ACTIVITY REPORT 2009 - 2013

Expedient

National Institute of Science And Technology for the Biorational Control of Pest-insect

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Sonia Maria Noemberg Lazzari - UFPR

Manfred Willy Muller - CEPLAC-Bahia
Jay Wallace da Silva e Mota - CEPLAC-Belém

Paulo Cesar de Lima Nogueira – UFS
Valéria Regina de Souza Moraes - UFS

SUMMARY

NATIONAL INSTITUTE OF SCIENCE AND TECHNOLOGY FOR THE BIORATIONAL CONTROL OF PEST-INSECT

INCT ASSOCIATE LABORATORIES

Headquarters:
Federal University of São Carlos
Centre for Exact Sciences and Technology
Chemistry Department
Coordinator: M. Fátima G.F. da Silva
Vice Coordinator: João B. Fernandes

Associate Laboratories:
National Institute of Science and Technology for the Biorational Control of Pest-Insect involves five states and seven institutions

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M. Fátima G. Fernandes da Silva
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Ronaldo Censi Faria
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Tiago Venâncio
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Carmen Lucia Cardoso

4. University of São Paulo USP

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Chemistry Department

Paulo César de Lima Nogueira
Valéria Regina de Souza Moraes

7. Executive Commission for the Development of Cacao -Pará

Jay Wallace da Silva e Mota

Executive Commission for the Development of Cacao -Bahia

Manfred Willy Muller

HISTORY - ESTABLISHMENT OF THE NATIONAL INSTITUTE OF SCIENCE AND TECHNOLOGY FOR THE BIORATIONAL CONTROL OF PEST –INSECT

The Natural Products Research Group of Federal University of São Carlos, SP-Brazil (UFSCar) was formed more than 30 years ago. The research interest of the group covered many aspects of General Phytochemistry. The State of São Paulo Research Foundation (FAPESP) made substantial contributions to our group develops new scientific strategies for the study of natural products by two Thematic Projects: “Study of the potential of some plant species and natural and synthetic products for the control of leaf-cutter ants”; coordinated by Prof. João B. Fernandes; and "Phytochemistry and chemical ecology: search for starter compounds for new insecticidal, fungicidal and bactericidal drugs for control of plant pests”, coordinated by Prof. M. Fátima G. F. da Silva.

In developing these studies the group had strong interaction with a number of other research groups, notably with: Center for the Study of Social Insects (CEIS), São Paulo State University (UNESP), Rio Claro, and Sylvio Moreira Citrus Center, Cordeirópolis, SP. More recently, the National Institutes of Science and Technology Program (INCT), launched in July 2008 by Ministry of Science and Technology - CNPq, permitted that our group was expanded. Thus, Professors J.B. Fernandes and M.F.G.F. da Silva aggregate in networks the best research groups of chemical ecological areas from five states and seven institutions in order to transform Brazil in the model country for control of insects with low impact to the environment, and created the National Institute of Science and Technology for the Biorational Control of Pest-Insect (NIST-BCPI).

Galipea jasminiflora

Dictyoloma vandellianum

Azadirachta indica

MISSION AND OBJECTIVES

MISSION:

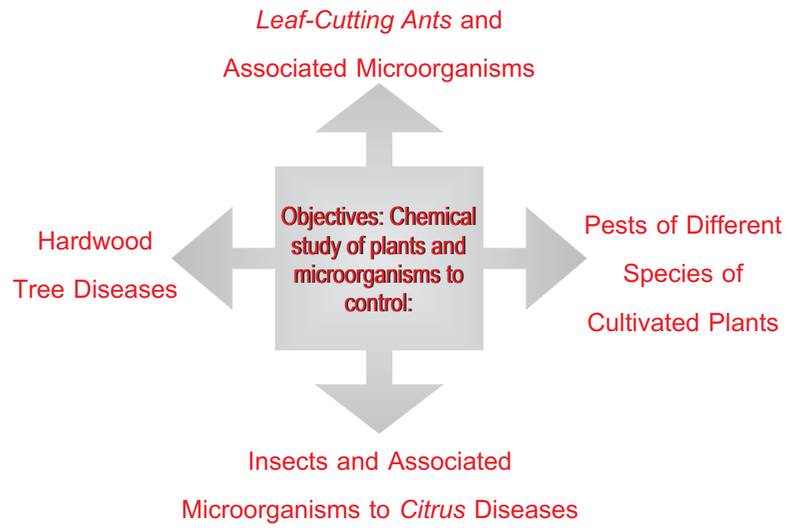
The efficient control of insects and the search for biologically active compounds that are closely related to human survival are important issues to be studied. Insects are the greatest mankind competitors with regard to food, besides being vectors of a number of diseases that affect humans, herds, and, plants.

The objective of this project was to carry out studies to control biorationally pest insect and microorganisms associated such as fungi, bacteria, and yeasts.

OBJECTIVES:

Development of methodology:

- Modification of insecticides structure to improve activity and solubility: Complexation of bioactive natural products with inorganic ions.
- Immobilization of enzymes in columns for High performance liquid chromatography.
- Development of enzymatic bioreactors for the evaluation of the insecticide activity in plant extracts.
- Nuclear Magnetic Resonance and its association with HPLC and mechanisms of action of insecticides.
- Toxicity of natural products and viability of use.



INTRODUCTION

The results presented illustrate the potential of an interdisciplinary program. They show interesting active compounds and new methodologies of assays, which will afford a highly efficient process for elucidation of multi-chemical defensive strategies in resistant plant cultivars. These methodologies also generated a more rational and scientific approach to pest insect control.

The assays of pesticide activity and inhibition of fungis and bacteria have been performed with plant extracts and natural products from plants or microorganism. The toxicity of a number extract and natural compounds to insects, fungis and bacteria were determined. The extracts and natural compounds showed moderate activity in comparison with commercial insecticides. Thus these compounds were assayed against other targets, which were published (see paper published). Neen oil from *Azadirachta indica* showed significant activity as insecticide.

However, if it is assumed that it is possible to modify the chemical structure of compounds to improve activity and selectivity, our results helped in directing the rational design of coumarins, alkaloids and flavonoids derivatives and the last as potent and effective insecticide, fungicide and bactericide.

Enzymes that degrade the polysaccharides of the vegetal (pectinases and amylases) in reducing sugars have been detected in symbiotic fungus and also have been found in the fecal liquid of the *A. sexdens rubropilosa*. These sugars constitute the main source of energy for the ants' nest. Therefore, the ants use symbiotic fungis to promote this process of degradation, once they are not capable to degrade the pectin directly.

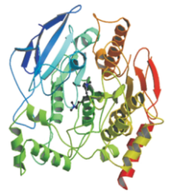
The enzyme acetylcholinesterase (AChE) is present in the central nervous system of insects, and hydrolyses the acetylcholine neurotransmitter in acetate and choline, thus finishing the synaptic transmission, playing a fundamental role in the transmission of the cholinergic nervous impulse. Two genes, Ace1 and Ace2, have been characterized in different classes of insects and two mutations in Ace1 have been associated with resistance in mosquitos. Enzymatic bioreactors were prepared using the enzymes acetylcholinesterase, butirilcholinesterase, and pectinase, and were used for studies of mechanism of action of substances, which presented inhibition activity against insects.

IMMOBILIZATION OF ENZYMES IN COLUMNS FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Acetylcholinesterase Bioreactors

IMERs-AChE (immobilized enzymes reactors) were developed and used for the evaluation of the enzyme activity on the variations of the procedure of the capillary pre-treatment. This process was successfully optimized. The assay with substances, which presented inhibition activity against insects were developed, and coumarins and complexes of bioactive natural products were the most active.

These results corroborate the possibility of using these bioreactors in the triage of collections of acetylcholinesterase inhibitor compounds and for studies of mechanism of action for bioactive natural insecticides.

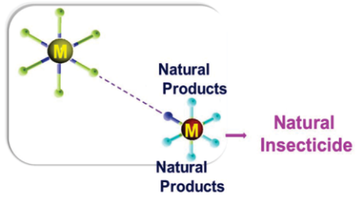


Others IMERs were developed for the large scale triage of inhibitors of butirilcholinesterase, pectinases and xanthine oxidase. However, these processes were not yet successfully optimized.

Modification of insecticides structure to improve activity and solubility

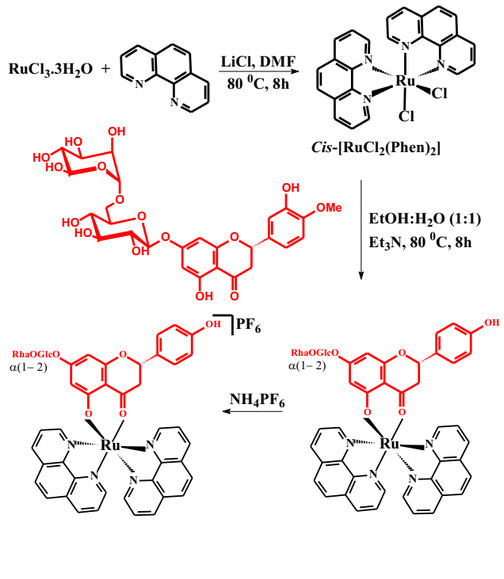
Quinoline alkaloids, acridone, xanthones, coumarins and piperamids were synthesized through methods described in the literature and/or new synthetic routes; however these compounds showed moderate activity in comparison with commercial insecticides. Thus these compounds were assayed against other targets, which were published (see paper published).

Complexes of bioactive natural products with inorganic ions were prepared, and they showed as potent and effective insecticide and bactericide.

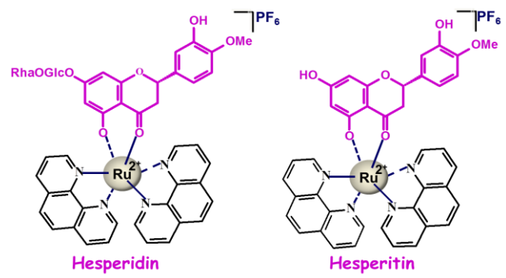


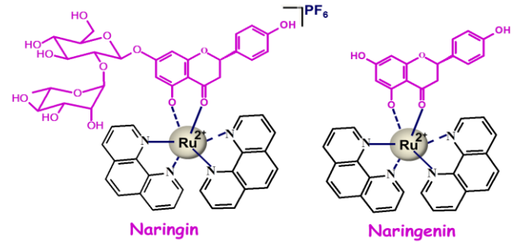
Flavonoids showed moderate activities against various insects and microorganisms, thus they were selected to be complexed with inorganic ions to improve their activities and solubility. Therefore, a series of piridinic complexes of Ru and Mg such as cis-[Ru(phen)(L)]+L (where L was hesperidin, hesperetin, naringin or naringenin) were prepared and characterized by spectroscopic (UV-Vis, FTIR, and RMN) and electrochemical (Cyclic and Differential-Pulse Voltametry) properties.

The complexes with Ru were synthesized as below:

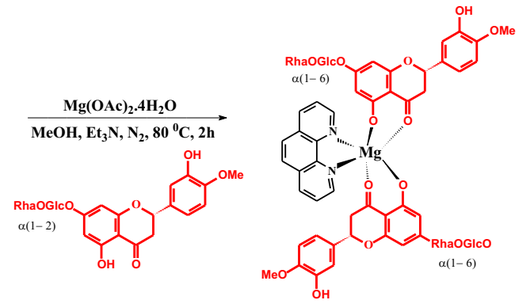


The following complexes with Ru have already been prepared:

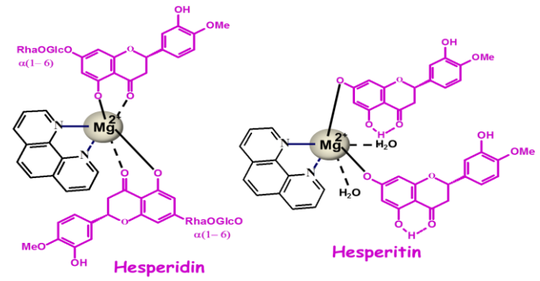


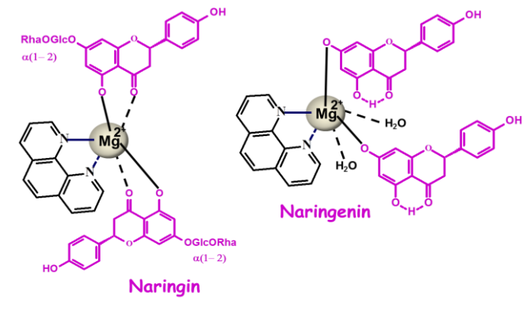


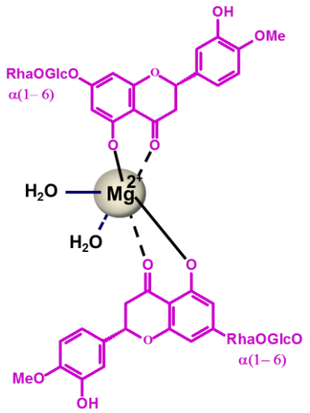
The complexes with Mg were synthesized as below:



The following complexes with Mg have already been prepared:







All complexes are stable in solid state, in most of the organic solvents tested and at various pH values. They are more hydrosoluble and liposoluble than the free flavonoids.

Effects of complexes on *Atta sexdens rubropilosa*

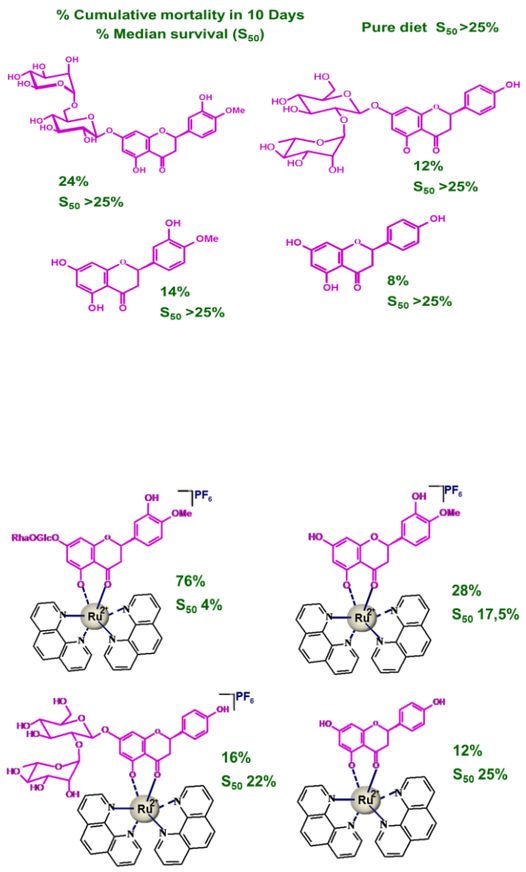
Leaf-cutting ants of the genera *Atta* and *Acromyrmex*, which are distributed from Argentina to the southern USA, cause serious damage to a wide variety of plants and are a serious crop pest in this area. They cut plant material and use it as the main substrate for the development of their symbiotic fungus *Leucoagaricus gongylophorus*, which is thought to be the only alimentary source for the ants larvae.

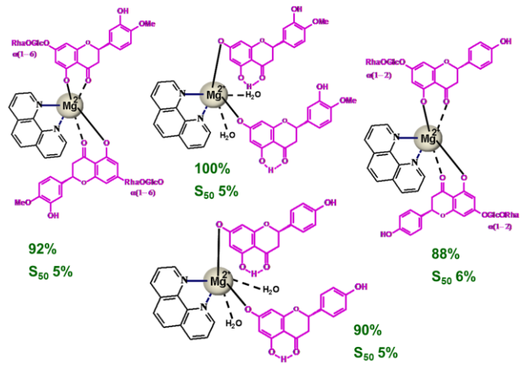
The fungus also may provide 9% of the energy requirements for adult workers. The workers seem to get most of their food sources from the products of leaf polysaccharide degradation by the symbiotic fungus.

Traditional control of these ants with insecticides, in spite of its efficiency, is still a problem because of their non-selective toxicity. As a consequence, the search for alternative methods to leaf-cutting ant control has intensified recently, trying to substitute traditional agrochemicals for others of shorter persistence, greater specificity and therefore less harmful to the environment.

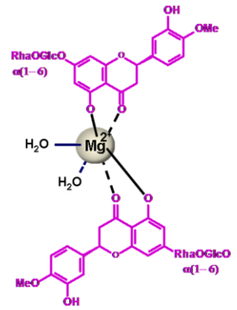
Some flavonoids and their complexes were assayed on *Atta sexdens rubropilosa*. In the assay were evaluated the Cumulative Mortality (CM) and median Survival (S_{50}: time at which 50% of the ants remained alive) of workers ants (*Atta sexdens rubropilosa*) subjected to bioassay by incorporation of hesperidin, hesperetin, naringin and their complexes and the insecticide sulfluramida (0.2%) into artificial diet.

The results are summarized below:

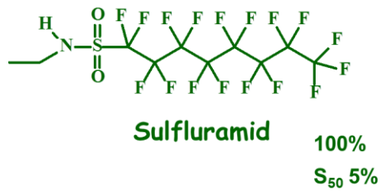




The below complex was not as efficient, indicating that the coordination to the Mg^{2+} ion and phenanthroline are important for modification of their physicochemical properties and consequent insecticide action.



Magnesium complexes were the most active, similar to insecticide sulfluramid.



The high toxicity of this commercial insecticide resulted in its removal from the market in several European countries. In Brazil the Ministry of Agriculture and Environment suggested that sulfluramid should also be removed from the market.

It has long been recognized that orange peel represents a promising source of hesperidin. A million metric tons of peel residues are generated as result of fruit processing, and thus, an extract of this residue could be considered for the isolation of hesperidin for synthesize the above complexes.

Magnesium (Mg) has been used in nutrient menu for different crops, and it is an important building block of the green plant pigment chlorophyll, which plays a key role in the use of sunlight to produce energy (photosynthesis).

Mg^{2+} complexes were powerful inhibitors of AChE of *Atta sexdens rubropilosa*, indicating their high selectivity to insects. Further, the complexes are essentially non-toxic to the aquatic bacterium *Vibrio fischeri* and to human HeLa cells.

Thus, the results suggest that the complex $[Mg(phen)(hesp)_2]$ or $[Mg(phen)(hespt)_2(H_2O)_2]$ could be useful for controlling *Atta sexdens rubropilosa* without harming beneficial organisms (Patent Br102012031380-4).

EFFECTS OF COMPLEXES ON APHID-GIANT-OF-PINE, *CINARA ATLANTICA* NYMPHS (HEMIPTERA: APHIDIDAE)

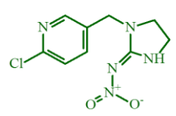
Cinara atlantica is a major pest of pines, causing up to 50% reduction in the overall productivity of wood in Brazil. All complexes were assayed on *Cinara atlantica* nymphs (Hemiptera: Aphididae). In the assay were evaluated the lethal concentrations of complexes and comparison with commercial insecticide imidacloprid.

The best results are summarized below:

$[Mg(phen)(hespt)_2(H_2O)_2]$

LC_{90} 233 µM; 24h

LC_{50} 72 µM; 24 h



Imidacloprid
(neonicotinoid)

LC_{90} 260 µM; 24h

LC_{50} 260 µM; 24h

The results suggest that the complex $[Mg(phen)(hespt)_2(H_2O)_2]$ could be also useful for controlling *Cinara atlantica* without harming beneficial organisms.

EFFECTS OF COMPLEXES ON BEDBUG TAN *Thaumastocoris peregrinus* (HEMIPTERA: THAUMASTOCORIDAE)

Thaumastocoris peregrinus is a major pest of Eucalyptus in Brazil.

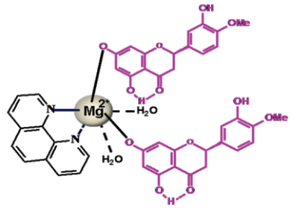
All complexes were assayed on *Thaumastocoris peregrinus* nymphs and adult. In the assay were evaluated the lethal concentrations of complexes and comparison with commercial insecticide imidacloprid.

Eggs Nymphs Adult

Nymphs

LC_{90} 496 µM; 24h

LC_{50} 74 µM; 24 h



$[Mg(phen)(hespt)_2(H_2O)_2]$

Adult

LC_{90} 323 µM; 24h

LC_{50} 63 µM; 24 h

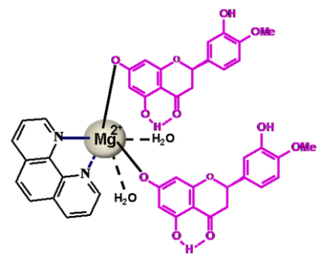
The results suggest that the complex $[Mg(phen)(hespt)_2(H_2O)_2]$ could be also useful for controlling *Thaumastocoris peregrinus* nymphis and adult without harming beneficial organisms.

EFFECTS OF COMPLEXES ON *Aedes aegypti*

Dengue and yellow fever are viral diseases that have major consequences in public health. Dengue and dengue hemorrhagic fever are considered the most important and disseminated viral diseases transmitted by mosquitoes. *Aedes aegypti* plays a crucial role in transmission of these infections. Dengue control is primarily based on the use of chemical insecticides against *A. aegypti*. However, insecticide resistance in dengue vectors has been reported from other areas for a long time. In this sense, the monitoring of *A. aegypti* insecticide resistance plays a key role in any vector control program.

In the assay were evaluated the lethal concentrations of complexes on larvae of *A. aegypti*.

The best results are summarized below:



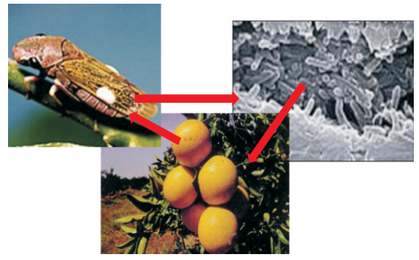
LC_{90} 17.45 µM; 24h

LC_{50} 4.47 µM; 24 h

The assays on other insects are in development.

EFFECTS OF COMPLEXES ON *Xylella fastidiosa*

Xylella fastidiosa, a Gram-negative bacterium, is transmitted by xylem-feeding leafhoppers (Homoptera, Cicadellidae) and colonizes the xylem of plants causing diseases on several economically important crops such as Citrus Variegated Chlorosis (CVC) in sweet orange. The generally accepted cause of the symptoms induced by *X. fastidiosa* is the occurrence of vascular occlusion inside the vessel leading to water stress. It was previously demonstrated that the bacterium is able to grow as a Biofilm, which may be an important factor for pathogenicity. CVC has been observed in all commercial sweet orange varieties, with transmission occurring mainly by xylem-feeding insects but also by graft propagation. Symptoms include leaf chlorosis, stunting, canopy dieback, and fruits that are small and useless for the juicing industry. To reduce losses and prevent dissemination of the pathogen, pruning, insecticide application, and healthy nursery trees have been used, but effective control has not been reported.



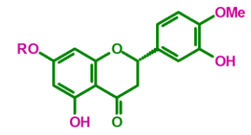
Needle-like crystallized material was often present in xylem vessels of *C. sinensis* infected by *X. fastidiosa*. A hypothesis was that the needle-like crystal is hesperidin. These crystals are not observed in healthy plants. Hesperidin is a common flavanone produced by citrus plants and also forms needle like crystals inside leaf petiole.

Hesperidin is most probably involved as a natural defense or in resistance mechanisms against *X. fastidiosa* in sweet orange varieties. However, it is not still clarity whether the ability to accumulated hesperidin and tolerance to CVC bacterium are correlated.

The HPLC-UV quantification method was applied to *C. sinensis* grafted onto *C. limonia* with and without CVC symptoms after *X. fastidiosa* infection. Hesperidin appears with a significant increase in symptomatic leaves. These data suggest that hesperidin plays a role in plant-pathogen interaction, probably as a phytoanticipin.

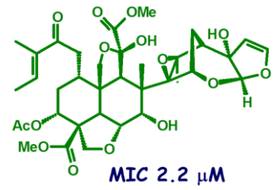
Some flavonoids and their complexes were assayed on the growth of *X. fastidiosa*. In the bioassay *in vitro* were evaluated the MIC. Preparing fresh isolated bacteria, young *Citrus sinensis* plants (6 months) are infected with *X. fastidiosa* 9a5c strain. Six month later the cells were isolated from petioles and stems of symptomatic plants and the assay was developed. All experiments were carried out with cells verified as *X. fastidiosa* by PCR with specific primers.

The best results on exponential phase are summarized below:

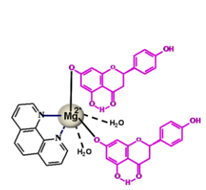


R = Rhamnosyl(α 1 → 6)glucose

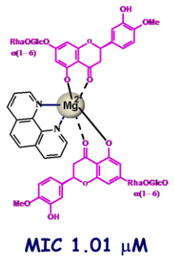
MIC 3.3 µM



MIC 2.2 µM



MIC 0.34 µM

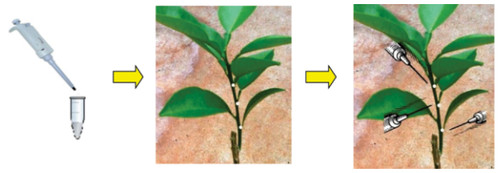


MIC 1.01 µM

MIC 0.19 µM

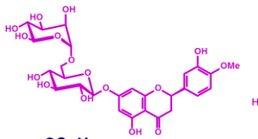
The results suggest that the complex $[Mg(phen)(ngnin)_2]$ or $[Ru(phen)_2(ngnin)]PF_6$ could be also useful for controlling *X. fastidiosa* without harming beneficial organisms.

Some flavonoids and their complexes were assayed on the growth of *X. fastidiosa in vivo*. Thirty grafts after 3 months of growth in greenhouse conditions were inoculated with cells of *X. fastidiosa* strain 9a5c, and after five months resulted in 90% of symptomatic plants.

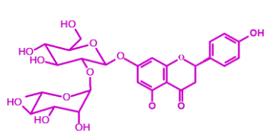


In the bioassay *in vivo* were evaluated the concentration needed to keep the bacteria alive 1%, examined by quantitative PCR.

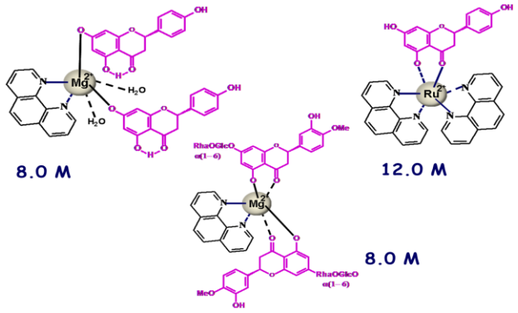
The best results are summarized below:



30 M



39 M



The results suggest that the complex [Mg(phen)(ngnin)$_2$] and [Mg(phen)(hesp)$_2$] could be useful for controlling *X. fastidiosa in vivo* without harming beneficial organisms.

Hesperidin is used worldwide as a food supplement, which does not hinder its use as pure or in complex to control the disease CVC citrus, ants and other insects of Brazilian agriculture.

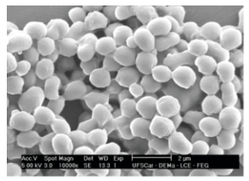
The metal complexes of hesperidin and hesperetin have intense blue luminescence, which is sensitive medium: strong in aqueous solution at pH greater than 8.0 and octanol (model phospholipid membranes) being suppressed with decreasing pH. This property photoluminescence of the compound will be explored in determining the mechanism of action considering that histological larvae, when analyzed by confocal microscopy should reveal where the compound is being accumulated inside the larvae, which should be confronted with the results experimental mechanism of action. These studies are in development for the larvae of leaf-cutter ant and *Aedes aegypti*.

The results described above for the control of agricultural pests, forest and urban areas, resulted in filing two patents in 2012, and these have the potential to be applied in the control of major pests and may be transferred to companies interested in applying them.

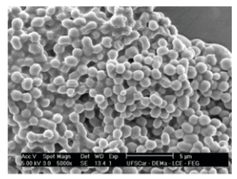
MODIFICATION OF INSECTICIDES TO IMPROVE ACTIVITY, SOLUBILITY AND STABILITY.

The Neem oil present low residence time in the field, which makes its application unfeasible. The low stability of the Neem active ingredients is due to the sensibility to sunlight and temperature. Thus, the goal of the project was to encapsulate the Neem oil enriched with azadirachtin. The extract with high contents of azadirachtin proved practical and easily incorporated into the oil. However, content of azadirachtin in the oil was affected by the temperature. The azadirachtin was degraded by UV irradiation, even being inserted in the oil. Thus, a new technique to increase efficiency of the Neem oil as insecticide was the production of Nanoparticles. Nananocapsules-NC and nanospheres-NS of Neem oil were produced using biodegradable and biocompatible polymers as PCL [poli-ε-(caprolactone)]. NP made of biodegradable polymers could be easily manufactured in a reproducible manner and represents an attractive alternative for improving the modulation of active compound realise, and stability.

All formulations presented macroscopic homogeneous aspect like a milky white bluish opalescent fluid (Tyndall effect) in agreement with the results previously reported in the literature for other nanoparticles systems. The Scanning Electron Microscope (SEM) micrographs of PCL nanocapsules and nanospheres of Neem for suspension were obtained and confirmed the homogeneous aspect of the nanoparticles.

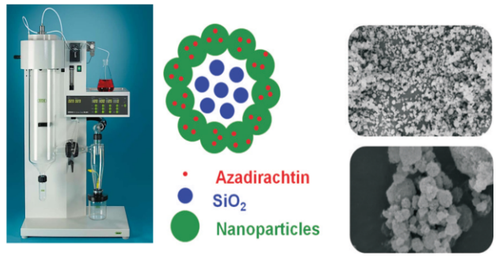


NC/PCL

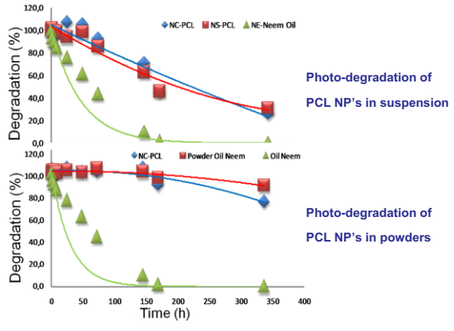


NS/PCL

After adding 3% (w/v) of colloidal silicon dioxide into the suspension of nanoparticles, the mixture was fed into a Mini-Spray-Dryer Büchi MSD 290 in order to obtain nanoparticles spray-dried powders. Morphology of PCL NC and NS of powders Neem was of homogeneous aspect.

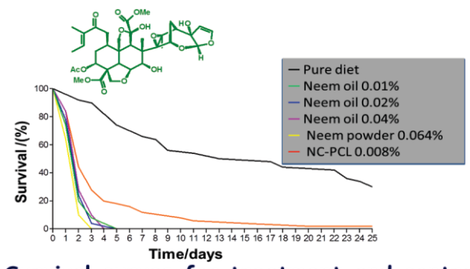


Azadirachtin degrades by UV irradiation, even being inserted in the oil. Then, studies of photo-degradation of PCL NP's in suspension and powders were evaluated. The total period of the test was 336 h, and both suspension and powder were more stable than Neem Oil.



Due to the promising results obtained with Neem oil, the Nanoformulations of Neem oil enriched with azadirachtin were assayed against *Atta sexdens rubropilosa*.

The NC-PCL presented similar activity of Neem oil, indicating that NC-PCL can be useful for controlling *Atta sexdens rubopilosa* in field.



Survival curves for treatment and control

NC-PCL is less susceptible than the oil to environmental influences as such heat, light, therefore, we can consider that this nanoformulation has better potential as an insecticide against leaf-cutting ant.

Due to the promising results obtained with Neem oil and NC-PCL, they were also assayed against other insects.

EFFECTS OF NEEM OIL AND THEIR NANOFORMULATIONS ON *Spodoptera frugiperda*

Spodoptera frugiperda is a major pest of many crops in the Americas and one of the most important pests of tropical maize, causing up to 34% reduction in the overall productivity of this crop in Brazil.

In the assay on *S. frugiperda* (after 10 days) the NC-PMMA and NS-PMMA presented better activity than Neem oil, indicating that they can be useful for controlling *S. frugiperda* in field. NC/NS-PMMA is less susceptible than the oil to environmental influences as such heat, light, therefore, we can consider that these nanoformulation have better potential as an insecticide.

Bioactivit on S. frugiperda (after 10 days)

Treatments	Mortality(%)	Weigh (mg)
NC-PLC control	2.08 ± 0.50	287.1 ± 29.2
NC-PLC	14.06 ± 1.50	50.7 ± 3.98
NC-PMMA	47.8 ± 1.31	11.3 ± 2.01
NS-PCL control	1.04 ± 0.25	345.8 ± 19.7
NS-PCL	9.38 ± 0.48	47.3 ± 6.53
NS-PMMA	45.0 ± 0.71	16.1 ± 1.46
Water (control)	3.13 ± 0.48	341,7 ± 16.7
Neem Oil	38.5 ± 1.38	18.0 ± 2.22

- PCL [poli -ε- (caprolactone)],
- PMMA (polimetilmetacrilate).

EFFECTS OF NEEM OIL AND THEIR NANOFORMULATIONS ON *Bemisia tabaci*

Bemisia tabaci (Genn) biotype B (Hemiptera: Aleyrodidae) has a wide range of host plants. The most common host species are the crops of: beans, tomato, and cotton.

In the assay on nymphs of *Bemisia tabaci* (*Genn*) biotype B (after 10 days), the Neem oil presented better activity than NC-PCL. However, NC-PCL is less susceptible than the oil to environmental influences as such heat, light, etc. Then, NC-PCL can have better potential for controlling *Bemisia tabaci* in field than Neem oil.

Bioactivit on on nymphs of *B. Tabaci* (after 10 days)

Treatments	Mortality(%)
NC-PLC control	2.00 ± 0.50
NC-PLC	40.1 ± 1.30
Water (control)	3.13 ± 0.48
Neem Oil	60.5 ± 1.40

EFFECTS OF NEEM OIL AND THEIR NANOFORMULATIONS ON *Tuta absoluta*

Tuta absoluta (Meyrick) (Lepidoptera: Gelechiidae) is a major pest of tomato tree, *Lycopersicon esculentum* Mill.

In the assay on *Tuta absoluta* (after 10 days) the NS-PMMA presented better activity than Neem oil, indicating that they can be useful for controlling *Tuta absoluta* in field. NS-PMMA is less susceptible than the oil to environmental influences as such heat, light, then, it has better potential for controlling *Tuta absoluta* in field.

Bioactivit on *Tuta absoluta* (after 10 days)

Treatments	Mortality(%)
NC-PLC control	2.00 ± 0.50
NC-PLC	21.1 ± 1.30
NC-PMMA	16.5 ± 1.10
NS-PMMA	43.5 ± 1.10
Water (control)	3.13 ± 0.48
Neem Oil	35.5 ± 1.40

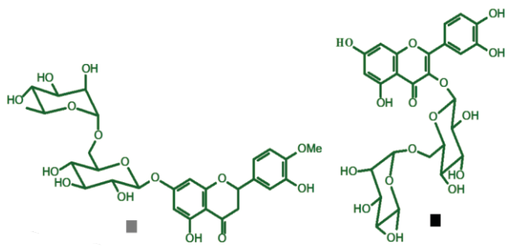
Finally, these results suggest that our INCT will provide soon nanocapsules and nanospheres of Neem oil to be used in integrated pest management system in Brazil. The nanoparticles preparation technique proved simple with reproducible results and the possibility of transferring to industrial scale. The method of nanoparticles preparation was patented in 2012, and this has the potential to be applied in the control of major pests and can be transferred to companies interested in applying it.

MICROORGANISMS ASSOCIATED TO CITRUS DISEASES AND RESISTANCE MECHANISMS

Xylella fastidiosa

Hesperidin is most probably involved as a natural defense or in resistance mechanisms against *X. fastidiosa* in sweet orange varieties. However, it is not yet clear whether the ability to accumulate hesperidin and tolerance to CVC bacterium are correlated. Thus, the purpose of this work was to develop a rapid and sensitive HPLC method for quantitative determination of hesperidin in Brazilian *C. sinensis* grafted on *C. limonia* cv. Pêra. The method was applied to test whether there was a differential accumulation of hesperidin in plants with CVC symptoms. The variations in hesperidin content were compared with the control plants, in which cells of *X. fastidiosa* were not inoculated. Preliminary HPLC studies showed variation in the second peak area, which was identified as rutin, thus it was also analyzed.

The HPLC-UV quantification method showed that the total content of rutin was low and practically constant in all analysis in comparison with hesperidin, which appears with a significant increase in symptomatic leaves. These data suggest that hesperidin plays a role in plant-pathogen interaction, probably as a phytoanticipin. Biosynthesis of this metabolite may represent a plant defense strategy in response to the pathogen attack, since this compound is reported to have antimicrobial activity on the growth of *X. fastidiosa*.

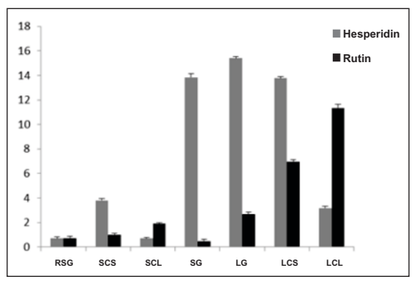


Variation in the contents of hesperidin and rutin in diferent parts of *C. sinensis* grafted onto *C. limonia* with (WS) and without (NS) CVC symptoms. RSG: negative control of rootstock stem; SG: negative control of stem of the graft; LG: negative control of leaves of the graft. Results are expressed as the averages of three experiments and three individual analyses (mean S.D.; g kg^{-1}).

The present HPLC-UV method is simple and accurate for the determination of hesperidin and rutin simultaneously in *C. sinensis*, *C. limonia*. In addition, the most efficient tool for detecting CVC disease is by polymerase chain reaction (PCR). However, PCR is expensive, and is subject to cross reaction and contamination. In this report, a diagnostic method was proposed for detecting CVC disease in asymptomatic sweet orange trees using the HPLC-UV method, which is not too costly and can screen many samples per hour using about 1 mg of leaves.

THE INFLUENCE OF ROOTSTOCK *Citrus limonia* ON SCION *C. sinensis* AFTER *Xylella fastidiosa* INFECTION

The influence of the rootstock on the content of bioactive compounds has been studied by numerous authors. In a review on grafts of the citrus, Cano and Bermejo (2011) showed that the effect of rootstock has been evaluated in relation to inorganic nutrient elements, essential oil and to other bioactive compounds as flavonoids. However, any one reference comparing plants developed from the germination of seeds and by grafting was found. The purpose of this work was also to apply the HPLC method for quantitative determination of hesperidin and rutin also in *C. sinensis* and *C. limonia* obtained from seed germination, to verify whether there was a differential accumulation of both flavonoids in grafted and seedling plants, and thus, whether rootstock induces resistance against *X. fastidiosa*. The results showed that the rootstock lead to increased hesperidin content that was 3.6 fold greater in the graft stem than that in the stem of *C. sinensis* seedlings. Increase in hesperidin content by rootstock can be related to the induced internal defense mechanisms. Graft alone can induce the production of hesperidin, but also supply with needed information to accumulate this flavonoid after inoculation with *X. fastidiosa*, and then reducing the susceptibility of sweet-orange to this bacterium.

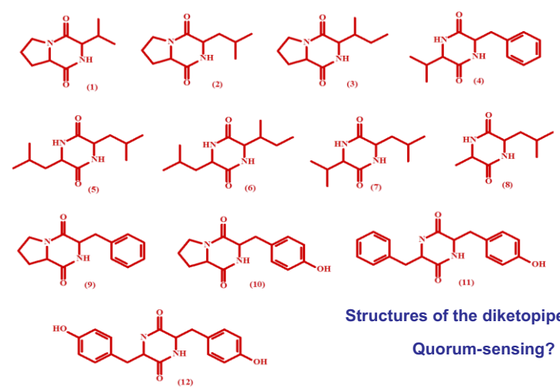


Variation in the contents of hesperidin and rutin in iferent parts of *C. sinensis*, *C. limonia* and their graft. RSG: negative control of rootstock stem; SCS: stem of *C. sinensis*; SCL: stem of *C. limonia*; SG: stem of the graft; LG: leaves of the graft, LCS: leaves of *C. sinensis*; LCL: leaves of *C. limonia*. Results are expressed as the averages of three experiments and three individual analyses (mean S.D.; g kg^{-1}).

Xylella fastidiosa CHEMICAL STUDY

The biofilm formation is considered the main mechanism of pathogenicity of the *X fastidiosa* bacterium. When cells reach the mature biofilm stage is activated intercellular communication system called "*quorum sensing*". This signaling allows the bacteria to regulate the expression of specific genes as, for example, secondary metabolite production, conjugal plasmids transfer, antibiotic resistance, biofilm maturation, virulence, swarming, and swarming motility.

However, the processes that mediate the formation and maintenance of these biofilms are still unknown. This project also describes the secondary metabolites identification of *Xylella fastidiosa* (9a5c) bacterium. Using GC-MS, LC-MS and LC-SPE-NMR allowed the identification of fatty acid derivatives, alcohol, and diketopiperazinic (DKP) alkaloids. This is the first chemical study of *X. fastidiosa* that boarded the alkaloids production. The role of DKP still remains largely unknown in microorganisms, so they can reveal major advances in the biological mechanism of bacteria and especially of *X. fastidiosa*. The technique of SPE-LC-NMR showed great promise for the identification of diketopiperazines related in this study.



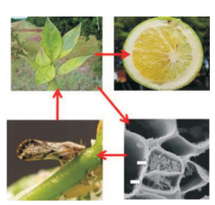
Structures of the diketopiperazines

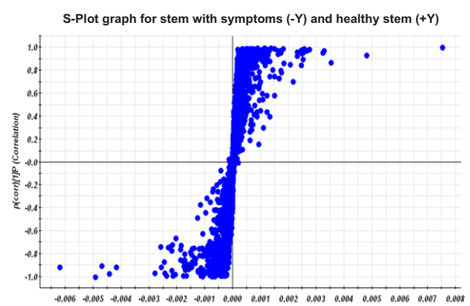
Quorum-sensing?

HUANGLONGBING (HLB) OR CITRUS GREENING

Huanglongbing (HLB) or Citrus Greening is one of the most destructive citrus diseases. The causal agent of this disease is a gram-negative plemorphic bacterium, which is limited to the phloem. The disease is attributed mostly to a new bacterium called *Candidatus Liberibacter americanus*.

C. sinensis grafted on *C. limonia* cv. Pêra with and without symptoms of HLB were examined in order to determine whether the secondary metabolites in this plant were associated with a chemical defense response. Extracts from wood, stem, roots and leaves were examined by HPLC-MS/MS using Acquity UPLC I-Class (chromatographic separation) and Synapt G2-S (MS), both from Waters. The software for chemometric analysis of this equipment (MarkerLynx) allowed us to analyze the extracts of all organs and showed which constituents varied in concentration in response to the presence of bacteria. Only one example was included using just the tool S-Plot software applied to extracts of stem with symptoms and without symptoms. In the graph ions below the horizontal line (negative values of Y) represents the most characteristic ions for the sample of stem with symptoms, while the ions above the horizontal line represent the most characteristic ions for the sample of the healthy stem. The farther from the axis, the ions are most representative of each group (in this case indicated by the rectangles in the figure). Thus, the ions present in the most extreme of this graph are the ones that characterize the stems with and without symptoms.



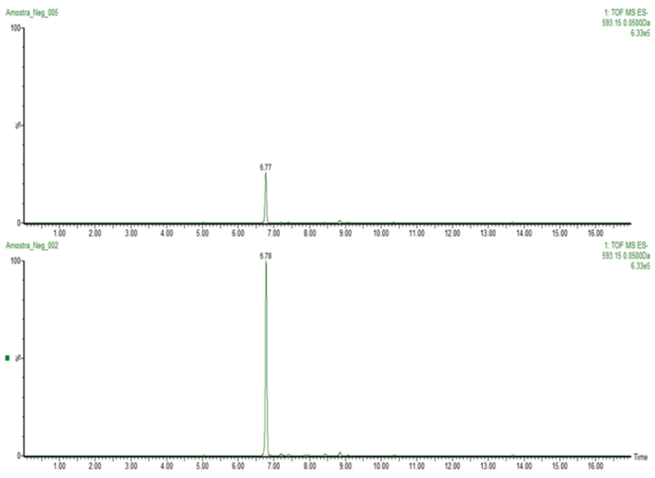


Ions present in the most extreme of S-Plot graph

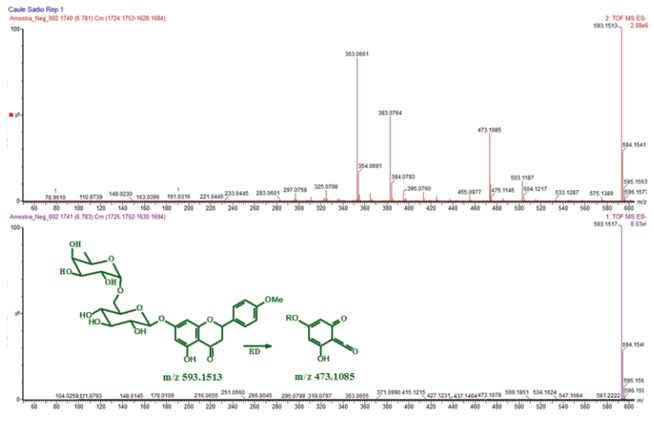
Primary ID	Retention Time	Mass	p[1]P	p(corr)[1]P	With symptoms	Without symptoms	Factor of Change	Uncertainty
13.54_763.5080	13.54	763.508	-0.151824	-0.969872	98.5053	43.3717	2.3	0.100
13.17_764.4085	13.17	764.4085	-0.210258	-0.91357	311.254	199.204	1.6	0.050
13.30_778.4247	13.3	778.4247	-0.161841	-0.903907	451.946	384.811	1.2	0.013
6.79_593.1503	6.78	593.1503	0.254497	0.999014	49.5569	200.018	4.0	0.003
1.04_315.0724	1.04	315.0724	-0.167895	-0.998406	88.9506	23.4375	3.8	0.116
0.73_265.0939	0.73	265.0939	-0.144581	-0.913394	146.849	93.7497	1.6	0.052

S-Plot showed the ion *m/z* 593.15 and RT 6.78 min are characteristic of the sample with symptoms.

Chromatograms for stem with symptoms (above) and healthy (below)



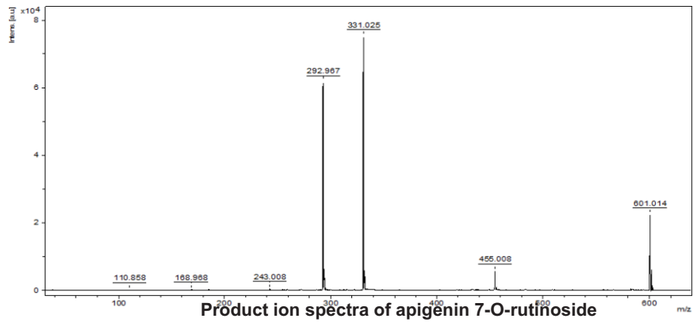
The chromatogram for each sample showed that in stem with symptoms (above) this ion is less intense, while in the sample of healthy stem (below) it is of higher abundance.



Since these experiments were obtained by MS^E, all compounds detected have a corresponding spectrum fragmentation, allowing more information about the structural compound of interest. Through the molecular ion *m/z* 593.15 and fragments obtained from these experiments were possible to identify the flavonoid dydimine. These data suggest that dydimine plays a role in plant-pathogen interaction. Further work is under way to establish whether dydimine possesses a role in plant-pathogen interaction.

Product ion spectra of dydimine

ALTERNARIA BROWN SPOT: *Alternaria alternata*



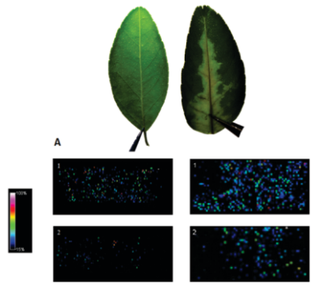
Product ion spectra of apigenin 7-O-rutinoside

The alternaria brown spot, caused by the fungus *Alternaria alternata,* has been considered one of the most serious fungal diseases in tangerine and its hybrid. Its presence was confirmed in the states of Minas Gerais, Rio Grande do Sul, Rio de Janeiro, and São Paulo.

A. alternate was inoculated in sweet orange (resistant) and “Murcott” tangor (susceptible) to evaluate the variation in the chemical profile through HPLC-DAD. The chemometric analyses of HPLC-DAD data showed that the response of citrus to fungus inoculated occur in leaves of “Murcott” Tangor. Then, to confirm this result an experiment were done in ^{1}H NMR 600 MHz, which showed the flavonoid apigenin 7-O-rutinoside with a significant increase in symptomatic leaves of “Murcott” tangor. This result stimulated an investigation of the distribution of this substance in intact leaves of “Murcott” tangor with and without symptoms, by MALDI-IMS. The results indicated an accumulation of this substance in symptomatic leaves.

This technique shows that the intensity of the ions (the *x, y* coordinates of the tissue examined) is correlated with a color scale, where red indicates high concentration of the analyte in the spot analysis, revealing thereby the amount of analyte present in a particular region. The distribution of this substance on the adaxial surface of the leaves T. 'Tangors' along the central rib of control leaves and with symptoms was obtained by extracting characteristic fragment ion *m/z* 601.014 $[M + Na]^+$. Images of two fragments characteristic of apigenin 7-O-rutinoside *m/z* 292.967 and *m/z* 331.025 are showed below.

By correlating the color scale with the signal strength, the images show a greater accumulation of apigenin-7-O-rutinoside in symptomatic leaves of 'Murcott', when compared to control leaves. This fact cannot be attributed to an uneven pressing of the sheet on board MS, nor an incomplete extraction, since the images were obtained from an uniform layer of matrix and showed a very homogeneous distribution over the surface of the tissues examined, indicating that the presence of the fungus *Alternaria alternata* induced plant, increasing the concentration of apigenin 7-O-rutinoside.



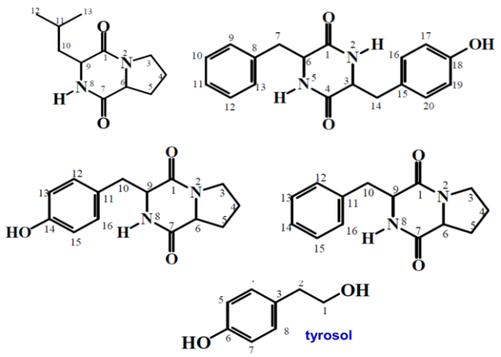
A: Image from extraction of ion m/z 292.697 B: Image from extraction of ion m/z 331.025. Where 1: adaxial face of T. ‘Murcott’ leaves with symptoms and 2: adaxial face of T.’Murcott' control leaves.

CITRUS BLACK SPOT: *Guignardia citricarpa*

The Citrus Black spot caused by the fungus *Guignardia citricarpa* cause significant losses in Valencia orange tree.

After optimization of the liquid culture medium (potato-dextrose, Czapeck and Czapeck enriched with 2% malt extract) and growth period (05 to 45 days) appropriate for *G. citricarpa,* this fungus was developed at a larger scale using potato-dextrose for 25 to 35 days. The crude extracts were submitted to fractionation by chromatography, which furnished 13 different compounds of different classes of secondary metabolites, namely four diketopiperazines [cyclo-(proline-leucine), cyclo-(phenylalaninetyrosine), cyclo-(proline-tyrosine), and cyclo-(proline-phenylalanine)], one nitrogen base (uracil), three nucleosides (uridine, 5-methyl-uridine, and inosine), one amino acid

(tryptophan), one aromatic alcohol (tyrosol), one furfuraldehyde (5-hydroxy-methyl-furfuraldehyde), one benzoic acid derivative (4-hydroxybenzoic acid), and one triglyceride. The isolation of tyrosol motivated new investigations on its possible role in pathogenicity events in Citrus, since there are some literature reports on its signaling and autoregulation activities in some endophytic fungi.

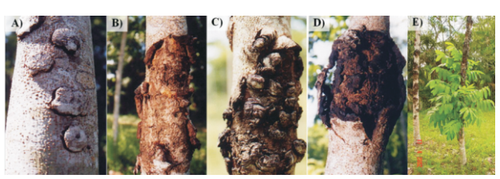


HARDWOOD TREE DISEASES

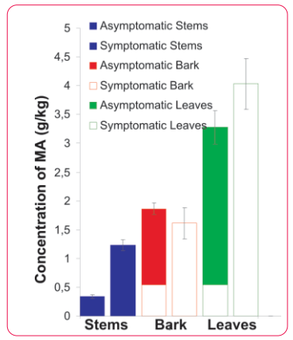
Canker in *Khaya Ivorensis* and Microorganism Associated

The Brazilian Agricultural Research Corporation - Embrapa - has been changing *Swietenia macrophylla* for trees from the *Khaya* genus (African mahogany). Such plants are not affected by the shoot borer *Hypsipyla grandella*. Recently, however, *Khaya* trees were infected by a new microbial pathogen.

K. ivorensis with and without symptoms of cankers, were examined in order to determine whether the secondary metabolites in this plant were associated with a chemical defense response. This study provides evidence that the limonoid methyl angolensate (MA) is present at higher concentrations in *K. ivorensis* with symptoms of cankers rather than in the plants without symptoms. HPLC-ESI-MS/MS method was developed for quantification of MA in all aerials parts of such plants. Methyl angolensate concentration did not change in the stem bark. Its amounts increased nearly fourfold in stems. Its amounts increased by 20% in leaves, when plants with symptoms were compared with those without symptoms. These data suggest that Methyl angolensate plays a role in plant-pathogen interactions, probably as a Phytoanticipin.



Canckers evolution on Khaya ivorensis



The fungus involved was identified as *Botryosphaeria rhodina*, based on morphology, and DNA sequences.

B. rhodina fungus obtained from the plant was inoculated in healthy plants of *K. ivorensis* in order to confirm Koch's postulate. After 7 months of experiment, the first signals of the disease appeared have been identified. However, the canckers evolution was not observed. The first signals were just a plant's response to the injury done to inoculate the fungus.

Lasiodiplodia theobromae anamorphic form of *Botryosphaeria rhodina* is recognized as the causal agent of several canckers diseases. Thus, fungus obtained from the plant was developed and obtained both form of fungus *B. rhodina* and *L. theobromae*. The last was inoculated in healthy plants and after 4 months of experiment, the first signal of the diseases appeared and in 6 months canckers evolution as above was observed. Tehfungus was again isolated form canckers and confirmed as *Lasiodiplodia theobromae* by PCR using specific primes. Therefore, Koch's postulate was confirmed and the new pictures show cancker evolution.

Canckers evolution on *Khaya ivorensis* A: Control plant where was inoculated only the culture medium; B; Plant with symptoms in which was inoculated *Losiodiplodia theobromae*, both after 6 months.

THE MICROORGANISM DATABASES ASSOCIATED TO LEAF-CUTTING ANTS

The microorganism databases contain information about lineages was developed. The survey conducted so far and the microorganism database can be accessed at http://estirpes.wii-records.com/.

Some species of ants collected were exhibited in WWW network to form a virtual library of *Attine* ants, or a virtual ants museum, which is exposed in http://omega.rc.unesp.br/formiga.

SCIENTIFIC RESULTS

The INCT-CBIP produced 157 scientific papers. Whereas in all the INCT-CBIP are 23 researchers would be an average of 6.8 papers per researcher, an average of 3 papers per year per researcher. According to CAPES, which evaluates graduate programs in Brazil, 3 papers per researcher per year are above the national average.

Papers Published

	National	International
Papers	30	127
Books	01	
Chapters of Books	06	04
Papers/Researchers = 157/23 = 6.8		

According to CAPES 3 publications per researcher per year is above the national average

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COOPERATION ACTIVITIES BETWEEN COMPANIES AND INCTS

COMPANIES

The “INCT” supports companies through quality control and technology transfer.

The following companies are supported by the patent held by the NPRG-UFSCar through the Neem pesticide analyses (see http://www.cbip.ufscar.br/):

Baraúna Industry and Commerce Ltda

Represented by Roberto A. Malimpence, (roberto@barauna.agr.br) city of Catanduva-SP.

Base Fértil Ribeirão Comercial Agrícola Ltda

Represented by CEO Carlos Elpidio Pereira, (financeiro@basefertilagricola.com.br) city of Cravinhos - SP.

DVA Technology – Serviços de consultoria em Tecnologia e Registros

Represented by Rogério de Castro, city Campinas, SP.

The acquisition of the unit LC-NMR has increased our interaction with the chemical-pharmaceutical companies in the region, which takes place on three different levels:

1. Request for NMR analysis as a service simply;
2. Characterization of compounds which involves the preparation of a report, and
3. Development of methodology of analysis processes and/or products where it is necessary to validate the methodology.

The companies are:

ABL ANTIBIÓTICOS DO BRASIL LTDA,
BIOAGRI LABORATÓRIOS LTDA
BIOINOVATION PRODUTOS BIOMÉDICOS S/A
BUNKER INDÚSTRIA FARMACÊUTICA LTDA
INSTITUTO TERAPÊUTICO DELTA
INSTITUTO VITA NOVA
EMS S/A
VALEANT FARMACÊUTICA DO BRASIL LTDA
VALLÉE S/A
MULTILAB IND. E COM. PROD. FARM. LTDA

INCT'S

National Institute of the Science and Technology of Genomics for Citrus Improvement - Marcos A. Machado:

We have been developing new methodologies for the control of citrus diseases using natural compounds that are more selective and less harmful to the environment.

National Institute of the Science and Technology of Semiochemicals in Agriculture - José Roberto Postali Parra:

The research group coordinated by Dr. José Djair Vendramim, ESALQ -Department of Entomology, has been establishing a close relationship with Dr. Parra by sharing equipment and holding discussions about methods for rearing certain insects.

National Institute of S&T of Structural Biotechnology and Medicinal Chemistry in Infectious Diseases - Glaucius Oliva:

The NPRG-UFSCar has maintained a close interaction with Dr. Glaucius Oliva’s group. In general, almost all substances isolated from plants, fungi, or bacteria by the NPG-UFSCar have been assayed target enzymes for a number of tropical diseases.

Hymenoptra-Southeast National Institute of S&T - Angélica Maria Penteado-Dias:

The NPRG-UFSCar has maintained a close interaction with Dr. Dias’ group. They have been exchanging information about the sustainable use of insect biodiversity, classification, and behavior as well as equipment use.

Group of coordinators focused on governance and cooperation between INCTs (nominated as I5+) : We have been discussing on governance in the national institutes, strengthening our scientific and technological collaboration, divulging scientific production, and focusing on teachers’ education.

I5+ INCT GROUP

“INCT” for Functional Complex Materials
(Dr. Fernando Galembeck, UNICAMP),
“INCT” of Drugs and Medicaments
(Dr. Eliezer Jesus Barreiro, UFRJ),
“INCT” for Continent-Ocean Materials Transfer
(Dr. Luiz Drude de Lacerda, UFC),
“INCT” for the Biorational Control of Insect-Pest
(Dr. M. Fátima G. F. da Silva, UFSCar), and
“INCT” of Energy and Environment
(Dr. Jailson Bittencourt de Andrade, UFBA).

TRANSFER OF KNOWLEDGE TO HIGH SCHOOL

The group from UFPR has visited public and private secondary schools taking the results of their research to the attention of students and teachers. The results of these visits can be viewed on the website developed by them:
http://www.flickr.com/photos/ufpr/sets/72157626402501187/detail/

The UFSCar team is working with the Institutional Scholarship Program Initiation to Teaching (PIBID), CAPES program which aims to promote the participation of students in the Bachelor of Chemical UFSCar in collaborative actions with teachers of Chemistry and Science public schools of São Carlos.
These activities have been carried out at UFSCar under the supervision of Dr. Clélia M.P. Marques and Dr. Vânia G Zuin.

Participation of INCT-CBIP has been:
- Evaluation of the potential and limitations of using WebQuest for teaching Organic Chemistry under the biorational control of pests-insect.
http://www.ufscar.br/gpqv/webquest

- Trade Knowledge
- It was created a Blog for interaction between students and staff PIBID: Blog: http://quipibidufscar.wordpress.com/; Blog PIBID Chemistry with contributions every 5 weeks.

- The results of these activities will form a chapter in the book that PIBID-UFSCar will launch with the theme: The challenge of university-school partnership in initiating teaching. Title: PIBIDIANOS and Basic Education Teachers: Influence of Collaborative Work in Initial and Continuing.

- The Project PIBID Chemistry - UFSCar have 12 fellows. They developed their initiation to Teaching in Public Schools in São Carlos: E. E. Prof. Adail Malmegrim Gonçalves, E. E. Dona Aracy Leite Pereira Lopes, E. E. Conde do Pinhal, E.M.E.B. Delila Galli and E. E. Prof. Orlando Perez.

- The success of these activities was recently recognized by UFSCar in honor of Prof. Vânia G. Zuin with the Award of Merit honor - honor the academic contributions, UFSCar. She was also invited to join the subcommittee Green Chemistry IUPAC, with emphasis on training and dissemination practices less impactful in the field of chemistry, as biorational control pests-insect. She had a publication about this activity at the Magazine of the IUPAC:
Using Green Chemistry in Teaching - A Brazilian Case Study
by Renan Bertolin, Milena Avancini, Andréia Matos, and Vânia Gomes Zuin*
CHEMISTRY International May-June 2013 Volume 35 No. 3
The News Magazine of the International Union of Pure and Applied Chemistry

- Finally, these initiatives have as the main objective to stimulate students to continue their studies showing how education can transform people, communities, society and the entire nation, and in addition to disseminate the results of research in development for INCT.

Science Fair at State School Adail Gonçalves, May 2010

Science Fair at State School Conde do Pinhal

These activities have been carried out at UFSCar under the supervision of Dr. Clélia M.P. Marques and Vânia G Zuin.

COMMITTEE MEETING

Committee meeting

First Committee meeting - 09/02/2009

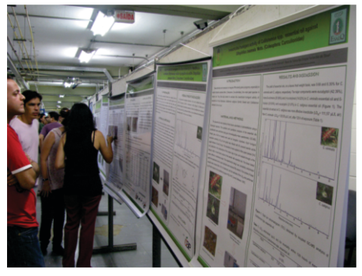
The meeting was held at the Institutional Support for the Scientific and Technology Development Foundation (FAI), UFSCar, in the meeting room facility with a video conference service.

Second Committee meeting - 30-31/11/2009

INCT-BCIP (National Institute of Science and Technology-Biorational Control of Insect-Pest) researchers attended this workshop, which was held at the PPGQ-UFSCar conference room.

The students from São Paulo state attended the meeting. The results of the studies that were carried out by students from other states were presented by their respective advisors, orally or in a poster format.

Third Committee Meeting 14-15/03/2011



Fourth Committee Meeting 5-6/03/2012

The Fifth Committee Meeting will take place 14-15/07/2013