffeine combined) of flush leaves among four greenhouse grown cacao cultivars. When comparing the cultivars based on each purine alkaloid separately, no statistically significant difference in the caffeine levels, which varied from 0.23 to 0.73 $mg \cdot g^{-1}$ dw, was observed. Theobromine levels were statistically higher in TSH leaves (1.57 $mg \cdot g^{-1}$ dw), followed by IMC (0.79 $mg \cdot g^{-1}$ dw), while AME (0.43 $mg \cdot g^{-1}$ dw) and SCA (0.27 $mg \cdot g^{-1}$ dw) had statistically similar lower content. For TSH and IMC genotypes, theobromine made up 75 % of total purine alkaloids while caffeine constituted 25 %. For SCA leaves, the proportions were just the opposite, with caffeine

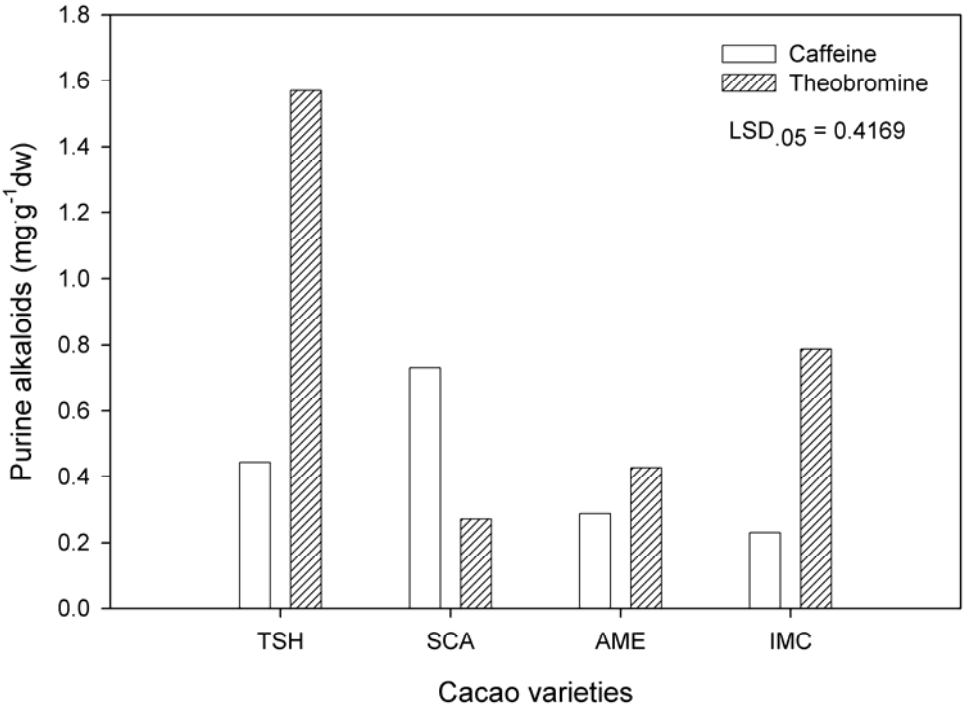
accounting for 75 % and theobromine 25 % of total purine alkaloids. AME flush leaves had 40 % caffeine and 60 % theobromine of the total purine alkaloid content (Figure 3).

Total procyanidin monomer content of cacao flush leaves averaged 3.48 $mg \cdot g^{-1}$ dw. (-)-Epicatechin averaged 2.33 $mg \cdot g^{-1}$ dw while catechin averaged 1.15 $mg \cdot g^{-1}$ dw. There was no significant statistical difference between cultivars when comparing their (-)-epicatechin content, which varied from 0.24 to 3.9 $mg \cdot g^{-1}$ dw, or their (+)-catechin content, which varied from 0.46 to 1.71 $mg \cdot g^{-1}$ dw. (-)-Epicatechin represented about 67 % of the procyanidin monomers for TSH, IMC and SCA, while (+)-catechin represented 67% of the procyanidin monomers in AME.

2.1.3.2. Levels of purine alkaloids and procyanidin monomers in flush leaves of greenhouse grown trees after application of chemical elicitors

BTH induced an increase of the procyanidin content of TSH flush leaves. AVG induced an increase of the purine alkaloid content of TSH flush leaves. When analyzing the components of each fraction separately it was observed that none of the chemical elicitors used increased caffeine or (+)-catechin contents of TSH flush leaves, but AVG induced an increase of the theobromine content by more than 60 % compared to control, while BTH induced an increase of (-)-epicatechin content by more than 60 % compared to control.

Figure 3. Purine alkaloid content of flush leaves of four greenhouse-grown cacao genotypes



The purine alkaloids caffeine and theobromine were not induced by application of elicitors to SCA flush leaves. Of the procyanidin monomers analyzed, only (-)-epicatechin increased by 67 % upon application of glutathione.

Caffeine, (-)-epicatechin, and (+)-catechin did not increase after elicitation in AME leaves. Only theobromine had a statistically significant increase of more than four fold upon application of BTH, rising from 0.43 $mg \cdot g^{-1}$ dw in control leaves to 1.9 $mg \cdot g^{-1}$ dw in BTH treated leaves.

Treatment with elicitors did not induce any changes in the purine alkaloid or procyanidin monomer content of IMC leaves.

2.1.3.3. Endogenous levels of SA and SAG in leaves of greenhouse-grown trees

Free salicylic acid content of IMC flush leaves was higher than the other cultivars analyzed. SAG content of AME flush leaves was similar to that of IMC flush leaves but significantly lower than SAG content of both SCA and TSH cultivars (Table 1).
SCA fully developed leaves had higher SA and SAG content than mature leaves of the other three varieties, which did not differ from each other (Table 1).

2.1.3.4. Levels of SA and SAG in leaves of greenhouse-grown trees after application of chemical elicitors

Free SA and SAG contents of TSH young and mature leaves did not vary upon application of chemical elicitors.

SAG was significantly lower in TSH mature leaves treated with AVG (SAG content of control leaves was 82.4 $ng \cdot g^{-1}$ fw and AVG treated leaves had 72.5 $ng \cdot g^{-1}$ fw SAG) ($LSD_{.05} = 8.16$).

Salicylic acid contents of SCA flush leaves did not change after elicitor application. Only free salicylic acid content of SCA old leaves decreased upon application of all four chemical elicitors (Figure 4).

Free SA and SAG content of AME flush leaves did not change upon chemical elicitor application.

Free salicylic acid content of AME fully developed leaves decreased upon application of BTH, glutathione and jasmonic acid. SAG content of AME mature leaves decreased upon application of BTH only.

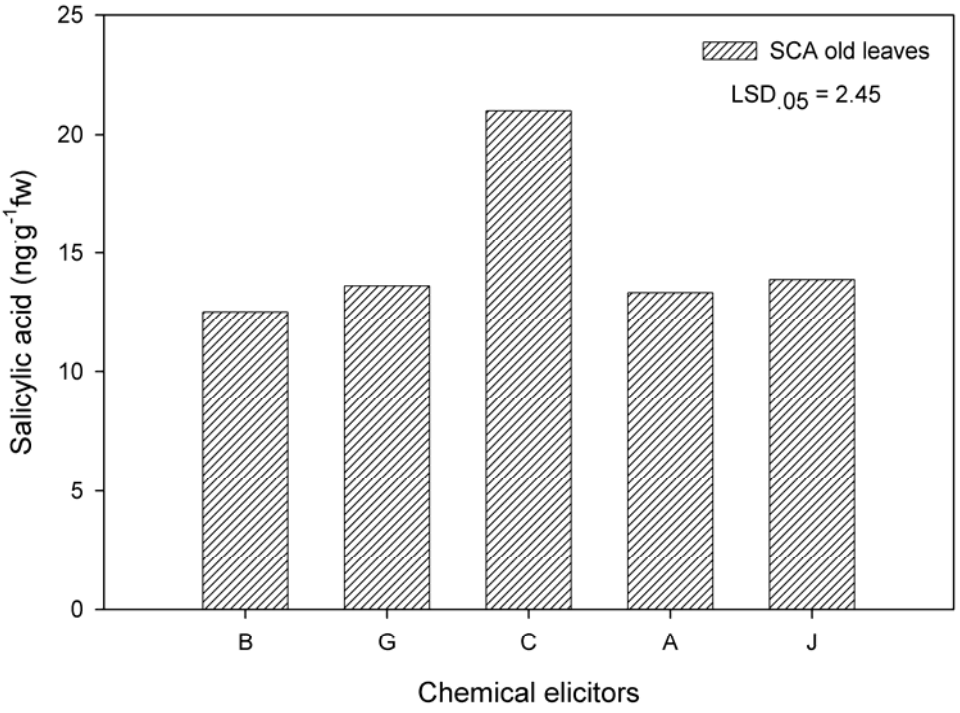
Free SA content of IMC flush leaves decreased upon application of BTH only. SAG content of IMC flush leaves decreased upon application of BTH and glutathione. Free salicylic acid content of IMC fully developed leaves increased upon application of AVG. SAG content of IMC mature leaves did not change upon application of elicitors.

Table 1. Leaf salicylic acid (SA) and salicylic acid glycoside (SAG) of four greenhouse-grown cacao genotypes IMC, AME, TSH and SCA.

Leaf Age	Cultivar	SA ($ng \cdot g^{-1}$ fw)	SAG ($ng \cdot g^{-1}$ fw)	Total (SA+SAG) ($ng \cdot g^{-1}$ fw)
Flush	IMC	23.1a*	130.7ab**	153.8
	AME	16.8b	99.7b	116.5
	TSH	16.5b	157.5a	174.0
	SCA	16.1b	167.2a	183.3
Mature	IMC	14.9b***	81.6b****	96.5
	AME	16.3b	81.4b	97.7
	TSH	14.0b	82.4b	96.4
	SCA	21a	105.6a	126.6

Mean separation within columns and by leaf age.
* $LSD_{.05} = 4.9$ ** $LSD_{.05} = 48.2$ *** $LSD_{.05} = 4.0$ **** $LSD_{.05} = 19.8$

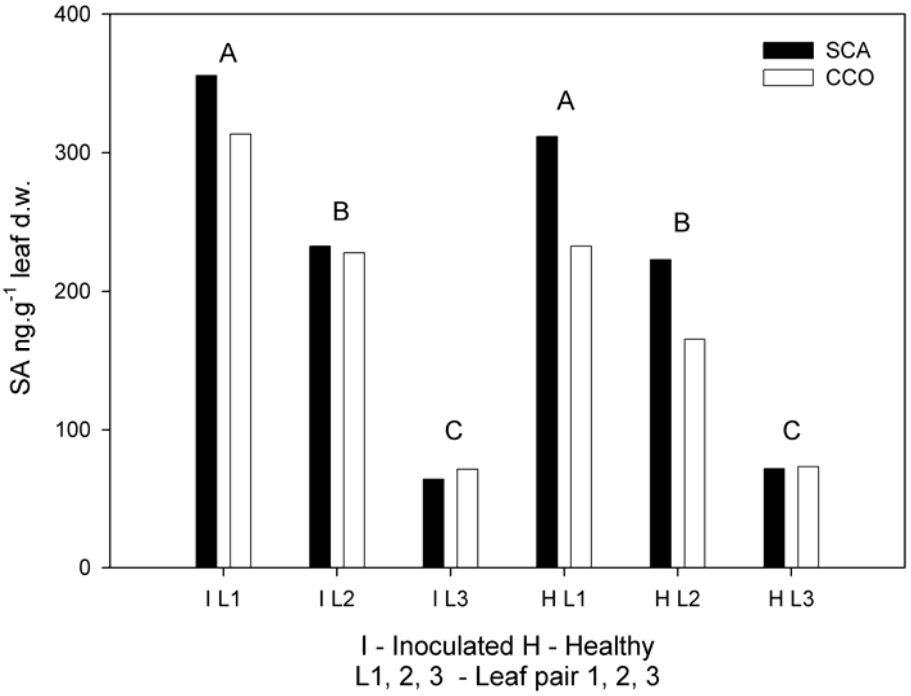
Figure 4. Free SA content in mature leaves of Scavina 6 (SCA) after treatment with chemical elicitors, benzothiadiazole (B), glutathione (G), control (C), aminoethoxyvinylglycine (A) and jasmonic acid (J)



2.1.3.5. Levels of SA and SAG in leaves of field-grown trees after pathogen inoculation

SA and SAG contents did not differ between the two cultivars tested (SCA and CCO). SA and SAG contents decreased with leaf age; SA and SAG contents of L1 were higher than L2, which in turn were higher than L3. Basidiospore inoculation did not alter SA and SAG contents in either cultivar (Figure 5; Table 2). All the results from this section about the levels of SA and SAG in leaves of field-grown trees after pathogen inoculation are summarized in Table 2.

Figure 5. Salicylic acid levels in young leaves



Different letters indicate significant difference among leaf pairs (IL1, IL2, IL3, HL1, HL2 and HL3) independent of genotype using F significance test at p 0.05.

Table 2. Leaf (L1) SA contents of field-grown trees inoculated (I) with basidiospores of *M. perniciosa* or uninoculated controls (C) and adjacent older leaf pairs L2 and L3*

Tissue	Cultivar	Trt	SA ($ng \cdot g^{-1}$ dw)	SAG ($ng \cdot g^{-1}$ dw)	SA+SAG ($ng \cdot g^{-1}$ dw)
Flush	SCA	I	217.4	1000.6	1218
		C	202.2	885.2	1087.4
	CCO	I	204.2	720.3	924.5
		C	157.2	517.0	674.2
L1	SCA	I	355.6	1711.8	2067.4
		C	311.9	1401.3	1713.2
	CCO	I	313.6	1099	1412.6
		C	232.6	743	975.6
L2	SCA	I	232.7	921.5	1154.2
		C	223	812.6	1035.6
	CCO	I	227.8	785.9	1013.7
		C	165.7	499.3	665
L3	SCA	I	64.2	368.6	432.8
		C	71.8	441.5	513.3
	CCO	I	71.3	276.1	347.4
		C	73.2	308.6	381.8

* No significant statistical differences were observed using the F test at P.05.

2.1.3.6. Changes in cacao procyanidin content throughout leaf development

The distribution of procyanidin oligomers, from monomer to decamer, found in cacao leaves combining all four varieties, is shown in Figure 6. The average monomer fraction including all leaf sizes was 21 $mg \cdot g^{-1}$ dw, about 30 % of total procyanidins. The following oligomers averaged respectively, from dimer to decamer, 16, 12, 11, 10, 8, 6, 4, 3, and 2 % of the total procyanidin content.

When comparing procyanidin contents of old versus flush leaves, the latter have significantly higher levels of both monomers and oligomers from dimer to decamer (Figure 7).

Total procyanidins of each leaf size for each cultivar is reported in Figure 8. AME leaves < 2 cm contained 55.7 $mg \cdot g^{-1}$ dw and there was a linear increase to the maximum of 96.1 $mg \cdot g^{-1}$ dw in leaves 15 to 20 cm long. Beyond that point procyanidin content started to decrease and reached its lowest level in the mature leaves, which contained 35.3 $mg \cdot g^{-1}$ dw. Similarly, total procyanidin content of TSH leaves < 2 cm was 54.5 $mg \cdot g^{-1}$ dw and reached a maximum in leaves 15 to 20 cm long with 85.4 $mg \cdot g^{-1}$ dw. TSH mature green leaves had the lowest level of total procyanidins with 33.7 $mg \cdot g^{-1}$ dw. IMC leaves < 2 cm contained 70.4 $mg \cdot g^{-1}$ dw total procyanidins, which peaked at 120 $mg \cdot g^{-1}$ dw in leaves 8 to 10 cm long. As IMC leaves developed further, the procyanidin levels decreased, reaching 27.7 $mg \cdot g^{-1}$ dw when leaves were fully mature.

Figure 6. Procyanidin content of cacao leaves

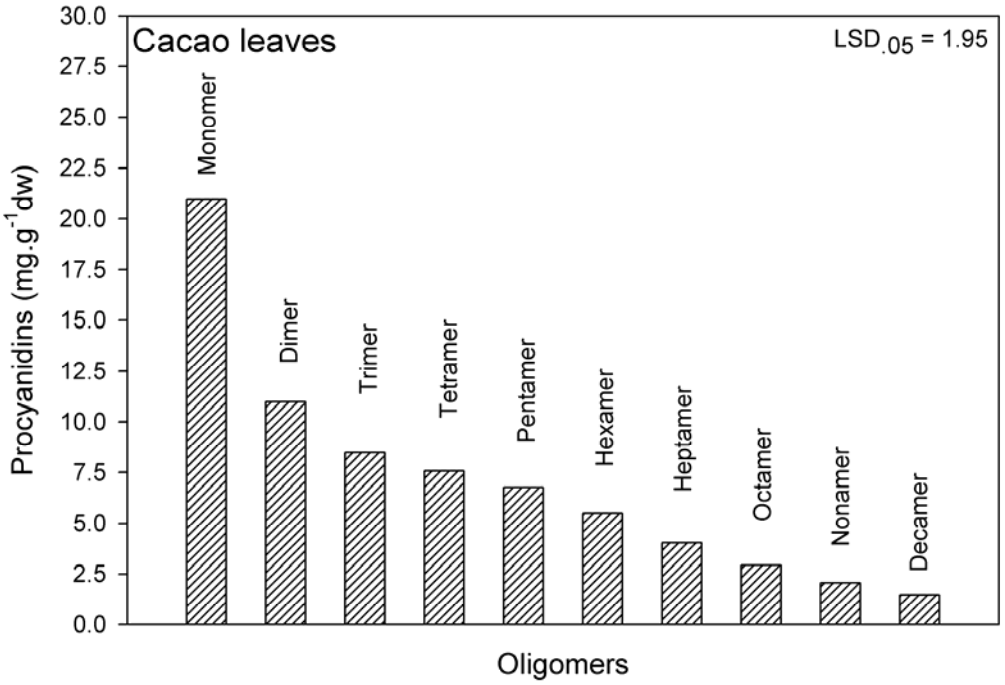


Figure 7. Comparison of total procyanidin content in flush and old leaves of cacao

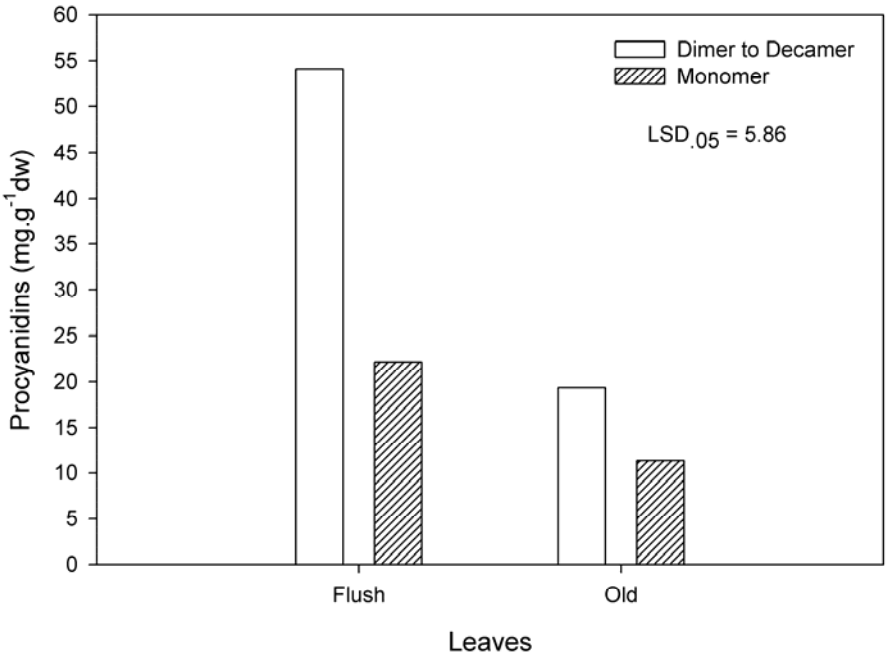
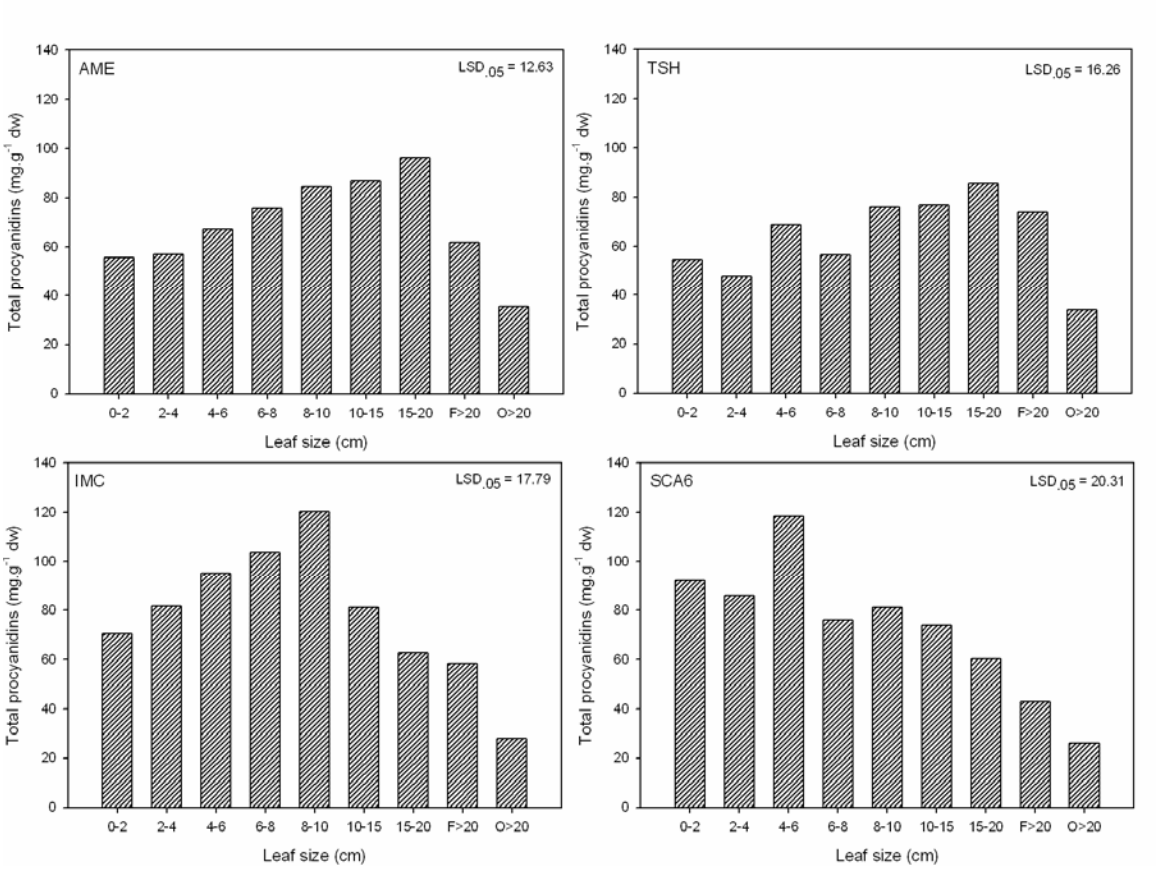


Figure 8. Total leaf procyanidin content throughout leaf development for four cacao varieties Amelonado, (AME), IMC 30 (IMC), TSH 565 (TSH) and Scavina 6 (SCA6)



SCA leaves < 2 cm had 92 mg·g^{-1} dw total procyanidins, and the highest levels were found in leaves 4 to 6 cm long at about 118.3 mg·g^{-1} dw. Similar to the other varieties, as SCA leaves aged the levels declined reaching 25.9 mg·g^{-1} dw when fully mature.

SCA, which is the most disease tolerant genotype, had the highest levels of monomers (31.1 mg·g^{-1} dw) and oligomers (67.6 mg·g^{-1} dw). These levels were significantly higher compared to the other more disease susceptible varieties, which had 39 % (AME) to 43 % (TSH) less total procyanidins in leaves that are susceptible to witches' broom disease (Figure 9).

2.1.3.7. Effect of pathogen inoculation on cacao leaf procyanidins

Table 3 shows the overall comparison of procyanidin monomers and oligomers present in SCA and CCO leaves before and after *M. perniciosa* basidiospore inoculation. Procyanidin monomers and oligomers significantly increased in L1 in SCA while only the oligomers were statistically higher in L2 of SCA leaves upon treatment.

Total procyanidin content of cacao leaves averaged 64 mg·g^{-1} dw. The average levels for each procyanidin fraction from monomer to decamer was the following: monomer 26.6 mg·g^{-1} dw (42 %), dimer 11.5 mg·g^{-1} dw (18 %), trimer 9 mg·g^{-1} dw (14 %), tetramer 6.7 mg·g^{-1} dw (10 %), pentamer 4.5 mg·g^{-1} dw (7 %), hexamer 3 mg·g^{-1} dw (5 %), heptamer 1.6 mg·g^{-1} dw (2 %), octamer 0.8 mg·g^{-1} dw, nonamer 0.4 mg·g^{-1} dw and decamer 0.002 mg·g^{-1} dw (octamer to decamer add up to 2 % of total procyanidins).

Figure 9. Procyanidin content of cacao flush leaves susceptible to witches' broom disease (< 6 cm) for four cacao varieties: AME, IMC, TSH and SCA

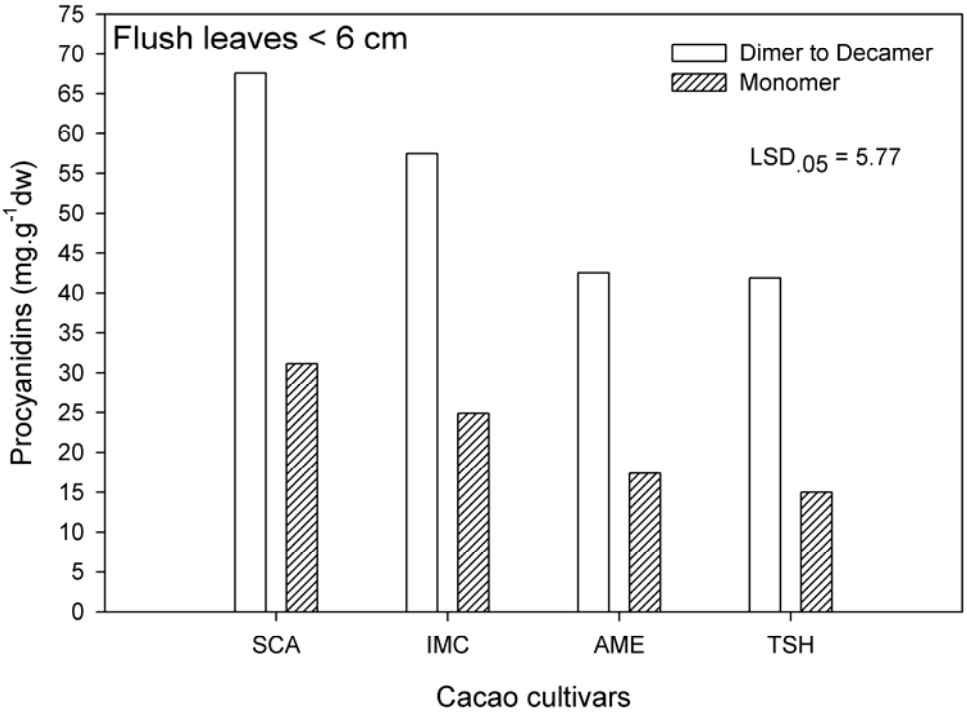


Table 3. Effect of inoculation with basidiospores of the pathogen *Moniliophthora perniciosa* on the procyanidin content (mg·g^{-1} dw) of flush leaves of field-grown disease resistant (SCA) and disease susceptible (CCO) cacao trees.

Cultivar	**SCA**				**CCO**			
Treatment	**Control**		**Inoculated**		**Control**		**Inoculated**	
Leaf pair	***Monomers***	***Oligomers***	***Monomers***	***Oligomers***	***Monomers***	***Oligomers***	***Monomers***	***Oligomers***
L1[z]	30.33	45.10	65.61	89.91	18.01	25.41	43.80	51.43
L2	17.17	28.50	39.03	65.85	13.30	24.20	38.65	45.36
L3	13.89	19.35	14.32	19.63	12.44	16.66	12.83	17.42

[z] inoculated leaf pair.
$LSD_{.05}$ = 30.06

F- test results presented in Table 4 indicate significant differences in the total procyanidin content for basidospore treatment ($Pr > F = 0.0036$) and leaf age ($Pr > F = 0.0021$), as well as for comparisons with the monomer and oligomer fractions separately. Although there is a trend for differences between cultivars when comparing the total procyanidin content ($Pr > F = 0.0930$), the only statistically significant difference ($Pr > F = 0.0467$) between cultivars was found when comparing the oligomer procyanidin fraction. Procyanidin oligomers, which represented 58 % of total procyanidins in the analyzed leaves, were statistically higher in SCA when compared to CCO.

Leaves from trees of both varieties inoculated with basidiospores had an average of 84 $mg \cdot g^{-1}$ dw total procyanidins, which was significantly higher than the 44.1 $mg \cdot g^{-1}$ dw total procyanidin content of leaves from control trees (treatment significance observed in Table 4).

Table 4 indicates a significant difference for the basidiospore treatment, but only SCA inoculated leaves had higher total procyanidins than SCA control leaves. CCO inoculated leaves did not differ statistically from CCO control leaves.

Taken together, all L1 samples from both cultivars, whether inoculated or not, had on average 92.4 $mg \cdot g^{-1}$ dw procyanidin, L2 leaves had 68 $mg \cdot g^{-1}$ dw and did not differ statistically from L1. In contrast, L3 leaves averaged 31.6 $mg \cdot g^{-1}$ dw procyanindins, which was significantly lower than the two younger pairs.

Independent of cultivar, the first leaf pair of control branches had levels similar (not statistically different) to the other two pairs; whereas in leaves from inoculated branches, the inoculated pair (L1) and the adjacent leaf pair (L2) had a total procyanidin content higher than the third leaf pair (L3). The inoculated leaf pair (L1) had statistically

higher total procyanidins than the L1 control, each with 125.4 $mg \cdot g^{-1}$ dw and 59.4 $mg \cdot g^{-1}$ dw respectively. Leaf pair 2 on the inoculated branch had statistically higher total procyanidins than the L2 control, each with 94.4 $mg \cdot g^{-1}$ dw and 41.6 $mg \cdot g^{-1}$ dw respectively. Leaf pair 3 on the inoculated branch had statistically similar total procyanidins as the L3 control, each with 32.1 $mg \cdot g^{-1}$ dw and 31.2 $mg \cdot g^{-1}$ dw respectively.

2.1.3.8. PCA analysis comparing caffeine, theobromine, (+)-catechin, (-)-epicatechin, salicylic acid, and salicylic acid glycoside levels of flush leaves treated with elicitors

The variables 1 to 6 were theobromine, (+)-catechin, caffeine, (-)-epicatechin, salicylic acid and salicylic acid glycoside. PCA 1 represented 60.61 % of total variation and it was mainly explained by the contributions from variables 1, 2, 3, 4, 6, while, PCA 2 represented 16.18 % of total variation and its main contribution came from variable 5. PCA 3 represented 11.59 % of total variation and the majority of the variation came from variable 6. Plotting PCA 1 with PCA 2, PCA 1 with PCA 3 or PCA 2 with PCA 3 separated the samples in clusters by genotype. In figure 10, where PCA 1 was plotted with PCA 2, AME and SCA clusters are grouped closer based on influence of the SA variable of the PCA 2. In figure 11, which shows a PCA 1 versus PCA 3 plot, the SCA cluster is separated from the AME cluster mostly based on the SAG contribution. PCA 2 and PCA 3 combined explain less then 18 % of the variation among the variables (Figure 12).

Table 4. Analysis of variance by F-test comparing leaf procyanidin contents under different treatments

Monomers	F Value	Pr > F	Oligomers	F Value	Pr > F	Total Proc.	F Value	Pr > F
Model	2.81	0.0157	Model	3.12	0.0092	Model	2.99	0.0115
Replicates	0.27	0.7636	Replicates	0.39	0.6791	Replicates	0.34	0.7142
Treatment	**11.27**	0.0029	Treatment	**9.78**	0.0049	Treatment	**10.60**	0.0036
Leaf age	**7.72**	0.0029	Leaf age	**8.58**	0.0018	Leaf age	**8.29**	0.0021
Cultivar	1.62	0.2169	Cultivar	**4.44**	0.0467	Cultivar	3.08	0.0930
Trt*age	2.83	0.0808	trt*age	2.39	0.1146	trt*age	2.62	0.0953
Trt*cult	0.03	0.8543	trt*cult	0.69	0.4167	trt*cult	0.30	0.5871
Age*cult	0.88	0.4274	age*cult	1.25	0.3057	age*cult	1.07	0.3600
trt*age*cult	0.13	0.8804	Trt*age*cult	0.19	0.8292	trt*age*cult	0.11	0.8921

Numbers is bold are statistically significant.

Figure 10. PCA analysis (PCA 1 x PCA 2) of six metabolites (variables) present in four cacao genotypes (AME, IMC, SCA and TSH) subjected to treatments with chemical elicitors (A – aminoethoxyvinylglycine, B – benzothiadiazole, C – control, G – gluthatione, J – jasmonic acid)

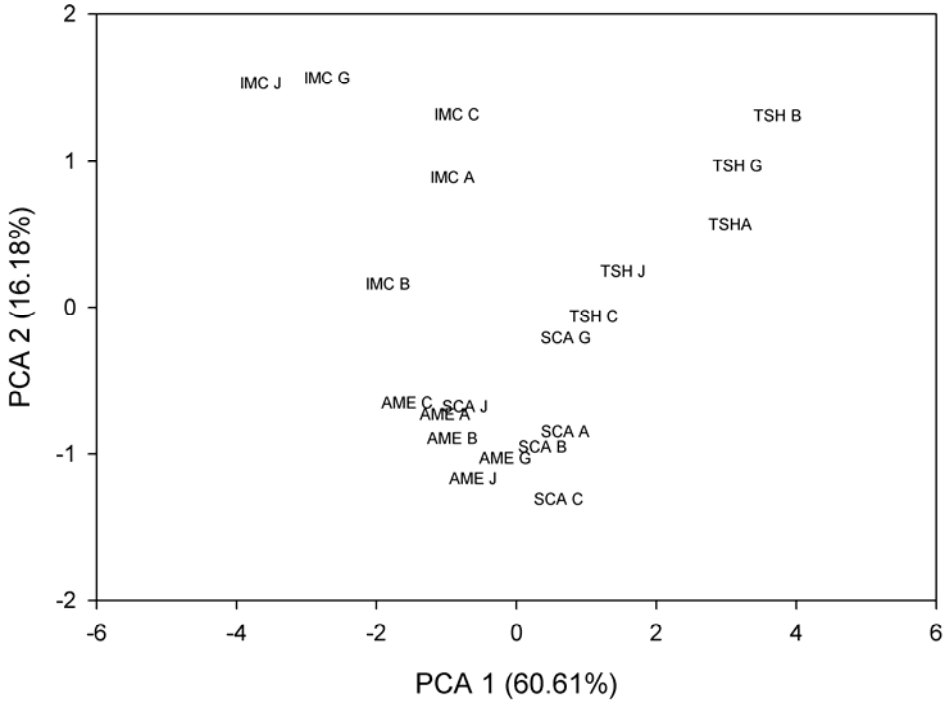


Figure 11. PCA analysis (PCA 1 x PCA 3) of six metabolites (variables) present in four cacao genotypes (AME, IMC, SCA and TSH) subjected to treatments with chemical elicitors (A – aminoethoxyvinylglycine, B – benzothiadiazole, C – control, G – gluthatione, J – jasmonic acid)

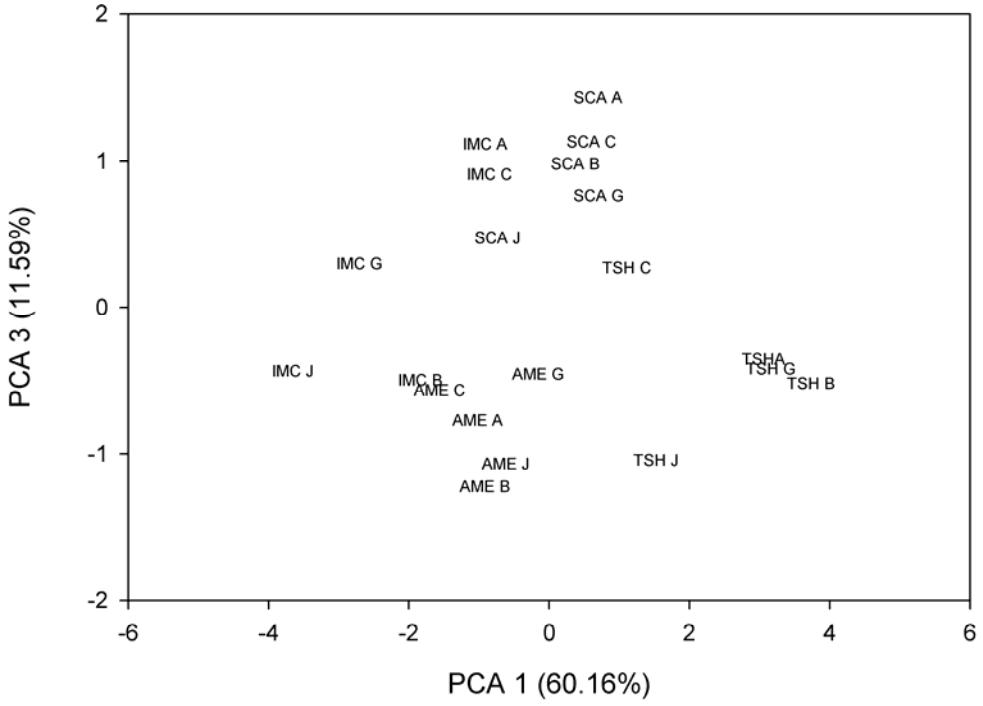
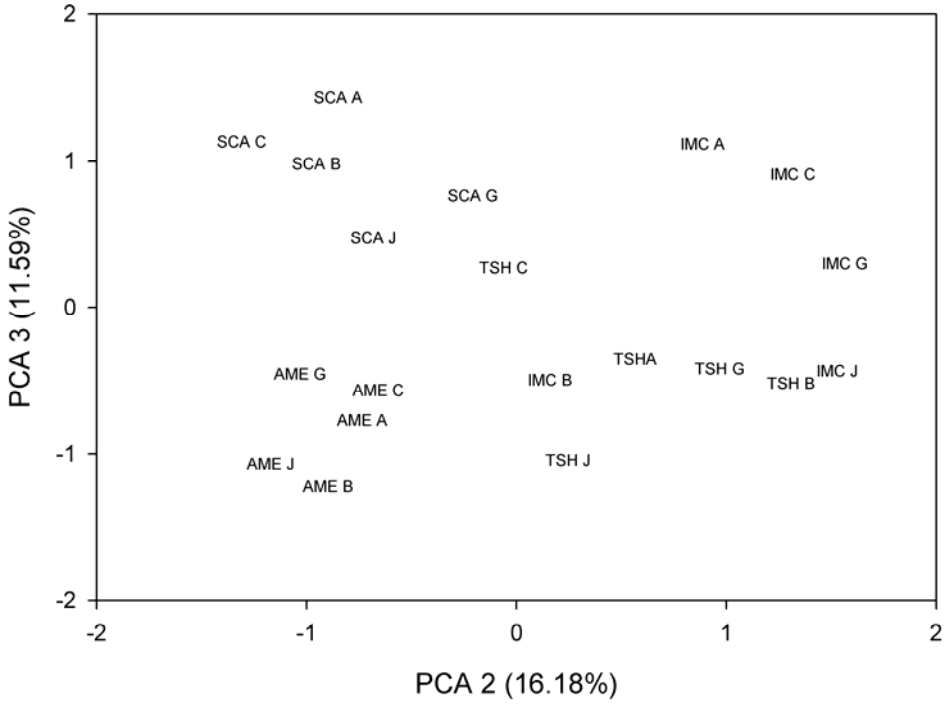


Figure 12. PCA analysis (PCA 2 x PCA 3) of six metabolites (variables) present in four cacao genotypes (AME, IMC, SCA and TSH) subjected to treatments with chemical elicitors (A – aminoethoxyvinylglycine, B – benzothiadiazole, C – control, G – gluthatione, J – jasmonic acid)



2.1.4. DISCUSSION

2.1.4.1. Effect of chemical elicitors on purine alkaloids and procyanidin monomers

In order to measure flavonoids several spectrophotometric methods have been employed including, Folin-Ciocalteu, acid/butanol assay, $AlCl_3$ assay, TEAC, FRAP. These are indirect measurements based upon the antioxidant ability of polyphenols (Luximon-Ramma et al., 2002). Direct measurements can be achieved using HPLC. Purine alkaloids and procyanidins have been analyzed simultaneously using reverse phase (RP) HPLC (Tokusoglu and Unal, 2002). Following the same direct approach , caffeine, theobromine, (-)-epicatechin and (+)-catechin were analyzed concurrently by RP-HPLC using a UV detector.

Theobromine levels (0.66 $mg \cdot g^{-1}$ dw) were found higher than caffeine levels (0.42 $mg \cdot g^{-1}$ dw) in cacao flush leaves. There were no differences in caffeine levels of flush leaves among the studied genotypes. Theobromine was higher in leaves of TSH followed by IMC, AME and SCA. While theobromine was 60 % in AME leaves it was 75 % of total purine alkaloid in TSH and IMC leaves, but was only 25 % in SCA leaves; thus caffeine levels were 40 % in AME, 75 % in SCA leaves and 25 % in TSH and IMC. Caffeine is known to be toxic to *M. perniciosa*, while theobromine is not (Aneja and Gianfagna, 2001). The higher proportion of caffeine in SCA leaves could be related to its higher disease resistance against *M. perniciosa* when compared to the other genotypes. However, Hammerstone et al. (1994) observed that caffeine levels are sometimes higher sometimes lower than theobromine depending on the leaf age. They found that the

youngest leaf on a branch had 1.73 $mg \cdot g^{-1}$ dw and 0.56 $mg \cdot g^{-1}$ dw of theobromine and caffeine respectively. With increasing leaf age, theobromine levels decreased but caffeine levels went up (0.65 $mg \cdot g^{-1}$ dw caffeine when theobromine is 0.57 $mg \cdot g^{-1}$ dw) before decreasing again to 0.28 $mg \cdot g^{-1}$ dw of caffeine and 0.15 $mg \cdot g^{-1}$ dw of theobromine in the oldest leaves. This could suggest that as leaves age, theobromine is converted to caffeine and then caffeine is later degraded to other compounds. Caffeine could have a defensive role in the plant when in sufficient concentration. Koyama et al. (2003) observed that more than 75 % of the purine alkaloids disappeared with leaf development and determined that purine alkaloid biosynthesis occurs only in young cacao leaves further suggesting a role in defense against some pathogens and insects, which preferentially attack these younger tissues. By the time caffeine levels drop, the leaf has acquired other defensive mechanisms and that is possibly the reason why older leaves are much less susceptible to disease.

Leal et al. (2007) found differential gene expression of a caffeine synthase gene and polyphenol biosynthesis related genes upon infection of cacao varieties with different levels of resistance to witches' broom disease. Scarpari et al. (2005) observed an increase in purine alkaloids during witches' broom development although they did not see changes in polyphenol levels. Aneja and Gianfagana (2001) also observed an increase in caffeine in cacao shoots infected with the fungus *Moniliophthora perniciosa*. Nojosa et al. (2003) suggested that phenolic compounds are involved in defense mechanisms of cacao against *M. perniciosa* because there are differences in the levels of total phenolics between resistant and susceptible clones in young and mature tissues. In this experiment cacao

flush leaves were found to contain 2.33 $mg \cdot g^{-1}$ dw (-)-epicatechin and 1.15 $mg \cdot g^{-1}$ dw (+)-catechin. There was no statistical significant difference between the studied cultivars. Beno-Moualem et al. (2001) showed induction of (-)-epicatechin synthesis in avocado by application of cytokinins. With grapes, BTH increased anthocyanin levels and improved resistance against the fungal pathogen *Botrytis cinerea* (Iriti et al., 2004). Verica et al. (2004) observed up-regulation of defense genes in cacao leaves treated with BTH.

During this project, the treatment with elicitors to leaves of greenhouse grown trees induced accumulation of theobromine and (-)-epicatechin only. BTH induced (-)-epicatechin in TSH leaves, and theobromine in AME leaves. AVG induced theobromine in TSH leaves and glutathione induced (-)-epicatechin in SCA leaves.

Cell culture and protoplast have been shown to respond similarly to whole plant systems when treated with elicitors (Gundlach et al., 1992; Edwards et al., 1991; Robbins et al., 1991). The detached leaf experiment also allows for more replication, thus is a controlled system unlike field or whole plant experiments which are subject to other variables that could interfere in the results. However, the results obtained upon application of chemical elicitors to cacao flush leaves did not show a clear pattern of induction across genotypes nor an inductive effect that was compound specific.

Although, a detached leaf assay with cacao has shown positive results in the past (Aneja and Gianfagna, 2001), for this study the results were variable. A chemical elicitor treatment to intact leaves while still on the tree may provide results that are more consistent.

2.1.4.2. Effect of chemical elicitors and pathogen inoculation in free SA and SAG of cacao leaves

Plant response to infection, wounding or other stresses caused either by biotic or abiotic factors is usually more effective in young, growing tissues. However, the salicylic acid responses observed upon chemical elicitor application happened mainly in older leaves. Older leaves are thicker and sturdier compared to the thin and tender young cacao leaves. They seem to senesce later than the flush leaves, which wilt and start to turn brownish at the edges much sooner when placed in the chemical elicitor solution after detachment and cutting. Possibly, for this reason older leaves were able to response to elicitors more effectively than young flush leaves. Except for young flush leaves of IMC all other genotypes did not vary in their SA or SAG contents upon application of chemical elicitors. IMC young leaves treated with BTH had reduced SA and SAG content, while glutathione treatment induced a reduction in the SAG content only. For the most part, chemical elicitor treatments reduced the SA contents of mature cacao leaves. SAG content of TSH mature leaves was reduced upon application of AVG, while free SA did not vary. Free SA content of SCA fully developed leaves was lower in all treatments, while SAG did not change. In AME mature leaves, BTH caused a reduction of free SA and SAG contents, while glutathione and jasmonic acid reduced free SA content of the treated leaves. Only IMC mature leaves had free SA levels increased when treated with AVG.

Plant disease resistance involves activation of a series of defense pathways that try to counterattack the pathogen. However, it may not be desirable for plants to launch

defenses that are metabolically costly if they are not effective. Therefore, plants have regulatory mechanisms to activate particular defenses according to the specific stimuli or stresses that they are subjected to. Some of these pathways can be opposing, down regulating each other, while others work in combination where the activation of one triggers another. For some plants, including cacao, it may not be advantageous to activate the SA pathway when attacked by necrotrophic pathogens. *M. perniciosa* is a hemibiotrophic fungus, with a biotrophic and necrotrophic phase. By avoiding the SA pathway cacao is preventing cell death. This allows the plant to avoid inducing the phase transition of the fungus from biotroph to necrotroph, which at the same time may provide for activation of other more effective defenses. This suggestion is supported by the negative results observed in induction of SA and SAG levels in young leaves followed by application with elicitors. While, SA and SAG levels increase in parallel after TMV infection in tobacco (Hennig et al., 1993), young cacao leaves maintained the same SA and SAG levels of the controls after *M. perniciosa* basidiospore inoculation, confirming that the plant's response to *M. perniciosa* infection is likely induced by a pathway other than SA (Figure 5).

Although there was no increase in SA in the inoculated leaves, the molecule could affect nearby tissues since it is mobile in the plant. This was not the case, as there was no increase in free SA or SAG levels found in non-inoculated leaves on the same flush as the inoculated ones (Figure 5).

Endogenous SA and SAG levels of both tested cultivars including the more disease tolerant SCA and the more susceptible CCO, did not differ statistically (Figure 5), reinforcing that SA levels are not indicative of resistance to *M. perniciosa* in cacao.

Although *M. perniciosa* preferentially infects the young meristematic tissue, both varieties of cacao had the highest levels of free SA and SAG in the youngest tissues independent of inoculation (Figure 5).

2.1.4.3. Leaf size experiment

Cacao flush leaves are thin and tender, and can be pale green or pink, and only when fully expanded become dark green, thicker and sturdier. Among the varieties studied, IMC 30 (IMC) was the only one with light green flush leaves, while the others, Scavina 6 (SCA), TSH 565 (TSH) and Amelonado (AME) had pink flush leaves.

The distribution of procyanidin oligomers, from monomer to decamer, found in cacao leaves combining all four varieties, averaged respectively, from monomer to decamer, 30, 16, 12, 11, 10, 8, 6, 4, 3, and 2 % of the total procyanidin content. The monomer fraction averaged 21 $mg \cdot g^{-1}$ dw. The levels of procyanidin monomers found in the analyzed leaves was relatively similar to that found in cocoa beans as reported by Kim and Keeney (1984) who found (-)-epicatechin ranging from 22 to 43 $mg \cdot g^{-1}$ dw in defatted unfermented beans, and determined that the monomer made up about 35 % of total bean procyanidins.

Fully developed leaves had significantly lower procyanidin contents than young leaves (Figure 7). These results followed the same trend observed by Osman et al. (2004) who measured only the monomer and found (-)-epicatechin content higher in the younger leaves.

Morphological and physiological differences between mature and flush leaves are evident and therefore analysis of cacao leaves in these two groups is obvious. However, there may be significant differences within flush leaves of different developmental stages. For that reason, flush leaves were separated into nine categories based on leaf size.

The levels of the very young leaves are intermediary and peak as leaves attain lengths of 4 to 20 cm; but as the leaves increase in size and develop further, the procyanidin content reaches its minimum.

Only the very young leaves up to about 5 cm are susceptible to infection by the fungus *Moniliophthora perniciosa*, the causal agent of witches' broom disease (Purdy and Schimidt, 1996). Of the four cacao varieties used in this study, SCA was the most disease tolerant variety, whereas AME was the most disease susceptible.

Both induced and constitutively present flavonoids have been associated with plant resistance to biotic and abiotic stresses (Harborne and Williams, 2000). Andebrhan et al. (1995) and Brownlee et al. (1990) demonstrated that purified cacao bean procyanidins and cacao shoot extracts containing procyanidins have antifungal effects against *Moniliophthora perniciosa*. Both extracts inhibited spore germination and germ tube elongation; however there was no effect on the growth of the saprophytic mycelia.

Leaves of SCA, the most disease resistant of the studied genotypes, had significantly higher levels of monomers and oligomers compared to the other more disease susceptible genotypes, at a stage when cacao leaves are susceptible to witches' broom disease (< 6 cm) (Figure 9).

2.1.4.4. Effect of pathogen inoculation in procyanidin levels

Plant response to infection usually involves activation of defenses triggered by signaling molecules, such as salicylic acid (SA), which result in phytoalexin accumulation and the hypersensitive reaction, a form of programmed cell death that will often stop disease development. It was showed earlier (Figure 5) that SA levels in cacao did not increase upon infection by *M. perniciosa*. In contrast, caffeine, which is toxic to *M. perniciosa*, does increase in cacao leaves upon chemical elicitor application and basidiospore inoculation (Aneja and Gianfagna, 2001). Purine alkaloid analysis upon pathogen inoculation was not done because although the analytical procedure allowed for detection of caffeine and theobromine, it was not possible to quantify them. Moreover this study had been previously done (Aneja and Gianfagna, 2001); but caffeine is likely only one of many phytoalexins used by the plant to defend itself. Leal et al. (2007) found genes that were differentially expressed in *Theobroma cacao* and associated with resistance to witches' broom disease, including a caffeine synthase gene, and genes related to the biosynthesis of proanthocyanidins. In figure 9, it can be seen that cacao leaves have as much procyanidins as cocoa beans, including monomers and oligomers from dimer to decamer. Earlier, Andebrhan et al. (1995) and Brownlee et al. (1990) had shown that cacao procyanidins have fungitoxic effects on basidiospore germination and germ tube elongation of *M. perniciosa*.

The overall comparison of all analyzed cacao leaves in this study (Table 3) shows that procyanidins increase upon infection with basidiospores of *M. perniciosa,* but the

increase was only statistically significant for the more disease resistant genotype Scavina 6.

Even though only leaf pair one (L1) had been inoculated with basidiospores, the next adjacent leaf pair (L2) also showed elevated levels of procyanidins indicating a systemic induction of procyanidins. The systemic increase of procyanidins occurred within 48 hours after inoculation with basidiospores, it was measured in the adjacent leaf pair (L2) only, and the systemic signal did not reach the next leaf pair (L3).

The results indicate enhanced procyanidin leaf content as a mechanism of defense used by *T. cacao* against infection by *M. perniciosa*. Moreover, Scavina 6, the more disease tolerant genotype had constitutively higher levels of procyanidin oligomers than CCO, which is more disease susceptible. These findings are consistent with the results of Leal et al. (2007), who observed activation of proanthocyanidins biosynthesis genes differentially expressed in genotypes with different levels of resistance to witches' broom disease. Selection of genotypes with high constitutive and inducible levels of procyanidins, and the development of chemical treatments to increase procyanidin content prior to infection, may reduce the incidence and severity of this disease in cacao.

2.1.5. CONCLUSIONS

Phytoalexins are one of several plant defenses against pathogens. Comparing genotypes with different levels of disease resistance based on levels of single compounds may not reflect their full defensive capacity because the effective response may require concurrent production of several compounds. The measurements of caffeine, theobromine, (-)-epicatechin and (+)-catechin, salicylic acid, and salicylic acid glucoside levels of cacao flush leaves varied within varieties before and after treatment with chemical elicitors. When analyzed separately it was impossible to determine whether any compound or elicitor treatment had a predominant role in disease resistance. However, the PCA analysis comparing the four studied cacao genotypes with different levels of disease resistance, each treated with four chemical elicitors, based on the content of six measured compounds, showed a trend in which the samples were clustered by genotype rather than by elicitor treatment. This clustering demonstrated a stronger genotype influence rather than elicitor treatment on the chemical profile content of cacao leaves. This example of chemical profiling allowed for the separation of genotypes with different levels of resistance based on their overall ability to produce phytoalexins.

In general, chemical elicitor or leaf inoculation with *M. perniciosa* basidiospores did not alter leaf salicylic acid or salicylic acid glucoside contents of young flush leaves. For some plants, including cacao, it may not be advantageous to activate the SA pathway when attacked by necrotrophic pathogens. *M. perniciosa* is a hemibiotrophic fungus, with a biotrophic and necrotrophic phase. By avoiding the SA pathway cacao is preventing cell death. This allows the plant to avoid inducing the phase transition of the fungus from

biotroph to necrotroph, which at the same time may provide for activation of other more effective defenses (Chaves and Gianfagna, 2006).

Cacao bean procyanidins have recently received much attention for their potential to improve human health (Schroeter et al., 2006; Selmi et al., 2006). However, the presence of these compounds in other plant parts as well as the role of these procyanidin oligomers in plant health has not been investigated. Flavan-3-ol monomers and oligomers from dimers to decamers were quantitatively determined by HPLC-FLD in leaves, throughout development, of four *Theobroma cacao* varieties. The levels of the very young leaves were intermediary and peaked as leaves attained lengths of 4 to 20 centimeters. As the leaves increased in size and developed further, the procyanidin content reached its minimum. Cacao flush leaves had higher procyanidin content than mature leaves.

Plant disease resistance involves activation of a series of defense pathways to counterattack the pathogen. Understanding the defense mechanisms by which the plant responds to pathogen attack will elucidate molecular markers useful for selecting genotypes with higher disease resistance attributes, and/or suggest alternative disease control strategies.

Witches' broom disease tolerant cultivar Scavina 6 had the highest levels of procyanidins when compared to Amelonado, TSH 565, and IMC 30, more disease susceptible genotypes, at a stage when leaves are susceptible to infection. Moreover, leaves of SCA 6 responded to *M. perniciosa* basidiospore infection by inducing accumulation of procyanidins in both the infected and adjacent uninfected leaves of

actively growing shoots, demonstrating an inductive response effect both locally and systemically.

Combined evidence for activation of procyanidin gene expression (Leal et al., 2007), antifungal effects of procyanidins against the witches' broom pathogen (Andebrhan et al., 1995; Brownlee et al., 1990), higher endogenous levels of procyanidins in leaves of a disease resistant genotype, including a peak in procyanidin levels during leaf development at a stage when leaves are susceptible to witches' broom disease, and the local and systemic increase in procyanidins in cacao leaves of a disease resistant genotype upon infection with basidiospores indicate a role for procyanidins in defense against *Moniliophthora perniciosa*. Therefore, procyanidin content of flush leaves is a potential marker for selecting for disease tolerance in cacao (Chaves and Gianfagna, 2007).

2.2. ENDOPHYTES AND THEIR METABOLITES

2.2.1. INTRODUCTION

Endophytes are microorganisms that grow within plant tissues without causing disease or negative impact on plant growth. What are the effects of the presence of the endophytes on the plant? Do they protect against pathogens? There are examples of endophytes associated with grasses that protect them against herbivory by producing alkaloids (White and Morgan-Jones, 1996). Fine fescues inoculated with endophytes suppressed dollar spot disease caused by *Sclerotinia homeocarpa* (Clarke et al., 2006). In some tree species, it has also been shown that insects avoid leaves with high endophytic incidence (Wilson and Carroll, 1997). In cacao, endophyte inoculation renders plants more resistant against *Phytophthora* sp. (Arnold et al., 2003). As a result, endophytes emerge as potential biological agents of disease control. In order to better understand and fully utilize endophytes for disease control it is important to establish the mechanisms by which they assist plants against pathogens.

Biological control using endophytes may occur through one or a combination of the following processes: antibiosis, competition, induction of defenses, and parasitism. Along with the ability to synthesize antimicrobial compounds, some endophytes produce antimicrobial compounds that are also mammalian toxins. While these toxins may be important in protecting the plants against pathogens, they are also a serious threat to human health. Among the most toxic and commonly present compounds in foods and feedstuffs are aflatoxins and ochratoxins. In general, contamination of plant products

such as grains, coffee berries or cocoa beans occurs post-harvest. However, the opportunistic organisms that infect crops post-harvest could be present endophytically in the plant during its development. Endophytes in some plant species can be transferred vertically to the next generation through seeds or horizontally to other plants by both spore and mycelium inoculation.

Before any endophytes can be seriously considered as biocontrol agents it must be first determined whether they can produce any beneficial compounds or toxins. A collection of endophytes found in coffee leaves (*Coffea arabica*), which consisted largely of *Aspergillus* and *Penicillium* species (Eurotiales: Trichocomaceae), was used to determine if the species and strains found as endophytes tended to be toxin producers. Moreover, the possibility exists that parasitoids used for biocontrol of coffee berry borer (CBB) and cocoa pod borer could carry spores of toxigenic fungi on their cuticle and serve as a source of fungal infection. To study this interaction, fungi that were isolated from a parasitoid wasp of the CBB were tested for ochratoxin production.

It has been proposed that some plant pathogens may have originated from endophytic strains that acquired pathogenicity factors and became capable of causing disease in the host (Aime and Phillips-Mora, 2005; Evans et al., 2003). At times, the separation between an endophyte, a pathogen, and an opportunistic organism, which is commonly a saprophyte (but given the right conducive environmental conditions can colonize plants under stress and cause plant death), serves as a means of classification because the difference from one role to another may be only temporal and circumstantial.

In order to study the interactions between endophytes and plant pathogenic fungi and to identify a potential biocontrol organism, dual culture plate assays, covered culture

assays, and bioassay tests for volatile antimicrobials were used with the aim of characterizing *in vitro* endophyte-pathogen interactions and possible antagonism. For the identification of mycotoxins and other metabolites released in culture, fungi were grown in different media, and after solvent extraction, samples were analyzed by HPLC and GC-MS.

2.2.2. MYCOTOXINS PRODUCED BY COFFEE ENDOPHYTES

Penicillium and *Aspergillus* species cause molding and rotting of grains and beans and are known to produce a diverse range of secondary metabolites along with toxins such as aflatoxins and ochratoxins (OT) (Figure 13). Ochratoxin A (OTA, 7-carboxy-5-cloro-8-hydroxy-3,4-dihydro-3R-methyliscoumarin-7-L-β-phenylalanine), one of the most widespread mycotoxins, is found in a number of processed foods including coffee and cocoa products (Bonhevi, 2004). Other compounds with similar chemical structure have been identified. One such compound, ochratoxin B (OTB, 7-carboxy-8-hydroxy-3,4-dihydro-3R-methylisocoumarin-7-L- β-phenylalanine), is a derivative of OTA that appears to be involved in the biosynthetic pathway of OTA as a precursor and/or metabolite (Harris and Mantle, 2001). Although OTB is not as toxic as OTA, both had significant oral LD50 in day-old chicks of 54 $mg \cdot kg^{-1}$ and 3.3 $mg \cdot kg^{-1}$, respectively (Cole and Cox, 1981).

Generally, crop contamination occurs post-harvest and the fungal infestation and OT levels vary with the drying process of the harvested plant material (Taniwaki et al., 2003). In order to avoid post harvest contamination, some important general protective measures include maintaining low moisture content, low temperatures, and insect-free conditions are recommended. Counter measures include quick air-drying or the use of other aeration systems to remove moisture and heat.

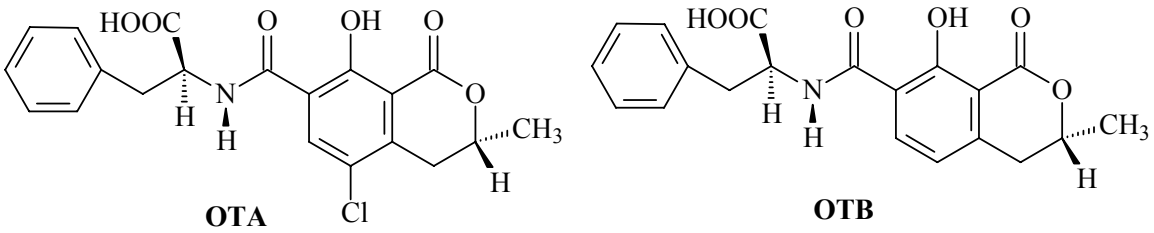
Although air, soil, and machinery are the major sources of inoculum, contamination can occur through other means. *Penicillium* spp. are common naturally occurring epiphytes on cacao (Hoopen et al., 2003). Cacao pods wounded by, machete, or

predators such as rats and squirrels or by cocoa pod borers are usually found highly contaminated with ochratoxigenic fungal species (Bastide et al., 2006). Endophytic fungal associations have also been shown to be the source of toxigens (Bacon et al., 2001). Similar problems occur in coffee plantations where coffee berries damaged by the insect coffee berry borer (CBB) become contaminated with toxigenic fungi (Vega and Mercadier, 1998).

Coffee and cacao while genetically unrelated are both tropical understory trees that grow in similar environments and both produce caffeine. Some endophytes are found in different tree species growing in the same environment (Arnold et al., 2003). Studies have shown that caffeine, present in both coffee and cacao, can inhibit the production of OT (Buchanan et al., 1981; Hasan, 1999). Caffeine inhibits toxin synthesis by restricting the uptake of carbohydrates, which are ultimately used for toxin synthesis (Buchanan and Lewis, 1984). Decaffeinated coffee beans have higher levels of toxins when compared to regular beans, presumably because the higher caffeine content reduces the growth of toxigenic contamination post-harvest (Soliman, 2002).

Since the endophytes from both coffee and cacao may be candidates for biocontrol of diseases and pests, the objectives of the following analyses were to screen a collection of fungi isolated as endophytes from (or associated with) coffee for their ability to produce mycotoxins, as well as to determine the effect of the culture media and caffeine on their ability to produce toxins.

Figure 13. Ochratoxins A and B



2.2.2.1. MATERIALS AND METHODS

2.2.2.1.1. Fungal isolates

50 isolates comprising *Penicillium* spp. and *Aspergillus* spp. isolated from coffee as endophytes, as well as fungi found associated with the coffee pathogen coffee berry borer (*Hypothenemus hampei*) and a wasp (*Prorops nasuta*) parasitic to this pathogen, were screened for the production of ochratoxins. An *Aspergillus ochraceus* isolate from Dr. James F. White Jr. laboratory collection at Rutgers University, was used as a positive control for OT.

2.2.2.1.2. Nutrient media

Isolates were grown in YES (2 % yeast extract and 20 % sucrose), YMA (yeast malt agar, Sigma-Aldrich), and potato dextrose agar (PDA, Difco).

2.2.2.1.3. Ochratoxin analysis

Ochratoxins A and B were measured using the method described by Bragulat et al. (2001). Three plugs (0.5 mm diameter x 0.5 mm length) of approximately 200 mg containing media and fungal mycelia were sampled at different time intervals after inoculation. Plugs were extracted with 0.8 mL methanol:formic acid (25:1) for one hour at 25^{o}C, then filtered through 13 mm nylon membrane filters. 10 uL of the filtrate were

injected in an HPLC equipped with a C18 reverse phase column (15 cm length x 4.6 mm i.d.; pore size 5 um; 3/8 inches outside diameter). The mobile phase was composed of acetonitrile:water:acetic acid (57:41:2), the flow rate was 0.8 $mL \cdot min^{-1}$, and the fluorescence detector was set at excitation wavelength of 330 nm and emission wavelength of 460 nm.

2.2.2.1.4. Aflatoxin analysis

Aspergillus oryzae NRRL 35191 isolated from coffee as an endophyte was analyzed for the production of aflatoxins using an AgraStrip aflatoxin test (Romer Labs, Union, MO), an immunochromatographic assay based on an inhibition immunoassay format with a detection limit of 4 ppb.

2.2.2.2. RESULTS AND DISCUSSION

Two *Aspergillus westerdijkiae* were isolated from coffee berry borer, a major pest in coffee plantations, and from a wasp, used as biological control agent against CBB (Vega and Mercadier, 1998); both isolates were found to produce ochratoxins.

Ochratoxin A is known to have antiinsect activity (Wicklow et al., 1996). This attribute is likely an advantage favoring selection of ochratoxigenic fungi associated with plants challenged by insect pests, and could be beneficial to the plant if it affects the insect pest. However, the ochratoxigenic fungus *A. westerdijkiae* was found associated with both the insect pathogen and its parasite. Therefore, caution should be exercised to

avoid spreading the toxigenic fungus or introducing it along with the biological control agent.

OT production has been shown to be inhibited by caffeine (Buchanan et al., 1981; Hasan, 1999). However, some isolates show the ability to degrade caffeine and produce higher levels of OTA when caffeine is added to the culture media (Tsubouchi et al., 1985). Similarly, OT production by *A. westerdijkiae* increased more then ten times when the fungus was grown in culture supplemented with 0.02 % caffeine. *A. ochraceus* also produced more OT in media supplemented with caffeine (Table 5). It is possible that toxigenic species in continuous contact with a high caffeine environment adapt and develop the ability to degrade caffeine and stimulate OT production. Adaptation may occur due to competition for organic nutrients. The bacteria *Pseudomonas putida* for example, growing in soil under coffee plants has developed mechanisms to degrade caffeine released by the plants (leaves, fruits and litter). While some microorganisms degrade caffeine, others, including *A. fumigatus*, *A. japonicus*, and *A. niger*, have also been reported to degrade ochratoxin A to less toxic products (Varga et al., 2000). Buchanan et al. (1981) observed a 98 % inhibition of OTA production when 0.3 % caffeine was present in the growing media. Tsubouchi et al. (1985) reported a strain of *A. ochraceus* with decreased OTA production in the presence of caffeine, while a different strain increased OTA production when grown under the same conditions. Suarez-Quiroz et al. (2004) observed a peak in OTA production by *A. ochraceus* in a nutrient media with 0.1 % caffeine. After peaking, OTA levels dropped with increasing caffeine levels.

Table 5. Concentration of OTs ($\mu g.g^{-1}$) produced by *Aspergillus* spp. (4 DAI) on media with and without caffeine

	Fungi	*A. ochraceus*		*A. westerdijkiae*	
Media	Caffeine	OTA	OTB	OTA	OTB
PDA	0%	10.96 (± 3.3)	3.23 (± 0.2)	0.25 (± 0.02)	0.31 (± 0.08)
	0.02%	1.42 (± 0.3)	1.78 (± 0.2)	0.15 (± 0.06)	0.53 (± 0.03)
	1%	0.52 (± 0.3)	6.83 (± 2.5)	0	0
YES	0%	4.04 (± 1.2)	3.55 (± 0.8)	0.26 (± 0.07)	0.27 (± 0.05)
	0.02%	32.83 (± 1.7)	18.99 (± 10.7)	3.40 (± 0.5)	3.22 (± 0.3)
	1%	8.13 (± 4.1)	63.64 (± 7.2)	0.22 (± 0.01)	0

During the current screening for ochratoxigenic fungi, 14 % of the tested isolates (Table 6) were found to be toxigenic. Taniwaki et al. (2003) sampled a number of *Aspergillus* isolates from coffee beans and of them, 75 % of the *A. ochraceus*, 3 % of the *A. niger*, and 77 % of the *A. carbonarius* isolates produced OTA.

One of the isolates identified to the species level, *A. oryzae*, is very similar to the aflatoxigenic fungus *A. flavus*. Although both species have the genes predicted to be involved in the aflatoxin synthetic pathway, they are only expressed in *A. flavus* (Keller et al., 2005). The analysis of culture filtrates of *A. oryzae* confirmed the absence of both OT and aflatoxin sustaining the species identity.

The *A. ochraceus* and *A. westerdjikiae* fungal isolates were inoculated in YMA, PDA, and YES to evaluate the effect of the media on ochratoxin production. Although the OTA levels were different in each media, the toxin was present in all three. The same was true for isolates that did not produce OT; when tested in the same three media, OT was not present in any of them. Although the chance that these isolates produce OT in another media cannot be ruled out, it is unlikely that the isolates negative for OT would produce it in other media or that isolates positive for OT would not produce it in other media.

A. ochraceus and both *A. westerdijkiae* isolates that produce OTA also produce OTB. *A. ochraceus* produced at least 10 times more OTB in YES media when compared to PDA (Table 5).

OT measurements were taken at different time intervals after inoculation. Isolates that did not produce OT after one week of inoculation never produced OT. Among those that produced OT, the levels increased with time and varied depending on the media. *A.*

ochraceus produced nearly the same amount of OTA at 4 days after inoculation (DAI) and at 35 DAI when grown in PDA. However when grown in YES and YMA, OT accumulated over time (Table 7). Accumulation of OTA over time was also seen with *A. westerdijkiae* CBB9-1 and *A. westerdijkiae* W6-1 when grown in YMA and YES media. When *A. westerdijkiae* CBB9-1 and *A. westerdijkiae* W6-1 were grown in PDA, OTA was not detected at 4 DAI but it was present at 35 DAI.

A. ochraceus under the same conditions can produce 10 times more ochratoxin A than *A. westerdijkiae*. OT production increased over time and can be measured as early as 4 DAI in YES and YMA for *Aspergillus ochraceus* and *A. westerdijkiae* CBB9-1.

Table 6. List of isolates analyzed for OT

	NRRL	Reference #	Identification	Isolated from	Country	OT
1	35193		*Aspergillus westerdjikiae*	*Prorops nasuta*	Colombia	+
2	35197		*Aspergillus westerdjikiae*	Coffee berry borer	Colombia	+
3	32580		*Penicillium brevicompactum*	Coffee	USA	
4	32582		*Penicillium brevicompactum*	Coffee	USA	
5	32574		*Penicillium brevicompactum*	Coffee	USA	
6	32576		*Penicillium brevicompactum*	Coffee	USA	
7	35214		*Penicillium brevicompactum*	Coffee	USA	
8	32579		*Penicillium brevicompactum*	Coffee	USA	+
9	32600		*Penicillium brevicompactum*	Coffee	USA	
10	35599		*Penicillium brocae*	Coffee	USA	
11	35434		*Penicillium citrinum*	Coffee	USA	
12	35438		*Penicillium citrinum*	Coffee	USA	
13	35449	i4-20	*Penicillium citrinum*	Coffee	USA	
14	32592		*Penicillium crustosum*	Coffee	Guatemala	
15	35217		*Penicillium crustosum*	Coffee	Colombia	
16	35466		*Penicillium cecidicola*	Coffee	USA	
17	35451		*Penicillium janthinellum*	Coffee	USA	
18	35363		*Penicillium coffea*	Coffee	USA	
19	35367		*Penicillium steckii*	Coffee	USA	
20	35463		*Penicillium steckii*	Coffee	USA	
21	35448	i4-6	*Penicillium citrinum*	Coffee	USA	
22	35460		*Aspergillus sp.*	*Prorops nasuta*		
23	35524		*Aspergillus sp.*	*Prorops nasuta*		
24	35436		*Aspergillus sp.*	*Prorops nasuta*		
25	35437		*Aspergillus sp.*	*Prorops nasuta*		
26	35191		*Aspergillus oryzae*	Coffee		
27	35185		*Penicillium brocae*	Coffee	Colombia	
28	35171		*Penicillium olsonii*	Coffee	USA	
29			*Penicillium olsonii*	Coffee	USA	
30	35186		*Penicillium diversum*	Coffee	USA	
31			*Penicillium olsonii*	Coffee	USA	
32	35167		*Penicillium olsonii*	Coffee	USA	
33			*Penicillium olsonii*	Coffee	USA	
34	35174		*Penicillium olsonii*	Coffee	USA	
35			*Penicillium olsonii*	Coffee	USA	
36			*Penicillium olsonii*	Coffee	USA	
37	32577		*Penicillium olsonii*	Coffee	USA	+
38	35166		*Penicillium olsonii*	Coffee	USA	+
39	35168		*Penicillium olsonii*	Coffee	USA	
40	35179	H81H2	*Aspergillus* sp.			
41	35213	H81P2	*Penicillium olsonii*			
42	35175	H81P4	*Penicillium olsonii*			
43	32583		*Penicillium sclerotiorum*	Coffee	USA	
44	32581		*Penicillium olsonii*	Coffee	USA	
45	35169		*Penicillium olsonii*	Coffee	USA	
46	35225	I1R22	*Penicillium citrinum*			
47	35176	M1S3	*Aspergillus tamarii*			
48	35178		*Penicillium crustosum*	Coffee	Mexico	+
49	35183		*Penicillium oxalicum*	Coffee	Colombia	+
50	35184		*Penicillium brevicompactum*	Coffee	Colombia	

Table 7. Ochratoxin A production (OTA μg.g^{-1} media ± STD dev.) by *A. ochraceus* and two *A. westerdijkiae* isolates from the parasitoid *P. nasuta* (W6-1) and the coffee berry borer, *Hypothenemus hampei* (CBB9-1) at 4 and 35 days after inoculation (DAI)

OTA	Media	YMA		PDA		YES	
Fungi	DAI	4	35	4	35	4	35
A. ochraceus		1.4±0.06	4.6±1.10	5.4±0.66	6.1±1.26	2.5±0.14	26±2.60
A. westerdijkiae W6-1		0	0.7±0.04	0	0.6±0.10	0	1.1±0.35
A. westerdijkiae CBB9-1		0.4±0.10	0.6±0.42	0	0.9±0.04	2.5±0.4	3.5±0.56

2.2.2.3. CONCLUSIONS

Among all tested fungal isolates, two *A. westerdjikiae* were OT producers. Although they produced less toxin than *A. ochraceus*, the levels were in the same order of magnitude. The *A. westerdjikiae* isolates that produced OTA also produce OTB. It is clear that media has an effect on OT production because quantitative differences were observed, although no qualitative difference was seen. OT levels varied according to the substrate and the presence of caffeine. Nutrient media containing higher levels of sugars induced higher levels of toxin production. The inhibitory effect of caffeine on toxin production is possibly related to restriction of carbohydrate uptake, previously suggested by Buchanan and Lewis (1984). Increased OT production, when intermediate levels of caffeine were added to the growing media, has also been reported in literature (Suarez-Quiroz et al., 2005; Tsubouchi et al., 1985), although no explanation has been provided. Caffeine below toxic levels may trigger toxin production while at higher levels completely disrupt fungal metabolism. Some toxigenic fungi may be able to break down caffeine and possibly use the degradation products as carbon and nitrogen sources.

2.2.3. *Aspergillus oryzae* FROM COFFEE, A NON-TOXIGENIC ENDOPHYTE WITH THE ABILITY TO SYNTHESIZE KOJIC ACID IN CULTURE

Penicillium and *Aspergillus* are cosmopolitan fungi capable of synthesizing a multitude of secondary metabolites with a broad range of functions (Cole and Schweikert, 2003; Keller et al., 2005). Some of these metabolites are known to play a role in the systems in which these fungi occur, and influence whether they are considered pathogens, endophytes, or opportunistic organisms. At times, the delineation between these categories is hazy and may be only temporal or circumstantial. For instance, an endophyte can grow asymptomatically or cause disease depending on the developmental stage of a plant or its physiological status (White and Morgan-Jones, 1996). Meanwhile, opportunistic organisms exploit weakened plants to cause disease. Schulz and Boyle (2005) suggested that these organisms exist on a continuum from saprophyte to endophyte to pathogen.

Some *A. oryzae* strains are known to produce kojic acid (KA; 5-hydroxy-2-hydroxymethyl-γ-pyrone; $C_6H_6O_4$), a metabolite with insecticidal, antibacterial and antifungal properties (Dowd, 1999; Dowd, 2002; Alverson, 2003; Burdock et al., 2001; US Environmental Protection Agency, 1997). KA is also utilized in cosmetics as a skin lightening agent. *A. oryzae* has been used for hundreds of years in the food industry for the preparation of many Asian foods such as miso, sake and shoyu and is generally regarded as safe (Bentley, 2006).

This study describes the identification of a non-toxigenic *Aspergillus oryzae* endophyte isolated from coffee that has the ability to synthesize KA in culture. Moreover, it has a potential to be inoculated and grow endophytically in cacao seedlings.

2.2.3.1. MATERIALS AND METHODS

2.2.3.1.1. Fungal isolate

Aspergillus oryzae (NRRL 35191) was found as an endophyte from coffee leaves, see Vega et al. (2006) for endophyte isolation methodology. In order to confirm the identity of this isolate, ITS and partial large subunit rDNA was amplified and sequenced. The sequence from NRRL 35191 indicates inclusion in the *A. oryzae*/*A. flavus* species group (Peterson et al., 2001). Because the ID gene of the *A. flavus*/*A. oryzae* complex is insufficiently variable to clearly distinguish the two taxa, the OMT12 locus was also amplified and sequenced as detailed by Geiser et al. (2000). This analysis was done by S.W. Peterson, USDA, Peoria, IL, USA.

2.2.3.1.2. KA extraction from fungal culture

Yeast extract sucrose (YES) medium (2 % yeast extract and 20 % sucrose) was inoculated with a $1x10^7$ spores mL^{-1} suspension of *A. oryzae* (NRRL 35191) and placed in a shaker (VWR analog orbital shaker OS-500) at 100 rpm for 7 days at 25 ^oC. The broth was filtered through glass wool and the filtrate was centrifuged at 10,000 x g for

five minutes. The pH of the supernatant was adjusted to 3.5 and extracted 3 times with 15 mL ethyl acetate. The organic phase was concentrated *in vacuo*.

2.2.3.1.3. Seedling inoculation and *in planta* extraction for KA and caffeine analysis

A cocoa pod from a tree grown at a Rutgers University greenhouse was cracked open, the seeds cleaned of mucilage, and the seed coat removed. Seeds were immersed in 70 % ethanol for one minute and then submerged in 10 % bleach solution for one hour, followed by rinsing 3 times with autoclaved water. Seeds were then placed inside a test tube (25 cm in length x 3 cm in diameter) containing half strength potato dextrose agar (PDA) with the radicle end up. Three week-old sterile cacao seedlings were inoculated with 0.5 mL of a $1x10^7$ spores mL^{-1} *A. oryzae* suspension placed on the radicle. After 3 weeks, seedlings were surface sterilized by rinsing with tap water, immersed in 70 % ethanol for one minute and 10 % bleach solution for one hour. The seedlings were then rinsed three times with autoclaved water, and the stem and roots sections were plated on PDA to assess for the presence of *A. oryzae*. Plates were kept at 25 ^oC. The remaining plant parts were ground in liquid nitrogen and extracted with 150 mL methanol, stirring overnight. The methanolic extract was filtered and concentrated *in vacuo*.

2.2.3.1.4. KA and caffeine analysis

The concentrated sample was derivatized with Tri-sil reagent (Pierce, Rockford, IL) and analyzed by GC-MS (Figure 14). A KA commercial standard (Sigma-Aldrich, St. Louis, MO) was converted with Tri-sil to its trymethylsylilated derivative and injected in the GC-MS (Column DB-1; The initial temperature of 70 ^{o}C was held for two minutes, then ramped up to 240 ^{o}C at 10 ^{o}C min^{-1}, and held for 30 minutes). KA was identified by matching the retention time of derivatized standard with the samples as well as by comparison of their mass spectra. Caffeine was identified along with KA in the same chromatogram by comparison of its mass spectra to the equipment's library NIST 98.

2.2.3.2. RESULTS AND DISCUSSION

Distinguishing *A. oryzae* and *A. flavus* species from one another can be difficult since both species have the genes predicted to be involved in the aflatoxin synthetic pathway, but the aflatoxin pathway genes are expressed only in *A. flavus* (Keller et al., 2005). When grown in YES media, *Aspergillus oryzae* NRRL 35191, did not produce ochratoxin A or B or aflatoxins B1, B2, G1 and G2, but produced kojic acid, identified as its trimethylsylilated derivative with the following fragmentation pattern 45(11), 73(58), 75(12), 147(23), 271(100), 272(26), 273(11), $M^{+\cdot}$ 286(1) (Figure 15). The production of koijc acid, and the absence of toxins, combined with the DNA analysis using the omt12 locus confirmed the identification of the endophyte as *A. oryzae*.

It has been hypothesized that *A. oryzae* evolved, after domestication, from *A. flavus*, and may have lost certain features important to survival in the natural environment (Kurtzman et al., 1986). However, our discovery of an *A. oryzae* isolate from healthy coffee leaves suggests that it may indeed be capable of independent growth and reproduction.

A. oryzae grew endophytically in cacao seedlings and the GC-MS profiles of the seedling extracts revealed the presence of KA in the inoculated seedlings when compared to the controls. *A. oryzae* inoculated seedlings also produced more caffeine than the non-inoculated seedlings (Figure 16). Caffeine has been shown to be part of the defensive mechanisms against pathogens in (Aneja and Gianfagna, 2001).

Aspergillus flavus grows saprophytically on a broad range of substrates but it is known as an opportunistic post-harvest plant pathogen that can cause disease in nut trees, cotton and corn. Although there are no reports of *A. oryzae* as a pathogen, we hypothesize that in the studied interaction, the cacao plant may recognize the endophytic *A. oryzae* as a pathogen and respond by producing caffeine, which is inhibitory to fungal growth (Aneja and Gianfagna, 2001). This hypothesis supports the idea of the endophytic continuum suggested by Schulz and Boyle (2005).

Due to the multitude of antipathogenic functions of KA, an endophytic non-toxigenic *A. oryzae* isolate that has the ability to produce KA may be useful as a protective agent against insects and pathogens. Although the mode of action of KA in cacao needs further investigation, it may involve both induction of plant defenses and direct antibiotic action against pathogens and insects. In addition, non-toxigenic *Aspergillus* isolates can reduce contamination of food crops by toxic isolates through

competitive exclusion (Cleveland et al., 2003). With further testing, through the inoculation of cacao seedlings and/or seeds, this isolate of *A. oryzae* has the potential to be used for this purpose as well.

2.2.3.3. CONCLUSIONS

Aspergillus oryzae was isolated as an endophyte from coffee leaves and was found to produce kojic acid (KA) in culture. When inoculated in cacao seedlings, *A. oryzae* grew endophytically and synthesized KA *in planta.* Cacao seedlings inoculated with *A. oryzae* produced higher levels of caffeine than non-inoculated ones. *A. oryzae* may be a useful endophyte to introduce to cacao since it grows non-pathogenically and induces the caffeine defense response that may make the plant more resistant to insects and diseases.

Figure 14. Kojic acid measured by GC-MS as trimethylsylate derivative.

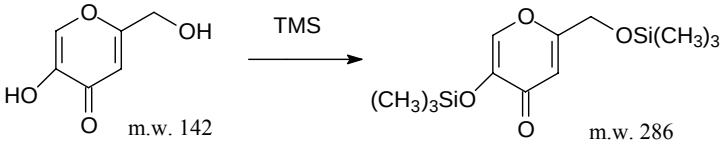


Figure 15. MS spectra of kojic acid after derivatization with TMS

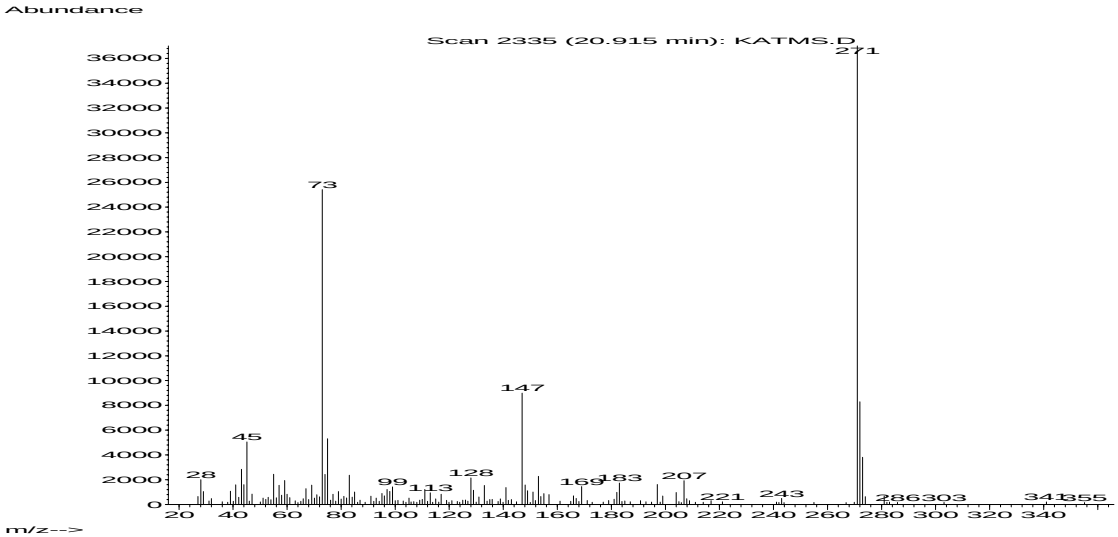
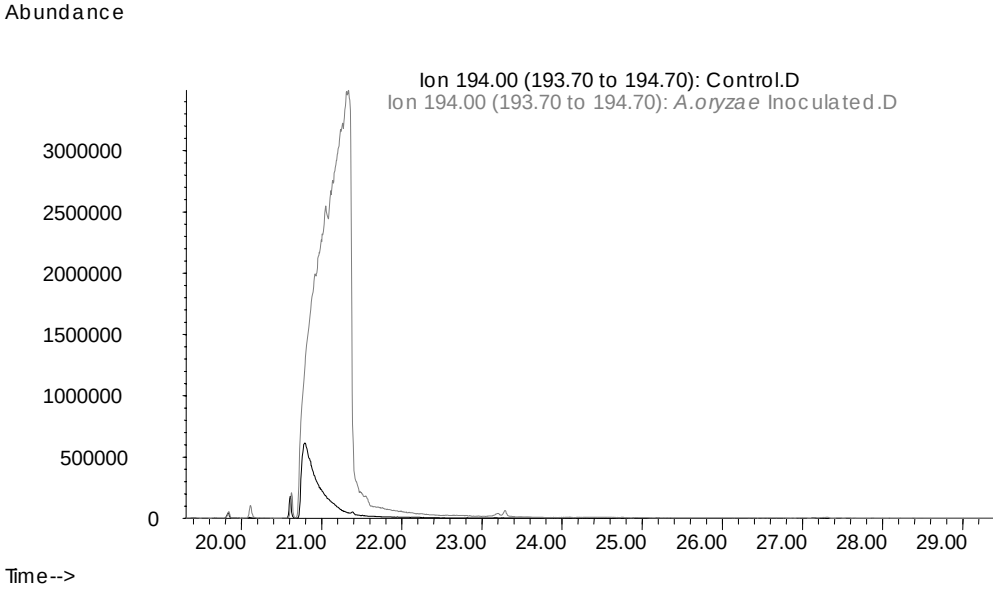


Figure 16. Overlaid extracted ion chromatograms of full scan analysis comparing cacao control seedling and a seedling inoculated with *A. oryzae*. Ion m/z 194 is the molecular ion and base peak of the mass spectrum of the caffeine molecule.



2.2.4. MORE ENDOPHYTES FROM COFFEE WITH POTENTIAL BIOCONTROL ACTIVITY AGAINST CACAO PATHOGENS

Cocoa pod borer (CPB), *Conopomorpha cramerella*, is the major cacao pest in South East Asia and causes 20 to 30 % yield losses. Cocoa pod borer is a Lepidoptera that bores through the pods to feed on the pulp, resulting in malformation of seeds and premature ripening of pods. Moreover, the wounds serve as an entry point for opportunistic organisms such as toxigenic fungi that contaminate beans and pose a threat to human health. CPB control strategies include pod stripping and ethephon application to eliminate flowers during the off-season and reduce inoculum (Prawoto and Atmawinata, 1995). Pesticides and plastic bag sleeves covering the pods have also been used. Biological control of CPB would be an interesting alternative based on the reduced environmental pollution and risk of harming other insect species by avoiding the use of pesticides.

In order to find a biological control agent, one alternative is to look for naturally occurring antagonists. Another approach is to use organisms that have proven successful in the control of other pathogens. For example, *Trichoderma asperellum*, *Metarhizium anisopliae*, *Paecilomyces fumosoroseus*, and *Beauveria bassiana*, have all shown to be pathogenic to fungi or insects (Kolombet et al., 2007; Vega et al., 1999; Shi and Feng, 2004; Wraight et al., 2000). Two important characteristics that the biocontrol agent must possess are efficacy and persistency. In addition, a successful biocontrol agent might use multiple mechanisms of pathogenesis and thrive under a wide range of environmental conditions.

B. bassiana has been successfully employed against coffee berry borer (CBB), a serious threat to coffee plantations. Although effective, *Beauveria bassiana* population declines during the drier periods, and only shaded and humid portions of coffee fields have a higher degree of parasitism (Costa et al., 2002). One hypothesis is that *B. bassiana* populations might be declining due to the sum of antagonistic interactions with the endophytes present in coffee. In order to better understand the interactions between *B. bassiana* and endophytes, twelve coffee endophytes were tested in combination with two *B. bassiana* isolates in bioassays.

2.2.4.1. MATERIALS AND METHODS

2.2.4.1.1. Fungal isolates

12 endophytes isolated from coffee leaves (Vega et al., 2006) as part of a survey of fungal endophytes in coffee were submitted to *in vitro* bioassays. Among the identified isolates tested against *Beauveria bassiana* were *Trichoderma asperellum*, *Penicillium steckii*, *P. citrinum*, *P. janthinellum* and an actinomycete.

2.2.4.1.2. Media effect

The actinomycete isolate was grown in four different media: PDA (pH 5.3), water-agar (2 % agar, pH 5.3), 1 % (1 % glucose, 1 % yeast extract, 2 % agar; pH 6.8) and ISP (4 $g \cdot L^{-1}$ yeast extract, 10 $g \cdot L^{-1}$ malt extract, 4 $g \cdot L^{-1}$ dextrose, 20 $g \cdot L^{-1}$ agar; pH 5.9).

2.2.4.1.3. Dual culture plate assays

In this screening method, both the endophyte and the insect pathogen were cultured simultaneously. The inoculation was performed by placing a 1 cm diameter agar plug with mycelium 2.5 cm from the center of an agar plate containing nutrient media (PDA or YES). A few days after inoculation, the plates were examined for fungal growth inhibition. The plates were incubated at room temperature and checked for zones of inhibition of mycelial growth when the fungal mycelium reached the edge of the plate. Some fungal species displayed an irregular pattern of growth, which made measurement of radial growth in culture difficult. Furthermore, when using plastic Petri dishes, the static caused spores to scatter about the plate. In order to avoid this inconvenience, spores were suspended in a solution made with 0.2 % melted agar and 0.05 % Tween 80. Spores were then spotted on the plate using an inoculation loop (Pitt, 1973).

2.2.4.1.4. Cellophane covered media bioassay

A sheet of sterile cellophane was placed on top of PDA media. An endophyte was then inoculated on the media on the center of the plate on top of the cellophane. The culture was allowed to grow for a few days until it approached the margins of the cellophane. The sheet was then peeled off, removing the endophyte. This was followed by a second inoculation of the media with a plant pathogenic fungus at the center of the plate. Observations of mycelial growth and development were recorded during the next few days.

2.2.4.1.5. Bioassay test for volatile antimicrobials

This bioassay used physical isolation of fungi allowing only for volatiles to interfere with the mycelial growth of the fungus being examined. Potato dextrose agar (PDA) was poured into all quadrants of Petri plates with the base plate separated into four quadrants, X-plate (Fisher Brand, USA). Each quadrant is 19 cm^2 and holds approximately 11 mL. A plug of agar containing an actively growing culture of *Beauveria bassiana* isolate was placed into one quadrant. The plate was sealed and incubated for four days at 23 ^oC prior to exposure of *Trichoderma asperellum* to the volatile organic compounds arising in the plates. The dividing walls in the plate prevent the diffusion of any compounds released in the media to have effects on the target organism. Growth of the fungi was measured and compared to its growth on a control plate (Strobel et al., 2001).

2.2.4.1.6. Analysis of the volatiles produced by endophytes

PDA was poured inside a two necked round bottom flask and allowed to solidify. The media was then inoculated with a spore suspension of *B. bassiana* and *T. asperellum* alone and in combination. The fungus was allowed to grow until it covered the media inside the flask. Then air was passed through the system (50 mL. min^{-1}) and the volatiles were collected by a cartridge containing a Tenax resin. The volatiles were then desorbed into the GC using a thermodesorption system and analyzed with the MS detector (Croft et al., 1993).

GC-MS conditions: GC HP 6890 series, MSD HP 5973 NIST 98; column J and W Scientific Inc. DB-1MS 50 m x 0.250 mm x 0.25 μm; initial temperature 70 ^{o}C held for two minutes; 10 $^{o}C \cdot min^{-1}$ until 240 ^{o}C held for 30 minutes; total run time 49 minutes; helium gas flow 1 $mL \cdot min^{-1}$; injector 250 ^{o}C; MSD 280 ^{o}C; mass scan started at three minutes and ranged from 20 amu to 550 amu, with a threshold of 150, scanning at 2.78 scans/second;

Desorption system conditions: short path thermal desorption model TD-4 Scientific Instrument Services Inc (Ringoes-NJ, USA). Gas purge time (helium chromatographic grade-airgas) 30 seconds; injection time one minute; desorption time five minutes; delay start 30 seconds; desorption temperature 250 ^{o}C; cooling trap temperature - 40 ^{o}C (CO_2 bone dry grade- siphon type) (from Airgas, Inc Radnor, PA, USA).

2.2.4.1.7. Effect of caffeine on fungal growth

Nutrient media (PDA) was supplemented with different concentrations of caffeine (0.01 %, 0.1 % and 1 %). Then coffee fungal endophytes and entomopathogenic fungi *Metarhizium anisopliae*, *Paecilomyces fumosoroseus*, and *Beauveria bassiana* from a USDA Beltsville, MD, collection (F.E. Vega), were inoculated on the plate and the mycelial radial growth recorded.

2.2.4.2. RESULTS AND DISCUSSION

A group of endophytes isolated from coffee were subjected to different concentrations of caffeine. The highest concentration of caffeine was inhibitory to growth of all of the isolates, although some more than others, most isolates failed to grow in 1 % caffeine. *B. bassiana*, *Metarhizium anisopliae* and *Paecilomyces fumosoroseus* isolates had their mycelial growth inhibited and morphological changes were evident when growing in high levels of caffeine in the media (Table 8 and Figures 17, 18, 19, 20, 21). Among the tested isolates, *Penicillium citrinum* (I4-6), *P. citrinum* (I4-20) and *Beauveria bassiana* IC-5486-Bb (Bb1) were the least inhibited by the presence of caffeine at 1 % in the media. It is then expected that most endophytic fungi will have difficulties in becoming established in crops where the caffeine levels are high. Therefore, when considering the introduction of a fungal endophyte as a biological control agent, it is preferable to use fungal isolates adapted to natural adverse environmental conditions, particularly elevated caffeine concentrations that can be as high as 0.6 to 0.8 % in cacao

beans and 1 % (*Coffea arabica*) to 2.4 % (*C. canephora*) in coffee beans (Ashihara and Crozier, 2001).

The three isolates that best responded to high caffeine levels in the media, *B. bassiana* isolate Bb1, and the endophytic *Penicillium citrinum* and *P. citrinum*, were then tested in dual plate assays.

When *B. bassiana* was challenged by *P. citrinum* (I4-6) and *P. citrinum* (I4-20), its growth rate was reduced by 13 % and 25 % respectively. Meanwhile each of the antagonists had its growth rate increased by 17 % (*P. citrinum*, I4-20) and 29 % (*P. citrinum*, I4-6) when grown in dual plates with *B. bassiana* (Table 9).

The two *Penicillium* isolates exerted fungistatic effect against *B. bassiana*, characterized by an inhibition zone between the *B. bassiana* and the endophyte on the dual plate assay (Figure 22).

Trichoderma asperellum was another endophyte from coffee tested in bioassays against *B. bassiana*. Rubini et al. (2005) had previously found *Trichoderma* sp. growing endophytically in cacao tissues as well. *Trichoderma* species are usually considered soil fungi that can also grow saprophytically in a number of substrates. In Africa, *T. asperellum* is a mycoparasite of the cacao pathogen *Phytophthora megakarya* and has been employed as biological control agent against this pathogen (Tondje et al., 2007). Other species of *Trichoderma* such as *T. harzianum* produce hydrolytic enzymes that may affect pathogens (Schirmböck et al., 1994; De Marco et al., 2003). An isolate of *T. harzianum* from a diseased cacao pod produces nonanoic acid, which has fungitoxic properties and, inhibits mycelial growth and spore germination of the cacao fungal pathogens *M. perniciosa* and *M. roreri* (Aneja et al., 2006).

Table 8. Caffeine effect on growth rates of fungal entomopathogens expressed as % increase or decrease (+ or -) compared to control

	Fungal Entomopathogens				
Caffeine %	Bb1	Bb4	Ma04	Ma06	Pr42
0.01	+7	+9	+2	-2	-1
0.1	-21	-3	+8	-34	-36
1	-68	-73	-86	-85	-88

Figure 17. Growth pattern of *Beauveria bassiana* isolate Bb1 in media containing caffeine (0-1%)

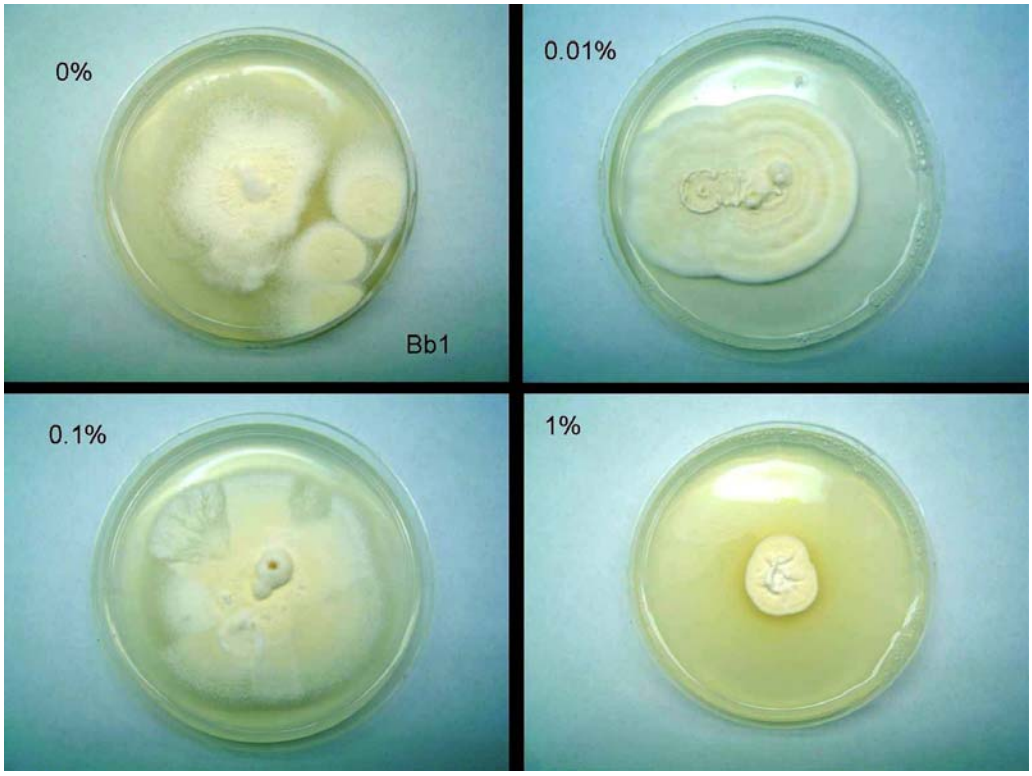


Figure 18. Growth pattern of *Beauveria bassiana* isolate Bb04 in media containing caffeine (0-1%)

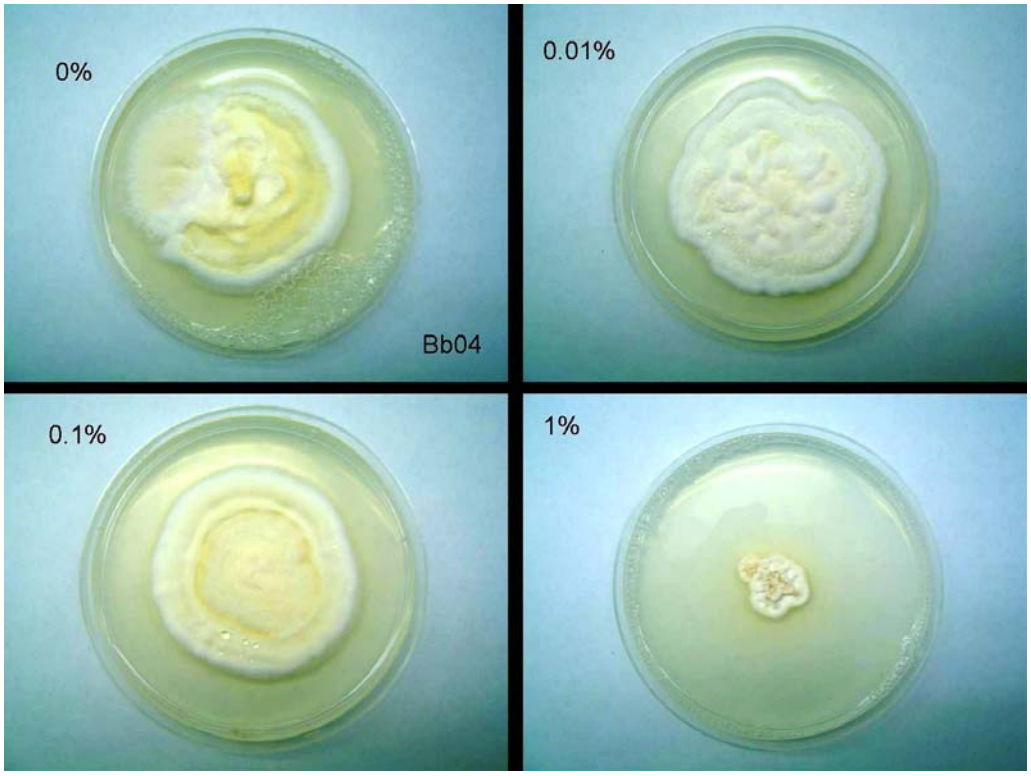


Figure 19. Growth pattern of *Metarhizium anisopliae* isolate MA04 in media containing caffeine (0-1%)

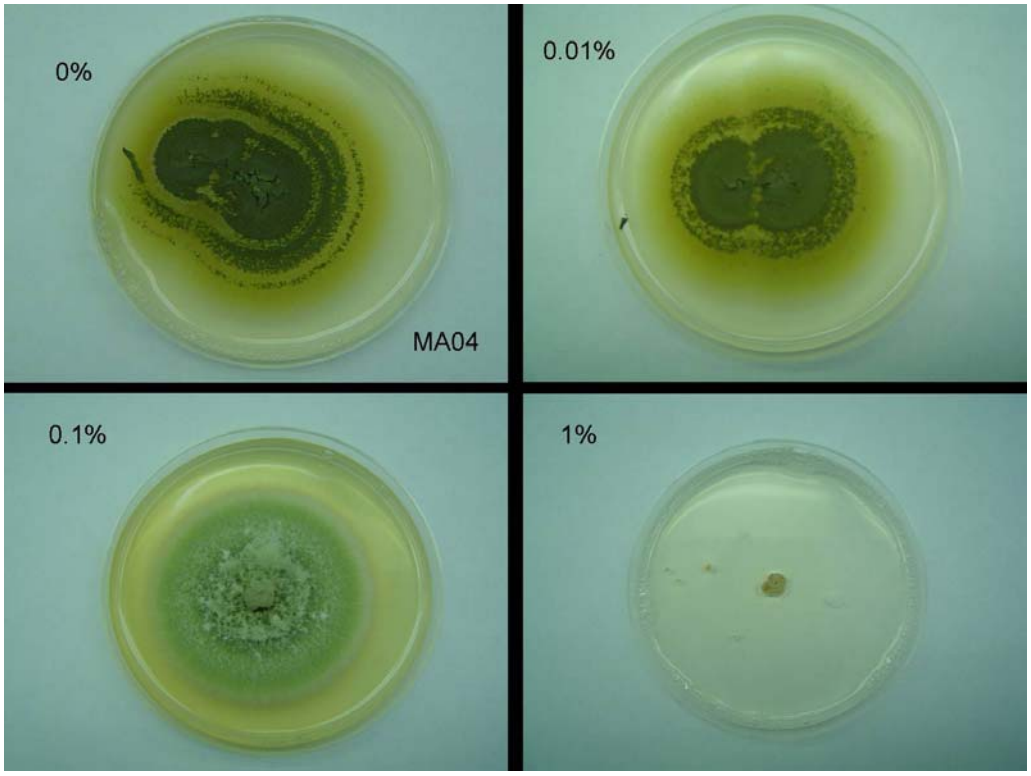


Figure 20. Growth pattern of *Metarhizium anisopliae* isolate MA06 in media containing caffeine (0-1%)

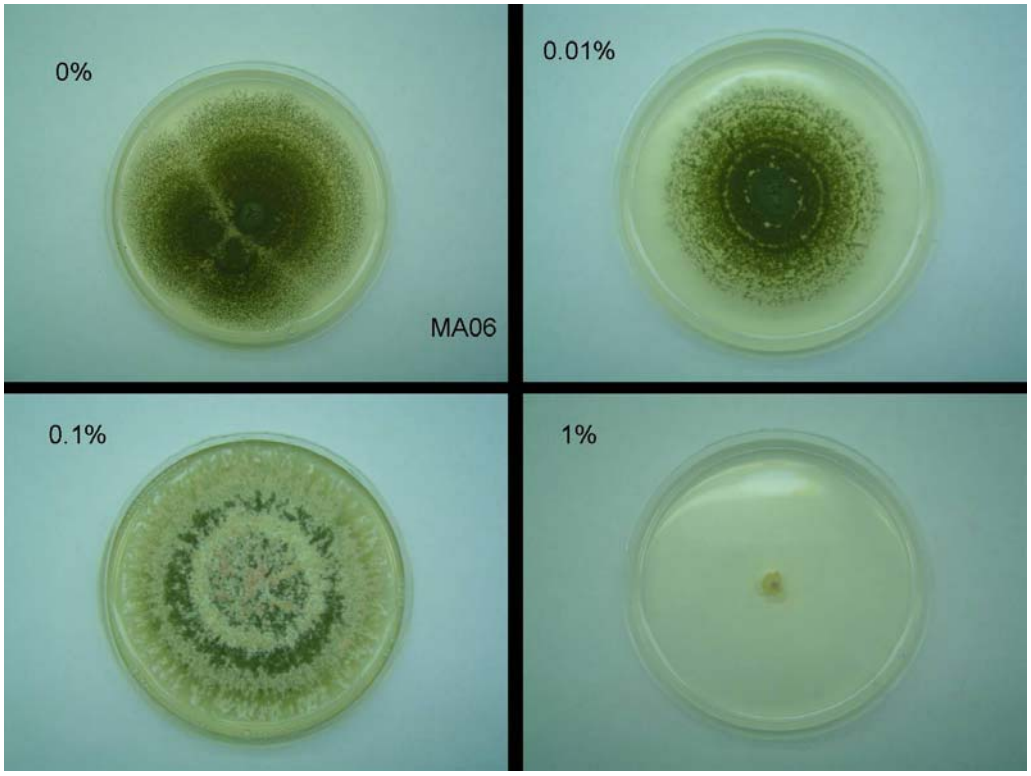


Figure 21. Growth pattern of *Paecilomyces fumosoroseus* isolate PR42 in media containing caffeine (0-1%)

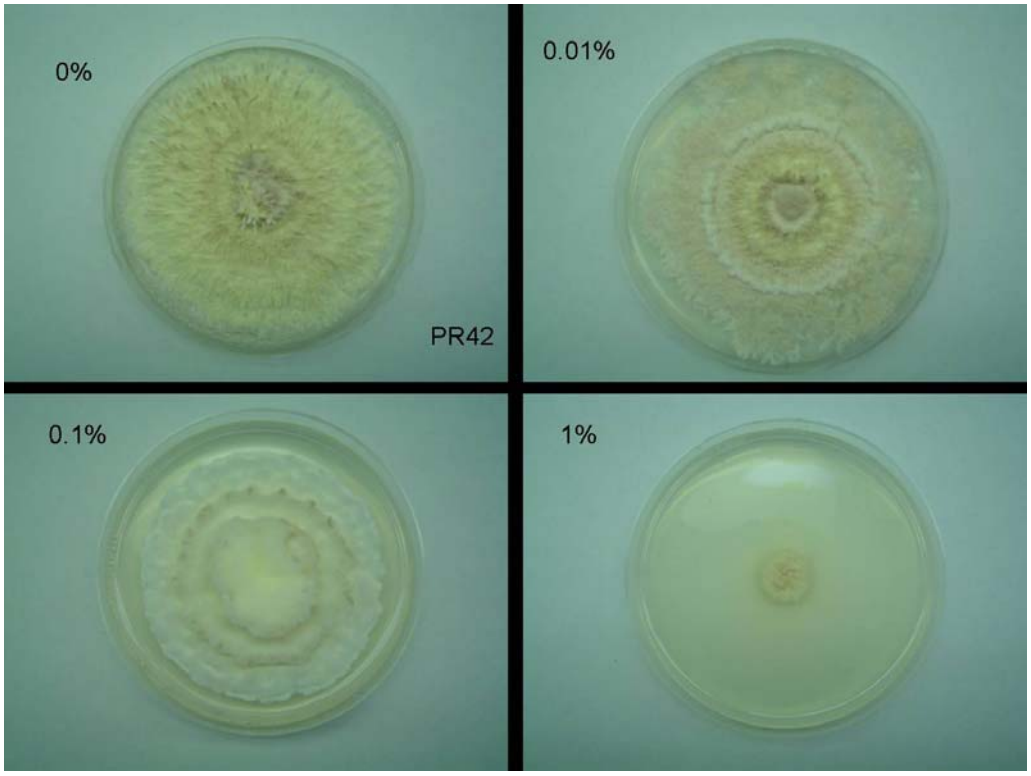
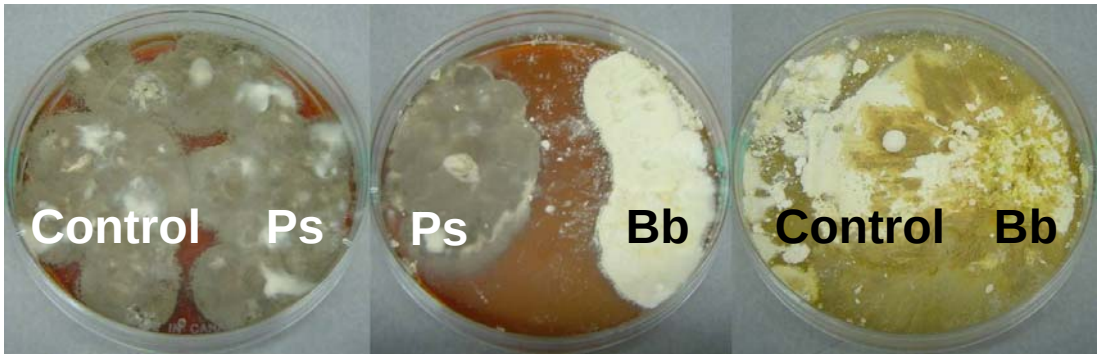


Table 9. Changes in fungal growth rate expressed in % increase (+) or decrease (-) compared to controls (grown in PDA)

Changes in growth rates	**1% caffeine**	**2% yeast extract and 20% sucrose**	**1% caffeine, 2% yeast extract and 20% sucrose**	**Antagonist**		**Antagonist, 1% caffeine, 2% yeast extract and 20% sucrose**	
				I4-20	I4-6	I4-20	I4-6
B. bassiana	-87	-8	-75	-25	-13	-7	+4
P. citrinum I4-20	- 11	+ 102	+47	+17		+2	
P. citrinum I4-6	-5	+168	+59	+29		+34	

Figure 22. Dual plate assay between *Penicillium citrinum* and *Beauveria bassiana*



T. asperellum was inoculated on top of a cellophane sheet on PDA media and was allowed to grow for two days. Then the cellophane was peeled off removing *Trichoderma aperellum*. *Phytophthora palmivora* was then inoculated in the center of the plate. Two days later, *P. palmivora* controls were fast growing while *P. palmivora* inoculated in *T. asperellum* pre-inoculated plates, failed to grow.

T. asperellum on dual plate assays exhibited mycoparasitism against *B. bassiana* and three cacao pathogens, *P. palmivora*, *Moniliophthora roreri* and *M. perniciosa* (Figure 23). Although *T. asperellum* is a fast growing isolate, its growth is completely inhibited by caffeine. In addition, volatile compounds produced by *B. bassiana* seem to have a fungistatic effect on *T. asperellum*. In an X-plate, *B. bassiana* was inoculated in ¾ of the plate while the remaining ¼ was inoculated with *T. asperellum*. In control plates, *T. asperellum* was inoculated in ¼ of the plate and the other ¾ were left with media only. In the control plate without *B. bassiana*, two days after inoculation, *T. asperellum* had crawled up the walls and invaded the media in the other quadrants (Figure 24). In plates where *B. bassiana* was present, *T. asperellum* was limited to the quadrant where it had been inoculated.

Although it is known that *Beauveria* spp. produce a number of high molecular weight bioactive compounds (Strasser et al., 2000), there is not much information about their volatile compounds and their effects on other organisms that interact with *Beauveria*.

Volatile profiles from *B. bassiana*, and *T. asperellum* grown separately and in combination were collected in adsorbent resin Tenax TA (most common and conventional adsorbent, Claeson et al., 2002) and analyzed by GC-MS (Table 10).

From *B. bassiana*, 31 compounds were identified. Also 32 volatile organic compounds (VOCs) produced by *T. asperellum* were characterized. When the two fungi were co-inoculated the VOC profile changed and a smaller number of VOCs (4) found were unique only to the combination of *B. bassiana* and *T. asperellum*. One compound ($M^{+\cdot}$ m/z 222, retention time 16.4 minutes) that could not be identified was the most prominent in the collection from the interaction between *B. bassiana* and *T. asperellum*. The compound is in the sesquiterpene region and was not present in the volatile profile of both fungi separately (Table 10). The identification of the majority of VOCs was done by matching the retention time of the standards to the retention time of the samples or by comparison to the equipment's library.

The fungal VOCs identified were distributed among organic acids, aldehydes, ketones, ethers, hydrocarbons and sesquiterpenes. Hexadecane and longipinene, only found in the samples of *Beauvera bassiana*, are known antifungal volatiles produced by fungi including *Trichoderma harzianum* that prevent development of several fungal soil pathogens (Jannet et al., 2005).

Among the most common VOCs reported from fungal cultures are hydrocarbons, ketones and terpenenoids. Many compounds found from *B. bassiana* and *T. asperellum* cultures have been previously described as fungal metabolites, uncliding 2-heptanone, 2-pentylfuran, acetic acid and hexanoic acid (Claeson et al., 2002). Other known microbial VOCs present in *B. bassiana* and *T. asperellum* collections were dodecane, tridecane, and 2-ethyl-hexanal (Claeson and Sunesson, 2005). Caryophyllene and 2-nonanone only found in the *Trichoderma asperellum* collection are among the volatile antimicrobial compounds produced by *Muscodor albus* (Strobel et al., 2001).

Beauveria bassiana is capable of biotransformation and depending on the substrate, novel compounds can be synthesized by *Beauveria* (Buchanan et al., 2000). In the case of the major unknown compound present during the interaction of *B. bassiana* and *T. asperellum*, it is possible that *B. bassiana* transformed some compound produced by itself or *T. asperellum* into a novel sesquiterpene which was not identifiable. The next step should be to try identifying this compound. Once identified, the metabolite should be tested for toxicity against *Trichoderma asperellum* in order to confirm a cause and effect relationship, regarding the growth inhibition of *T. asperellum*, observed in the x-plate assay with *B.bassiana*. Moreover, utilization of potentially active compounds as substrates for *Beauveria bassiana* could lead through biotransformation to powerful metabolites with antibiotic properties.

More experiments should follow up, using different substrates and comparing different fungi to determine VOCs specific to a fungal species or compounds produced by fungi in general, but substrate specific.

Another tested isolate obtained from coffee was an actinomycete. Actinomycetes are known to produce antibiotics, including some called actinohordins. Some actinohordins antibiotics can be red or blue in color depending on the pH of the substrate and can be easily identified visually. Depletion of glucose is known to abolish blue pigment synthesis. Growth limitation by nitrogen, phosphate or other trace elements may also result in synthesis of actinohordin (Figure 25). This current isolate when grown in a media containing yeast extract, malt extract, dextrose, and agar, at pH 6, produced a purple-bluish coloration in the media (Figure 26). It is very likely that the color in the media is due to the presence of actinohordin. Further characterization would be necessary

to confirm its identity. The molecule produced by the actinomycete isolate could also be a new antibiotic with similar structure to actinohordin. Even if identified as the same compound, the discovery of another isolate growing endophytically in coffee capable of synthesizing an important antibiotic deserves further investigation.

Figure 23. Dual plate assays between *T. asperellum* (Tasp) and *M. perniciosa* (Mp), and *M. roreri* (Mr)

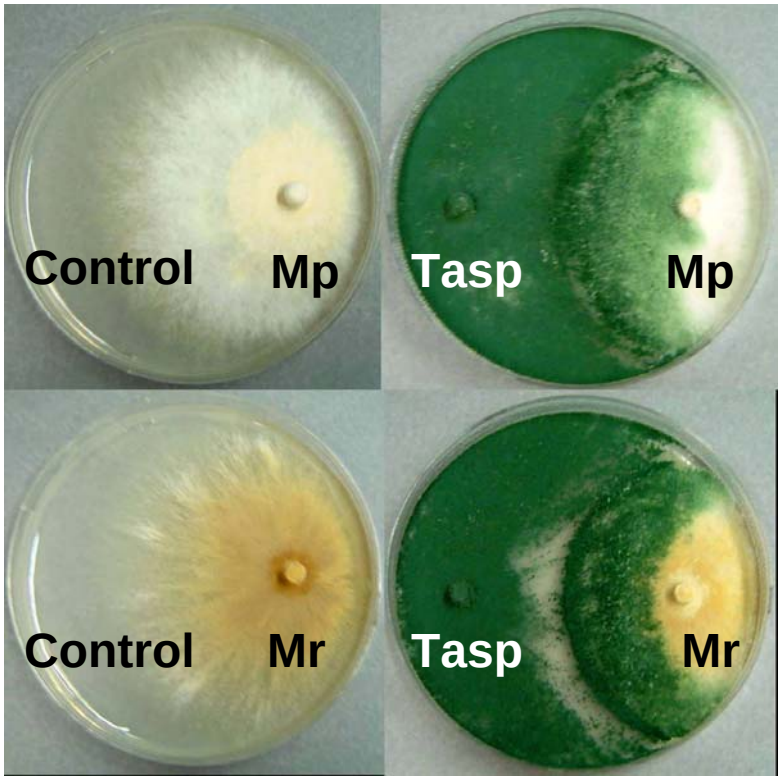


Figure 24. X-plate assay between *T. asperellum* (Tasp) and *B. bassiana* (Bb1 and Bb2).

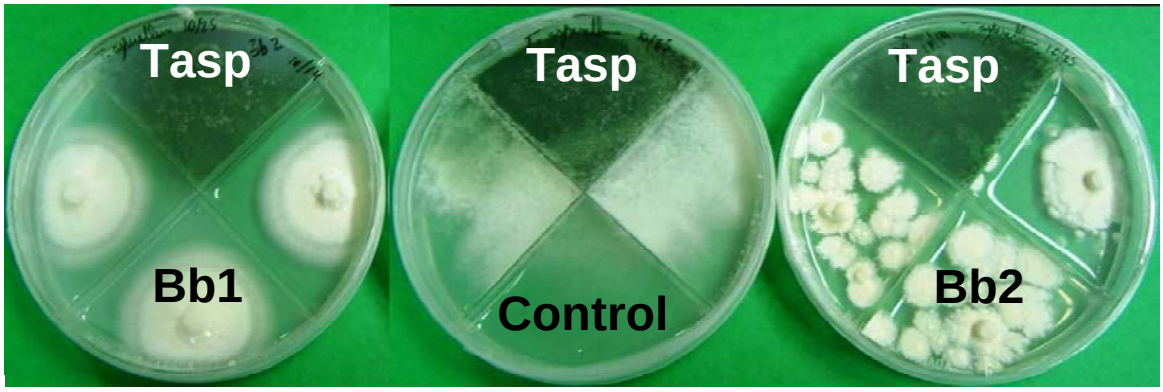


Table 10. Volatiles produced by *Beauveria bassiana* and *Trichoderma asperellum*

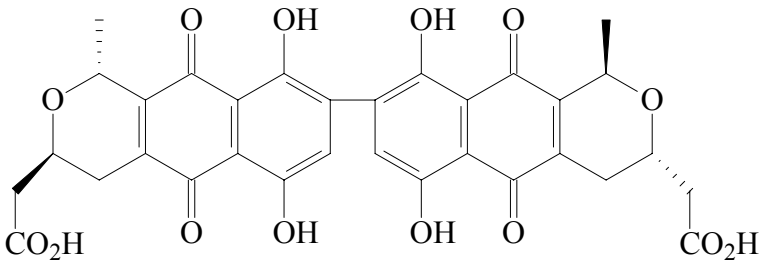
Rt.	Organic Acids	Origin	Fragments m/z (Abundance%)	Match (%)
5.0	acetic acid	T; B; X	29(10), 42(16), 43 (100), 45(90), M+. 60(66)	91
6.6	butanoic acid	B	27(22), 39(16), 41(27), 42(27), 43(26), 45(18), 60(100), 73(36), M+.88(2)	93
7.9	pentanoic acid	B	41(18), 43(12), 45(13), 55(12), 60(100), 73(39), 87(2), M+.100(<1)	86
9.3	hexanoic acid	T; B	27(17), 29(14), 39(15), 41(30), 43(22), 45(17), 60(100), 73 (48), M+.116(<1)	86
11.6	2-ethyl hexanoic acid	T	29(21), 41(39), 57(100), 73(23), 87(23), 88(95), 101(19), 116(15), M+.144(<1)	94
	Aldehydes and Ketones			
6.4	hexanal	T; B;	27(41), 29(49), 41(89), 43(75), 44(100), 56(93), 57(66), 72(24), M+. 100(1)	97
7.1	2-hexenal	B	27(43), 39(61), 41(100), 42(57), 55(84), 57(46), 69(69), 83(54), M+.98(16)	97
7.4	4-heptanone	T; B;	27(14), 39(10), 41(23), 43(100), 58(9), 71(99), 86(2), 99(3), M+. 114(22)	91
7.6	3-heptanone	T; B;	27(14), 29(35), 41(22), 57(100), 72(20), 85(31), M+. 114(13)	94
7.7	2-heptanone	T; B; X	27(10), 29(9), 39(10), 41(14), 43(100), 58(70), 59(12), 71(20), M+.114(8)	91
8.0	heptanal	T; B; X	29(63), 41(94), 42(58), 43(100), 44(81), 55(66), 57(59), 70(84), M+.114(4)	96
8.1	3,4,5-trimethyl-2-cyclopenten-1-one	B	53(18), 79(17), 81(21), 95(6), 96(5), 109(100), 110(7), 123(4), M+.124(47)	90
8.4	2-ethyl-2-hexenal	T	39(43), 41(72), 43(49), 55(100), 67(51), 69(33), 97(84), 111(37), M+. 126(77)	83
8.7	2-ethyl-hexanal	T; B;	27(17), 29(24), 41(43), 43(41), 57(100), 72(92), 85(5), M+.128(1)	91
8.9	benzaldehyde	T; X	29(25), 39(7), 50(27), 51(45), 52(11), 77(100), 78(19), 105(98), M+.106 (99)	97
9.1	3,5-heptadienone	T	43(33), 57(50), 99(100), 100(7), M+.128(27)	86
9.2	2-octanone	T; B	27(7), 41(14), 43(100), 58(91), 59(17), 71(18), 85(7), 113(4), M+.128(5)	91
9.4	octanal	B	27(42), 29(61), 41(90), 43(100), 56(68), 57(90), 69(35), 84(56), M+.128(1)	94
10.5	acetophenone	T	51(24), 77(76), 105(100), M+. 120(33)	95
10.6	3-ethyl-2-cyclopenten-1-one	T	27(33), 39(42), 57(100), 67(46), 81(69), 95(13), 109(14), M+.110(64)	93
10.9	2-nonanone	T	43(74), 58(100), 59(24), 71(20), 82(4), 84(4), 85(4), 127(3), M+. 142(7)	95
11.1	nonanal	T; B; X	29(47), 41(84), 43(72), 44(47), 55(55), 56(60), 57(100), 70(39), M+. 142(<1)	96
12.5	2-decanone	T; B	29(15), 41(29), 43(98), 57(24), 58(100), 71(37), 85(8), 96(6), M+.156(6)	95
12.7	decanal	T; B; X	29(54), 41(98), 43(95), 44(51), 55(73), 57(100), 70(53), 71(49), M+.156(<1)	95
13.9	2-undecanone	T; B	43(92), 58(100), 71(37), 85(10), 95(4), 112(5), 127(3), 155(2), M+.170(5)	95
14.2	undecanal	T; X	29(48), 41(87), 43(100), 55(66), 57(84), 68(40), 71(41), 82(51), M+.170(<1)	98
	Eters			
8.6	1-methoxy-4-methyl-benzene	X	27(21), 39(25), 51(35), 77(48), 79(32), 91(15), 107(46), 121(79), M+.122(100)	90
9.0	3-methyl-2(5H)-furanone	B	39(48), 41(100), 53(11), 69(75), M+.98(72)	94
9.5	2-pentyl-furan	T; B; X	27(11), 39(11), 41(14), 53(17), 81(100), 82(26), 94(5), 95(6), M+.138(20)	94
10.1	5-ethyldihydro-2(3H)-furanone	B	29(34), 42(20), 55(15), 56(19), 57(18), 70(12), 85(100), M+.114(2)	94
12.6	2-n-heptylfuran	T	27(10), 41(17), 53(16), 67(8), 81(100), 82(31), 95(21), M+.166(17)	93
	Hydrocarbons			
12.8	dodecane	B	29(26), 41(46), 43(83), 57(100), 71(51), 85(29), 112(5), 113(6), M+.170(5)	91
14.1	1-tridecene	T; B	41(79), 43(100), 55(79), 56(50), 57(67), 69(54), 70(43), 83(50), M+.182(22)	95
14.3	tridecane	T; B; X	27(13), 29(23), 41(45), 43(87), 55(26), 57(100), 71(57), 85(33), M+.184(4)	98
15.5	1-tetradecene	T	41(100), 43(71), 55(95), 56(67), 57(70), 69(70), 70(65), 83(74), M+.196(2)	95
15.6	tetradecane	T; B; X	29(21), 41(42), 43(80), 57(100), 71(59), 85(38), 99(9), M+.198(4)	98
15.7	1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-cyclohexane	B	41(66), 67(76), 68(73), 79(60), 81(100), 91(47), 93(96), 107(62), M+.204(2)	99
16.8	1-pentadecene	T; B	41(90), 43(100), 55(93), 56(54), 57(86), 69(68), 83(69), 97(56), M+.210(2)	99
16.9	pentadecane	T; B	29(18), 41(39), 43(73), 57(100), 71(63), 85(39), 99(13), M+.212(4)	97
18.1	hexadecane	B; X	41(36), 43(71), 57(100), 71(62), 85(44), 99(13), M+.226(4)	97
	Sesquiterpenes			
15.8	isocaryophyllene	B, X	69(45), 91(100), 93(66), 105(72), 107(51), 133(53), 161(48), M+.204(40)	93
16.1	caryophyllene	T	41(84), 69(100), 79(70), 91(75), 93(95), 105(52), 107(45), 133(79), M+.204(7)	96
16.2	2,6-dimethyl-6-(4-methyl-3-pentenyl)-bicyclohept-2-ene	T	41(56), 55(29), 69(45), 77(28), 91(39), 93(100), 107(31), 119(95), M+.204(2)	98
16.4	unknown	X		
15.3	longipinene	B; X	41(80), 55(50), 77(36), 91(70), 105(59), 119(100), 133(45), M+.204 (19)	93
17.0	unknonwn	T	28(100), 41(92), 43(83), 55(58), 57(80), 93(58), 119(79), 121(52), M+.204 (11)	
17.2	cedrene	X	65(), 105(), 133(), 161(), 189(), M+. 204()	90
17.5	unknonwn	X	105(), 129(), 145(100), 187(), M+. 202()	

B - *Beauveria bassiana*

T - *Trichoderma asperellum*

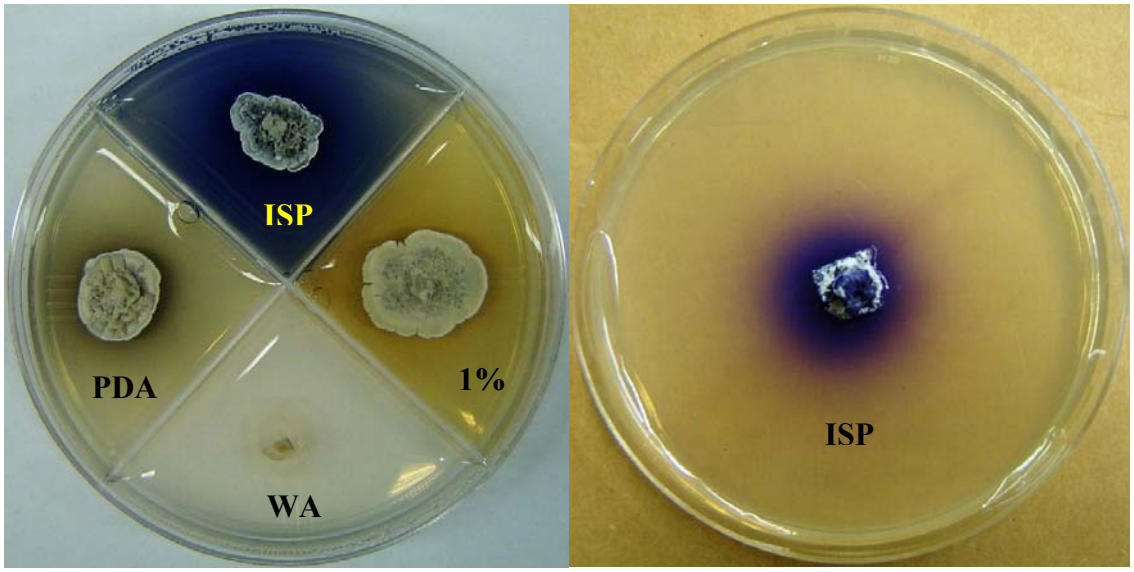
X - Interation *B. bassiana x T. asperellum*

Figure 25. Chemical structure of Actinohordin



Actinorhodin

Figure 26. Effect of the substrate on metabolite production by the actinomycete isolate



2.2.4.3. CONCLUSIONS

Media composition has an effect on the metabolites produced by the fungi studied. Caffeine, besides affecting the growth of the endophytes, affected their ability to produce toxins.

Volatiles played a role in the interactions between the fungal isolates. Fast growth rates may give advantage to some fungi, but their ability to grow under adverse conditions and produce secondary metabolites is also important.

The interactions between studied microorganisms ranged from parasitism to production of volatile organic compounds, antibiotics or toxins. The environment, to which the organisms are subjected, influences their fitness and should be considered when selecting biological control isolates. In a complex chemical warfare between microorganisms, understanding the factors influencing their ability to produce secondary metabolites enables researchers to provide favorable conditions for the target beneficial organism to succeed.

2.2.5. ESTABLISHMENT OF *Beauveria bassiana* AS AN ENDOPHYTE IN COCOA PODS

The entomopathogen *Beauveria bassiana* (Ascomycota: Hypocreales) has a potential to be used in cacao since it has been shown to grow endophytically in *Theobroma cacao* seedlings and is an efficient entomopathogen (Posada and Vega, 2005).

Several studies have tried to establish the fungal entomopathogen *Beauveria bassiana* as an endophyte to control insect pests in different plants species (Bing and Lewis, 1991; Jones, 1994; Wagner and Lewis, 2000; Leckie, 2002; Cherry et al., 2004; Quesada-Moraga et al., 2006; Gómez-Vidal et al., 2006; Akello et al., 2007). The stigma in a flower is a natural opening through which fungi can penetrate, as demonstrated by plant pathogenic fungi such as *Ustilago nuda* and *U. tritici* in cereals and *Monilinia oxycocci* in cranberries (McManus et al., 1999; Agrios, 2005). This experiment had two objectives: (1) determine if *Beauveria bassiana* could infect cacao pods via conidial spraying of the flowers; (2) determine if the fungus can colonize the pods as they develop, possibly protecting them against insects and mycotoxigenic fungi.

2.2.5.1. MATERIALS AND METHODS

2.2.5.1.1. Counting spores

A hemocytometer was used to estimate the amount of spores per mL in a spore suspension. Then the appropriate dilutions were made to obtain the desired concentration.

2.2.5.1.2. Application of *Beauveria bassiana* to cacao flowers and pods

A spore suspension of *B. bassiana* ($1x10^7$ conidia mL^{-1}) in 0.1 % Triton X-100 was applied with a hand held sprayer (Jet-Pak spray system, Sprayon Products, Inc., Cleveland, OH) to flowers of 15-year old greenhouse grown cacao trees. *Beauveria bassiana* treatments were applied immediately after hand pollination of recently opened flowers. Four trees were used for the inoculation with *B. bassiana* and four were used as controls. The flowers used as control were sprayed with sterile autoclaved water plus 0.1 % Triton X-100. After spraying, flowers were individually covered with plastic bags. In the case of the flowers sprayed with *B. bassiana* the increased moisture within the plastic bag was expected to encourage germination of *B. bassiana* conidia. The experiment was repeated four times; each time 120 flowers were treated, half with *B. bassiana* and half with water. Pods and peduncles were evaluated for presence of *B. bassiana* on a monthly basis after the treatments were applied (for a total of 5 months). The pods were divided in four sections and cut open; tissue samples were taken at random from the outer and inner part of each section of the pod (Figure 27).

Pod tissues were sterilized by submerging in 0.5 % bleach for two minutes, 70 % ethanol for two minutes, and then rinsed in sterile water. Tissues were dried with sterile tissue paper and the edges were removed, followed by plating 3x4 mm sections in 5 cm Petri dishes with YMA plus antibiotics (Posada and Vega, 2005).

2.2.5.2. RESULTS AND DISCUSSION

In the *B. bassiana* inoculation experiment, from a total of 480 flowers that were hand pollinated and sprayed, only 40 set fruit (8.33 %). Usually the fruit set achieved with hand pollination ranges from 30-50 %, in this experiment the fruit set was surprisingly lower. The *B. bassiana* treatment could have affected fruit set, although the controls (sprayed with water and surfactant) were also low. Perhaps even the spray with water and surfactant after pollination could have affected the flowers, or washed the pollen away and reduced the chance of fruit setting. Another control where flowers were hand pollinated and not sprayed at all would have helped to determine whether the spraying with water and surfactant influenced fruit set or not. Out of the 40 fruits formed, 10 wilted and only 30 resulted in pods that could be sampled. For cacao, this is a typical percentage of fruit that fail to develop to maturity. For *B. bassiana* treatment, 16 pods were sampled, while for controls, 14 pods were sampled (Table 11).

B. bassiana was recovered from the outer section of the pods in the second and third evaluation, two and three months post-inoculation. In the second evaluation, one pod out of four (25 %) was positive for *B. bassiana*, which was isolated from the third

quarter of the pod, and the fungus grew from one of the six pieces of tissue placed on YMA (Figure 27).

In the third evaluation, one out of five pods (20 %) was positive for *B. bassiana*, which was recovered from the fourth quarter of the pod, and the fungus grew in four of the six pieces of tissue placed on YMA. Tissues from a peduncle attached to the pod were positive for *B. bassiana* in the second evaluation. The evaluations of surface sterilized water did not yield any microorganisms thus assuring that the external surface of tissues was sterile.

This is the first report of the successful establishment of a fungal entomopathogen in fruits, as an endophyte, inoculated via flowers. This finding demonstrates a novel method for inoculating plants with a biological control agent. The recovery of *B. bassiana* from the pod and peduncle tissues demonstrates its ability to colonize and move through the pod. Further evaluations need to be done in an attempt to increase the *B. bassiana* establishment efficiency. The data indicate that although it is possible to establish *B. bassiana* via floral inoculation, the lower fruit set and poor percentage of successful inoculations need to be overcome before *B. bassiana* in the pods can be considered for testing as a novel biocontrol method against the cocoa pod borer.

2.2.5.3. CONCLUSIONS

B. bassiana was isolated from various sections of the cacao pod and peduncle two and three months after the flowers had been hand-pollinated and sprayed in the greenhouse with a *B. bassiana* conidial suspension. These results indicate that spraying flowers with conidia can result in the establishment of a fungal entomopathogen as an endophyte, and that the fungus can move within the pod as it grows. This finding could lead to an innovative way to manage insect pests in biocontrol programs.

Figure 27. Cocoa pod sections and parts sampled for determining the presence of *Beauveria bassiana*

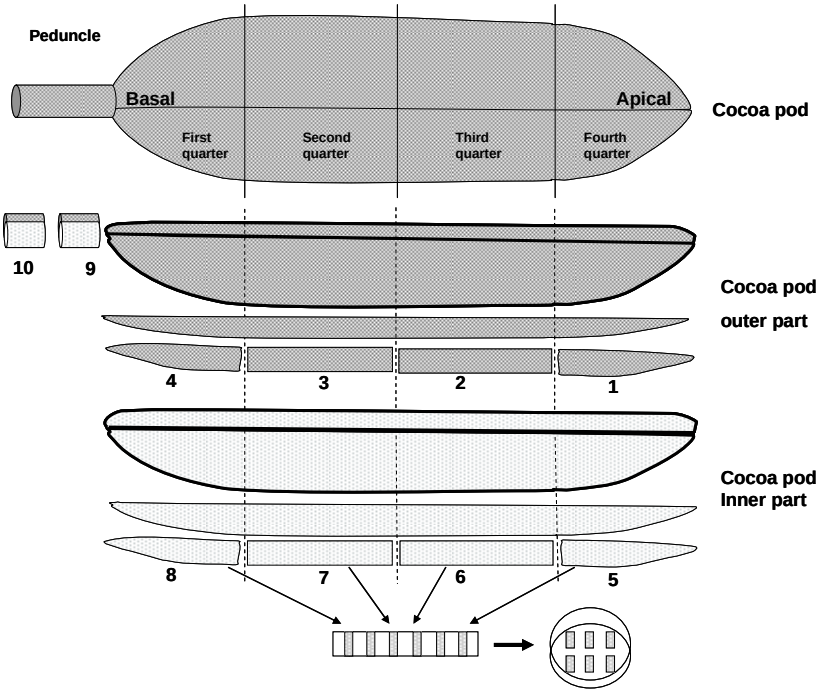


Table 11. Percentage of *B. bassiana* recovered from pods sprayed with a *B. bassiana* spore suspension after flower pollination

	B. bassiana Treatment					Control				
		Pod		Peduncle	Pod+Ped		Pod		Peduncle	Pod+Ped
Endophytic Fungi	+Bb Inside	+Bb Outside	+Bb Ins+Out	+Bb	+Bb	-Bb Inside	-Bb Outside	-Bb In+Out	-Bb	-Bb
First evaluation										
B. bassiana	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Second evaluation										
B. bassiana	0.0	**0.8**	**0.8**	**0.8**	**1.7**	0.0	0.0	0.0	0.0	0.0
Third evaluation										
B. bassiana	0.0	**5.2**	**5.2**	0.0	**5.2**	0.0	0.0	0.0	0.0	0.0
Fourth evaluation										
B. bassiana	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Fifth evaluation										
B. bassiana	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

2.3. PATHOGEN

2.3.1. INTRODUCTION

Among the pathogens attacking cacao, those affecting pods deserve primary consideration due to direct crop losses. The three major fungal pod diseases of economic relevance are Witches' Broom (WB), Frosty Pod (FP) and Black Pod (BP). The causal agents for these diseases are *Moniliophthora perniciosa*, *Moniliophthora roreri* and *Phytophthora* sp. respectively. BP disease, although present throughout the world, is a major problem in Africa where species of *Phytophthora* that are more virulent infect cacao. WB and FP are present only in the Americas; FP does not occur in Brazil at this time, but only in the northwestern portion of South America and in Central America. WB disease, the major constraint for cocoa bean production in South America and the Caribbean, a serious threat to the other cacao growing regions of the world still free of the pathogen, is the focus of this study.

Witches' broom disease caused by *Moniliophthora* (= *Crinipellis*) *perniciosa* (Stahel) Aime & Phillips-Mora, belongs to kingdom Fungi, phylum Basidiomycota, class Basidiomycetes, order Agaricales, family Tricholomataceae. *M. perniciosa* is a hemibiotrophic fungus that infects young meristematic tissue (actively growing shoots, flowers and developing fruits) and grows intercellularly as a monokaryotic biotroph. Subsequent to infection, the plant develops abnormal growth patterns including hypertrophy and hyperplasia, with excessive and unorganized growth of apical parts, loss of apical dominance, and proliferation of clustered short lateral shoots with undeveloped

leaves that resemble a broom. Throughout this period, the fungus becomes necrotrophic (dikaryotic) and infected plant parts turn necrotic. Once plant tissues are dead, basidiocarps emerge and release spores that start a new infection cycle (Figure 28).

Control strategies commonly employed against cacao pathogens include use of resistant cultivars, pruning and pod removal or pod stripping, fungicide applications and use of biological control agents. Phytosanitation is more successful with frosty pod disease because only pods have to be removed, whereas with witches' broom, infected branches, which are not always easily accessible, must be removed as well. Although there are disease tolerant cacao varieties, the genetic and physiological variability within the pathogen, as well as the absence of a genetic cross resistance to multiple diseases, makes cacao selection difficult. Therefore, a better understanding of the interactions between pathogens and cacao will help define more efficient disease management practices. In view of identifying the biochemical mechanisms of pathogenesis and disease resistance, *M. perniciosa* isolates were grown in culture media for the analysis of fungal metabolites and enzymes with possible roles in the disease process; the pathogen was also challenged with plant metabolites and fungal endophyte extracts.

Figure 28. *Moniliophthora perniciosa* basidiocarps formed in cherelles (young fruit of cacao) from a farm in Pichilingue, Ecuador

2.3.2. pH EFFECT ON GROWTH OF *M. perniciosa* AND *M. roreri*

M. perniciosa and *M. roreri* were grown in PDA at different pH levels in order to determine the optimum pH for growth of these pathogens in culture. *M. roreri* grew well on media with pH levels ranging from 5.6 to 4.4. At pH 3.8 its growth was completely inhibited (Figure 29). *M. perniciosa* had its growth rate slowed at pH 4.4, and this inhibition was stronger at pH 3.8 (Figure 30). The results show that relatively low pH is inhibitory to the growth of these cacao pathogens, presenting a potential mechanism of control.

Figure 29. Effect of pH on the growth of *M. roreri*

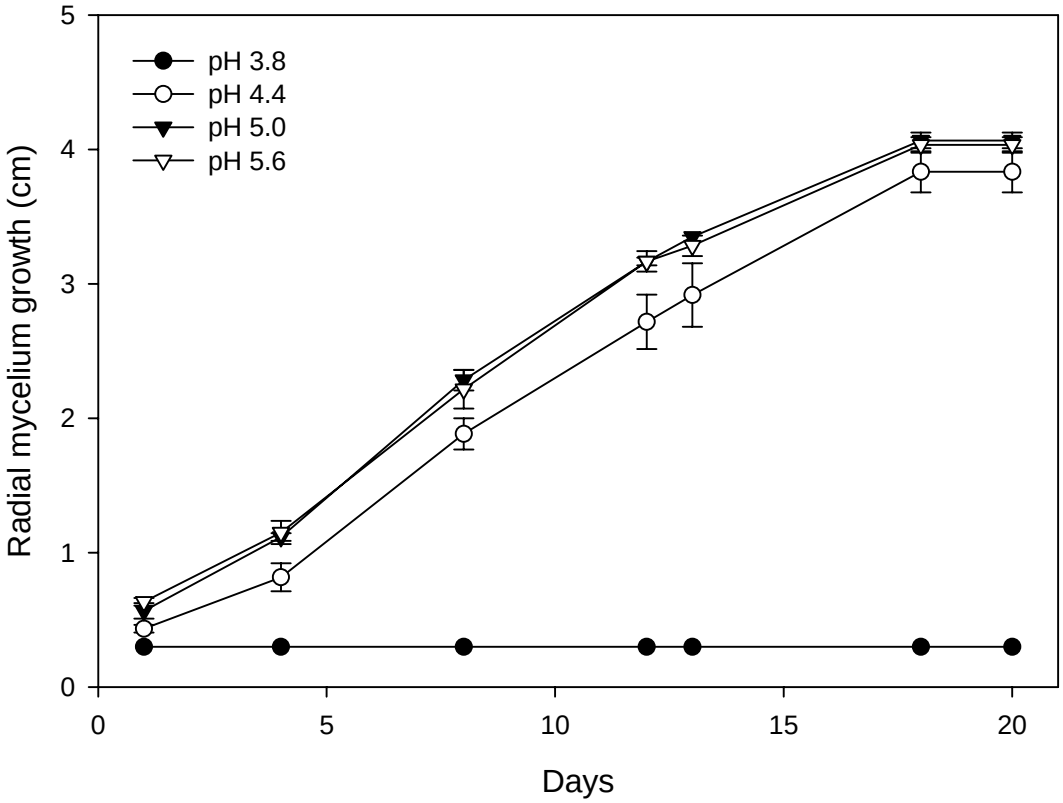
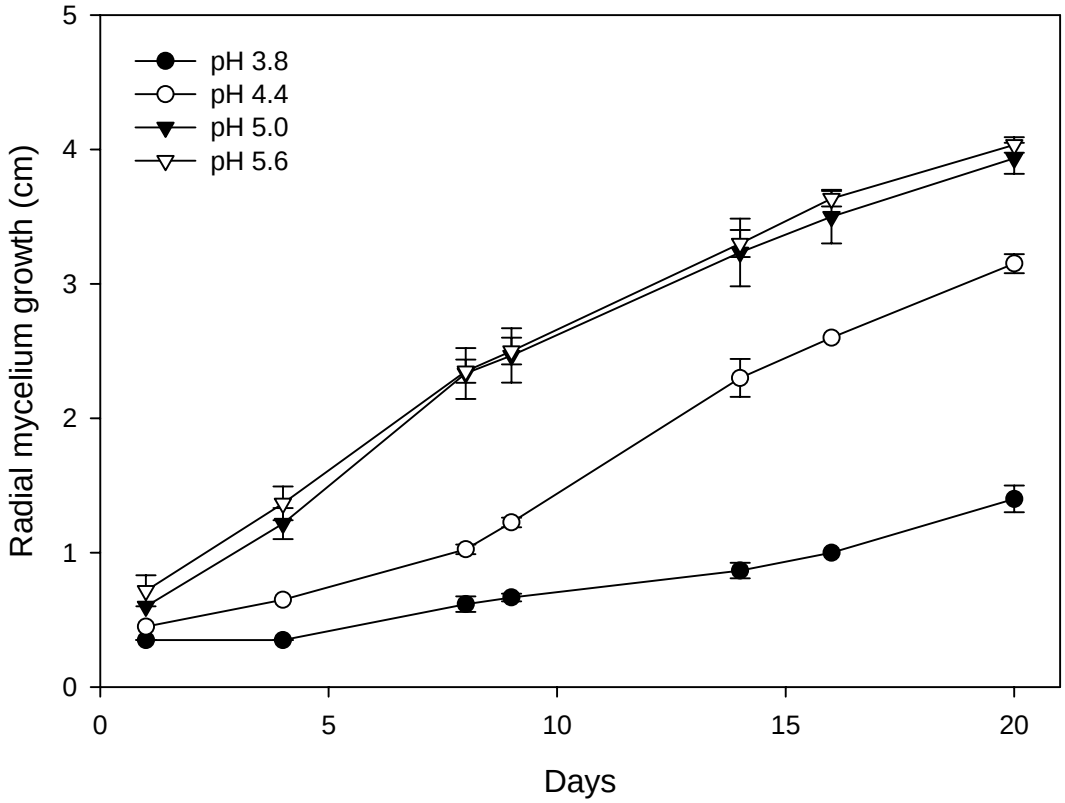


Figure 30. Effect of pH on the growth of *M. perniciosa*



2.3.3. ANASTOMOSIS GROUPS OF *Moniliophthora perniciosa* ISOLATES

M. perniciosa have been found associated with other plant species such as Bignoniaceae lianas, *Solanum paniculatum*, and *Heteropterys acutifolia*. *M. perniciosa* isolates have been classified according to host range: C-type pathogenic to cacao, L-type associated with Bignoniaceae lianas, S-type found in Solanaceus hosts, and H-type associated with the shrub *H. acutifolia*. The C-types have been further divided by geographic region. The C- and S-biotypes are non-outcrossing and form broom symptoms on hosts, whereas the L-biotype is outcrossing and asymptomatic. Wheeler and Mepsted (1988) also classified isolates based on their ability to induce symptoms. Phylogenetic analysis of several regions of the rRNA locus revealed near identity between C- and S-biotype isolates from diverse loci, but the L- and H-biotypes formed separate groupings. Differences could not be found among *M. perniciosa* C-type isolates using 20 random amplified polymorphic DNA (RAPD) markers, while vegetative compatibility experiments showed genetic differences within basidiospores from the same basidiocarp when single spore cultures were plated together. It is likely that a small number of genes control vegetative compatibility. Therefore, RAPD banding patterns that target relatively few areas of the genome possibly did not include the genes involved in vegetative compatibility (Andebrhan, 1994). Andebrhan et al. (1999) determined that *M. perniciosa* isolates found in Bahia belong to two groups based on RAPD analysis.

Here ten *M. perniciosa* isolates collected from infected brooms in Bahia, Brazil were subjected to vegetative compatibility assays in order to distinguish isolates and find

correlations between mycelial characteristics or other aspect of fungal physiology that could be linked to pathogenicity.

2.3.4. RESULTS AND DISCUSSION

A single *M. perniciosa* isolate, when inoculated at two sides of a Petri plate, has the two fronts of mycelial growth merge in a homogeneous mycelial mass. The same growth pattern occurs when two vegetatively compatible isolates are co-inoculated on a plate (Figure 31). However, when mycelia of two incompatible isolates come upon each other, a pigmented thick layer forms at the point where they merge (Figure 32). A third type of interaction is an intermediate one, in which a pigmented layer is absent in between the isolates and they do not merge as evenly as in the compatible case (Figure 33). Andebrhan et al. (1999) determined that there are two groups of isolates infecting cacao in the Bahian region where these isolates were collected.

Based on the results of the vegetative compatibility experiments, three groups of isolates were observed (Figure 34). One group was composed of isolates PS10, PS12, 16avd1 and Hrvd1, which were compatible among each other and partially compatible with isolates of group 2, composed of Alf42, PS9 and 1130. Isolates from group 2 had intermediate compatibility among each other. Both groups 1 and 2 were incompatible with isolates of the third group, 278, 1103 and PS6, which were compatible among each other. This set of isolates also varied in growth rates that ranged from 0.3 to 0.6 cm/day, where isolates from group 1 had higher rates (Figure 35).

Figure 31. Vegetatively compatible isolates (1103 left and 278 right)

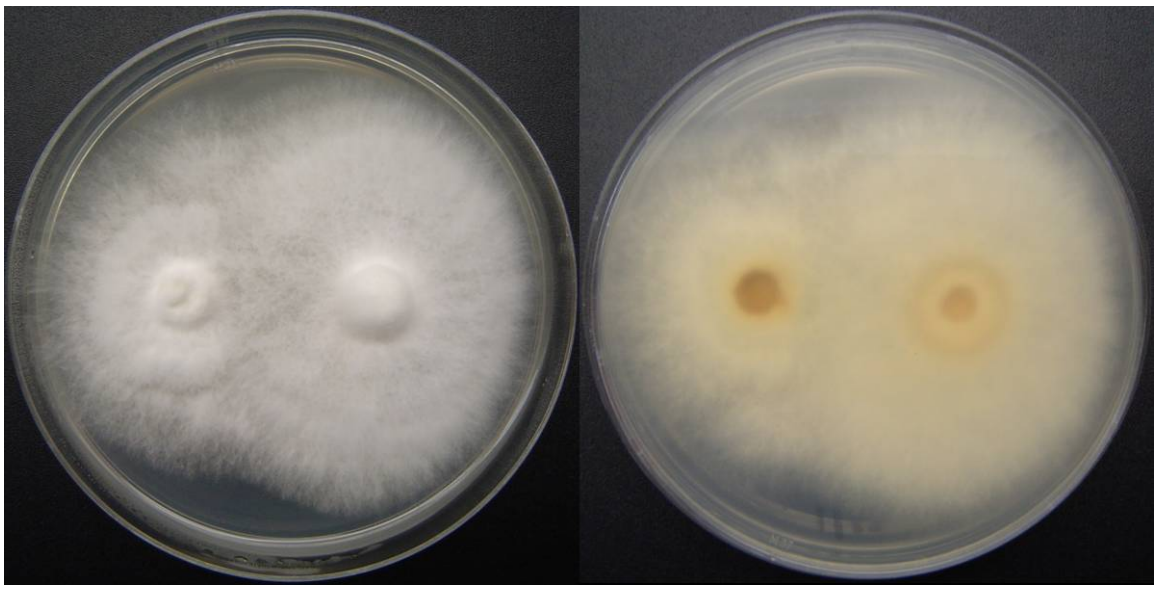


Figure 32. Vegetatively incompatible (PS6 left and Alf42 right)

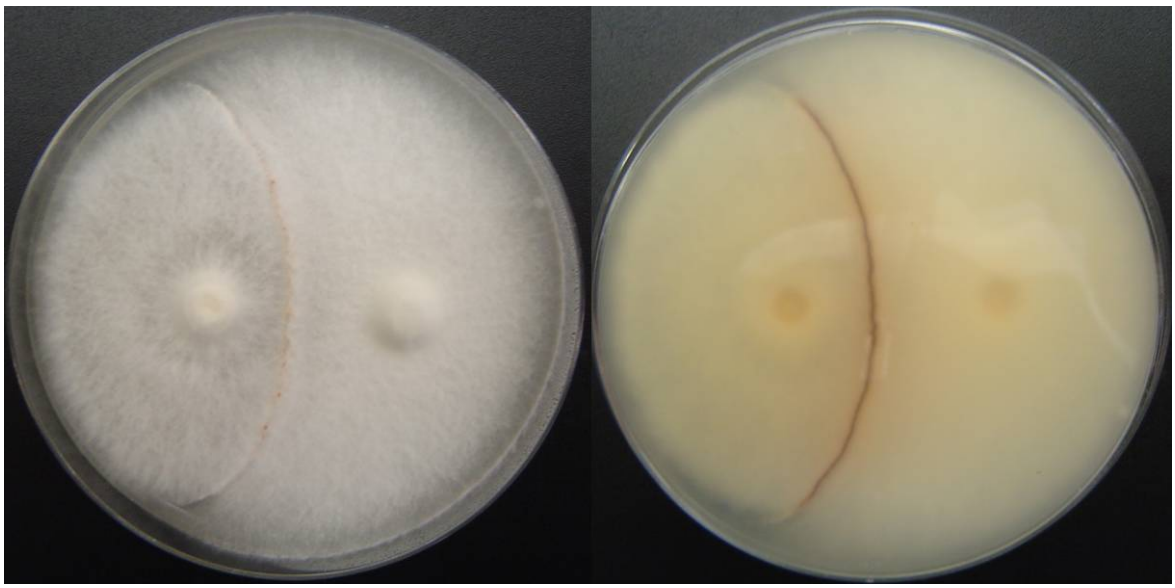


Figure 33. Intermediate vegetative compatibility (PS9 left and PS12 right)

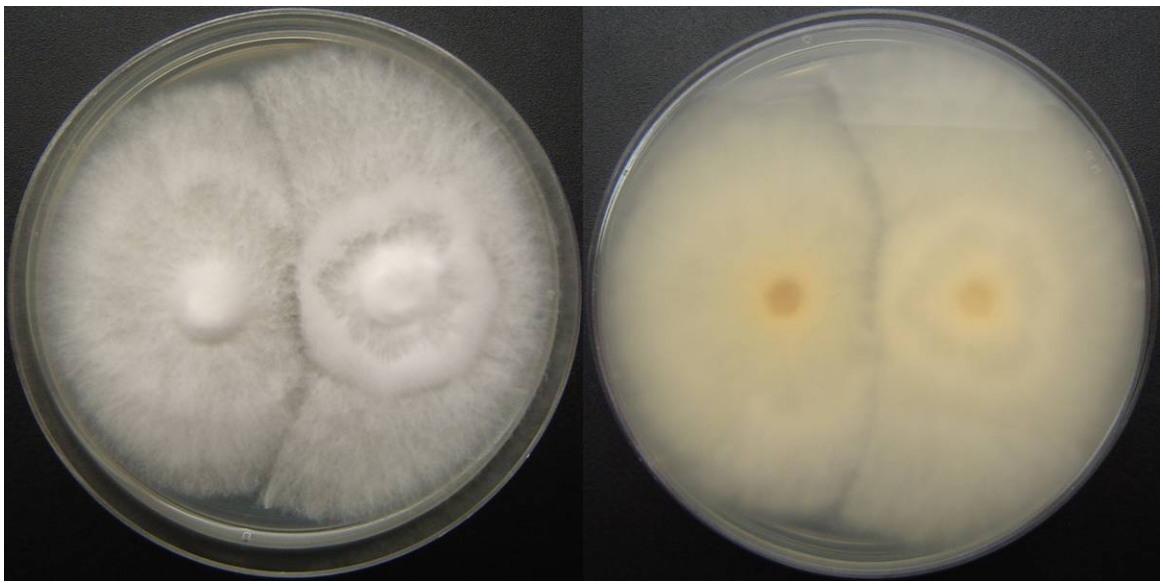


Figure 34. Vegetative compatibility groups of *M. perniciosa* isolates (Red – Incompatible; Yellow – Intermediate compatibility; Green – Compatible)

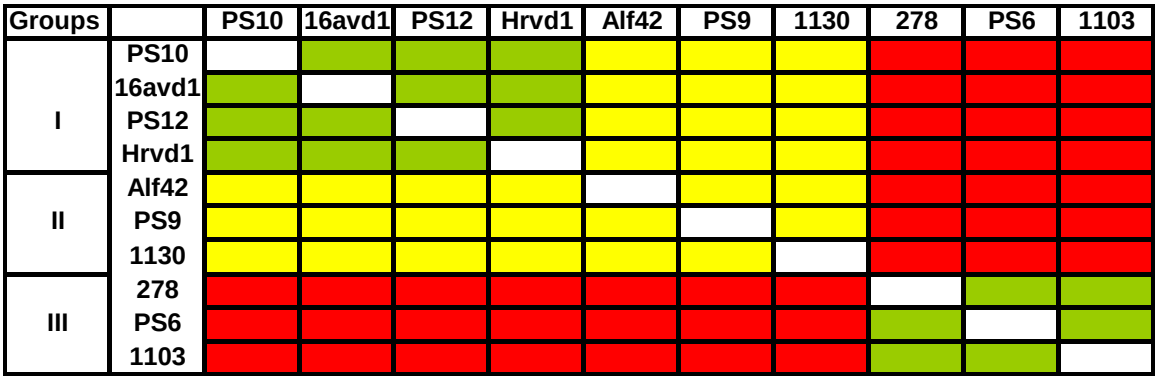
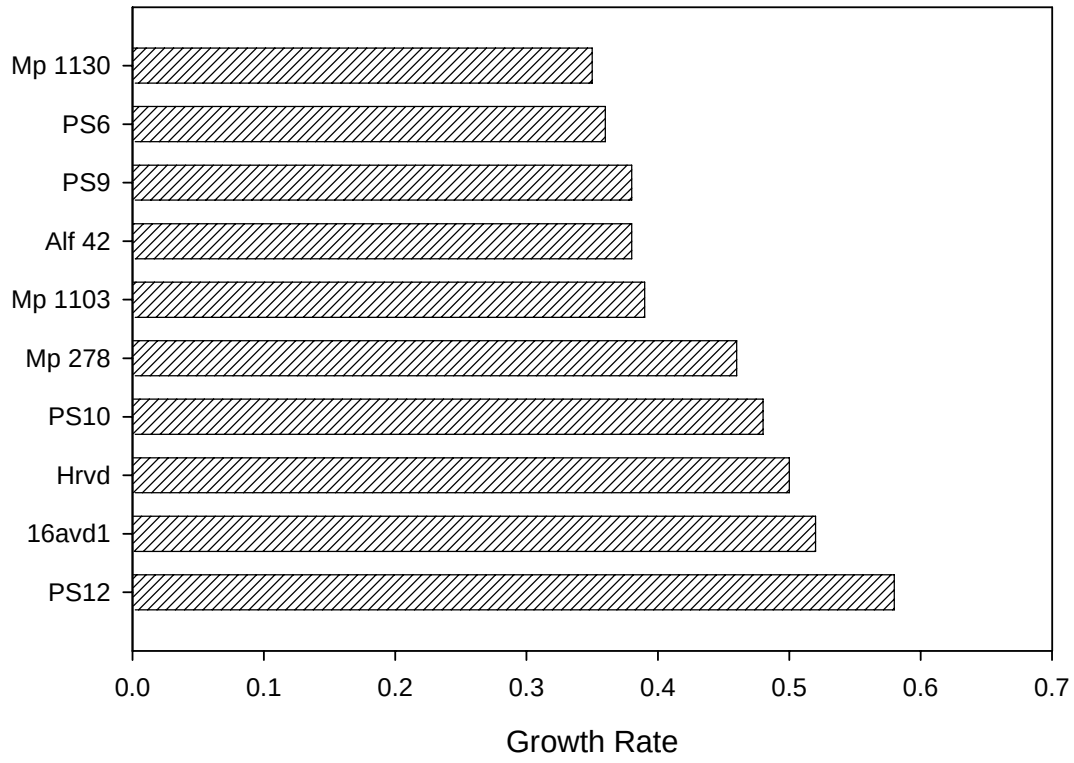


Figure 35. *M. perniciosa* isolate growth rates (cm/day)*



* No significant statistical differences were observed using the F test at P.05.

2.3.5. METABOLITES PRODUCED BY *M. perniciosa*

Although most steps of *M. perniciosa* infection biology are known (Frias et al., 1991), a few key points remain unclear. The symptoms appear to be hormonally induced, yet application of hormones to uninfected actively growing leaves fails to induce broom development. Another aspect not well understood that could be linked to symptom development, is when and how the pathogen switches from a biotrophic to a necrotrophic phase. Scarpari et al. (2005) conducted a thorough study of the biochemical changes in cacao during the development of witches' broom; however, salicylic acid (SA) levels were not analyzed. SA, an extensively documented plant hormone, triggers systemic acquired resistance (Durrant and Dong, 2004), thermogenesis (Raskin et al., 1989), and regulates coupled cell enlargement and cell death (Rate et al., 1999; Vanacker et al., 2001). In cacao, SA and chemical elicitors, including an SA analogue, benzothiadiazole, induce chemical changes similar to those observed in infected tissues when applied to young shoots (Aneja and Gianfagna, 2001). The hypothesis here is that chemical changes leading to broom development (cell enlargement and death) involve regulation via the SA pathway and that the fungus may be the source of SA.

2.3.5.1. MATERIALS AND METHODS

2.3.5.1.1. Chemical reagents

All solvents used were HPLC grade. BSA (N,O-bis(trimethylsilyl)-acetamide) (Pierce, USA) was used as derivatizing reagent prior to GC-MS analysis. SA was used as the standard for HPLC and GC-MS analyses.

2.3.5.1.2. Fungal cultures

M. perniciosa isolates 278, Alf42, 1103, PS12, 16avd1, 1130, Hrvd1, PS9, PS6 and PS10 collected in Bahia, Brazil, from diseased cacao tissues were grown from mycelia in potato dextrose broth for three weeks. The mycelia were then filtered off using glass wool, and the broth was lyophilized and stored at -80 ^{o}C until analysis.

2.3.5.1.3. Plant material

Infected brooms and healthy cacao shoots were collected, freeze-dried and stored at -80 ^{o}C.

2.3.5.1.4. SA extraction and HPLC analyses

SA extraction and HPLC analyses followed the method of Verberne et al. (2002). A Shimadzu LC-10AT equipped with a RF-10A spectrofluorometric detector and a C18 column (140 mm x 4.6 mm x 0.5 μm particle size) was used. The fluorescence detector was set at an excitation wavelength of 305 nm and emission of 407 nm; the flow rate was 0.8 $mL \cdot min^{-1}$ using a mobile phase of 90 % 0.2 M sodium acetate buffer pH 5.5 and 10 % methanol.

2.3.5.1.5. GC-MS analyses

The samples analyzed by HPLC were dried in nitrogen, resuspended in acetonitrile and derivatized with BSA (reaction vial was heated at 70 ^{o}C for 15 minutes). Samples were injected in the GC without further purification. GC-MS analyses were performed using a Hewlet Packard (HP) gas chromatograph 6890 series, equipped with an Agilent column DB-1MS (60 m x 250 μm i.d. x 0.25 microns film thickness) using helium (chromatographic grade, from Airgas, NJ, USA) as carrier gas with a flow rate of 1 $mL \cdot min^{-1}$. Oven temperature was set at 70 ^{o}C, held for two minutes and increased to 240 ^{o}C at 10 $^{o}C \cdot min^{-1}$ and held for 30 minutes. Spectral data was obtained from an HP mass spectrometer 5973 series with ionization energy of 70 eV and mass scan range from 40 to 550 amu at 1.97 $scans \cdot s^{-1}$. The MS source temperature was set at 230 ^{o}C.

Compounds were identified by comparison to commercially available standards or the equipment's library (NIST 98).

2.3.5.1.6. SA application to cacao tissues

SA was applied to young and old leaves, flowers, trunk and fruits (2 months old) of cacao using a hand held sprayer. Plant parts were sprayed until their surface was completely covered with solution. Spray solution contained 20 mM of SA in distilled water and 0.05 % Tween 80. Control spray solution contained 0.05 % Tween 80 in distilled water.

2.3.5.1.7. Statistical analysis

Statistical analysis of the data was performed using SAS version 9.1 for Windows (SAS Institute, USA).

2.3.5.2. RESULTS AND DISCUSSION

M. perniciosa is a hemibiotrophic fungus, with a biotrophic and necrotrophic phase. As such, cell death assists the pathogen to complete its life cycle. For this reason, cacao avoids the SA pathway. In summary, SA levels in young leaves treated with elicitors were not statistically different from control treatments. Young cacao leaves maintained the same SA levels as the controls after pathogen inoculation, locally and systemically. Endogenous SA levels of both tested cultivars, including the more disease tolerant SCA and the more susceptible CCO, did not differ statistically. Cacao had the

highest levels of SA in the youngest tissues, which are known to be susceptible to disease.

Some pathogens produce plant growth hormones (auxin, ethylene) and derivatives (coronatine, a JA analogue) as virulence factors that may act by disrupting the tightly regulated plant defense metabolism, altering physiological processes and favoring the pathogen to thrive. All tested *M. perniciosa* isolates, when grown in liquid culture media, produced SA at varying levels ranging from about 0.3 to 2.2 $ng \cdot g^{-1}$ of fresh weight of mycelia (Figure 36). There was an inverse relationship between growth rate *in vitro* and SA content *in planta*. Vegetative compatible isolates from group 1, were the fastest growing isolates, and produced the lowest average content of SA.

SA was identified by matching the retention time of the standard with the compound in the sample at specific excitation and emission wavelengths. A calibration curve was prepared by injecting different concentrations of the commercially available SA standard. In order to confirm SA's identity, samples extracted for the HPLC analysis were examined by GC-MS. GC-MS runs also confirmed the presence of two other aryl compounds, mandelic acid and phenyllactic acid (Figure 37). SA was identified as its trimethylsilylated derivative obtained after reaction with BSA. The retention time and mass spectra of the compound present in the fungal extract were identical to the trimethylsilylated SA standard. The major fragment ions were: m/z 73(78.2), 91(11.4), 135(28.2), 149(7.4), 179(8.6), 209(6.8), 267(100), 268(22.4), 269(10.3) and $M^{+\cdot}$ 282(<1) (Figure 37). Another phenolic compound characterized by GC-MS was 2-hydroxy-2-phenylacetic acid (also known as mandelic acid) with the distinctive fragmentation: m/z 73(65.3), 147(33.1), 163(11.3), 179(100), 253(15.2), 281(2.5) and $M^{+\cdot}$ 296(<1) (Figure

37). A third compound identified was 2-hydroxy-3-phenylpropanoic acid (also known as phenyllactic acid) with the following fragmentation pattern: m/z 59(5.5), 73(100), 147(74.5), 193(93.7), 205(5.9), 220(19.7), 267(14.1), 295(10.8) and $M^{+\cdot}$ 310(<1) (Figure 37). Identification of the two latter compounds was based on their fragmentation pattern and by comparison to the equipment's library. These molecules have been previously identified as intermediates in the metabolic pathway of the white rot fungus, *Bjerkandera adusta*, leading to synthesis of aryl metabolites (Lapadatescu et al., 2000), and have been found in the basidiomycete *Oudemansiella mucida* (Zouchova et al., 1982). These SA derivatives could be intermediates in the synthesis of SA as well as active compounds that could interfere with the plant defenses by mimicking SA. The jasmonic acid pathway, primarily responsible for defense responses against herbivores, is negatively regulated by SA (Kunkel and Brooks, 2002; Thaler et al., 2002). Therefore, activation of SA may render the plant susceptible to attack from other pathogens by what Cui et al., (2005) called systemic induced susceptibility (SIS). In the SIS activated by SA, the pathogen alters a defensive pathway to induce vulnerability to pathogen-vectoring herbivores that would, in one hypothesis, increase the spread of the pathogen. In addition, *Arabidopsis thaliana* mutants impaired in the JA pathway are more susceptible to necrotrophic fungal pathogens including *Alternaria brassicicola*, *Botrytis cinerea*, and *Pythium* sp. (Kunkel and Brooks, 2002). Based on these results, one hypothesis for further investigation in cacao is that SA blocks the JA pathway, rendering the plant susceptible to *M. perniciosa* infection.

There are evolutionary speculations that *M. perniciosa* evolved from an endophyte to a pathogen. It may have acquired the mechanism of producing SA to alter

plant defenses to its advantage, and as a consequence, modified itself from a biotrophic and endophytic organism to a hemibiotrophic pathogen.

At a certain stage in its development, the fungus may be the source of the SA, which stimulates cell growth and enlargement, producing the broom symptoms. The rising SA levels induce cell death and the brooms become necrotic. The symptoms remain localized in the young tissues due to their sensitivity to SA. When high concentrations of SA were applied to different plant parts, only flowers and young leaves showed necrosis and abnormal growth (Figure 38). In addition, the analyzed broom tissue had SA levels five times higher than healthy tissue (Figure 39). Healthy shoots had 224.2 ng of $SA \cdot g^{-1}$ dry weight stem tissue, while brooms had statistically higher ($F\ p < 0.01$) levels of 1,212.3 ng of $SA \cdot g^{-1}$ dry weight stem tissue. Although the possibility that the higher SA levels are produced by the plant cannot be ruled out, the results suggest that the fungus promotes an increase in SA levels, which may play a role in pathogenesis (Figure 40) and provide a target for disease control.

The fungal isolates used in this study were collected from diseased cacao tissue. Thus, it is possible that non-pathogenic strains do not produce SA and therefore have a symbiotic interaction with cacao.

Following Chaves and Gianfagna (2006), Kilaru et al. (2007) obtained similar results that support and confirm the findings described above. Moreover, the mechanism of pathogenicity by *M. perniciosa* likely involves cell death regulated by calcium oxalate and hydrogen peroxide (Ceita et al., 2007). Plant resistance seems to be related with the ability of the plant to avoid calcium oxalate accumulation and by activation of hydrogen peroxide quenching enzymes (Ceita et al., 2007).

Figure 36. Salicylic acid produced by *M. perniciosa* isolates in culture

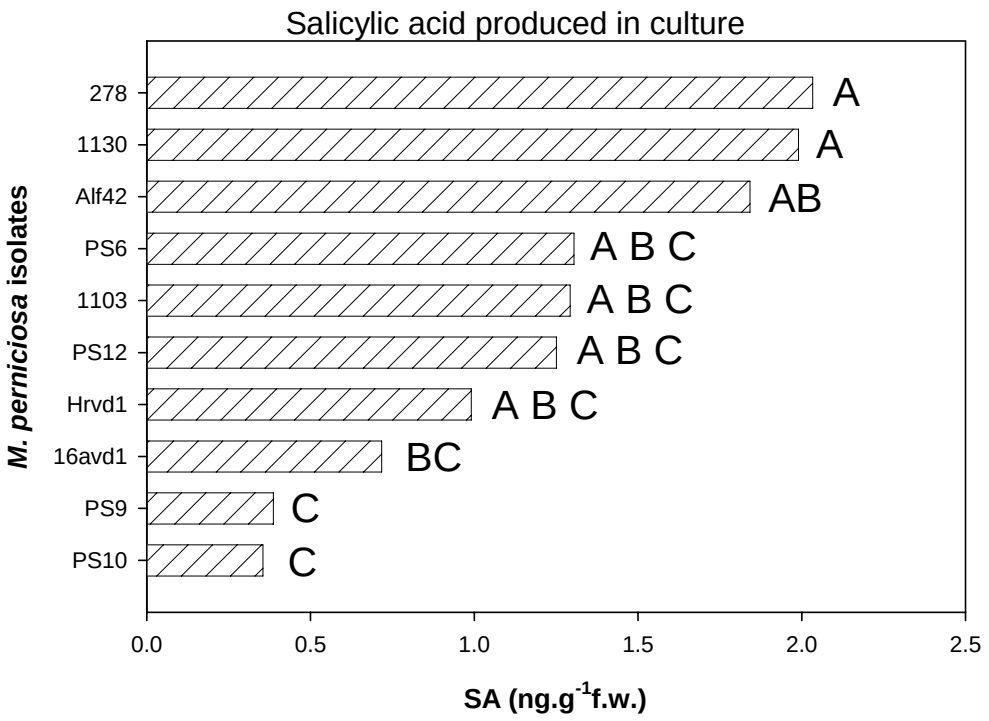


Figure 37. Mass spectra of trimethylsilylated fungal metabolites salicylic acid, mandelic acid and phenyllactic acid

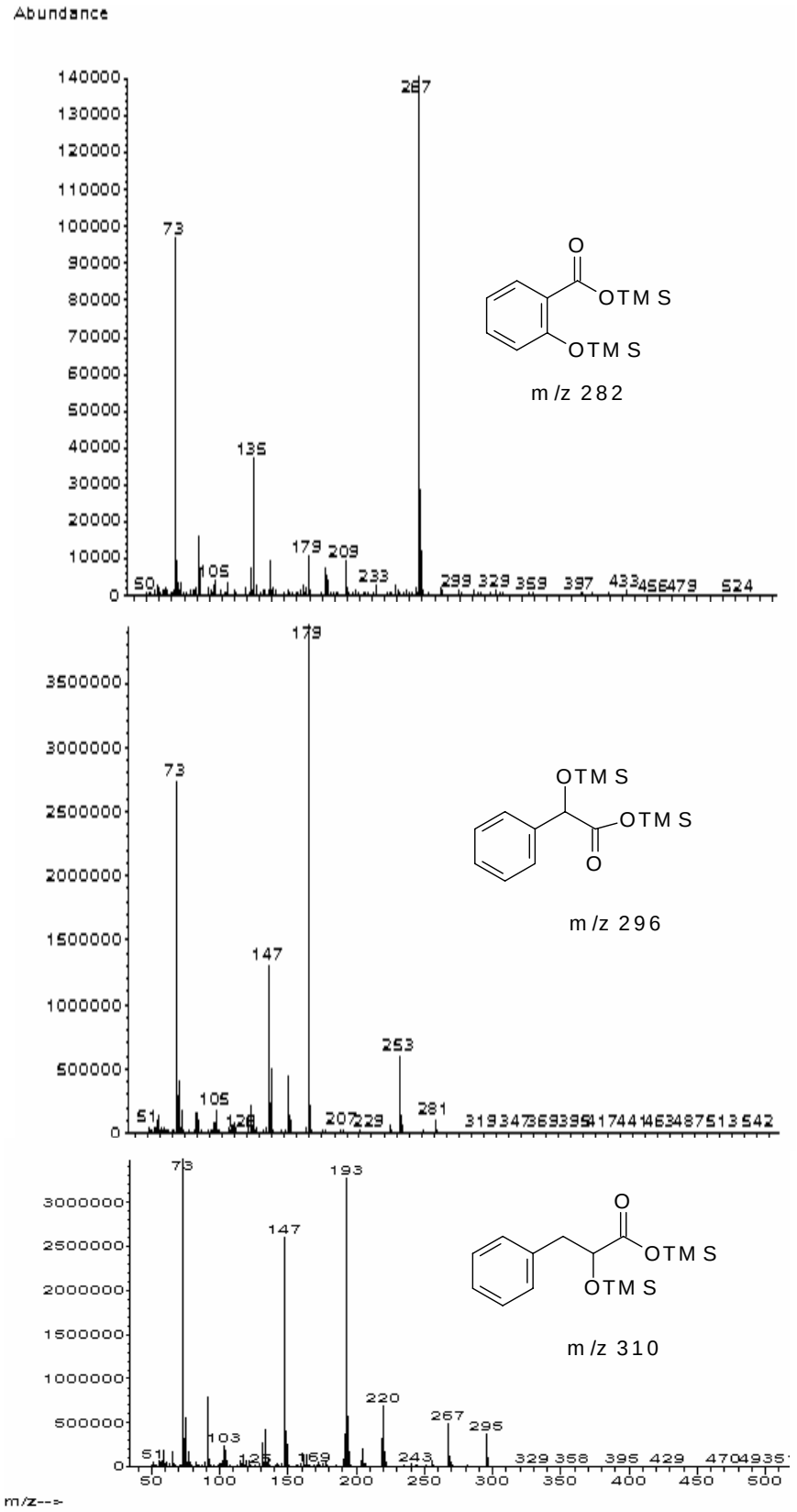

Abundance
287
73
135
179
209
105
233
OTMS
OTMS
m/z 282
179
73
147
253
105
281
OTMS
OTMS
m/z 296
73
193
147
220
267
295
103
OTMS
OTMS
m/z 310
m/z-->

Figure 38. Salicylic acid treatment to young cacao leaves

Figure 39. HPLC chromatogram of salicylic acid in brooms and healthy stems. *Different letters following means indicate significant difference at $p < 0.01$.

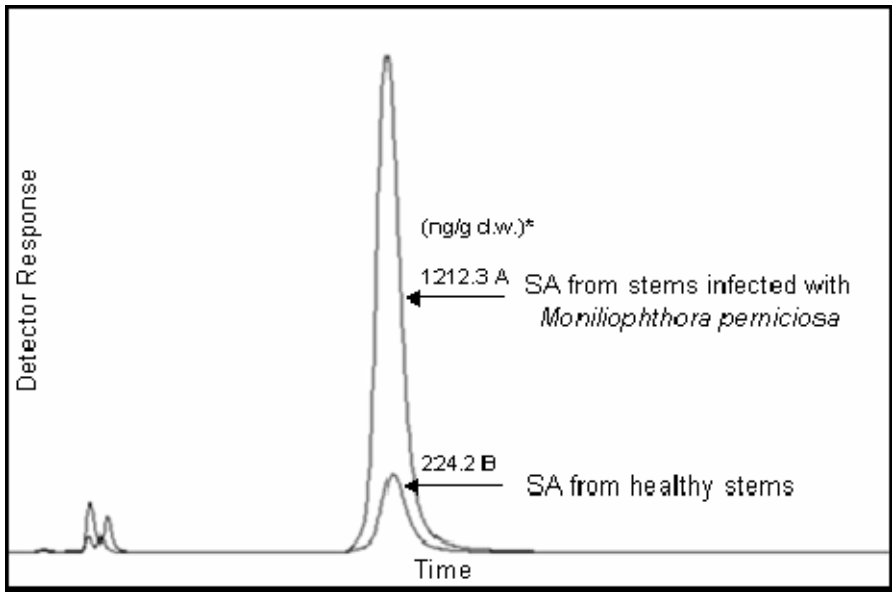
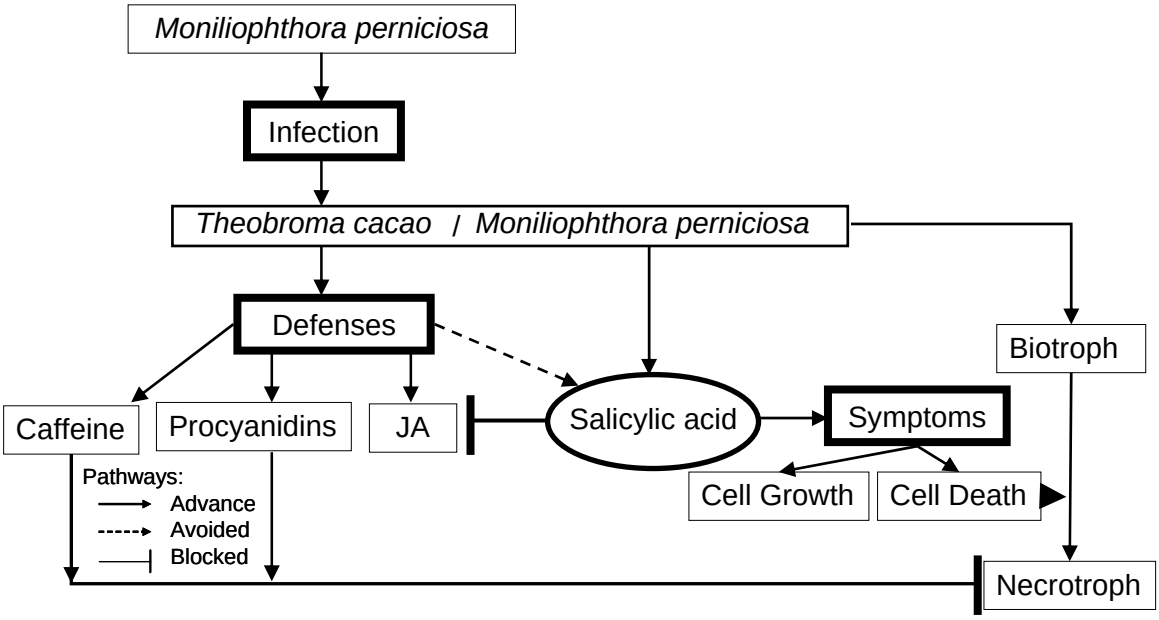


Figure 40. Flow chart with proposed role for salicylic acid in pathogenicity



2.3.5.3. CONCLUSIONS

Salicylic acid (SA), phenyllactic acid and mandelic acid were identified as products of fungal metabolism by *M. perniciosa* during the dikaryotic growth stage. Levels of SA were quantified in infected and uninfected plant tissues as well as in filtrates of fungal cultures. When SA levels were measured in plant tissues, infected brooms had five times more SA compared to healthy shoots. Moreover, application of SA to leaves induced curling and necrosis. In contrast, leaf inoculation with basidiospores or application of chemical elicitors did not alter leaf SA content, suggesting that in this infection system, the actively growing mycelia is the source of the SA rather than the plant.

When plants respond to infection by necrotrophic pathogens through the activation of cell death, the pathogen growth is accelerated instead of retarded. The necrotrophic pathogens *B. cinerea* and *S. sclerotiorum* induced oxidative burst and cell death in *Arabdopsis*, and their pathogenicity was directly related to the generation and accumulation of superoxide and hydrogen peroxide (Govrin and Levine, 2000). For necrotrophic fungal pathogens, including *M. perniciosa*, SA may be a component of pathogenesis and not part of the defense response of plants. Therefore, it is possible that plants capable of responding to infection more effectively by activating cell death, when challenged by a necrotrophic pathogen, tend to be more disease susceptible. This can explain why cultivars tolerant to non-necrotrophic pathogens are susceptible to necrotrophs like *M. perniciosa*.

2.3.6. *M. perniciosa* ENZYMES INVOLVED IN DISEASE

Lipoxygenases (LOX; EC 1.13.11.12) are iron-containing dioxygenases widely distributed in plants, animals and fungi. In plants, lipoxygenases are known to be active in defense response. LOX gene expression is regulated by different effectors such as JA, abscisic acid, and by different forms of stress, such as wounding, water deficiency, or pathogen attack. In mushrooms and other fungi such as *Penicillium* sp., the combined activities of LOX and HPLS (hydroperoxide lyase) convert linoleic acid and linolenic acid into volatile C8 and non-volatile C10 compounds. One of these C8 compounds, 1-octen-3-ol, responsible for the characteristic mushroom aroma, is also known to be an insect attractant (Ritchie, 1994) and an inhibitor of spore germination (Chitarra, 2003).

Another metabolite of the LOX pathway produced along with 1-octen-3-ol is the bioactive substance 10-oxo-trans-8-decenoic acid (ODA). ODA is a fungal hormone that stimulates mycelial growth, stipe lengthening, and basidiocarp initiation (Champavier, 2000; Money, 2002). It is also very inhibitory to other fungi such as *Penicillium* spp. and *Aspergillus* spp. (Mau and Li, 2002; Okull et al., 2003). The lypoxygenase pathway involved in the development of basidiomycetes, including the witches' broom pathogen, is a possible target for plant defenses because blocking the production of ODA may prevent basidiocarp development and the formation of infective basidiospores.

It has been shown in previous sections that procyanidins are part of cacao's biochemical defensive response to pathogens. (-)-Epicatechin and other flavonoids have been reported to inhibit various lipoxygenases such as rabbit reticulose 15-lipoxygenase-1, recombinant human 5-lipoxygenase and soybean 15-lipoxygenases (Sadik et al., 2003).

It is possible that cacao polyphenols may target fungal lipoxygenases to inhibit fungal reproduction. *M. perniciosa*, however, is successful in causing disease in cacao. Therefore, it must have its own defenses against procyanidins.

In a pathosystem involving avocado and the hemibiotroph *Colletotrichum gloeosporoioides*, Guetsky et al. (2005) found that laccase secretion by *C. gloeosporoioides* lowers (-)-epicatechin levels, which in turn increases lipoxygenase activity, metabolizing an antifungal compound (conjugated diene, 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene). This contributes to the activation of quiescent infections, thus inducing susceptibility in avocado. Furthermore, *C. gloeosporioides* isolates that showed reduced capability to metabolize (-)-epicatechin were not able to colonize ripe fruits that naturally lack the antifungal compound. Laccase activity has been reported to be associated with normal mycelial growth in a range of fungi and in the development of rhizomorphs and sclerotia in certain basidiomycete and ascomycete taxa, as well as with basidiome production. In some basidiomycetes, expression of laccase genes and/or accumulation of laccase occur during vegetative growth, but both expression and activity decline following basidiome initiation.

Laccases (EC 1.10.3.2), polyphenol oxidases (PPO), catechol oxidase (EC 1.10.3.1) and tyrosinases (EC 1.14.18.1), are phenolic oxidative enzymes that cause browning in many fruits and vegetables, and can be produced by a range of fungi.

Polyphenol oxidases are also often referred to as tyrosinase, phenolase, catechol oxidase, or catecholase. PPO are copper containing enzymes (Chen et al., 1991) that catalyze the hydroxylation of monophenols to o-diphenols and oxidate o-diphenols to o-quinones. These products may condense and react non-enzymatically with other phenolic

compounds, amino acids, proteins, and other cellular constituents to produce colored polymers and pigments.

Basidiomycetes have several oxidases that can use phenolic substrates. In *Agaricus bisporus*, tyrosinase, laccase, and peroxidase were detected. Tyrosinase was present in larger amounts and was the main enzyme responsible for browning reactions (Zhang and Flurkey, 1997; Jolivet et al., 1998).

These enzymes have considerable overlap in terms of their substrate affinities. Any substrate with characteristics similar to a p-diphenol can be oxidized by laccases. In addition, some fungal laccases can also oxidize monophenols and ascorbic acid (Mayer, 2002). They can be differentiated from one another by testing the effects specific substrates and inhibitors on enzyme activity. For example, L-tyrosine (L-3-[4-hydroxyphenyl]-alanine) is not used by laccases but by tyrosinases only.

Laccase is a copper containing enzyme that oxidizes phenolic compounds to reactive semiquinones that, depending upon the degree of auto-oxidation can yield quinines, which in turn polymerize forming insoluble complexes. The reactive semiquinone may also react with O_2 to yield a superoxide radical ($O_2^{-\cdot}$) that may lead to a depolymerization processes. This contrasts with other polyphenoloxidases that lead to formation of quinines rather than semiquinones. Distinguishing the products of these reactions may be very complicated due to the variability of the products formed, and it requires highly sophisticated and complex analytical procedures and equipment. Spectrophotometric measurements of enzyme activity using different substrates, however, are advantageous in that they are straightforward and require only basic instrumentation.

Here phenoloxidative enzymes secreted in the growing media by *M. perniciosa* isolates were extracted and analyzed with different substrates and inhibitors to determine their activity and possible role in pathogenesis.

2.3.6.1. MATERIALS AND METHODS

2.3.6.1.1. Fungal cultures

Fungi were stored in 15 % glycerol at -80 ^{o}C to preserve viability and limit variations due to mutation, a consequence of continuous subculturing.

2.3.6.1.2. Effects of ODA on *M. perniciosa*

M. perniciosa was inoculated in media containing 10-oxo-trans-8-decenoic acid (ODA) alone and in combination with 1-octen-3-ol. In addition, two endophytes used as biological control agents of *M. perniciosa* were challenged with ODA.

2.3.6.1.3. *M. perniciosa* growth assay in culture supplemented with (-)-epicatechin

M. perniciosa growth was evaluated in culture media supplemented with either (-)-epicatechin, the main polyphenol in cacao, or kojic acid, a PPO inhibitor and fungal metabolite, and both compounds combined.

2.3.6.1.4. (-)-Epicatechin degradation

(-)-Epicatechin degradation was analyzed by RP-HPLC using a fluorescence detector. Mobile phase A (90:8:2) (water:methanol:acetic acid) and mobile phase B (90:8:2) (methanol:water:acetic acid) were used in a gradient starting at 100 % A. At five minutes, A concentration was 75 % and B 25 %, at ten minutes A and B were 50 %, and at 20 minutes A was 25 % and B 75 %. The flow rate was 0.8 $mL \cdot min^{-1}$. The fluorescence detector was set at ex. 290 nm and em. 320 nm, while the UV detector scanned from 200 to 400 nm (Zhu et al., 2003).

2.3.6.1.5. Growth of *M. perniciosa* in culture broth for enzyme extract

Ten plugs, 0.6 cm in diameter containing mycelia of *M. perniciosa* growing on potato dextrose agar (PDA) were placed in 250 mL erlenmeyer flasks containing 100 mL of potato dextrose broth (PDB). The flasks were placed on a shaker at 100 rpm for three weeks. Mycelial balls were filtered off, washed with deionized water, and both broth and mycelia were freeze-dried. The freeze-dried samples were combined with ten times the volume of ice cold extraction buffer containing 1 mM phenylmethylsulphonylfluoride (PMSF) in 0.01M phosphate buffer. The suspension was vortexed for one minute and left on ice for five minutes. It was then centrifuged at 4 ^{o}C for ten minutes at 8,000 x g. An aliquot of the supernatant was separated for use to determine protein content. The remainder of the supernatant was used as the enzymatic extract.

2.3.6.1.6. Protein determination

Protein was determined by the Bradford method using bovine serum albumin as a standard. Using Bio-Rad dye reagent, the absorbance was measured at 595 nm (Bradford, 1976).

2.3.6.1.7. Gel electrophoresis

Non-denaturing gels were prepared using the Mini Protean (Bio-Rad) according to the method of Laemmli (Laemmli, 1970). Molecular weights were determined by using standards (Bio-Rad). The marker used was the wide molecular weight range from Sigma (6,500-205,000). Concentrations were adjusted to the sample with lowest protein content and 5 µL of a 60 % sucrose solution was added to 20 µL of the adjusted samples. Then the polyacrilamide gel (6 % stacking gel and 12 % resolving gel) was loaded with 25 µL of sample and ran at 150 V, using a running buffer consisting of 150 $g.L^{-1}$ tris base, 720 $g.L^{-1}$ of glycine, pH 8.8. Gels were rinsed with buffer (50 mM sodium phosphate buffer pH 6.8), then stained using coomassie brilliant blue or (-)-epicatechin solution in buffer phosphate pH 6.8. To test the inhibitors, gels were soaked in a solution containing the inhibitor in buffer phosphate pH 6.8 before immersion in the substrate solution.

2.3.6.1.8. Enzyme assays

Enzyme activity was determined at 25 ^{o}C using 2,2'-azino-bis-ethylbenthiazoline (ABTS), 3,4-dihydroxy-L-phenylalanine (L-dopa), tyrosine, (-)-epicatechin, and cacao seed extract as substrates, in 0.01 M citrate buffer at pH 4 to 5.5 and sodium phosphate buffer at pH 6 to 9. Oxidation of ABTS was monitored at 420 nm (Shi et al., 2002), (-)-epicatechin at 390 nm, and L-dopa at 475 nm (Baurin et al., 2002).

2.3.6.1.9. Polyphenoloxidase inhibition

The enzyme assays described above were also performed in the presence of a polyphenoloxidase inhibitor, kojic acid, which is a natural compound produced by one of the endophytes isolated from coffee and described in previous sections of the dissertation.

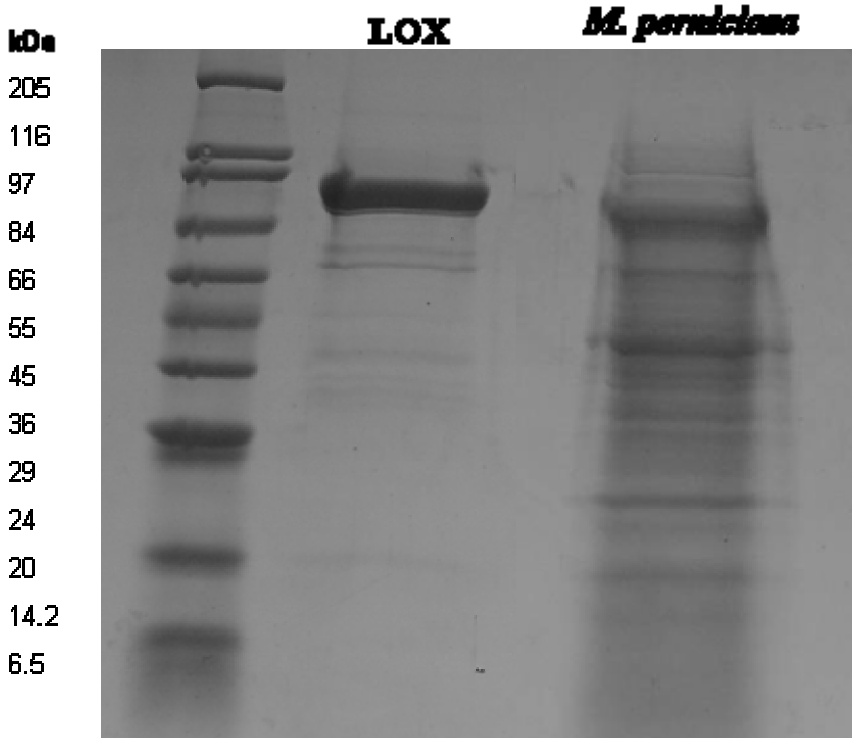
2.3.6.2. RESULTS AND DISCUSSION

The actual role of ODA on fungal development is not certain, and the effects vary according to the concentration applied and the fungal species. ODA improved *M. perniciosa* basidiocarp formation (Hebbar, P.K. unpublished results). This fungal hormone, when present in the growing media did not stimulate mycelial growth of *M. perniciosa* significantly. The addition of 1-octen-3-ol alone or in combination with ODA also did not significantly alter *M. perniciosa* mycelial growth (Table 12). Perhaps in the case of *M. perniciosa*, ODA acts by influencing fruiting body formation and stipe elongation and is not as important for mycelial growth. When tested against *Trichoderma stromaticum* and *T. koningopsis*, which are used as biological control agents against *M. perniciosa*, ODA inhibited growth rates slightly. The LOX enzyme responsible for production of ODA was extracted from mycelia of *M. perniciosa* and identified in a protein gel by comparison to commercial LOX (Figure 41). Further investigation comparing the levels of this enzyme in basidiocarps and mycelia may help clarify the role of ODA and LOX in fungal development.

Table 12. Growth rates (cm/day) of *M. perniciosa* in media containing ODA and 1-octen-3-ol

	ODA	1-octen-3-ol	ODA + 1-octen-3-ol
1.250 mM	0.3018	0.2890	0.3388
0.625 mM	0.2947	0.2839	0.3009
0.125 mM	0.2979	0.2676	0.2710
0 mM	0.3007	0.3007	0.3007

Figure 41. Protein gel of *M. perniciosa* mycelial extract compared to commercial soybean LOX



M. perniciosa inoculated on culture media (PDA) supplemented with (-)-epicatechin had similar growth rates to that of PDA alone (Figure 42). In addition, the presence of the fungus induced browning/darkening of the media containing (-)-epicatechin (Figure 43). No such browning effect was seen in the absence of the polyphenol in the media. The browning observed in the media indicates the oxidation of polyphenols through the action of fungal polyphenoloxidases. Kojic acid, a polyphenoloxidase inhibitor, when applied to the media, slightly inhibited growth of *M. perniciosa*. However, when applied in combination with (-)-epicatechin, the inhibition was stronger and almost no browning of the media occurred (Figures 42 and 43).

The oxidation of (-)-epicatechin seen in the plate assay was determined by measuring the degradation of (-)-epicatechin. Two *M. perniciosa* isolates (PS10 and 278) inoculated in PDB containing (-)-epicatechin completely degraded the polyphenol within hours of inoculation (Figure 44).

Mycelial growth of *M. perniciosa* is inhibited in media containing procyanidins and kojic acid combined, and no browning is observed. Therefore, it suggests that when phenoloxidases from *M. perniciosa* are inhibited by kojic acid, the polyphenols from cacao are now available to inhibit the growth of the pathogen. Similar to *C. gloeosporioides* in avocado (Guetsky et al., 2005), *M. perniciosa* virulence or pathogenicity may be reduced if the ability to oxidize cacao polyphenols is impaired.

Determinations of pH optima for the fungal enzymatic extract secreted in the media compared to that of commercial PPO and laccase were made in an attempt to distinguish the oxidative enzyme involved in the polyphenol degradation (Figure 45).

Figure 42. Growth rates of *M. perniciosa* in media containing (-)-epicatechin and KA

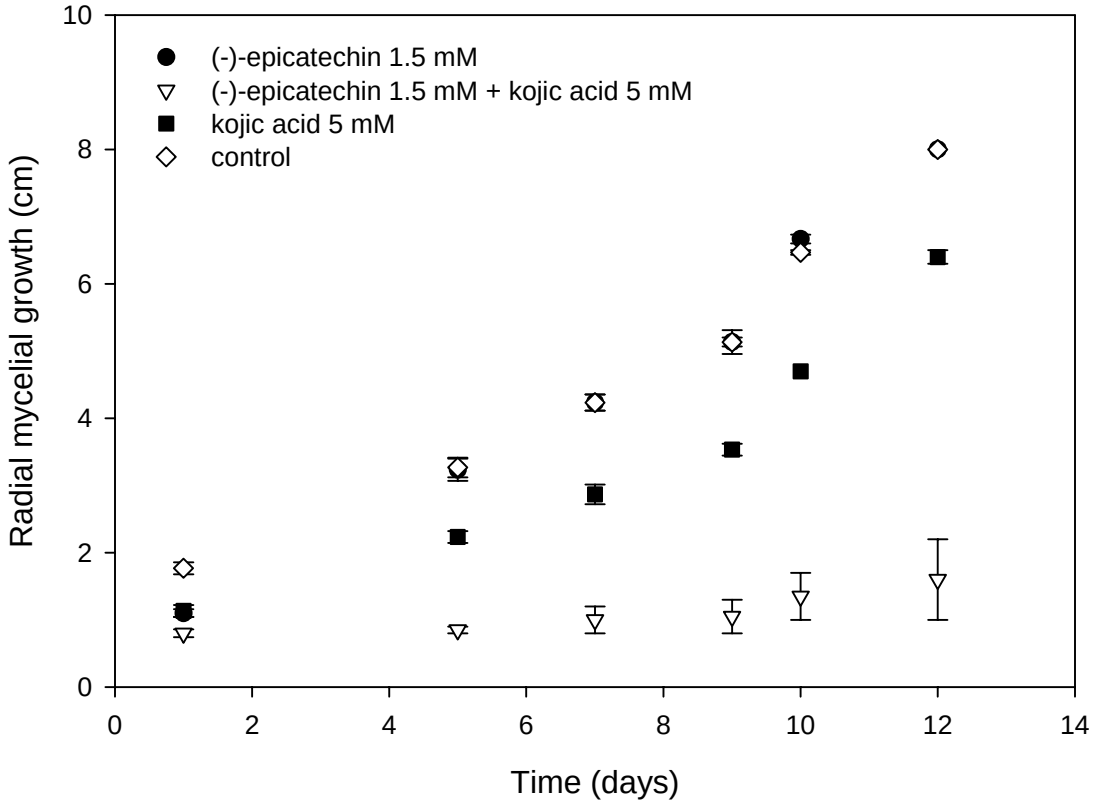


Figure 43. *M. perniciosa* growth assay in the presence of (-)-epicatechin (bottom left), kojic acid (top right), (-)-epicatechin and KA (bottom right), and media alone (top left)

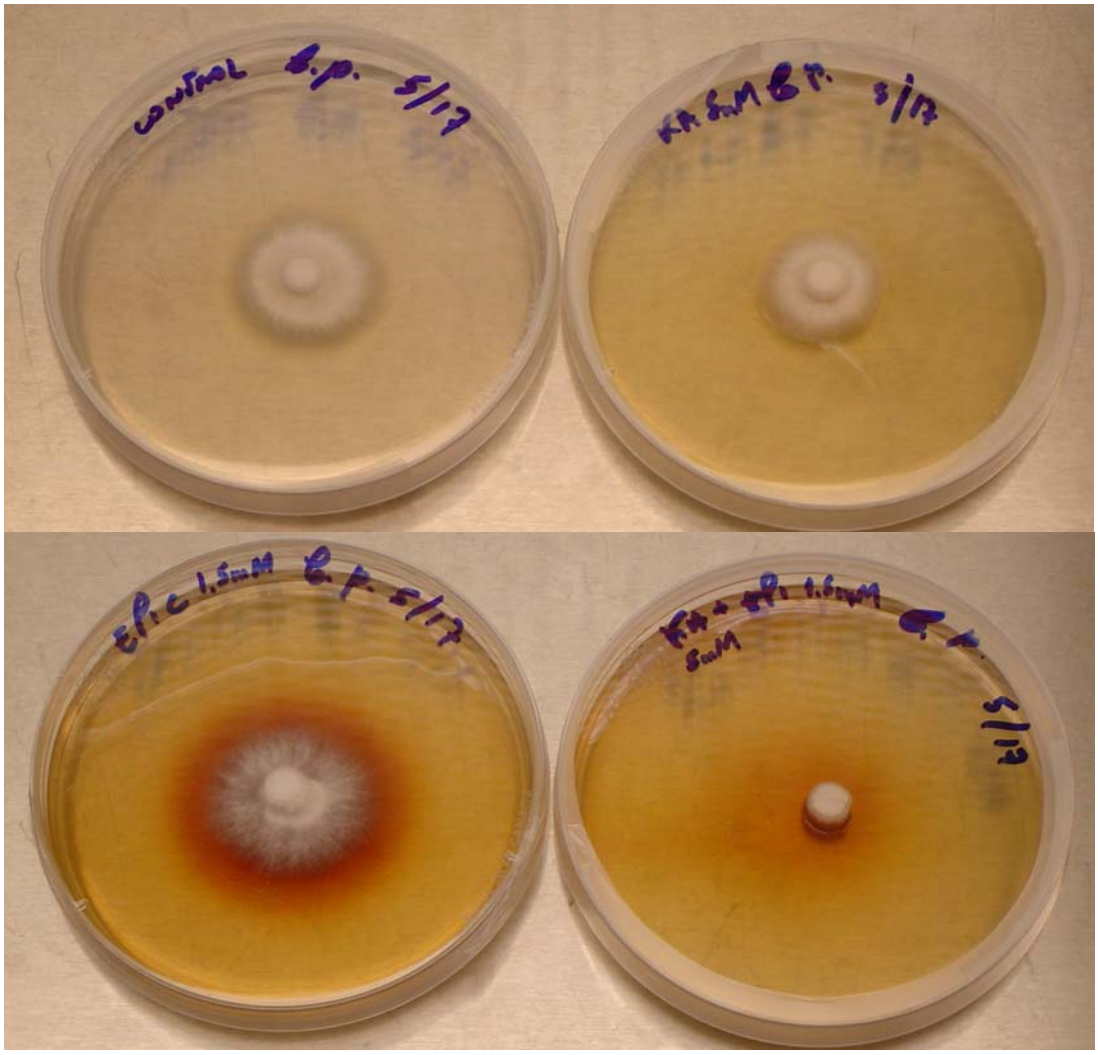


Figure 44. (-)-Epicatechin degradation by *M. perniciosa* isolates

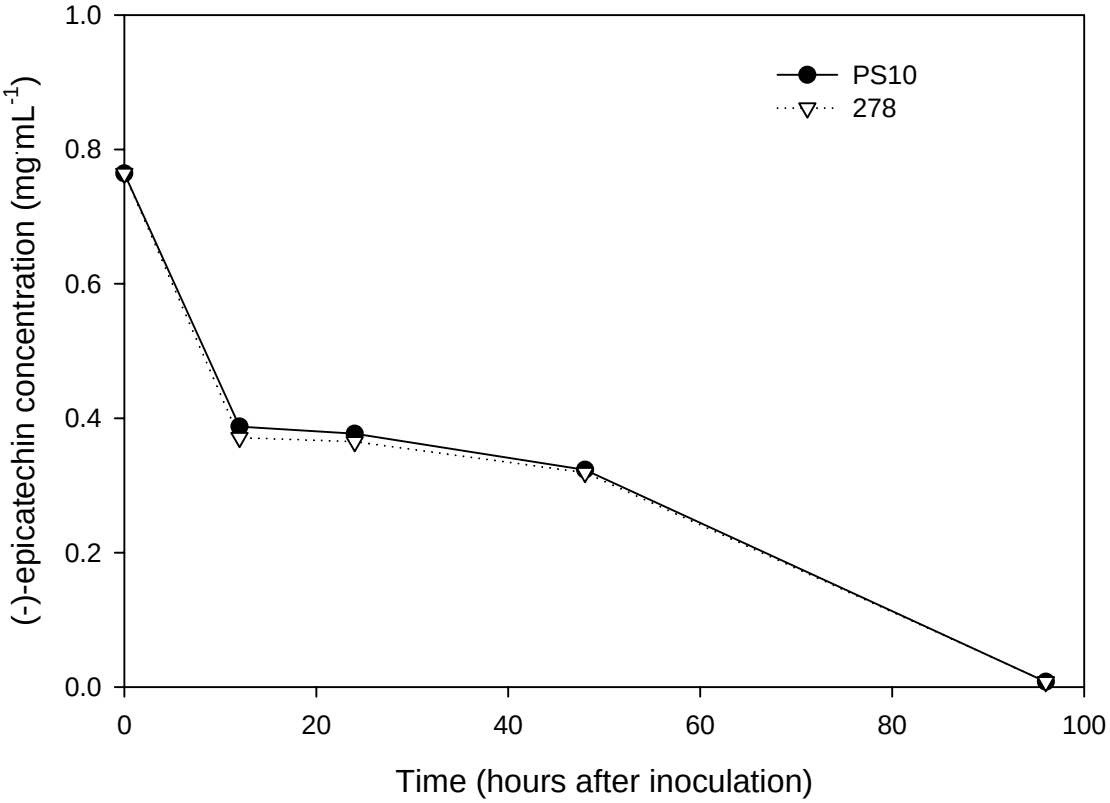
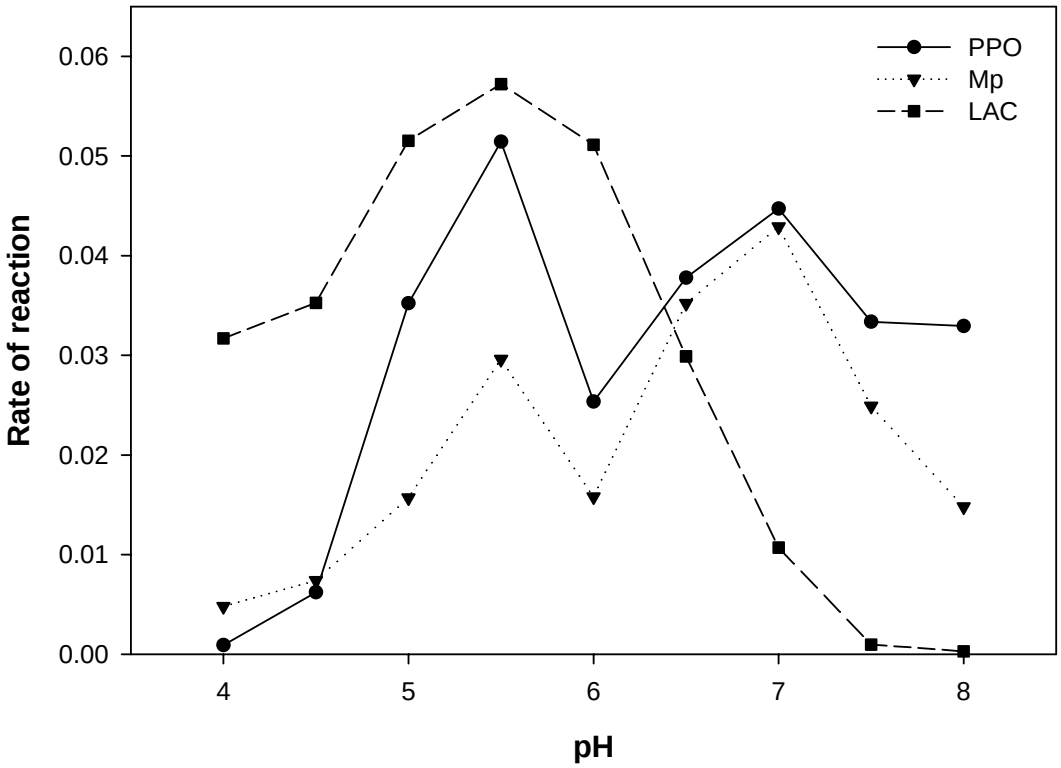


Figure 45. Comparison of pH optima for activity of commercial laccase (LAC), PPO and *M. perniciosa* (Mp) extracellular enzyme extract using (-)-epicatechin as substrate at 25^{o}C



All three analyzed enzymes had a peak in activity at pH 5.5. While laccase activity only peaked at pH 5.5, PPO and *M. perniciosa* extract also peaked at pH 7. It is possible that both enzymes are present in the fungal extract, and it is also possible that the commercial PPO may not be completely pure and contain laccases, as shown by Rescigno et al. (2007).

Studies show variation in the optimum pH and the relative activity of the phenol oxidative enzyme towards different substrates. In Guetsky et al. (2005) findings, laccase activity was highest at pH 6 and laccase gene expression was highest in the presence of (-)-epicatechin at pH 5.6.

Gauillard and Richard-Forget (1997) analyzed PPO extracts of pears and found the molecular mass to be 43,000 Da. Fan and Flurkey (2004) identified PPO isoforms from mushroom without latent activity below pH 5.5. Crude extracts had isoforms ranging from 50 to 230 kDa (after denaturing SDS PAGE), while purified isoforms had mw of 70 kDa.

M. perniciosa extracts showed bands at around 45 kDa, while the commercial PPO showed a protein band around 84 kDa (Figure 46). Clearly shown on another activity gel (Figure 47), the fungal enzymatic extracts exhibited a protein band very distant from the commercial PPO when revealed with an (-)-epicatechin solution. The fungal enzyme maintained its activity after heating at 60 ^{o}C for 5 minutes. But when placed under denaturing conditions by heating at 100 ^{o}C for 5 minutes, activity was completely abolished.

When a commercial laccase, commercial PPO, and the fugal enzymatic extract were compared in the same activity gel, the laccase and the fungal extract exhibited

bands that co-migrated to the same point (Figure 48). Although there was some staining at the commercial PPO lane at the same place as the other two enzymes, the stronger band for PPO migrated more slowly than the other two (Figure 48). It is possible that commercial PPO has different isoforms or that it has some laccase contamination.

Enzymatic extracts of *M. perniciosa* isolates and commercial enzymes responded differently according to the substrate used in the enzyme activity assays. While all three enzymes reacted with (-)-epicatechin, only commercial laccase and *M. perniciosa* media extract oxidized ABTS, and only commercial PPO oxidized tyrosine (Figure 49). It is most likely that the enzyme from *M. perniciosa* is a laccase rather than a polyphenol oxidase; however, it is not possible to rule out the presence of PPO in fungal extracts due to the two peaks of pH optima for the fungal extracts.

When using cacao's main polyphenol, (-)-epicatechin, as susbtrate, all tested *M. perniciosa* isolates induced browning, which indicated oxidized (-)-epicatechin. This suggests that in order to avoid the plant polyphenols, the fungus secretes polyphenoloxidative enzymes that oxidize these compounds. If not oxidized, polyphenols could block fungal lipoxygenases, preventing ODA (basidiocarp hormone) synthesis that ultimately could lead to preventing basidiocarp formation.

In order to avoid browning a few mechanisms can be used, including reduction of quinones formed during oxidation, or the inhibition or inactivation of PPO (Hsu et al., 1988). Reducing agents, such as ascorbic acid, cysteine, and sulfur dioxide reduce the ortho-quinone to their ortho-phenol precursor. This mechanism however, is temporary because the reducing agents themselves are irreversibly oxidized during the process. Kojic acid, a polyphenoloxidase inhibitor, seems to act through a few different

mechanisms. It functions as a reducing agent, as an inhibitor to the enzyme, or by interfering with the oxygen uptake required for the enzymatic browning (Son, 2001; Burdock, 2001; Chen et al., 1991).

The temporary inhibition of enzyme activity caused by ascorbic acid was observed when enzymatic extracts of *M. perniciosa* were incubated with (-)-epicatechin and different concentrations of ascorbic acid (Figure 50).

When kojic acid, ascorbic acid, and citric acid were added, each one separately at 3.33 mM, to *M. perniciosa* enzymatic extract and 3.33 mM of (-)-epicatechin, all inhibitors reduced polyphenoloxidase activity (Table 13).

Table 14 shows the rate of oxidation using different concentrations of L-dopa as substrate. Cacao procyanidin extract containing monomers and oligomers from dimer to decamer were also oxidized by the fungal enzymatic extract.

Figure 46. Protein gels with commercial polyphenol oxidase (PPO) and media and mycelial extracts of *M. perniciosa* (isolate PS12). Left panel stained with commassie blue and right panel with (-)-epicatechin.

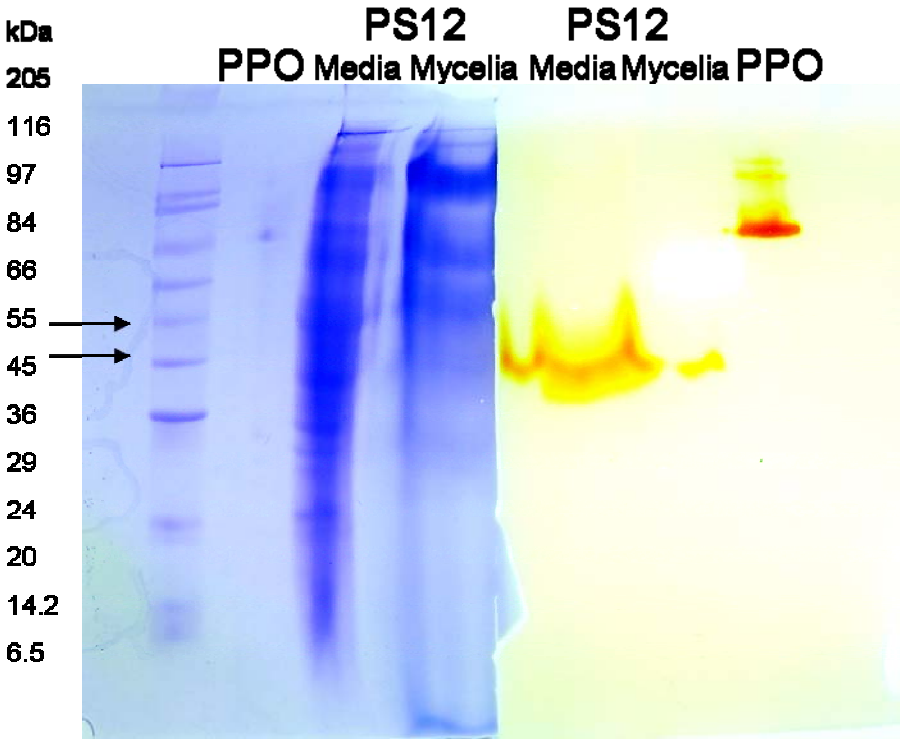


Figure 47. Activity gel stained by immersing non-denaturing protein gel in an (-)-epicatechin solution

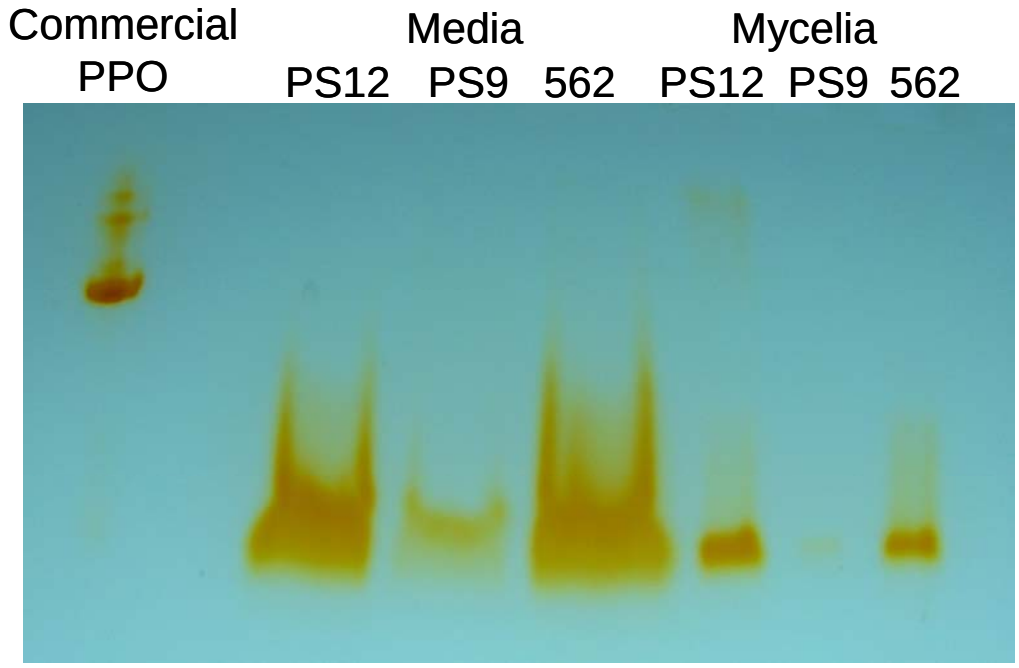


Figure 48. Activity gels comparing commercial laccase (LAC) and PPO to *M. perniciosa* enzyme extract (Mp)

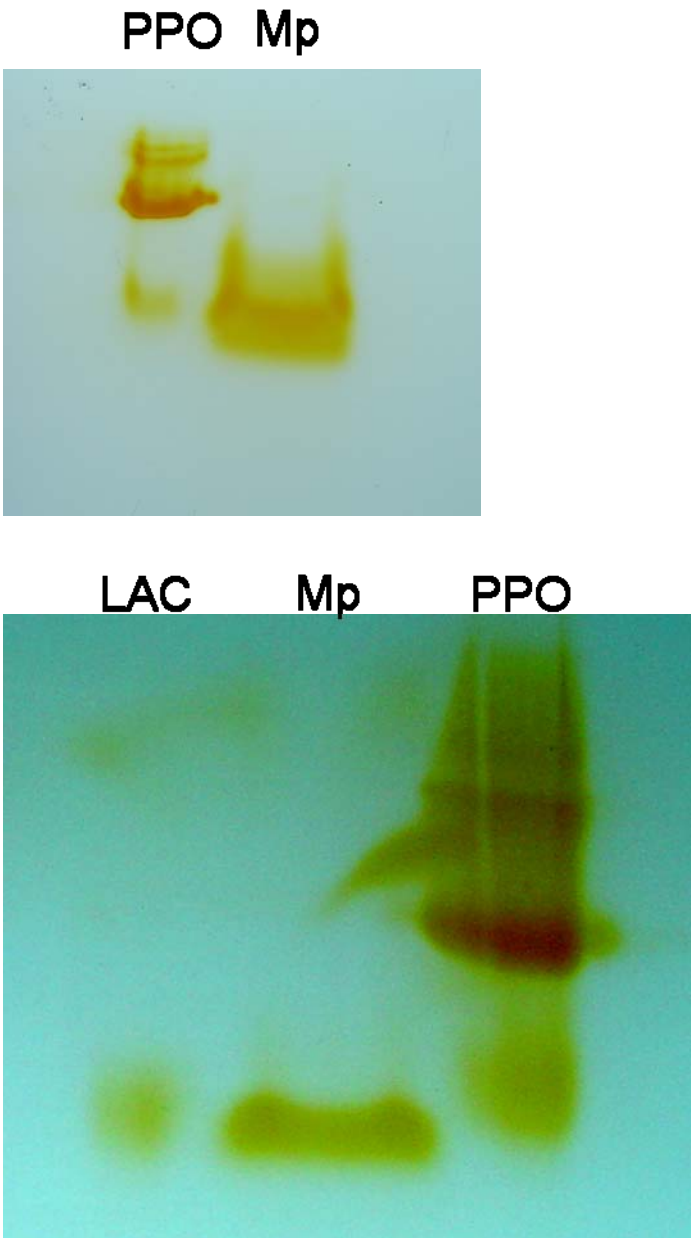


Figure 49. Enzyme activity of *M. perniciosa*, PPO and laccase using ABTS as substrate

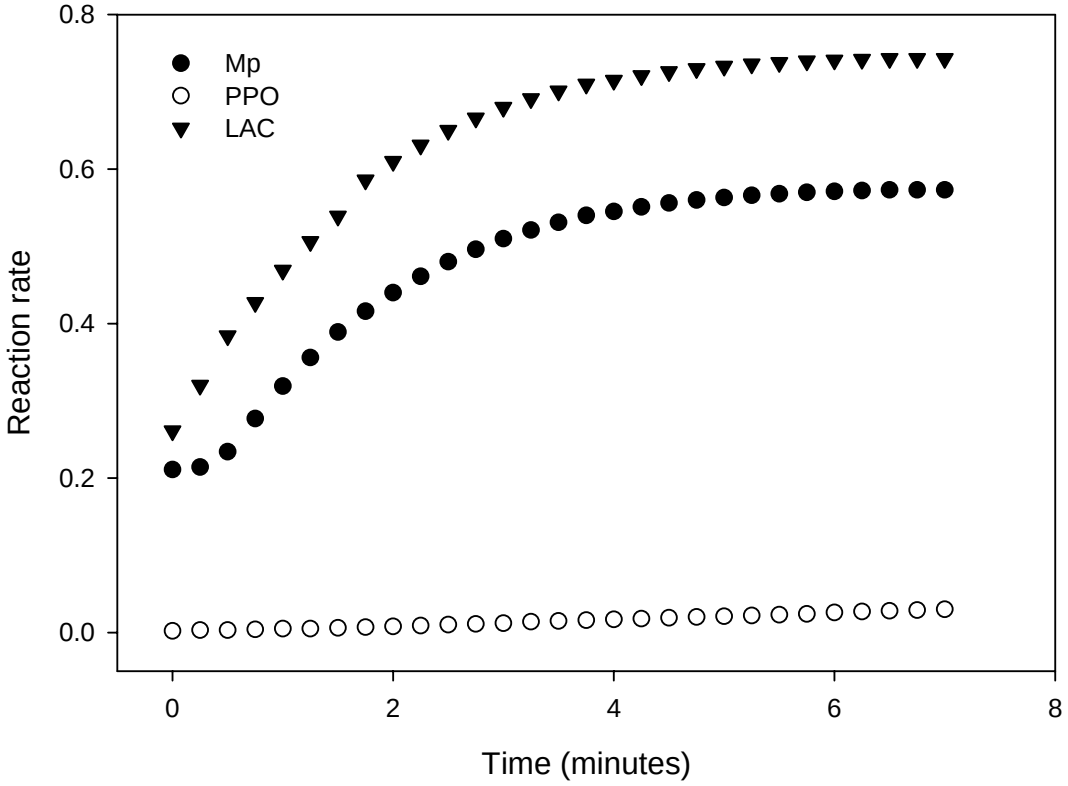


Figure 50. Effect of different concentrations of ascorbic acid in the inhibition of polyphenoloxidase activity of *M. perniciosa*

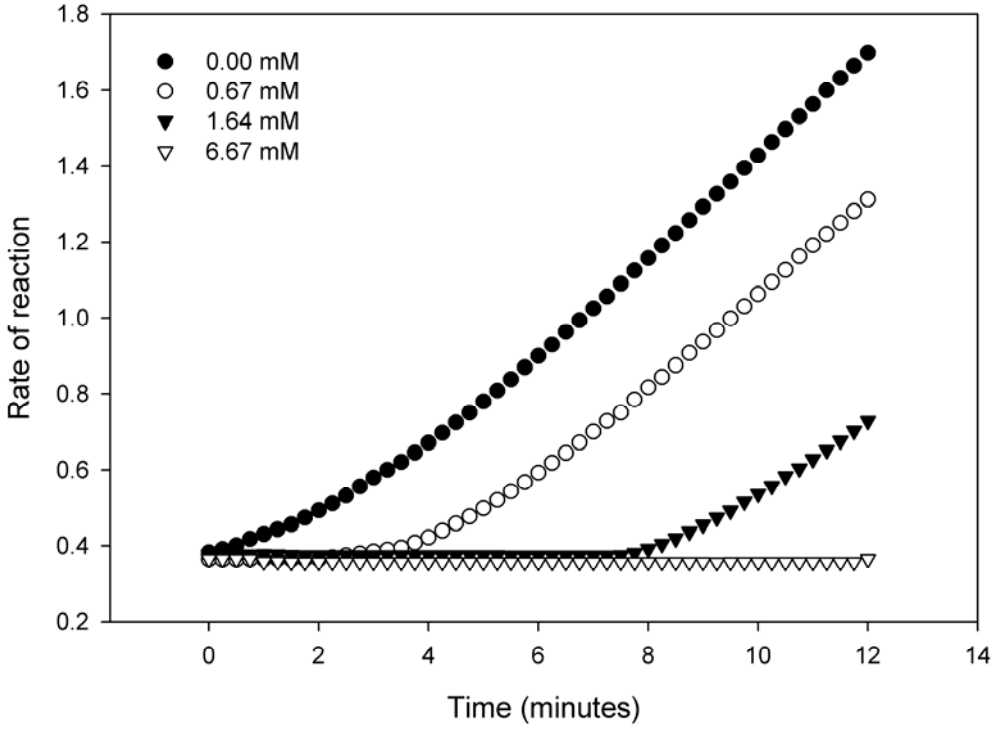


Table 13. Effect of polyphenoloxidase inhibitors, kojic acid (KA), ascorbic acid (AA) and citric acid (CA) tested at 3.33 mM on enzyme activity using (-)-epicatechin as substrate

	% Inhibiton
No inhibitor	0
KA	50%
AA	100%
CA	100%

Table 14. Rates of oxidation of L-dopa using *M. perniciosa* enzymatic extract

Substrate concentration	Reaction rate
4.17 mM	4.4 μmol L^{-1}·min^{-1}
3.33 mM	4.1 μmol L^{-1}·min^{-1}
1.67 mM	3.4 μmol L^{-1}·min^{-1}
0.83 mM	2.4 μmol L^{-1}·min^{-1}

2.3.6.3. CONCLUSIONS

Although the fungal hormone ODA did not affect mycelial growth, further investigation is needed to determine whether the lipoxygenase pathway is important for fungal development. Laccase is one of the polyphenoloxidative enzymes produced by *M. perniciosa* as a virulence or pathogenicity factor and is therefore a potential target for disease control. The main procyanidin found in cacao, (-)-epicatechin, can prevent pathogen growth in the presence of a polyphenoloxidase inhibitor that blocks the ability of the fungus to detoxify polyphenols, rendering them toxic to fungal growth. Application of polyphenoloxidase inhibitors to cacao may affect the ability of the pathogen to thrive in plants with high levels of polyphenols. In future studies, other polyphenoloxidase inhibitors should be tested. In addition, mutant lines of the pathogen could be developed, specifically with mutations in genes that are found to encode for polyphenoloxidases. The mutants would then be used to inoculate cacao, where disease symptoms and (-)-epicatechin levels would be monitored.

3. PRODUCTION

3.1. INTRODUCTION

Crop yield had been the major focus of cacao research until the late 1980's, when fungal disease outbreaks began to cause severe crop losses. Brazil, once the number two producer and exporter of cacao beans in the world, has changed its status to importer to match domestic industrial demands due to major reduction in crop production caused by witches' broom disease. However, even if losses due to pathogens persist in restricting production, it is imperative to revisit the issues of low fruit set and low yields and determine ways to improve pod production in order to maximize the potential of the cacao producing countries around the world.

Cacao has a cauliflorous habit with flowers growing from the trunk. Flowers are perfect with both male and female parts. The androecium consists of five stamens, each bearing two anthers, and five staminodes (sterile stamens). The gynoecieum is composed of an ovary with five united carpels, and a stile and stigma forming the pistil. The perianth consists of five sepals and five modified petals, the petals each bearing a ligule.

Cacao flowers begin to open in late afternoon, and continue throughout the evening so that the flower is fully open just before dawn the next day. The stigma is receptive for pollination during morning hours, and pollen remains viable for 24 hours. If the flower is not pollinated it abscises within 48 hours.

One factor limiting cacao yields is pollination. The ratio of fruit set per number of flowers produced is very low (Young, 1994). Trees typically bear about 4,500 flowers in

a six-month period, with less than 1 % of naturally pollinated flowers yielding fruits (Ibrahim and Hussein, 1987). Hand pollination can increase fruit set, confirming that natural pollination is a limiting factor for fruit yield. Therefore, efforts to enhance yields must focus on increasing pollination efficiency.

Cacao trees can be self compatible or self incompatible and pollination by flying insects results in approximately 25 to 50 % of cross pollination on self compatible trees (Wood and Lass, 1987). The major natural cacao pollinators are ceratopogonids, commonly referred as biting midges and gall midges from the dipteran family Ceratopogonidae, genus Forcipomyia and Culicoides. These insects seem to be attracted to fragrance emitted from cacao flowers (Young, 1994). Erickson et al. (1992) suggest that *Theobroma* spp. exhibit floral thermogenicity to assure thermal stability, essential for the release of specific ratios of floral volatile compounds. The increase in flower temperature is likely related to increased respiration levels using the alternative oxidase respiratory pathway (cyanide-resistant respiration) (Seymour, 2001), known to be triggered by salicylic acid (SA) (Raskin et al., 1989).

Volatile organic compounds (VOC), temperature, respiration rates, and salicylic acid content of flowers of greenhouse grown cacao trees were measured in an effort to further investigate floral attractants and better understand their regulatory mechanisms. Fruit set upon hand pollination was also determined.

3.2. MATERIALS AND METHODS

3.2.1. Volatile Organic Compounds

Flowers collected at 9:00 AM were placed in a 250 mL two-necked round bottom flask. The flask consisted of a closed system with air pumped in through one neck at a flow rate of 50 mL· min^{-1} and out through the other neck, passing through a moisture trap (composed of cotton plugs and drierite) into an adsorbent resin (Tenax) cartridge, for a collection time of 30 minutes. VOCs adsorbed in the Tenax columns were desorbed using a short-path thermal desorption system (Scientific Instrument Services) (Hartman et al., 1993) into a cryotrap prior to GC-MS analysis.

GC-MS conditions: GC HP 6890 series, MSD HP 5973 NIST 98; column J and W Scientific Inc. DB-1MS 50 m x 0.250 mm x 0.25 μm; initial temperature 70 ^{o}C held for two minutes; 10 ^{o}C · min^{-1} until 240 ^{o}C held for 30 minutes; total run time 49 minutes; helium gas flow 1 mL · min^{-1}; injector 250 ^{o}C; MSD 280 ^{o}C; mass scan started at three minutes and ranged from 20 amu to 550 amu, with a threshold of 150, scanning at 2.78 scans/second;

Desorption system conditions: short path thermal desorption model TD-4 Scientific Instrument Services Inc (Ringoes-NJ, USA). Gas purge time (helium chromatographic grade-airgas) 30 seconds; injection time one minute; desorption time five minutes; delay start 30 seconds; desorption temperature 250 ^{o}C; cooling trap temperature - 40 ^{o}C (CO_2 bone dry grade- siphon type) (from Airgas, Inc Radnor, PA, USA).

In order to test volatiles emitted from different flower parts, 150 TSH 565 flowers were separated into three parts: petals, sepals, and reproductive components (including, stamens, ovary, pistil, staminodes) and their VOCs were analyzed separately. Flower pedicel was discarded.

Flowers were also treated with 200 μM salicylic acid aqueous solution, then enclosed to collect their VOCs.

3.2.2. Temperature measurements

Temperature measurements were taken from closed and open flowers from three different *T. cacao* genotypes, Amelonado, TSH 565 and IMC 30, while the flowers were still on the trees. The trees were grown in greenhouses under environmentally controlled conditions. Temperature measurements of three different plant tissue surfaces, flowers (open and closed), bark, and leaves (young and mature) from nine trees of the three mentioned genotypes, were taken twice a day (at 9:00 AM and 5:30 PM) for seven days. Temperatures were measured using a portable infrared thermometer model Raynger ST3 (standard laser) from Raytek Corporation. Ambient temperature was also recorded for the seven day period.

3.2.3. Respiration

Open and closed cacao flowers were collected at 9:00 AM. Tomato flowers were used as an external control for comparing levels of respiration. Cacao flowers were then

treated with SHAM (salicylhydroxamic acid), a specific inhibitor of the alternative oxidase pathway, potassium cyanide (KCN), used as inhibitor of the cytochrome oxidase pathway, and salicylic acid (SA), known to trigger the alternative oxidase pathway. A 10 mM solution of SHAM was prepared in 0.3 % ethanol, a 1 mM solution of KCN was prepared in a 20 mM HEPES buffer pH 8, following the method described by Millennar et al. (2001), and a 200 μM SA aqueous solution was prepared.

For the chemical treatments, flowers were placed in a 50 mL plastic syringe containing SHAM, KCN, or SA solution. A vacuum was created inside the syringe, and the plunger was pulled to force the solution to penetrate the flower. The flowers were then rinsed with deionized water and placed in a 50 mL filtering flask with a rubber stopper on top and a septum on the side arm, which were sealed with Teflon tape. The flasks were placed on a shaker and gas samples were taken with a plastic syringe and hypodermic needle that was pierced on a rubber stopper after collection in order to avoid gas leakage from the syringe. Two samples of each flask were taken every 15 minutes for two hours, one sample for CO_2, the other for O_2 analysis. The gas samples were then injected in a Shimadzu GC 8A IT gas chromatograph with two injectors connected to two columns, one for CO_2 (Alltech; Length 6ft; O.D. 1/8”; I.D. 0.085”; stationary phase – PORAPAK Q; mesh range 80/100) and the other for O_2 (Supelco; 3’ x 14”; stationary phase – molecular sieve; mesh range 60/80). The column temperature was set to 30 ^{o}C, and the thermal conductivity detector (TCD) set a 100 mA.

3.2.4. Salicylic acid analysis

Cacao flowers from Amelonado, IMC 30 and TSH 565 were used in this experiment. Buds and open cacao flowers were sampled before and after spraying with 1mM SHAM solution.

SA and SAG analysis was previously described on page 15.

3.2.5. Hand pollination

Recently open flowers were pollinated at 9:00 AM using a forceps. Staminodes surrounding the stigma were clipped off to facilitate access to the stigma. All stamens were removed and used to self-pollinate each flower by rubbing the anthers against the stigma.

3.3. RESULTS

3.3.1. Volatile organic compounds

47 VOCs produced by cacao flowers were identified (Table 15). The compounds belonged to the chemical classes: aldehyde, ketone, alcohol, amide, organic acid, sesquiterpene and hydrocarbon. The most abundant group was the hydrocarbon with 29 compounds varying from 8 to 17 carbons, while alcohol, amide and sesquiterpene each had only one representative compound identified. Although the analysis focused on qualitative information, it was observed that 1-pentadecene, followed by 7-tetradecene, tridecane, and pentadecane were the compounds found in higher amounts. Salicylic acid treatment of the flowers induced the production of long chain organic acids, tetradecanoic acid, pentadecanoic acid, 3-hexadecenoic acid and hexadecanoic acid, not normally present otherwise.

All three, sepals, petals, and reproductive parts produced volatile organic compounds. In order to compare the relative levels of volatiles emitted by each flower segment, the 12 most abundant compounds were selected. 3-hexadecene, 1-pentadecene and pentadecane were produced in higher levels in the sepals, while undecane, dodecane, 2-methy-dodecane, 6-tridecene, tridecane, 7-tetradecene, and tetradecane tended to be higher in petals. 4-dodecene and 3-methyl-tridecane were produced at similar levels in the three measured flower parts (Figures 51, 52 and 53).

Table 15. Volatile organic compounds produced by cacao flowers

R.t.	Aldehydes	Fragments m/z (Abundance%)	Match %
6.5	hexanal	27(42), 29(52), 41(81), 43(63), 44(100), 56(87), 57(67), M+. 100(2)	95
7.18	2-hexenal	29(58), 39(74), 41(100), 42(63), 43(70), 55(84), 57(54), 69(66), 83(56), M+.98(21)	97
7.9	heptanal	27(50), 29(66), 41(100), 42(59), 43(98), 44(93), 55(68), 57(58), 70(94), M+.114(2)	96
9.5	octanal	29(50), 41(93), 42(42), 43(100), 44(61), 55(52), 56(69), 57(77), 81(39), M+.138(5)	91
11.2	nonanal	29(55), 41(99), 43(83), 44(50), 55(59), 56(59), 57(100), 69(36), 70(35), M+. 142(<1)	93
12.65	decanal	29(55), 41(100), 43(90), 44(47), 55(69), 57(93), 68(38), 70(43), 71(38),M+. 156(<1)	83
8.9	benzaldehyde	28(80), 43(34), 51(44), 77(100), 105(85), 106(85), M+.116(3)	96
	Ketones		
7.6	2-heptanone	27(12), 29(18), 39(13), 41(19), 43(100), 58(62), 59(10), 71(17), M+.114(11)	83
7.7	1-(1-cyclohexen-1-yl)-ethanone	39(31), 43(100), 53(17), 55(13), 79(29), 81(85), 109(31), M+.124(22)	90
9.9	5-ethenyldihydro-5-methyl-2(3H)-furai	29(34), 42(20), 55(15), 56(19), 57(18), 70(12), 85(100), M+.114(2)	81
	Alcohols		
10	2-ethyl-1-hexanol	28(28), 41(42), 43(40), 55(31), 57(100), 70(22), 83(22), M+.(130)(<1)	91
	Amides		
8.3	N,N-diethylformamide	27(21), 28(24), 30(84), 44(46), 58(100), 86(35), M+.101(83)	93
	Organic acids		
9.2	hexanoic acid	27(27), 39(26), 41(49), 43(53), 45(20), 60(100), 73 (48), M+.116(<1)	78
19.8	tetradecanoic acid	28(33), 41(98), 43(100), 55(83), 57(72), 60(65), 69(46), 71(37), 73(92), M+.228(20)	98
21	pentadecanoic acid	29(45), 41(94), 43(100), 55(82), 57(72), 60(91), 69(40), 71(47), 73(82), M+. 242(26)	91
22	9-hexadecenoic acid	29(39), 41(100), 43(86), 55(98), 57(51), 67(39), 69(59), 73(42), 83(46),M+.254(2)	92
22.3	hexadecanoic acid	29(35), 41(89), 43(100), 55(78), 57(70), 60(73), 69(43), 71(35), 73(87), M+.256(26)	98
	Sesquiterpenes		
16.1	longifolene	28(100), 29(17), 32(17), 41(30), 43(30), 55(27), 57(19), M+.(204)	99
	Hydrocarbons		
6.7	octane	41(44), 43(100), 55(22), 56(25), 57(33), 70(13), 71(24), 85(33), M+.114(10)	87
8.1	nonane	28(76), 29(37), 31(31), 41(47), 43(100), 57(72), 85(22), M+. 128(4)	93
9.8	decane	28(60), 29(28), 41(50), 43(100),56(25), 57(80), 71(31), 85(24), M+.142(6)	95
10.3	indene	28(27), 39(12), 41(12), 63(12), 89(13), 115(94), M+.116(100)	94
10.4	3,7-dimethyl-1,3,7-octatriene	27(19), 39(29), 41(35), 53(19), 77(38), 79(47), 80(36), 91(49), 93(100), M+.136(7)	96
10.8	2-methyl-decane	29(23), 41(50), 42(24), 43(100), 57(85), 71(53), 75(42), 85(27), M+. 156(1)	96
11.1	4-undecene	28(100), 32(32), 41(35), 43(30), 55(44), 57(30), 69(27), M+.154(8)	96
11.3	undecane	41(55), 43(97), 55(24), 56(23), 57(100), 71(42), 85(26), M+.156(5)	96
12.6	3-dodecene	28(57), 41(77), 43(57), 55(100), 56(55), 57(39), 69(70), 70(51), 83(33), M+.168(22)	98
12.7	4-dodecene	28(100), 29(48), 41(82), 43(82), 55(88), 56(50), 57(57), 69(54), 70(51), M+.168(12)	98
12.8	dodecane	29(28), 41(56), 43(95), 57(100), 71(54), 85(27), 112(6), M+.170(4)	96
13.8	2-methyl-dodecane	28(38), 29(34), 41(70), 43(100), 55(42), 57(92), 71(46), 85 (31), 125(29), M+. 184(1)	95
14	2-undecanone	29(13), 41(27), 43(90), 58(100), 59(34), 71(26), M+. 170(5)	91
14.1	6-tridecene	41(71), 43(52), 55(100), 56(58), 57(37), 69(68), 70(49), 83(46), M+.182(22)	98
14.3	tridecane	41(46), 43(83), 55(21), 57(100), 71(57), 85(34), M+.184(5)	98
15.2	2-methyl-tridecane	28(49), 41(51), 43(99), 57(100), 71(54), 85(36), M+.198(1)	96
15.3	3-methyl-tridecane	29(23), 41(42), 43(47), 56(27), 57(100), 71(41), 85(31), M+. 198(1)	96
15.4	7-tetradecene	41(87), 43(68), 55(100), 56(63), 57(48), 69(93), 70(58), 83(61), 97(43), M+.196(23)	99
15.5	1-tetradecene	29(47), 41(100), 43(95), 55(91), 56(56), 57(76), 70(47), 71(38), 83(44), M+.196(10)	99
15.6	tetradecane	41(45), 43(82), 57(100), 71(59), 85(37), 99(8), 112(4), M+.198(4)	98
16.5	2-methyl-tetradecane	41(49), 43(100), 55(26), 56(20), 57(96), 69(16), 70(11), 71(53), 85(37), M+.212(1)	95
16.6	1,6-tridecadiene	28(24), 29(30), 39(30), 41(100), 43(59), 54(59), 55(84), 56(20), 57(31), M+.180(7)	94
16.7	1-pentadecene	41(75), 43(66), 55(97), 56(63), 57(55), 69(100), 70(71), 83(89), 97(76), M+.210(46)	93
16.9	pentadecane	29(21), 41(46), 43(82), 57(100), 71(62), 85(41), 99(10), 113(5), M+.212(4)	96
17.9	3-hexadecene	41(87), 43(83), 55(100), 56(60), 57(61), 69(85), 70(56), 83(69), 97(55), M+. 224(18)	99
18	1-hexadecene	28(40), 41(54), 43(88), 55(51), 56(26), 57(35), 69(37), 71(100), 83(29), M+.224(3)	96
18.1	hexadecane	29(25), 41(49), 43(82), 57(100), 71(62), 85(38), 99(10), M+.226(4)	98
19	8-heptadecene	41(91), 43(90), 55(100), 56(57), 57(69), 69(81), 70(55), 83(74), 97(60), M+.238(16)	99
19.2	heptdecane	29(22), 41(43), 43(78), 55(20), 56(18), 57(100), 70(16), 85(33), M+.240(3)	96

Figure 51. Levels of tridecane, 7-tetradecene and 1-pentadecene emitted by petals, sepals and reproductive parts of cacao flowers

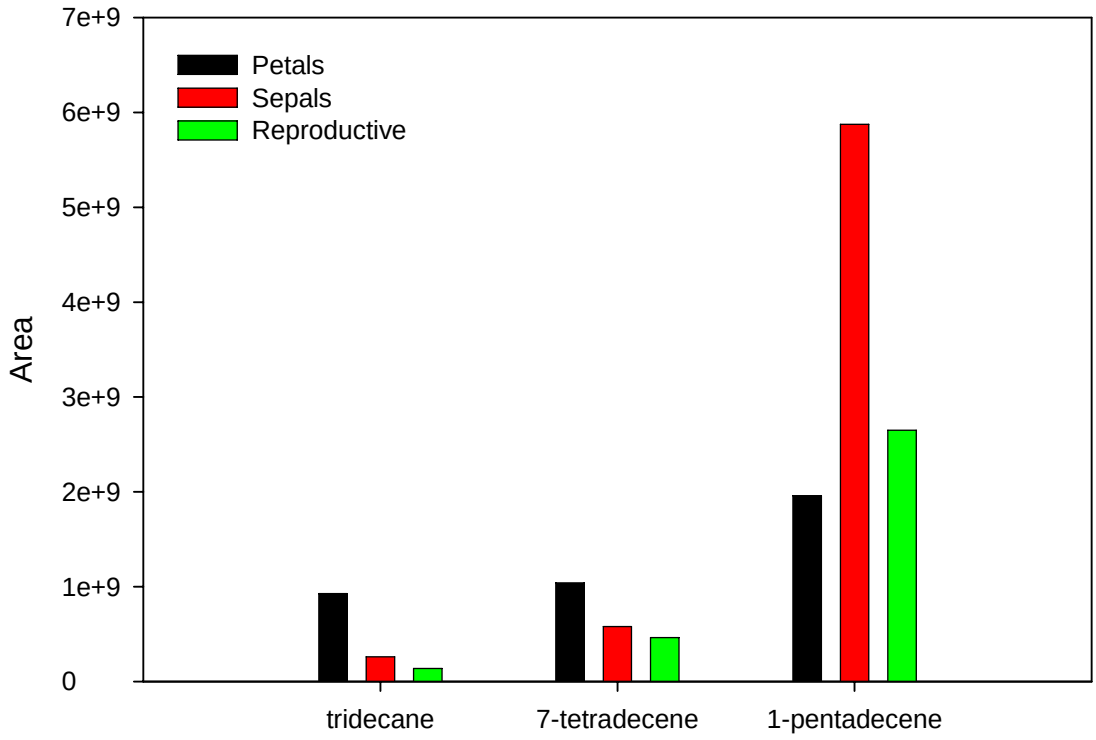


Figure 52. Levels of undecane, dodecane, 6-tridecene, tetradecane and pentadecane emitted by petals, sepals and reproductive parts of cacao flowers

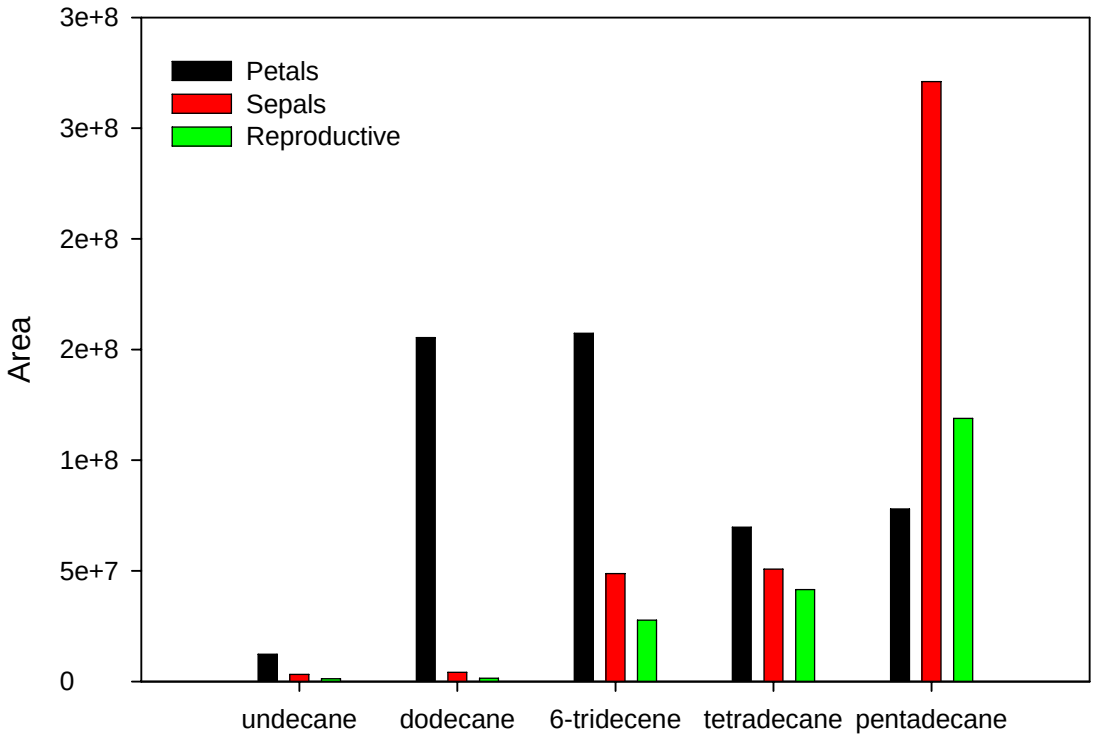
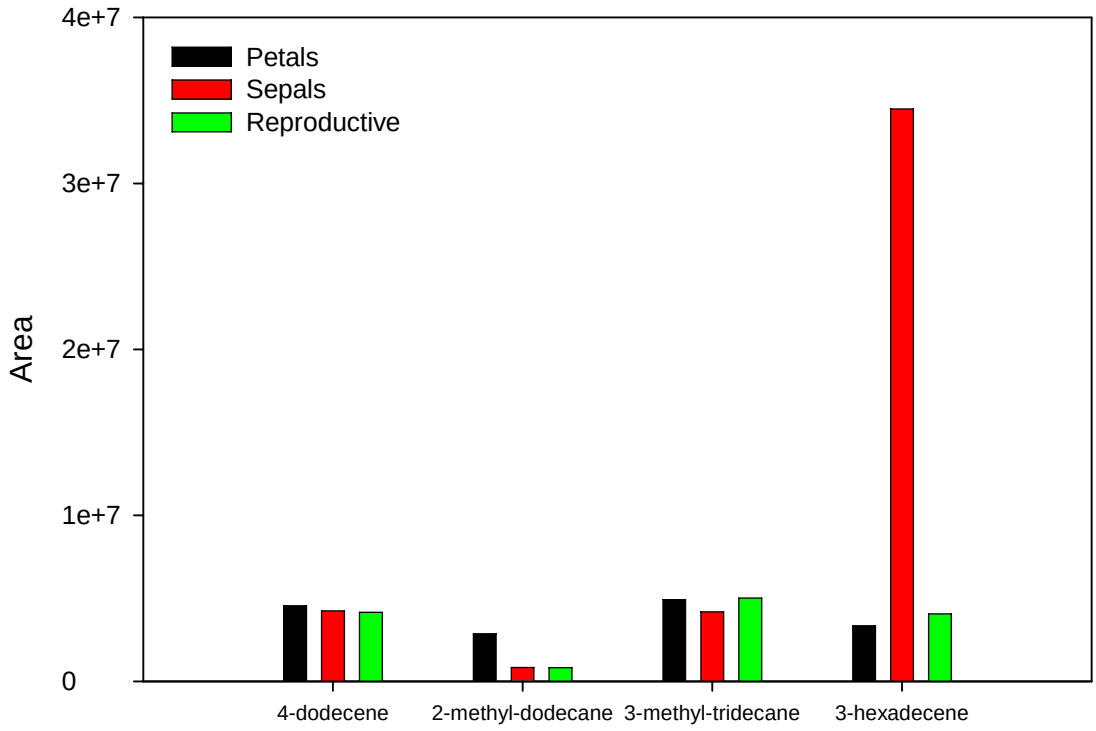


Figure 53. Levels of 4-dodecene, 2-methyl-dodecane, 3-methyl-tridecane and 3-hexadecene emitted by petals, sepals and reproductive parts of cacao flowers



3.3.2. Temperature measurements

Measurements indicated that flower temperatures in the morning (24.13 ºC) were 1.91 ºC higher than the surrounding bark and 3.00 ºC higher than nearby leaves, but 1.08 ºC lower than the ambient temperature (Table 16). When the temperature data was analyzed for differences between open and closed flowers and young versus old leaves at different times of the day, no differences were observed (data not shown). In the afternoon, flower temperature (22.76 ºC) was higher than leaf temperature by 0.9 ºC but lower than bark and ambient temperatures (Table 17). All tissues measured had temperatures lower than ambient temperature. Salicylic acid treatment increased flower temperature by 2 ºC.

Table 16. Temperature measurements of flowers, background, and plant surfaces of *T. cacao* in the morning and afternoon (SE, standard error)

	Mean temperature (ºC) ± SE		
	9:00 AM	5:30 PM	Daily average
Ambient	25.21 ± 0.49 (n=5)	23.52 ± 0.93 (n=5)	24.80 ± 0.59 (n=10)
Flower	24.13 ± 0.12 (n=365)	22.19 ± 0.24 (n=79)	23.79 ± 0.11 (n=444)
Bark	22.22 ± 0.25 (n=60)	22.76 ± 0.47 (n=43)	22.44 ± 0.19 (n=103)
Leaves	21.08 ± 0.12 (n=78)	21.29 ± 0.16 (n=118)	21.21 ± 0.11 (n=196)

3.3.3. Respiration

Respiration rate was determined using gas chromatography (Figure 54) to measure the increase in CO_2 and decrease in O_2 in the head space where flowers were enclosed. Open flowers respired more than closed ones, expressed in Figure 55 as μmoles of CO_2 emitted per gram of flower dry weight. No differences were observed in the levels of flower respiration among the three cacao varieties investigated (data not shown).

SHAM lowered the levels of CO_2 evolution in closed and open flowers, suggesting the presence of the alternative oxidase pathway (Figures 56 and 57). When KCN was added, the levels of respiration were dramatically reduced; the remaining respiration present may have been due to the alternative oxidase pathway (Figures 56 and 57).

Tomato flowers had respiration rates similar to cacao flowers (Table 17).

Figure 54. Chromatogram showing CO_2 peak at 0.816 minutes

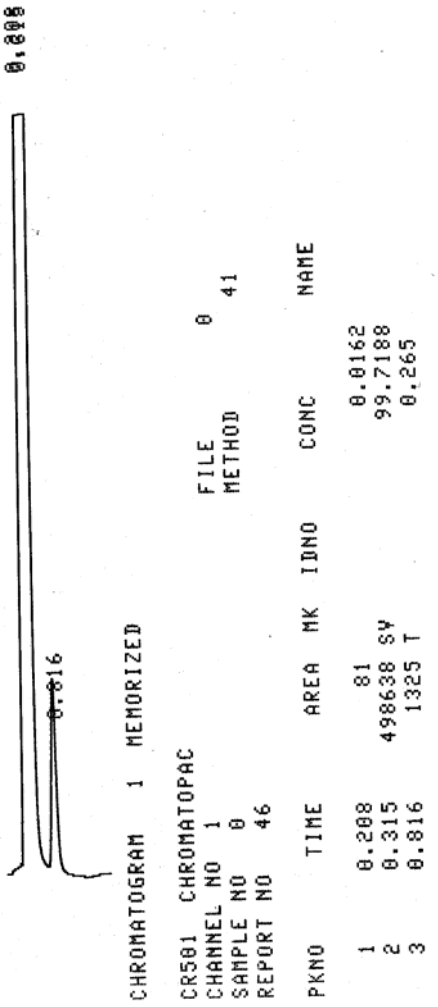


Figure 55. CO_2 evolution in opened and closed cacao flowers

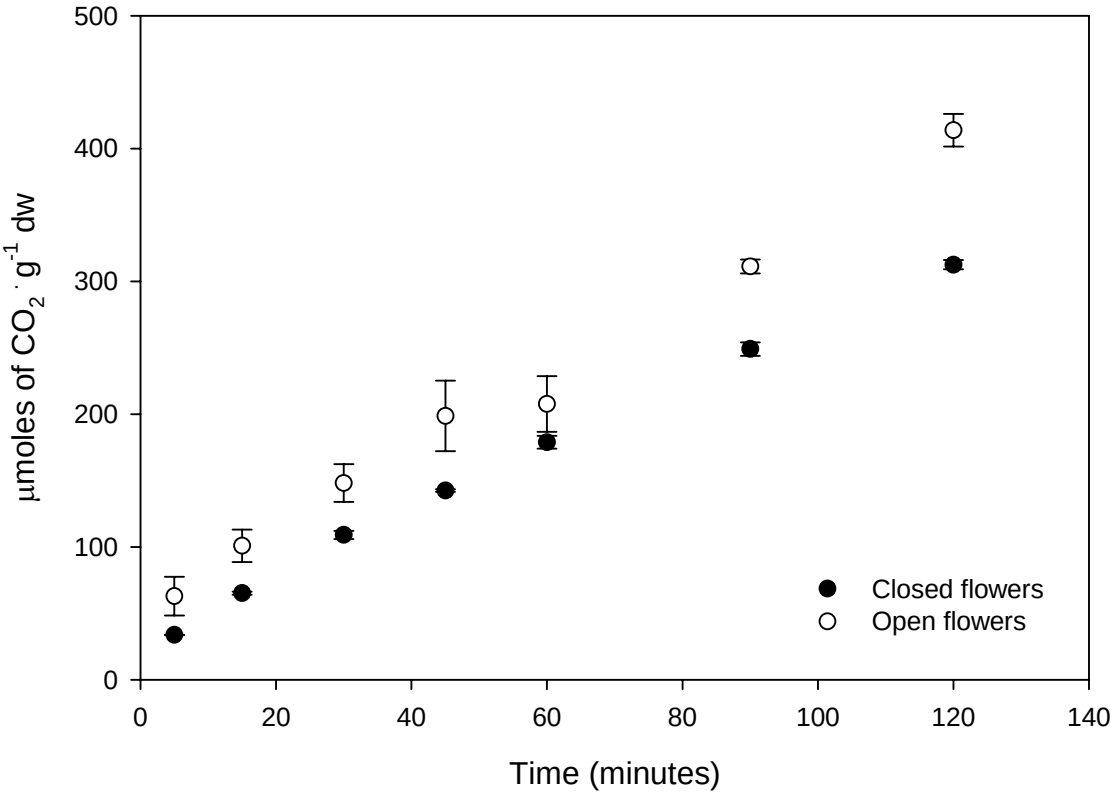


Figure 56. Closed cacao flower respiration expressed as CO_2 evolution

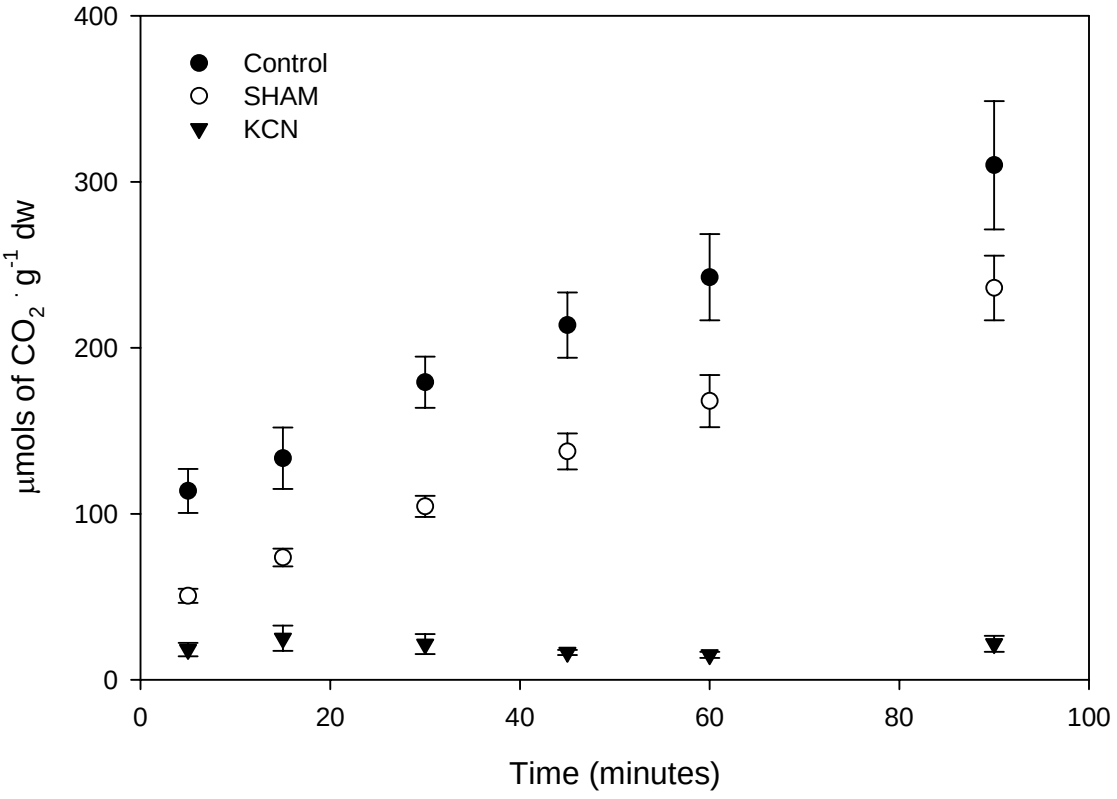


Figure 57. Open cacao flower respiration expressed as CO_2 evolution

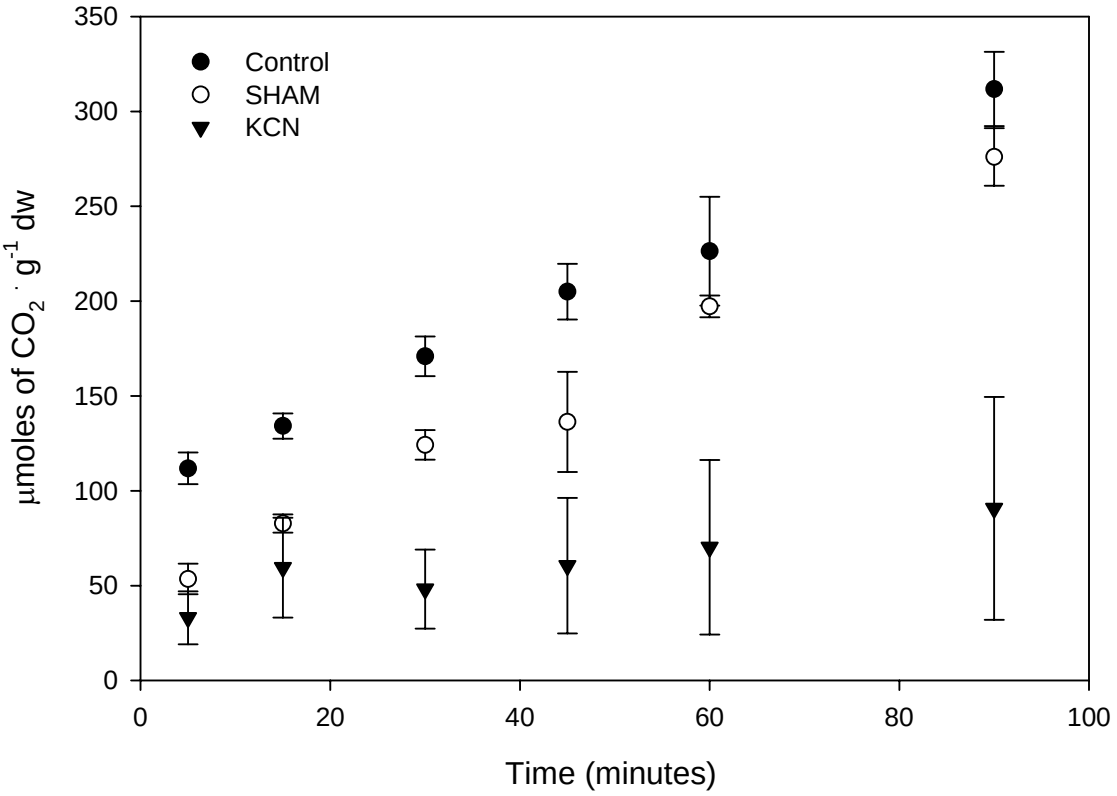


Table 17. Percentage of accumulated CO_2 produced by enclosed cacao and tomato flowers

	% CO2	
Hours	Cacao	Tomato
0	0.21	0.2
0.5	0.35	0.38
1	0.59	0.66
1.5	0.71	0.75
2	0.53	0.72
16	1.71	4.02
40	9.91	12.85

3.3.4. Salicylic acid analysis

Salicylic acid and salicylic acid glucoside were measured in buds and open cacao flowers before and after spraying with 1mM SHAM solution (Table 18). LSD analysis of the data, showed significant differences for SHAM treatment, cultivar, and the interaction treatment versus cultivar for SA and SAG contents (Table 19).

Buds and open flowers did not differ in their SA and SAG contents (Table 19). While flowers of all analyzed genotypes, Amelonado, TSH 565 and IMC 30 had higher SA and SAG levels after treatment with SHAM (Table 20). Amelonado had the lowest SAG content and TSH had the highest SA content among the three genotypes (Table 21).

Table 18. Salicylic acid (SA) and salicylic acid glucoside (SAG) contents of open and closed cacao flowers before and after treatment with SHAM

Flower	Cultivar	SA ($ng.g^{-1}$ fw)		SAG ($ng.g^{-1}$ fw)	
		- sham	+ sham	-sham	+sham
Bud	AME	22.3	123.9	90.1	351.8
	TSH	22.5	549.7	84.4	1042.5
	IMC	24.3	74.0	105.6	1014.5
Open	AME	22.6	77.7	81.2	238.0
	TSH	24.1	380.6	141.0	1037.1
	IMC	21.2	65.1	131.8	683.4

Table 19. LSD analysis of the SA and SAG contents of cacao flowers

SA	F Value	Pr > F	SAG	F Value	Pr > F
Model	24.59	<0.0001	Model	17.37	<0.0001
Replicates	0.51	0.6058	Replicates	0.76	0.4810
Treatment	**109.67**	<0.0001	Treatment	**142.6**	<0.0001
Flower age	4	0.0579	Flower age	1.49	0.2345
Cultivar	**48.52**	<0.0001	Cultivar	**19.96**	<0.0001
Trt*age	3.92	0.0604	trt*age	2.88	0.1039
Trt*cult	**48.17**	<0.0001	trt*cult	16.9	<0.0001
Age*cult	1.81	0.1872	age*cult	1.01	0.3819
trt*age*cult	2	0.1596	trt*age*cult	0.81	0.4564

Numbers in bold are statistically significant

Table 20. Effect of treatment on salicylic acid (SA) and salicylic acid glucoside (SAG) contents of cacao flowers by cultivar

AME	SA Mean	$LSD_{.05}$ = 22.08	SAG Mean	$LSD_{.05}$=55.37
-sham	22.461	B	85.65	B
+sham	100.796	A	294.92	A

TSH	SA Mean	$LSD_{.05}$ = 140.86	SAG Mean	$LSD_{.05}$ = 403.57
-sham	23.29	B	112.7	B
+sham	465.14	A	1039.8	A

IMC	SA Mean	$LSD_{.05}$ = 18.26	SAG Mean	$LSD_{.05}$ = 90.71
-sham	22.727	B	118.68	B
+sham	73.271	A	846.34	A

Mean separation within columns and by treatment.

Table 21. Effect of cultivar on salicylic acid (SA) and salicylic acid glucoside (SAG) contents of cacao flowers

	SA		SAG	
	Mean	$LSD_{.05}$ = 46.181	Mean	$LSD_{.05}$ = 132.16
AME	61.63	B	190.28	B
TSH	244.22	A	576.24	A
IMC	48.00	B	482.51	A

3.3.5. Hand pollination

Out of 132 flowers pollinated, 57 set fruit (43 %) and of these, 17 wilted, resulting in 40 pods reaching maturity. This represented 30 % of the total flowers pollinated and 70 % of those that set fruit.

3.4. DISCUSSION

Young and Severson (1994) had been the only ones to report the volatile profile of cacao flowers. They collected these volatiles using steam distillation, a procedure which may lead to artifacts and may extract compounds that are not naturally emitted by the flowers. Using the Tenax cartridges, only naturally emitted volatiles, which accumulate in the headspace of the container enclosing the flowers, are blown out with the air which is pumped in the vessel and then adsorbed in the Tenax resin as they come out.

Although, a few of the VOCs identified here had been reported by Young and Severson (1994), here many more were identified including, hexanal, 2-hexenal, heptanal, octanal, benzaldehyde, 2-heptanone, 1-(cyclohexen-1-yl)-ethanone, 5-ethenyldihydro-5-methyl-2(3H)-furanone, n,n-diethylformamide, hexanoic acid, longifolene, octane, nonane, indene, 3,7-dimethyl-1,3,7-octantriene, 2-methyldecane, 4-undecene, 3-dodecene, 4-dodecene, 2-methyl-dodecane, 2-undecanone, 2-methyltridecane, 3-methyltridecane, 1,6-tridecadiene (Table 15).

When the most abundant VOCs from cacao flowers, pentadecene, pentadecane and a few other, were tested individually in insect attraction trials, none was effective (Young, 2007). However, flower extracts placed in insect traps were successful in attracting more cacao pollinators compared to controls (Young, 2007). It is possible that compounds present in smaller amounts not previously identified and therefore not utilized in the insect attraction trials are the actual attractants of cacao pollinators.

Regarding the temperature measurements, the results suggest that cacao flowers regulate their temperature using the alternative oxidase pathway, thereby corroborating previous reports that indicate cacao flowers can increase their temperature to levels higher than their surrounding background tissues during morning hours. Cacao flower temperatures were 1.91 ºC higher than the surrounding bark, similar to findings by Erickson et al. (1992) who observed cacao flowers with 2.1 ºC higher than the bark. Cacao flowers, due to their cauliflorous nature, are more exposed to shaded conditions and lower temperatures than leaves. Therefore, the fact that flower temperature is higher than bark and leaves reinforces the presence of floral thermoregulation. The current results also support the idea that greater differences between flower temperature and their surrounding background tissues occur at morning hours when natural pollination is thought to occur (Erickson et al., 1992).

Measurements showed no differences between temperatures of open and closed flowers, but flower temperature differed from other plant parts as well as the ambient temperature. Flower temperature also differed at certain times of the day (Table 16). Nonetheless, the differences in temperature, although small, are biologically relevant. Honeybees, for example, are able to differentiate thermal differences down to 0.25 ^{o}C

(Lacher, 1964). In contrast to the findings of Erickson et al. (1992) however , cacao tissue temperatures were lower than ambient temperature. Erickson et al. (1992) conducted the experiments under field conditions, while this experiment was conducted in a greenhouse with controlled environmental conditions but with possibly lower humidity and greater radiational cooling than under field conditions. Pollination biology involves a number of fascinating aspects related to pollinator attraction. While pollinators are attracted to flowers by visual or sensory stimuli (temperature, odor, CO_2), some plants provide pollinators with food (nectaries) and shelter. Attractants, including nectaries and color, have been suggested to be involved in pollinator attraction in cacao (Brew, 1987; Young et al., 1984). It has been shown that midges, known pollinators of cacao, are attracted to CO_2. It was also demonstrated that CO_2 in combination with a common fungal volatile, 1-octen-3-ol, induced a synergistic effect in attracting the insects (Ritchie et al., 1994). Cacao pods infected by fungi (*Moniliophthora roreri* and *Phytophthora* spp.) were reported to be a major breeding site for the midge pollinators of cacao (Young, 1986). These insects have also been found in rotten cocoa fruit, rotten banana soft stalks, and leaf litter, and are more abundant during the rainy season (Hurtado-Mejia and Velez-Angel, 1999).

Respiration levels were higher in open flowers than in closed ones (Figure 54). However, there were no temperature differences between open and closed flowers. Therefore, the increased respiration in open flowers compared to closed ones is not likely directly related to the use of the alternative oxidase pathway, but to the greater metabolic requirement of open flowers.

Plant respiration is the source of ATP and provides carbon intermediates used in the synthesis of many cellular constituents. Plant respiration can occur through the cytochrome pathway and the alternative pathway, which constitute the respiratory electron-transport pathways of plant mitochondria. The cyanide resistant respiration enzyme, alternative oxidase, responsible for the alternative pathway, catalyzes a four electron reduction of oxygen to water and is specifically inhibited by salicylhydroxamic acid (SHAM). When electrons pass to the alternative pathway from the ubiquinone pool two site of energy conservation (complexes III and IV) are bypassed and no ATP is formed. Since there is no energy conservation site on the alternative pathway between ubiquinone and oxygen, the free energy that would normally be stored as ATP is lost as heat (Moore and Siedow, 1991). This heat induces in some plants volatilization of compounds that serve as insect attractants.

Thermoregulation seems to occur through the alternative oxidase pathway since SA increased respiration rates while SHAM reduced them (Figure 58). Moreover, flowers treated with SA had an increase in temperature of up to 2 ^{o}C.

In this experiment the respiratory quotient (RQ; RQ = O_2rate/CO_2rate) observed was greater than one, for control flowers and flowers treated with SA, indicating that organic acids contribute to the substrates used for respiration (Table 22) (Hopkins and Huner, 2004).

Interestingly flowers treated with 200 μM of salicylic acid maintained their normal appearance, whereas control flowers after 84 hours of treatment were brown (Figure 59). SA may be acting on the inhibition of ethylene biosynthesis and/or reversing the effects of abscisic acid (Aneja et al., 1999).

Figure 58. Effect of SA, SA plus SHAM, SHAM and KCN treatments on cacao flower respiration

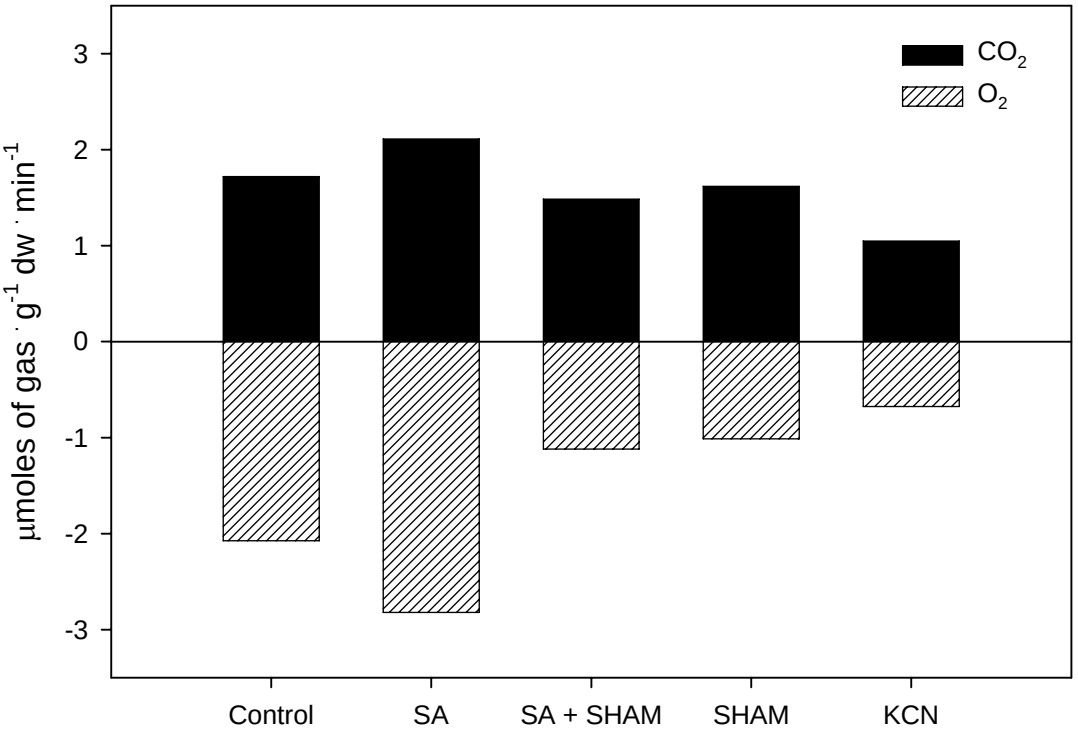
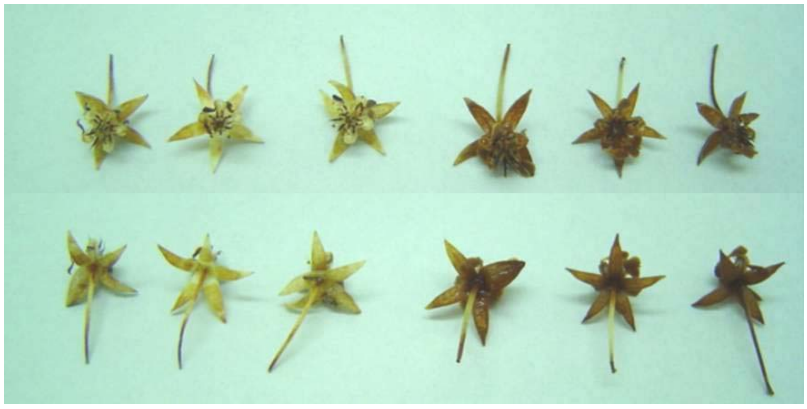


Table 22. Respiratory quotient (RQ) of flowers treated with different chemicals

	Control	SA	SA + SHAM	SHAM	KCN
RQ	1.2	1.3	0.75	0.63	0.65

Figure 59. Flowers treated with 200 µM of salicylic acid (3 on the left) compared to controls (3 on the right), after 84 hours of treatment



The presence of SA in cacao flowers is another evidence for the alternative respiration present in cacao flowers. SA and SAG contents of open cacao flowers did not differ from buds. Although there were differences among cultivars, Amelonado had the lowest levels of SAG of the three, while TSH 565 had the highest SA levels. SAG is though to be a storage form of SA. When present in higher levels in the self-incompatible gentopytes (TSH and IMC) this storage compound could be involved in producing more SA to affect the release of floral attractants (CO_2 and volatiles) more effectively than in Amelonado (self-compatible) to due the incompatible genotypes' higher dependency on cross-pollination.

The alternative respiratory pathway is specifically inhibited by SHAM and triggered by SA. The application of SHAM to open and closed flowers induced an accumulation of SA and SAG. One explanation for the SA accumulation is that flowers keep producing SA in order to activate the alternative pathway, which fails to occur due to the presence of the inhibitor. The other option is that SHAM is degraded to SA *in planta*. This hypothesis have been suggested by Chivasa et al., (1997) who observed an induction of PR-1 protein accumulation upon application of SHAM, similar to the effect caused by SA application.

3.5. CONCLUSIONS

Many new volatile organic compounds released by cacao flowers were identified. It is possible that these compounds, some of which are present in smaller amounts than compounds previously reported in the literature, can contribute to the attraction of cacao pollinators. Cacao flowers raised their temperatures higher than background tissues during morning hours when pollination is known to occur. Flower temperature regulation occurs through the alternative respiration pathway. Endogenous and inducible SA and SAG were found in cacao flowers, and their levels varied between self-compatible and self-incompatible genotypes suggesting a possible role in pollination biology.

Further studies, such as electroantennograms and wind tunnels, using the newly identified volatiles in combination with CO_2 are the next steps in trying to correlate the identified compounds with pollinator attraction.

Purification of alternative oxidase enzyme and determination of the effects of SA and SHAM on its expression levels should be performed to continue the investigation on the alternative respiration pathway in cacao flowers.

4. PROCESSING

4.1. INTRODUCTION

Although pests and diseases, the reliance on pollinators, and poor fruit set all contribute to lower crop yields, bean quality will always be one of the main concerns in cacao production systems. The principal criterion for assessing the quality of cocoa for manufacturers is flavor. Flavor is developed and assessed after roasting, but the plant genotype, as well as the fermentation and drying processes are factors that influence the composition of aroma precursor compounds.

Once ripe, cacao pods are harvested, cracked open with a machete, and the beans, naturally covered with a mucilaginous pulp, are stacked in a pile. Banana leaves are then placed on top of the pile in order to avoid wetting from constant rain. In some cocoa-growing regions, beans are placed in heaps, boxes, baskets or trays. Every other day the bean pile is mixed or turned to provide aeration and to allow uniform fermentation of all beans. After a few days (commonly varies from three to seven days), beans are spread out and dried under the sun, then roasted, ground and processed.

During fermentation, sucrose is converted into glucose and fructose, which are then converted into alcohol and acids by yeasts and bacteria. Acids migrate from the pulp to the beans, killing the embryo, disrupting cell walls, and liberating their contents (Schwan and Wheals, 2004). Enzymes are also active during fermentation, oxidizing polyphenols to quinones, which then polymerize or bind to proteins, giving beans a dark color. Maillard reactions, non-enzymatic chemical reactions between amino acids and

sugars, also may contribute to the browning of the beans. However, this reaction is thought to occur after fermentation and drying, during roasting (Hansen et al., 1998).

Currently, chocolate manufacturers are rediscovering the health benefits of cocoa and increasing the levels of cacao in their products. In effect, they are increasing the procyanidin contents of their products and therefore turning candies into nutraceutical products. Health benefits associated with the consumption of cacao bean products are mainly derived from procyanidins. These compounds are potent antioxidants, and a substantial amount of research indicates a series of beneficial properties including their action as vasodilating agents shown to improve cardiovascular function (Keen et al., 2005; Schroeter et al., 2006).

Fermentation is essential to produce precursor aroma compounds that, after roasting, become the familiar flavors of cocoa powder and chocolate. However, during the fermentation process, cocoa polyphenols are oxidized and the health benefits of the cocoa bean are lost. Although some oxidation of cocoa polyphenols is necessary due to their contribution to the bitter taste of unfermented beans, it would be desirable to limit this process, particularly for products designed for the health food market.

Most of the work on cacao bean fermentation was done in the 1950's and 1960's. With the new analytical tools available today, more accurate data can be obtained, particularly on the procyanidin contents of beans. In this section, research on the effect of adding an inhibitor of polyphenol oxidases to a cacao bean fermentation under laboratory conditions is described to determine if losses of polyphenols during fermentation could be limited without affecting the sugar and amino acid content needed for the development of flavor precursors.

4.2. MATERIALS AND METHODS

4.2.1. Fermentation

Cacao pods from greenhouse grown Amelonado trees were harvested, cracked open and the mucilaginous beans placed in 250 mL beakers. The beakers were placed in an incubator at 40 ^{o}C and bakers' yeast was added as a fermentation starter culture. An aqueous solution of kojic acid (10 mM) was added along with the yeast to one of the beakers while the control received only yeast and water. Every day the pile of beans was turned and transferred to another beaker. Periodically bean samples were removed for chemical analysis during the time course of the experiment. After 14 days beans were removed from the incubator freeze-dried and kept at - 80 ^{o}C.

4.2.2. Effect of kojic acid on yeast

Yeast suspensions applied to the cocoa bean fermentation were plated on PDA media containing 10 mM of KA and on PDA without KA.

4.2.3. Bean sample preparation

Seed coat was removed from freeze-dried beans before grinding them in a mortar and pestle into a fine powder. Beans were defatted with hexane, extracted with

acetone:water:acetic acid (70:29.5:0.5), filtered, and concentrated, prior to the different chemical analyses.

4.2.4. Sugar analysis

Sugars were analyzed using the anthrone method measuring the absorbance at 620 nm and using sucrose as standard (Yemm and Willis, 1954).

4.2.5. Amino acids analysis

Total amino acids were analyzed using the ninhydrin method, absorbance measured at 570 nm and used phenylalanine as standard (Rosen, 1957).

4.2.6. Procyanidin analysis

Procyanidins were measured using the method described on page 17.

4.3. RESULTS AND DISCUSSION

The major flavonoids in cocoa beans are procyanidins, oligomers of flavan-3-ols ranging from monomers to decamer (Kelm et al., 2006). Unfermented cocoa beans contain approximately 6 to 8% (dry weight basis) of phenolic compounds and the major phenolic compound in cocoa bean is (-)-epicatechin with 2 % dw (Wollgast and Anklam, 2000; Porter et al., 1991). Kim and Keeney (1984) found 21.89 to 43.27 $mg.g^{-1}$ of (-)-epicatechin in dry defatted unfermented samples and dry defatted fermented beans had 2 to 10 $mg.g^{-1}$. According to Bonhevi and Coll (1997), optimally fermented cocoa beans should have a maximum total polyphenol content of 58 $mg.g^{-1}$, tannins 31 $mg.g^{-1}$ and (-)-epicatechin 3 $mg.g^{-1}$.

Cacao beans have a strong polyphenol oxidase system capable of removing 80 % of the total polyphenols in 15 minutes (Forsyth, 1952). Misnawi et al. (2003) showed that partly fermented cocoa enriched with polyphenoloxidases increased significantly procyanidin degradation, resulting in lower levels of procyanidins in the beans.

Levanon and Rossetini (1965) showed that polyphenolic compounds and sugars decreased over time during the fermentation process. Ardhana and Fleet (2003) found lower sugar levels in fermented beans compared to unfermented beans. In contrast, Brito et al. (2000) found an increase in the sugar content of fermented beans compared to unfermented ones. Rohan and Stewart (1967) observed a loss of almost 100 % of reducing sugars and 50 % of the free amino acids and during the roasting process of cocoa. Meanwhile, Rohan (1964) found that the amount of free amino acids present in cocoa beans increases during fermentation.

On page 69, it was shown the identification of kojic acid, a natural compound synthesized in fermentation cultures of *Aspergillus oryzae*, isolated as an endophyte from coffee. This natural product is commercially available and used in the food processing industry, and in the cosmetic industry as an antioxidant (Bentley, 2006). KA inhibits PPO from a number of organisms including a PPO of the cacao pathogen *M. perniciosa* (See page 152). When KA was added to cacao bean fermentations conducted in the laboratory, there were dramatic visual differences in the appearance of the cacao beans during fermentation. KA treated beans were lighter in color with more purple anthocyanin pigment evident, whereas untreated beans were darker in color (Figure 60). Cut tests indicate that the oxidation of cocoa polyphenols is reduced (Figure 61). Yet KA had no effect on growth of the yeast (Figure 62).

Although total procyanidins decreased during fermentation in the treated and control beans, kojic acid treated beans by the end of the experiment had about two-fold higher procyanidin levels that non-treated beans (Figure 63). The monomer fraction including (-)-epicatechin and (+)-catechin decreased from 153.6 $mg.g^{-1}$ in unfermented beans to 22.4 $mg.g^{-1}$ in untreated beans and 42.1 $mg.g^{-1}$ in KA treated beans six days into fermentation. While oligomers (dimer to decamer) levels were 42 $mg.g^{-1}$ in non-treated beans they were 99 $mg.g^{-1}$ in KA treated beans six days into fermentation.

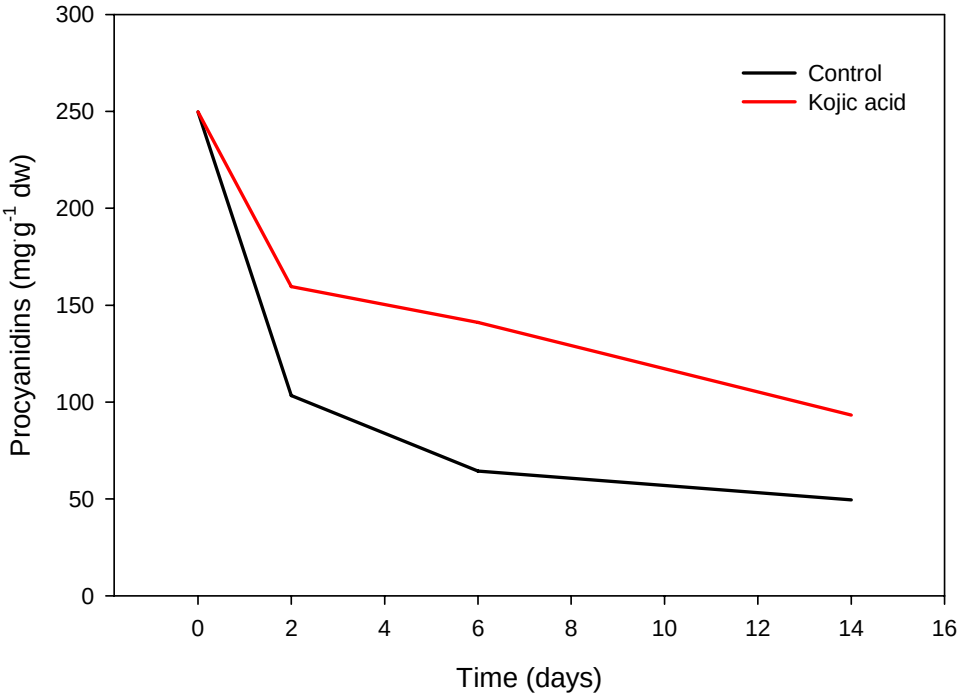
Amino acids levels increased during fermentation (Figure 64), while sugars levels decreased (Figure 65). KA treated beans had higher levels of both sugars and amino acids when compared to control beans (Figures 64 and 65).

Figure 60. Beakers containing beans during fermentation (left – control, right – KA treated)

Figure 61. Cut test showing on the left control beans and on the right KA treated beans after 7 days of fermentation.

Figure 62. Yeast streaked on media containing 10 mM of kojic acid in PDA (right) compared to PDA alone (left)

Figure 63. Total procyanidin content of beans during fermentation*



* No significant statistical differences were observed using the F test at P.05.

Figure 64. Total amino acids in cacao beans during fermentation

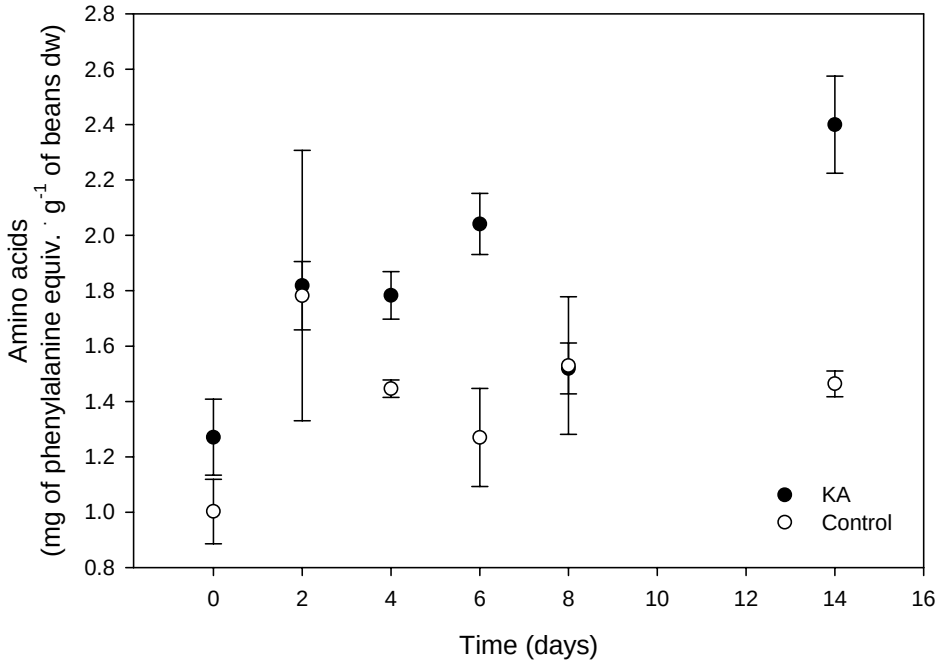
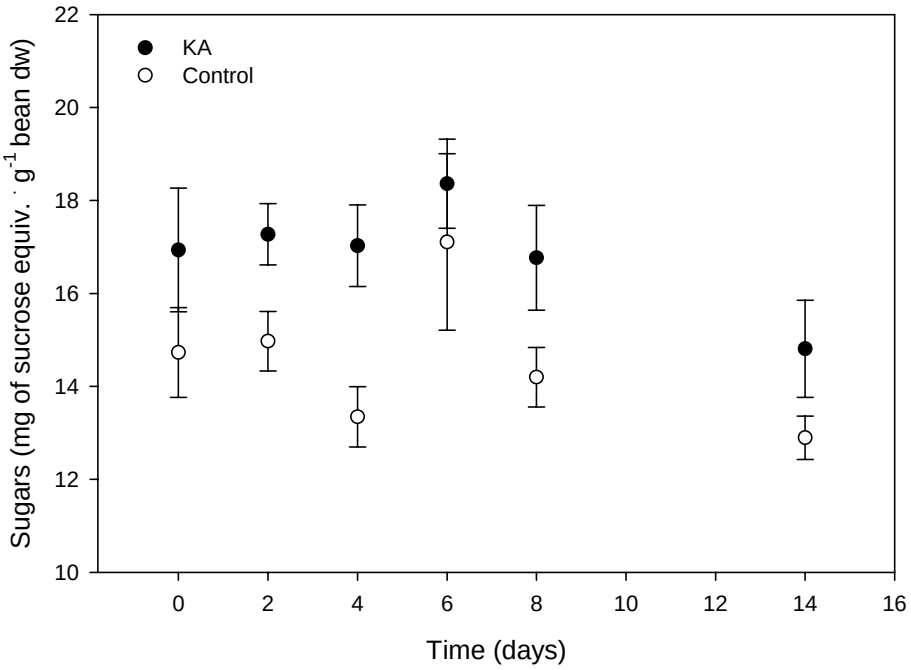


Figure 65. Total sugar content of beans during fermentation



4.4. CONCLUSIONS

Although cacao bean fermentation has been studied for decades, even today, the process is typically uncontrolled and great variation exists in fermentation methods and monitoring between farms. All of these factors may affect the quality of the final product. This part of the processing is not under control of manufacturers but of small farmers in poor regions of the world including, West Africa, Indonesia and Malasia. Moreover, it is not likely that anything will change, since altering cultural practices is usually difficult unless some simple alternative methods are available, and/or relevant financial benefits are involved.

The results of this study suggest that addition of kojic acid, which is a natural product and food additive safe for consumption, during the fermentation of cocoa beans can maintain higher procyanidin content of fully fermented beans. This simple, environmentally and user friendly, low cost, hazard free procedure would lead ultimately to cocoa powder with higher procyanidins, which could be used as a component in cocoa health food products. Follow up fermentation experiments using larger scale standard methods will help determine if the application of a polyphenol oxidase inhibitor will produce fully fermented cocoa beans with higher levels of polyphenols.

5. SUMMARY AND CONCLUDING REMARKS

During this project, problems associated with three main aspects of cacao were investigated: disease, production and processing. The dissertation was divided into 3 sections: (1) Studies on plant-endophyte-pathogen interactions allowed for the identification of new possible mechanisms of disease control; (2) Studies on cacao flower physiology indicated ways to improve pollination and therefore increase fruit set and crop yield; and (3) Investigations on the fermentation step of cacao processing permitted discovery of a method for maintaining higher levels of compounds valued by cacao manufacturers for chocolate production.

Plant defenses, including, endogenous and inducible levels of caffeine, theobromine, (-)-epicatechin and (+)-catechin, salicylic acid, salicylic acid glucoside were evaluated in genotypes with different levels of disease resistance in order to determine a broad spectrum defense response, which may require concurrent production of several compounds. Cluster analysis demonstrated a stronger genotype influence rather than elicitor treatment effect on the chemical profile of cacao leaves. This example of chemical profiling allowed the separation of genotypes with different levels of resistance based on their overall ability to produce phytoalexins.

Flavan-3-ol monomers and oligomers, from dimers to decamers, were quantitatively determined by HPLC-FLD in leaves throughout development in four *Theobroma cacao* genotypes. The levels of the very young leaves were intermediary and peaked as leaves attained lengths of 4 to 20 centimeters. As the leaves increased in size

and developed further, the procyanidin content reached its minimum. Cacao flush leaves had higher procyanidin content than mature leaves.

Witches' broom disease tolerant genotype Scavina 6 had the highest levels of procyanidins when compared to Amelonado, TSH 565, and IMC 30, more disease susceptible genotypes, at a stage when leaves are susceptible to infection. Moreover, leaves of SCA 6 responded to *M. perniciosa* basidiospore infection by inducing accumulation of procyanidins in both the infected and adjacent uninfected leaves of actively growing shoots, demonstrating an inductive response effect both locally and systemically.

Combined evidence for activation of procyanidin gene expression (Leal et al. 2007), antifungal effects of procyanidins against the witches' broom pathogen (Andebrhan et al., 1995; Brownlee et al., 1990), higher endogenous levels of procyanidins in leaves of a disease resistant genotype, including a peak in procyanidin levels during leaf development at a stage when leaves are susceptible to witches' broom disease, and the local and systemic increase in procyanidins in cacao leaves of a disease resistant genotype upon infection with basidiospores indicate a role for procyanidins in defense against *Moniliophthora perniciosa*. Therefore, procyanidin content of flush leaves is a potential marker for selecting for disease tolerance in cacao.

Laccase is one of the polyphenoloxidative enzymes produced by *M. perniciosa* as a virulence or pathogenicity factor and is therefore a potential target for disease control. The main procyanidin found in cacao, (-)-epicatechin, can prevent pathogen growth in the presence of a polyphenoloxidase inhibitor that blocks the ability of the fungus to detoxify polyphenols, rendering them toxic to fungal growth. Application of polyphenoloxidase

inhibitors to cacao may affect the ability of the pathogen to thrive in plants with high levels of polyphenols.

Salicylic acid (SA), phenyllactic acid and mandelic acid were identified as products of fungal metabolism by *M. perniciosa*. Levels of SA were quantified in infected and uninfected plant tissues as well as in filtrates of fungal cultures. Infected brooms had five times more SA compared to healthy shoots. Moreover, application of SA to leaves induced curling and necrosis. In contrast, leaf inoculation with basidiospores or application of chemical elicitors did not alter leaf SA content, suggesting that in this infection system, the actively growing mycelia is the source of the SA rather than the plant. For necrotrophic fungal pathogens, including *M. perniciosa*, SA may be a component of pathogenesis and not part of the defense response of plants. Therefore, it is possible that plants capable of responding to infection more effectively by activating cell death, when challenged by a necrotrophic pathogen, tend to be more disease susceptible.

Among all tested endophytic fungal isolates, two *Aspergillus westerdjikiae* were found to be ochratoxin producers. Media composition had an effect on the metabolites produced by these fungi. Caffeine, besides affecting the growth of the endophytes, affected their ability to produce toxins. Caffeine below toxic levels may trigger toxin production, while at higher levels completely disrupts fungal metabolism. Some toxigenic fungi may be able to break down caffeine and possibly use the degradation products as carbon and nitrogen sources.

An *A. oryzae* isolate was found to produce kojic acid (KA) in culture. When inoculated in cacao seedlings, *A. oryzae* grew endophytically and synthesized KA in planta. Cacao seedlings inoculated with *A. oryzae* produced higher levels of caffeine than

non-inoculated ones. *A. oryzae* may be a useful endophyte to introduce to cacao since it grows non-pathogenically and induces the caffeine defense response that may improve plant resistance to pathogens.

The interactions between studied microorganisms ranged from parasitism to production of volatile organic compounds, antibiotics or toxins. The environment to which the organisms are subjected influences their fitness and should be considered when selecting biological control isolates. In a complex chemical warfare between microorganisms, understanding the factors influencing their ability to produce secondary metabolites enables researchers to provide favorable conditions for the target beneficial organism to succeed.

B. bassiana was isolated from various sections of the cacao pod and peduncle two and three months after the flowers had been hand-pollinated and sprayed in the greenhouse with a *B. bassiana* conidial suspension. These results indicate that spraying flowers with conidia can result in the establishment of a fungal entomopathogen as an endophyte, and that the fungus can move within the pod as it grows. This finding could lead to an innovative way to manage insect pests in biocontrol programs.

Many new volatile organic compounds released by cacao flowers were identified. It is possible that these compounds, some of which present in smaller amounts than compounds previously reported in the literature, can contribute to the attraction of cacao pollinators. Cacao flowers raised their temperatures higher than background tissues during morning hours when pollination is known to occur. Flower temperature regulation occurs through the alternative respiration pathway. Endogenous and inducible SA and

SAG were found in cacao flowers, and their levels varied between self compatible and self incompatible genotypes suggesting a possible role in pollination biology.

The fermentation step of cacao processing is much uncontrolled and demands improvements. Addition of kojic acid, which is a natural product food additive safe for consumption, to fermenting cocoa beans allowed for reduced procyanidin losses in fully fermented beans. This simple, environmentally and user friendly, low cost, hazard free procedure would lead ultimately to cocoa powder with higher procyanidins, which could be used as a component in cocoa health food products.

Cocoa is and has always been a highly valued crop in the world. Old medicinal properties are being rediscovered, bringing about new applications for cacao. Yet cacao production is limited by factors such as pathogens and low productivity. The basis for unraveling these issues lies in identifying their mechanisms. One approach is through the analysis of the complex chemical interactions between the plant itself and various pathogens affecting cacao production.

In nature, many compounds serve multiple purposes. Exploring known compounds for novel activities uncovers new involvements for these metabolites. Cacao procyandins are not only important in human health but participate in the plant's immune system. It is critical to maintain a holistic perspective of the various agricultural, processing, and manufacturing aspects of the crop in order to determine its success.

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