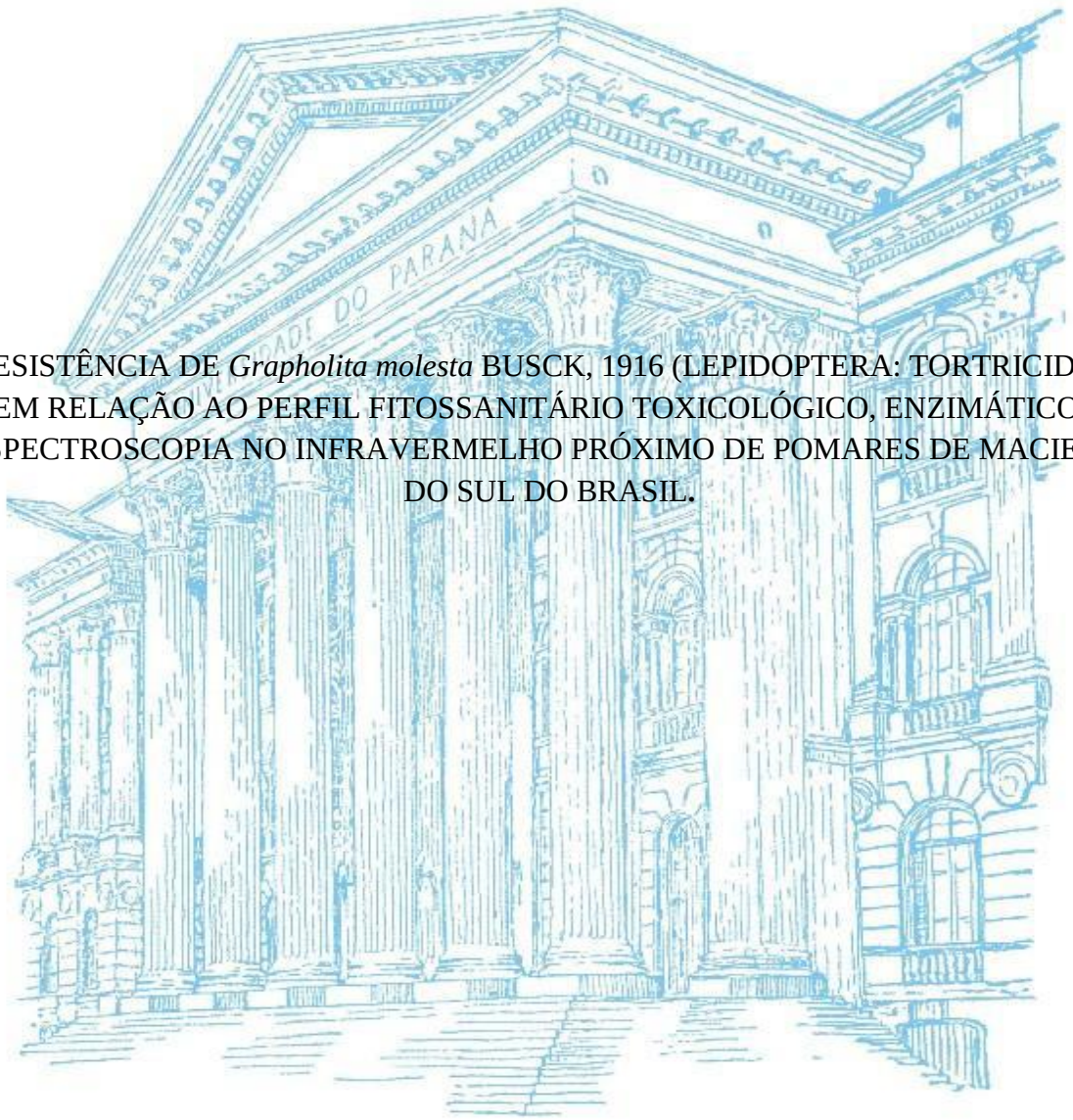
UNIVERSIDADE FEDERAL DO PARANÁ

ROSANGELA TEIXEIRA



RESISTÊNCIA DE *Grapholita molesta* BUSCK, 1916 (LEPIDOPTERA: TORTRICIDAE) EM RELAÇÃO AO PERFIL FITOSSANITÁRIO TOXICOLÓGICO, ENZIMÁTICO E ESPECTROSCOPIA NO INFRAVERMELHO PRÓXIMO DE POMARES DE MACIEIRA DO SUL DO BRASIL.

CURITIBA, 2013

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Tese apresentada como requisito parcial à obtenção do título de Doutora em Ciências Biológicas, pelo Programa de Pós-Graduação em Ciências Biológicas, Área de Concentração em Entomologia, da Universidade Federal do Paraná.

Orientador: Prof. Dr. Lino Bittencourt Monteiro

Co-Orientadora: Dr[a]. Helena Cristina da Silva-Assis

CURITIBA
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Tese aprovada como requisito parcial para obtenção do grau de "Doutor em Ciências", no Programa de Pós-graduação em Ciências Biológicas, Área de Concentração em Entomologia, da Universidade Federal do Paraná, pela Comissão formada pelos professores:

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Prof. Dr. Mario Antonio Navarro da Silva
(UFPR)

Curitiba, 24 de fevereiro de 2014

DEDICO

À Deus

Aos meus pais Jorge e Ivanaura

À meu marido Valdenir

À minha filha Ana Julia

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Agradeço a Deus pela vida e por estar ao meu lado sempre iluminando meu caminho e minhas conquistas.

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RESUMO

O objetivo geral deste trabalho foi avaliar a susceptibilidade de populações de *Grapholita molesta* a inseticidas, em pomares de macieira do Sul do Brasil com diferentes manejos fitossanitários, assim como qualificar estas populações com uso da espectroscopia no infravermelho próximo. Os insetos foram coletados em quatro pomares da região Sul do Brasil sendo elas: Porto Amazonas (PR) São Joaquim e Fraiburgo (SC) e Vacaria (RS), além disso, foram utilizadas duas populações como referência; uma susceptível mantida em laboratório há 18 anos e, uma com alta frequência de resistência, obtida pelo uso de inseticidas por 18 gerações. Foi realizado um levantamento das estratégias fitossanitárias adotado nos pomares de coleta, que foi utilizado para traçar o perfil fitossanitário de cada pomar. Foram realizadas avaliações toxicológicas (Cl_{50} e Cl_{90}) e enzimáticas (Acetilcolinesterase (AChE), Glutationa S-transferase (GST), α e β Esterases (α EST, β EST) de todas as populações coletadas, comparadas com as populações de referência, para avaliar a resistência a quatro grupos químicos utilizados na região, sendo eles: Organofosforados (Clorpirifós), Carbamatos (Carbaril), Deltametrina (Decis) e Diacilhidrazina (Tebufenozide). As influências do manejo fitossanitário sobre uma população de campo, comparada com uma de laboratório, foram avaliadas quanto a bioensaios toxicológicos e enzimáticos. Neste estudo, uso do espectrofotômetro no infravermelho próximo (NIRS) foi utilizado para caracterizar as populações de campo e de laboratório. Como resultados foram definidos quatro perfis fitossanitários, o qual o manejo mais inadequado (MI) foi o que teve maior relação com a resistência das populações. O manejo influenciou a população de campo quanto à resistência. Todas as populações avaliadas apresentaram alta frequência de resistência a clorpirifós e Carbaril (Cl_{50}) quando comparado com a população de referência. As populações resistentes apresentaram alta atividade de GST e α Esterases, além de não inibir a atividade de AChE. O NIRS separou as populações em três grupos, um deles sendo a susceptível (PS) outro com a RS e RT, e o terceiro agrupando SC e PR. Na análise de susceptibilidade a população coletada a campo foi selecionada pelas técnicas de manejo utilizadas nos pomares de coleta, sendo menos susceptível quando comparada a população de laboratório. Conclui-se que as populações avaliadas mostraram alta frequência de resistência a inseticidas organofosforados e carbamatos, este aumento está condicionado ao sistema de manejo adotado em cada pomar, além disso, as populações foram separadas pelo infravermelho, mostrando uma tendência de uso desta técnica para separar populações susceptíveis e com grau de resistência.

Palavras-Chave: Monitoramento de resistência; Manejo de pragas; NIRS; Susceptibilidade.

ABSTRACT

The aim of this study was to estimate the resistance of *Grapholita molesta* populations to insecticides and characterize the plant management of apple orchards in southern Brazil in plant profiles, as well as evaluate the use of near infrared spectroscopy to characterize these populations. The insects were collected in four orchards in southern Brazil which are: Porto Amazonas (PR) São Joaquim, Fraiburgo (SC) and Vacaria (RS). In addition, two populations were used as reference, one of them maintained in the laboratory for 18 years and the other reared with tough dosed of insecticides for 18 generations. A survey data collected in orchards, which was used to trace the plant profile of each orchard, was developed. Toxicological (LC_{50} and LC_{90}) and enzyme (acetylcholinesterase, glutathione S-transferase, α and β Esterases) of all populations collected, compared with the reference populations were performed to assess the strength of four chemical groups used in the region, with them. They are: Organophosphate (Chlorpyrifos) Carbamates (Carbaryl), Deltamethrin (Decis) and Diacilhidrazida (tebufenozide). The influences pest management over a field population, compared to a laboratory one, was evaluated for toxicity and enzymatic bioassays. In this study, use of near infrared spectrophotometer (NIRS) was performed to characterize the populations of field and laboratory. As result, four plant profiles, which the more inadequate management (IM) had the highest relationship with the resistance of populations, were defined. The management influenced the field population for resistance. All populations tested were resistant to chlorpyrifos and Carbaryl (LC_{50}) compared with the reference population. The resistant populations showed high activity in GST and α Esterases, and did not inhibit the activity of AChE. The NIRS characterize the populations susceptible and resistant to PLS analysis, separating into three groups: one of them was susceptible (PS), the other with RS and RT, and the third grouped SC and PR. In the analysis of the susceptibility field population proved to have been selected by the management techniques used in the orchard, being less likely when compared to laboratory population. In conclusion populations evaluated showed a high level of resistance to organophosphate and carbamate insecticides, this increase is subject to the management system adopted in each orchard, furthermore, the populations were separated by infrared, showing a trend in using this technique to separate populations and likely degree of resistance.

Keyword: Monitoring of resistance; Pest management; NIRS; Susceptibility.

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SUMÁRIO

INTRODUÇÃO GERAL

A produção de maçãs no Brasil teve um aumento de mais de 6.000% nas últimas três décadas. De importador, o país passou a abastecer todo o mercado interno e exportar 15% de sua colheita. A cultura de macieira, assim como outras fruteiras de clima temperado, enfrenta vários problemas de ordem fitossanitária, principalmente relacionado a insetos-pragas e doenças. Dentre os insetos nocivos a macieira, mariposa oriental, *Grapholita molesta* (Lepidoptera: Tortricidae), e as moscas-das-frutas, espécies pertencentes ao gênero *Anastrepha*, são as que causam as maiores perdas de frutos.

G. molesta apresenta um ciclo de vida completo com desenvolvimento de ovo, larva, pupa e adulto, Os ovos são diminutos e são postos isoladamente na fase interior da folha, nas brotações e nos ramos novos e nos frutos, as lagartas recém-eclodidas são branco-acinzentadas com cabeça preta, quando bem desenvolvidas apresentam coloração branco-rosada apresentam cinco instar de desenvolvimento, as pupas são frágeis e ficam abrigadas em casulos de seda tecido pelas larvas em fendas da casca dos troncos, nas axilas dos ramos ou no solo. As primeiras mariposas surgem na primavera, oriundas de larvas que passaram o inverno em diapausa. São insetos crepusculares, com atividade de migração, alimentação, acasalamento e postura concentrada entre 17 e 22 horas. Seu ciclo de vida completo varia de 23 a 58 dias, podendo ocorrer de cinco a oito gerações anuais.

É uma das principais pragas da cultura do pessegueiro e, devido às proximidades com outras rosáceas, adaptou-se a macieira, tornando praga importante no Brasil a partir dos anos 80. Esta praga na forma de larva podem atacar ramos e frutos, nos ramos alimentam-se dos primórdios foliares e depois penetra na medula, formando galerias, o ponteiro atacado, seca e fica enegrecido, é comum às larvas abandonarem o ponteiro atacado e se instalarem em outro em busca de alimento, assim elas podem atacar de três a sete ponteiros da mesma planta. Nos frutos as larvas de primeiro instar penetram preferencialmente pela região do pedúnculo ou do cálice e vão se alimentar da polpa próximo à região carpelar, no ponto de penetração das larvas é possível observar uma deposição de excremento, facilitando a identificação do dano.

O controle desta praga é realizado com uso de inseticidas em cobertura, principalmente de amplo espectro de ação, as aplicações se iniciam após o período de floração, para ter ação sobre os primeiros adultos emergidos, evitando uma alta densidade populacional, são realizadas aproximadamente nove aplicações anuais de inseticidas. Porém, apesar do número de pulverizações, ainda são observados um aumento na flutuação antes de

10 dias após pulverização, podendo estar relacionado à seleção de indivíduos resistentes.

A resistência de *G. molesta* já foi observada em vários países. A tolerância a inseticidas pode ser diagnosticada por bioensaios clássicos de concentração-mortalidade, determinada para uma população susceptível, entretanto a expressão do status de resistência é limitada. Assim, a combinação dos bioensaios clássicos com a análise bioquímica, permite identificar as enzimas específicas associadas com níveis de resistência em uma praga de interesse. Neste projeto as análises enzimáticas foram realizadas em: acetilcolinesterase (AchE); esterases (α e β) e glutathiona S-transferase (GST). AchE está relacionada ao mecanismo de ação de fosforados e carbamatos a partir da inibição da acetilcolina, enquanto que α e β esterase e GST estão envolvidos no metabolismo secundário dos insetos. Neste processo, a insensibilidade ao sitio alvo e metabolismo elevado, são os principais mecanismos de resistência.

A partir da definição de populações apresentando resistência metabólica, foi testado a Espectroscopia no Infravermelho Próximo (NIRS) como uma técnica para qualificar populações resistentes em função do fenótipo de cada população. Esta técnica tem sido utilizada para caracterização de populações, separação de espécies, filogenia em díptero, porém, para caracterização de populações resistentes ainda não existe trabalhos.

Visando obter informações para um melhor entendimento da resistência de *Grapholita molesta* a inseticidas, este trabalho teve como objetivo avaliar a relação do manejo adotado no pomar e a tolerância a tratamentos químicos, utilizados em pomares de macieira no Sul do Brasil. Esta pesquisa está estruturada em três capítulos:

Capítulo I. Relação da resistência de *G. molesta* aos inseticidas com o perfil fitossanitário de macieira no Sul do Brasil. As análises toxicológica, bioquímica das populações coletadas foram relacionadas com ações de manejo fitossanitário realizado nos últimos cinco anos, em quatro pomares de macieira.

Capítulo II. Avaliou-se a eficiência da analise de espectroscopia no infravermelho próximo (NIRS), para a separação de populações resistentes de populações susceptíveis, comparada com analises bioquímica.

Capitulo III. Verificação da suscetibilidade de populações coletadas em pomares de três estados do Sul do Brasil comparada com uma população suscetível de laboratório com análises toxicológicas, bioquímicas e físico-químicas.

CAPÍTULO I

RESISTÊNCIA DE *Grapholita molesta* (Busk, 1916) (LEPIDOPTERA: TORTRICIDAE) A INSETICIDAS EM FUNÇÃO DO PERFIL FITOSSANITÁRIO DE POMARES DE MACIEIRA NO SUL DO BRASIL

Artigo encaminhado à Revista Crop Protection

Resistance of *Grapholita molesta* (Busk, 1916) (Lepidoptera: Tortricidae) to insecticide according to phytosanitary profile of apple orchards in the Southern Brazil

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Abstract

The oriental fruit moth is the most important pest of peach worldwide. In Brazil it is adapted to apple orchards, becoming a major pest. Insecticide application has been the only tool used to control for many years. Excessive use of these compounds promotes selection pressure on populations, with the possibility of acquiring resistance. The objective of this work to study the relationship between resistance of *G. molesta* and orchards phytosanitary profile in Southern Brazil through toxicity (four insecticide chemical groups) and enzyme bioassays (Glutatione S-Transferase (GST), esterases (α and β esterases) and acetylcholinesterase (AchE)). A survey was conducted on the phytosanitary strategies in orchards, with capture of pest individuals in the regions of highest apple production in each state in southern Brazil: Vacaria (RS), Fraiburgo, São Joaquim (SC) and Porto Amazonas (PR). The reference population (ER) had been kept in laboratory for over 15 years. In addition, we analyzed populations of *G. molesta* reared in laboratory for toxicological bioassays with different insecticide groups. Mortality was evaluated for seven days. The examination GST enzyme, esterase and AChE were performed with larvae of third and fourth instars. Results indicate that the populations collected in Vacaria, Fraiburgo and São Joaquim showed tolerance to carbaryl and chlorpyrifos, while those from Porto Amazonas showed to be susceptible. To deltamethrin the populations from Fraiburgo and Vacaria were more tolerant. In evaluating the enzymatic RS population a significant increase in the activity of the enzymes AChE, GST and α esterase was observed. Analyzing the field data it was realized that the phytosanitary strategies used in the orchards influence the tolerance of the population in relation to insecticides applied.

Indexing Terms. Resistance management; Oriental fruit moth; Susceptibility

Resumo

A *Grapholita molesta* é a praga principal da cultura do pessegueiro em todo o mundo e no Brasil se adaptou em macieira, tornando-se uma das principais pragas. Por muitos anos o uso de inseticida foi à única ferramenta utilizada para seu controle. O uso excessivo dos mesmos compostos promove uma pressão de seleção às populações, com possibilidade de adquirir resistência. Objetivou-se com este trabalho realizar o monitoramento de resistência de populações de *G. molesta* coletadas em pomares de macieira na região Sul do Brasil, por meio bioensaios toxicológicos (inseticidas de quatro grupos químicos) e enzimáticos (Glutationa S-Transferase (GST), esterases (α e β esterases) e Acetilcolinesterases (AchE)). Um levantamento de estratégias fitossanitárias foi realizado nos pomares de coleta da praga, nas regiões de maior produção de maçã em cada estado do Sul do Brasil: Vacaria (RS), Fraiburgo e São Joaquim (SC) e Porto Amazonas (PR). A população de referência foi multiplicada em laboratório por mais de 15 anos. Os bioensaios toxicológicos foram realizados com neonatas e a mortalidade foi avaliada aos sete dias. O exame enzimático com GST, esterases e AchE foi realizado com larvas de terceiro e quarto instares. O resultado indicou que as populações coletadas em Vacaria, Fraiburgo e São Joaquim apresentaram tolerância a clorpirifós e carbaril enquanto que Porto Amazonas se mostrou mais sensível que as demais. Para deltametrina as populações coletadas em Fraiburgo e Vacaria se mostraram mais tolerantes nos bioensaios toxicológicos, para tebufenozide não houve diferença estatística entre as populações. Na avaliação enzimática a população RS apresentou um aumento significativo na atividade das enzimas AchE, GST e α esterase. Analisando os dados de coleta de campo, se percebeu que as estratégias fitossanitárias utilizadas no pomar influenciam na tolerância da população em relação aos inseticidas aplicados.

Termos de indexação. Manejo de resistência; Mariposa oriental; Susceptibilidade

Introduction

The oriental fruit moth, *Grapholita molesta* (Busk, 1916) (Lepidoptera: Tortricidae), is the most important peach pest worldwide (Shearer & Usmani, 2001; Llanos & Marin, 2004; Timm et al., 2008). In Brazil, the first peach occurrence of *G. molesta* was recorded in 1929 (Gonzales, 1986). *G. molesta* adaptation occurred due to the small distance to other Rosaceae orchards and it was found on apple orchards by the early 80s (Lorenzato, 1988; Hickel, 1993). The same occurred in the eastern U.S. in the 90s (Felland & Hull, 1998; Bergh &Engelman, 2001).

Initially, larvae attacks earlier producing crop plants, and after harvest the adults migrate to the later ones, e.i. apple cultivars. The larvae damages are similar in both fruit species, it forms galleries in branches and fruits (Reis et al., 1988; Natale et al., 2003).

G. molesta management is made with botanical insecticides (Stearns 1920), organophosphates (Rothschild & Vickers, 1991, Kovanci & Walgenbach, 2005), carbamates

(Rothschild & Vickers, 1991), pyrethroids (Kanga et al. 2003) and neonicotinoids (Elbert et al. 2008). In Southern Brazil, *G. molesta* and *Bonagota salubricola* (Meyrick, 1937) (Lepidoptera: Tortricidae) control requires up to 10 treatments per year with broad-spectrum insecticides (Monteiro et al., 2009). Besides, other two to three sprayings are carried out with phosphorous insecticides for fruit fly, (*Anastrepha fraterculus*, Wiedmann, 1830, Diptera: Tephritidae,) control. Despite the high use of pesticides, there are reports of control failures (Monteiro et al., 2008) and an increase in the prevalence of oriental fruit moth in the last 10 years, which may be related to spread of resistant individuals (Jones et al., 2010) in orchards, as reported in North America (Kanga et al., 1997; 2003) and South America (Sigwart et al., 2011). However, few studies have been conducted in Southern Brazil on *G. molesta* resistance to insecticides in apple.

The objective of this study was to compare the resistance status of four populations of *G. molesta* collected in apple orchards in different Southern Brazil counties through toxicological bioassays with four insecticides (chlorpyrifos, deltamethrin, tebufenozide and carbaryl) and biochemical analysis to evaluate the enzymatic activity of glutathione s-transferase, esterases (α and β) and acetylcholinesterase.

Materials and Methods

Insects

Individual *Grapholita molesta* specimens were sampled in apple orchards, selected from the economic importance and the historical insect fluctuation. The orchards were located in Porto Amazonas (Paraná State - PR) (25º32’8”S, 49º53’33’’ W, 854 m) - population named PR; Vacaria (Rio Grande do Sul State - RS) (28º 30' 44" S, 50º 56' 02" W, 971 m) - population named RS; São Joaquim (Santa Catarina State - SC) (28º15’S e 49º54’ W, 1.360 m) - population named SC1; and Fraiburgo (SC) (27° 01' 34" S, 50° 55' 17" W, 1048 m) - population named SC2. The larvae were collected in 2010 and maintained on artificial diet for multiplication (Guennelon et al., 1981) and were re-infested in 2011. Larvae used for toxicological bioassays were between the fourth and fifth laboratory generation, depending on replica. The insects were homogenized for biochemical analysis immediately after field collection and maintained in a freezer (-80ºC). The reference susceptible population (ER) has been maintained on artificial diet for 18 years without new genetic material introduction.

Orchards phytosanitary profile

The studied orchards were analyzed regarding their management performances during five years (2006 to 2011) and served to characterize the orchards` pesticide profiles. These

management practice performances were carried out according to a multicriteria scoring technique proposed by Rodrigues et al. (2003), based on a system of indicators and applied change coefficients. In this approach, positive values express factors or measures of good agricultural practices, while negative values indicate factors that promote selection of resistant insect individuals. The profile was set from six management indicators, which are described in Table 1:

I. Pest Monitoring. The use of insecticides based on monitoring allows the rationalization of sprays with less environmental impact (Salles, 1991), unlike a prescribed spray model (pre-defined timetable). Simultaneous *G. molesta*, *B. salubricola* and *Anastrepha* sp. monitoring was considered a very positive factor (+3). When monitoring was performed for a single species it was scored as moderately positive (+1). It is considered completely negative (-3) when monitoring was not carried out and sprays followed a calendar;

II. Mating disruption to control *G. molesta*. This strategy provides significant reduction in insecticide applications against Oriental fruit moth (Kovanci et al., 2005). Therefore, orchards that used formulations for periods of 90 to 120 days of the year, for two years or more, were considered positive, respectively +1 and +3. Orchards that did not use sexual confusion were scored as negative (-3) ;

III. Biological control of *Panonychus ulmi* (Koch, 1836) (Acari: Tetranychidae). Apple producers in southern Brazil multiply *Neoseiulus californicus* (McGregor, 1954) (Acari: Phytoseiidae). The bio agent is used as a biomarker to select insecticides with less impact (Monteiro, 1994). The use of biological control favors the relationship between pests and natural enemies. So, the ones that used only phytoseiid *P. ulmi* as control were positive (+3) and when they needed acaricides in 50% of the orchard area, score was +1.

IV. Spraying insecticides frequency. The frequency is related to the number of insecticides used to control Tortricidae and *A. fraterculus*. Apple orchards pest unbalance has relations with phytosanitary strategie choices (Kanga et al., 2003). The larger the number of insecticide sprays, the greater is the negative impact, ranging from +3 to -3. Positive values represent environment balanced orchards;

V. Rotation of insecticide chemical groups. Spraying with the same insecticide chemical groups is directly related to resistant individuals' selection (Kanga et

al., 1997). Thus, phosphate exclusive use, or insecticides with the same mechanism action, to control Tortricidae and fruit flies was deemed negative (-1 and -3). On the other hand, when chemical control was accomplished with three and four different groups it was considered positive, +1 and +3, respectively;

VI. Capturing insects after spraying. The number of *G. molesta* captured was checked on the 10th day after insecticides spraying. The insecticide residual effect and efficiency was registered for each population. Orchards that had captures over 20 *G. molesta* individuals (control level) received a negative value (-3) and -1 when range 11 to 19 adults. Captures ranging from 0 to 10 were scored positive (+1 to +3). The values of captured individuals express the average of each orchard per year.

The sum of change coefficients, considering the positive and negative values of the six indicators, calculated cumulatively for five years of study, defined the profiles of orchards in a scale with maximum +90 and minimum -90 (i.e., coefficient ±3*6 indicators *5 years). This level of performance was sectioned into five intervals of equal amplitude that characterized the quality of these pest management profiles, as follows: i. Integrated Pest Management (IPM), whose sum of indicators resulted in values 55 to 90; ii. Good Agricultural Practices (GAP), in the range of 19 to 54; iii. Conventional Management (CM), in the range of performance between -18 to 18; iv. Unbalanced Management (UM), in the range of -19 to -54; and v. Inadequate Management (IM), in the range of -55 to -90 (Table 2). In addition to plant health, the orchards are influenced by environmental conditions, according to the variation of latitude (25 to 28) and altitude (854-1360 m). The possible influences of these environmental factors were not considered in the plant profile.

Bioassays

Bioassays that assess the toxicity of insecticides were performed in ELISA microplate (96 wells) wells filled with 150µl of an artificial diet (Soybean-Wheat Germ Insect Diet, Stonefly Industries, TX, EUA). The volume used was 6µL of each concentration of insecticide solution. Chlorpyrifos (Lorsban® 480 BR, Dow AgroSciences), deltamethrin (Decis®, Bayer CropScience), carbaryl (Sevin® SL, Bayer CropScience) and tebufenozide (Mimic® 240 SC, Dow AgroSciences), were tested in seven concentrations, defined from the

pilot tests. After 20 minutes of drying at 22 ± 2 ° °C, one neonate was placed in each well per concentration (n = 24) and replica (n= 168) and held in three replicas (n= 504), each one with its respective control. Neonates were handled with a fine-tip brush. The microplate wells were sealed with parafilm to prevent the caterpillar leakage and dehydration of the diet. The mortality was observed after seven days, and the dead ones were those who did not respond to the touch of the brush. The corrected mortality was calculated by the highest dosage by Abbott (Abbott, 1925), and a statistical analysis was performed by the probit test program POLO PLUS. The ratio resistance (R/R) was calculated for each insecticide. R/R carried out through the statistical program, calculation of CL_{50}/CL_{95} collected between population and reference, furthermore, was carried a simple correlation analysis was performed between the corrected mortality and LC_{50}.

Enzymatic activities

The acetylcholinesterase (AChE), glutathione S-transferase (GST) and esterases (α e β Est) analyses were carried out with *pools* (n = 10), with five larvae, from the 3th to 5th instar (n = 50). The pools were homogenized in the ratio of 1:10 (weight: volume) in potassium phosphate buffer 0.1 M (pH 7.0) with a microhomogenizer and centrifuged for 20 min (10.000 x g at 4°C). The supernatant was used for the determination of enzymatic activities.

The activities in the glutathione S-transferase were measured based on the Keen *et al.* (1976) method at 340 nm. Overall, 20 µL of the extract in the microplate and 180 µL of a solution containing (0.6 mM GSH a, 0.5 mM CDNB a) were added. The activities of the acetylcholinesterase were measured at 405 nm by the Ellman *et al.* (1961) method that was modified to the microplate by Silva de Assis (1998). 50 µL of the extract and 200 µL of DTNB were added (5. 5 – ditio-bis-2-nitrobenzoate 0.75 mM) in the microplate and 50 µL substrate acetiltiocoline at 10 mM. The esterase activity was measured in *end point* at 570 nM according to the method proposed by Brazil (2006). For α esterase, it was added 10 uL of 1:30 diluted sample in each well added 200 µL l alpha-naphthyl acetate / Na phosphate, maintained at rest at room temperature for 15 minutes. After it was added 50 uL of fast blue, and after five minutes, held the reading. The background was prepared with only water and the positive control was added 10 uL of a solution of alpha-naphthol in 0.5 mg / uL (~ 3.5 nmoles / uL). For β esterase, concentrations of reactants were the same as used for α. However it was used as base the reaction of β-naphthyl acetate / Na phosphate, and the positive control was done with β-naphthol 0.5 mg / uL.

The protein concentration was determined by the Bradford method (1976) by using bovine serum albumin as a standard. A microplate spectrophotometer TECAN A 5082 was used for measurements, and the results of the enzymatic activities were expressed in $nmol.min^{-1}.mg\ protein^{-1}$.

Statistical analysis

Data were expressed as mean ± standard error of the mean. Analysis of variance (ANOVA) was utilized by determining the differences of the results in the groups. Means were compared by the Tukey test significant difference ($P < 0.05$). Relationships between efficacies and resistance mechanisms were investigated through correlations test and $\chi 2$, analyses conducted in SAS program.

Results

Phytossanitary profile of orchards

The profile of the studies apple orchards is presented in Table 2, separating them into three performance levels. The performance of the orchard in Porto Amazonas (PR population) qualified as Good Agricultural Practices (GAP). The orchard in San Joaquim (SC1 population) and Fraiburgo (SC2 population) were characterized as being typically Conventional Management (MC) and the orchard in Vacaria (RS population) presented Inadequate Management (MI).

There was a positive relationship between the use of mating disruption and reduced insecticide use in the orchard PR in the first four years. There was no orchard ranked absolute reduction (+3) of *G. molesta* on the 10th day after spraying. Besides, the largest catches occurred in the orchard typified as MI. The change coefficient for the Rotation of insecticides indicator was the main negative factor to reduce the performance scores in the first three years, except in the orchard SC1.

Toxicology Bioassays

Mortality, among three replicates of each population, was not different, so data was grouped into a single statistical analysis of each population (n= 504).

The Cl_{50} values for chlorpyrifos showed that populations of *G. molesta* RS, SC1 and SC2 had significantly higher mortality compared to PR and reference (ER) (Table 3). The ratio resistance (R/R) calculated for the four populations prevailed between 13 and 23 times

larger than the ER, PR and population the lightest reason. Regarding Cl_{50}, there were no differences among populations. Analyzing χ^2, the equality among populations hypothesis in relation to ER was rejected. There was a negative correlation (r = -0.93) between Cl_{50} and the correct mortality.

Bioassays with carbaryl showed that populations RS, SC1 and SC2 were significantly different of PR and ER. Behavior equal to that was shown for chlorpyrifos (Table 3). The R/R populations were higher in RS, SC1 and SC2, ranging between 16.4 and 19 times. The PR had the lowest value. Data confirmed in $\chi 2$, rejecting $p < 0.05$. The corrected mortality was similar for all populations ranged 81 to 85%.

For deltamethrin, SC2 population was different when measured LC_{50} (Table 3) and it had the largest R/R. The analysis showed that the LC_{95} of the RS population was not different from SC2 one. Both showed greater tolerance for the chemical compound LC_{95} and LC_{50}. RS' R/R was the largest (95%) and it was four times higher than other populations. The analysis showed the $\chi 2$s SC2 population superior, with greater heterogeneity, rejecting the hypothesis of equality. The corrected mortality was similar among populations, varying from 88 to 94%, with a negative correlation between LC_{50} and corrected mortality (r = -0.97) among populations (Table 3).

The population SC2 was more tolerant to tebufenozide than the others and was similar to ER (Table 3). No differences between populations were found for LC95. Compared with the reference population for R/R, SC2 had the highest value of 1.10. The other populations were similar to values close to 0.70. The corrected mortality ranged from 75 to 86% among populations, being lower for PR and higher for RS (Table 3).

Enzymatic activities

The enzymatic activity of GST in the RS population was greatest among those tested. It was 4.1 times greater than the reference population ($F = 8.27$, df = 6, $P < 0.0001$) (Figure 1). The acetylcholinesterase activity was statistically higher for CR and PR, populations collected in Santa Catarina (SC1 and SC2). They showed AchE activity similar to that obtained in the reference population ($F = 1.53$, df = 6 $p = 0.1282$) (Figure 2). The αEst activity was significant for RS however SJ population activity was lower than in the reference population ($F = 10:57$ df = 6 $P < .0001$) (Figure 3). The βEst activity was not different between all populations (Figure 3).

Discussion

Resistance to insecticides is the development of the capacity of a pest population to tolerate concentrations that would be lethal to most individuals of the species. To avoid this, the genetic selection process needs to be interrupted by non-chemical control methods or by uses of new active ingredients with different modes of action (Georghiou, 1994). Those are not the case for phytosanitary strategies in orchards in southern Brazil, as organophosphates were the most used compounds. Despite the increase in the Spray frequency indicator in Vacaria (RS population) there was no decrease in the population dynamics of *G. molesta* on the 10th day after treatment. This suggests that management was inadequate, motivated by the lack of monitoring and consequent decision making as prescribed timetable.

The applications of insecticide field aims to achieve the first stage larvae, before it enters the fruit after the insecticide has no more contact only exit for pupation, the moment and application technology are also important factor in the management of *G. molesta*.

An alternative way to reduce the resistance potential is a combination of mating disruption for oriental fruit moth to conventional management (Kovanci et al., 2005). In Porto Amazonas orchard, there was a reduction in the Spray frequency indicator over *G. molesta*. However the indicator Rotation of insecticides remains negative due to the use of phosphorous insecticides for fruit flies control. In this case, although the indicator Rotation of insecticides has been negative, reducing the frequency of spraying, there was a positive factor for this population, with influence on the number of catches on the 10th day after spraying. This indicates the weakness of individuals selection potential, as mentioned by Thomson et al. (2001). This combination of chemical control and sexual confusion allows the population to remains low, as reported by Rodriguez et al. (2012) in Spain. On the other hand, the Fraiburgo orchards not reproduce these results, partly due to the late maturity of cultivars that were planted in the region, delaying harvest around 45 days compared to Porto Amazonas. This difference in the crop cycle coincides with the increase in population fluctuation of other pest control being achieved with organophosphate insecticides. It showed negative influence in the indicator Rotation of insecticides. The Porto Amazonas plant profile was positive and confirmed the bioassays results, which allowed discriminate the PR population more susceptible to organophosphates and carbamates. The history influence on the phytosanitary status toxicology, as mentioned by Rodriguez et al. (2010) was also detected. The rates of lethal concentration (50%) indicated that the remaining populations had opposite results compared to that seen in PR, which ranged from 16 to 24 times more tolerant. Phosphorous insecticides continued use in recent decades showed Tortricidae resistant populations in

Brazil (Siegwart et al., 2011), Spain (Rodriguez et al., 2010, 2012) and Canada (Kanga et al., 2003). The RS population was more tolerant to deltamethrin, with R/R (95%) four times higher than other populations, although spraying with pyrethroid has been limited to an annual application. One can explain this result by the chemical use over the years in this orchard, which is one of the oldest in Vacaria (Siegwart et al., 2011). Jones et al. (2011) reported that survival in bioassays is not necessarily related to resistance evidence, but the unexpected levels of natural tolerance. It may be the case of the SC2 population, which had the R/R (50%) twice as high. However, no history of deltamethrin in orchard applications over the last five years was recorded. Besides, Tortricidae pyrethroids tolerance was already detected (Slirme et al. 1998; Reyes et al. 2007). The populations were sensitive to tebufenozide, despite evidences of metabolic resistance in various Lepidoptera species (Souphanor & Bouvier, 1995; Cao & Han, 2006). Nevertheless, the increasing insecticide groups application of the action broad-spectrum ones may accelerate selection processes.

The study of the biochemical mechanisms demonstrated high activity for Ache, GST and αEST. All biochemical results indicate the RS as the population with the highest activity for these three enzymes, corroborating the results of toxicological bioassays. In the case of the PR population, AchE activity not was inhibited, even though it had received fewer insecticide applications and proved it was susceptible. This may be related to the insecticides rotation, which has action on acetylcholine. Ache has been consistently associated with resistance to organophosphate (Li et al. 2,007; Kakani et al., 2008) as well as GST and αEST and not only to the organophosphorus but to other chemical groups (Rodriguez et al. 2,010; Siegwart et al., 2011; Reyes et al., 2012). Considering that the population RS had high results in the three enzymes, it may have been not only tolerance to a chemical group. So in future work, it is important to include synergisms tests between chemical groups, for populations that already showed tolerance to certain insecticides.

Finally, indicators that originated the plant profile have demonstrated that the RS population had more negative values to the CA. It indicates a trend of tolerance proved by biochemical and toxicological bioassays.

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6. Tables e Figures.

Table1. Components that characterize the profile of plant apple orchards in southern Brazil, using a system change coefficient (developed according Rodrigues et al. (2002).

	Coefficients of alteration (CA)			
Components	3	1	-1	-3
Monitoring[1]	*G. molesta e A. fraterculus*	*G. molesta ou A. fraterculus*	irregular	absent
Mating disruption[2]	3cycle	2 cycle	1 cycle	absent
Phytophagus mite control[3]	Phytoseiid mite	Phytoseiid mite/acaricide	One acaricide	> 2 acaricide
Frequency of insecticide[4]	4 a 6	7 a 9	10 a 13	> 13
Rotation of insecticide[5]	4	3	2	1
Flutuation post-treatment[6]	0 a 5	5 a 10	11 a 19	> 20

1 - Application of insecticides based on monitoring of *G. molesta* e *A. fraterculus*

2 - Presence of a formulation of mating disruption for minimum period of four months per year, with a reduction of insecti

3- Biological control of *P. ulm* i inoculativo with or without predatory mites. Acaricide use in orchards

4 - Number of insecticide applications per time interval

5 - Number of groups chemicals used to control insects

6 - Number of *G. molesta* on the 10th day after spraying with insecticides

Table2. Sum of coefficients alteration of profile components of tree apple orchards in Southern Brazil, assessment in five production cycles (2006-2011).

Production cycle	Components	Orchards			
		RS	SC1	SC2	PR
	Monitoring	-1	3	3	3
	Mating disruption	-3	-1	-3	3
2006/07	Phytophagous mite control	-3	3	-3	3
	Frequency of insecticide	-3	-3	-1	1
	Rotation of insecticide	-1	-1	3	-3
	Fluctuation post-treatment	-3	1	-1	1
	Subtotal coeficientes	**-14**	**2**	**-2**	**8**
	Monitoring	-1	3	3	3
	Mating disruption	-3	-1	-3	3
2007/08	Phytophagous mite control	-3	3	-3	3
	Frequency of insecticide	-1	-3	-1	1
	Rotation of insecticide	-1	-1	3	-3
	Fluctuation post-treatment	-1	1	-1	1
	Subtotal coefficients	**-10**	**2**	**-2**	**8**
	Monitoring	-3	3	3	3
	Mating disruption	-3	-1	-3	3
	Phytophagous mite control	-3	3	-3	3
2008/09	Frequency of insecticide	-3	-3	-1	1
	Rotation of insecticide	-1	1	1	-3
	Fluctuation post-treatment	-1	1	1	1
	Subtotal coefficients	**-14**	**4**	**-2**	**8**
	Monitoring	-3	3	3	3
	Mating disruption	-3	-1	-3	3
	Phytophagous mite control	-3	3	-3	3
2009/10	Frequency of insecticide	-3	-3	-1	1
	Rotation of insecticide	-1	-1	1	1
	Fluctuation post-treatment	-1	1	1	1
	Subtotal coefficients	**-14**	**2**	**-2**	**12**
	Monitoring	-3	3	3	3
	Mating disruption	-3	-1	-3	-3
	Phytophagous mite control	-3	3	-3	1
2010/11	Frequency of insecticide	-3	-3	-1	1
	Rotation of insecticide	-1	-1	1	1
	Fluctuation post-treatment	-1	1	1	1
	Subtotal coefficients	**-14**	**2**	**-2**	**4**
	Total	**-66**	**12**	**-10**	**40**
	Profile phytosanitary Orchard*	**IM**	**CM**	**CM**	**BAP**

*Refers IM= Inadequate management, BAP=Best Agricultural practices, IPM=Integrated Pest Management, CM=Conventional management.

Table3. Cl_{50} and Cl_{95} of four insecticides in fields populations of *G. molesta* compared with susceptible population

Population	Corrected mortality (%)	χ2/gl	CL_{50}µg/g 95%	TCL	CL_{95}µg/g	[A]R/R95	Slope ± SE
			Chlorpyrifos				
ER	91.49	0.83/4	0.49 (0.27-0.92)		1262.00 (256-1548.10)		048±0.05
RS	81.44	3.90/4	9.78 (8.29-11.43)	19.95*	50.59 (36.48-83.72)	0.04	2.30±0.27
PR	87.50	0.08/4	6.84 (5.89-7.83)	13.95*	39.13 (28.96-61.09)	0.03	2.17±0.23
SC2	80.12	0.33/4	11.43 (9.69-13.25)	23.32*	57.66 (43.23-88.42)	0.04	2.34±0.25
SC1	83.33	0.56/4	9.77 (8.43-11.24)	19.93*	59.94 (43.03-98.63)	0.04	2.08±0.22
			Carbaryl				
ER	85.41	0.27/4	1.13 (0.68-1.99)		769.99 (216-4941.70)		058±0.05
RS	85.21	1.96/2	21.61 (18.25-25.31)	19.12*	129.19 (91.53-216.40)	0.16	2.11±0.24
PR	81.94	0.91/2	10.91 (8.40-14.23)	9.65*	231.77 (125.00-589.00)	0.30	1.23±0.14
SC2	85.50	1.07/4	21.41 (17.76-25.33)	18.67*	124.72 (88.55-212.42)	0.16	2.14±0.25
SC1	85.00	1.84/4	19.03 (16.35-22.02)	16.84*	96.85 (71.50-152.14)	0.12	2.32±0.25
			Deltamethrin				
ER	91.29	0.05/4	10.97 (8.03-14.26)		168.25 (105.90-333.81)		1.38±0.15
RS	92.26	1.49/4	5.06 (2.67-8.00)	0.46	817.43 (294.02-4287.17)	4.85*	074±0.09
PR	91.66	1.39/4	14.83 (12.25-17.85)	1.35	142.13 (98.97-233.00)	0.84	1.67±0.14
SC2	88.89	4.30/4	29.80 (23.57-36.62)	2.64*	180.20 (116.42-401.20)	1.07	2.10±0.22
SC1	94.80	2.25/4	10.17 (7.79-12.79)	0.92	127.17 (84.00-227.45)	0.75	1.50±0.15
			Tebufenozide				
ER	78.00	1.45/4	11.73 (9.81-13.89)		69.02 (48.43-119.66)		2.13±0.25
RS	86.00	0.46/2	7.89 (6.12-9.76)	0.67	83.29 (50.17-201.59)	1.20	1.60±0.23
PR	75.50	0.41/4	8.27 (6.66-10.05)	0.70	112.25 (62.65-315.47)	1.62	1.45±0.21
SC2	81.83	0.34/4	13.00 (11.26-14.97)	1.10	60.86 (45.55-93.25)	0.88	2.45±0.26
SC1	83.33	1.19/4	8.93 (7.71-10.25)	0.76	52.73 (38.34-84.81)	0.76	2.13±0.23

[A]R/R 95 ratio of resistance between population reference and population field

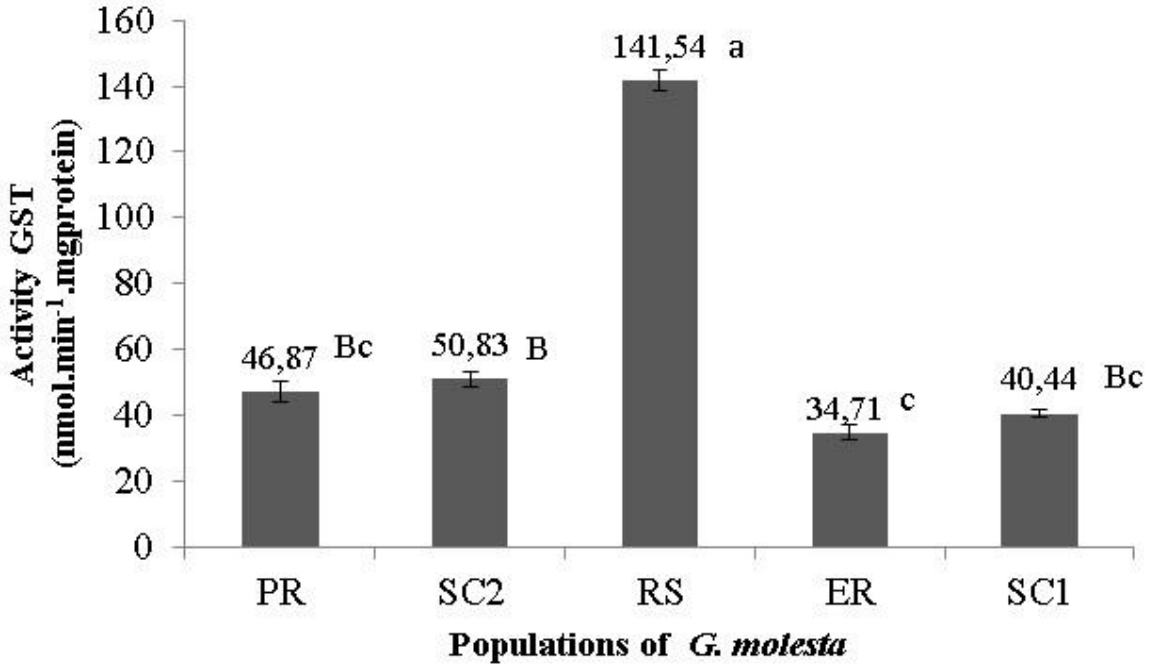

Figure1. Enzimatic activity of Glutathione S-transferase expressed in nmol.min.mgprotein of field populations *G molesta* in plant apple orchards in Southerm Brasil. Different letters mean stastistical significance.

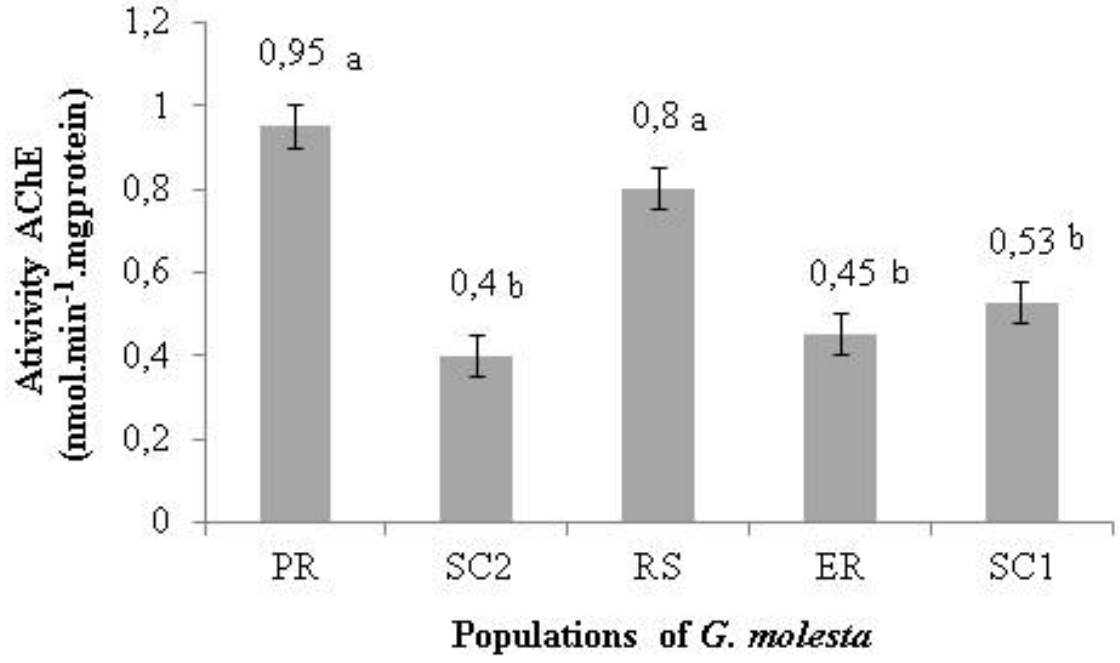

Figure2. Enzimatic activity of Acetylcholinesterase (AchE) expressed in nmol.min.mgprotein of field populations *G. molesta* in apple orchards in Southerm Brasil. Different letters mean stastistical significance.

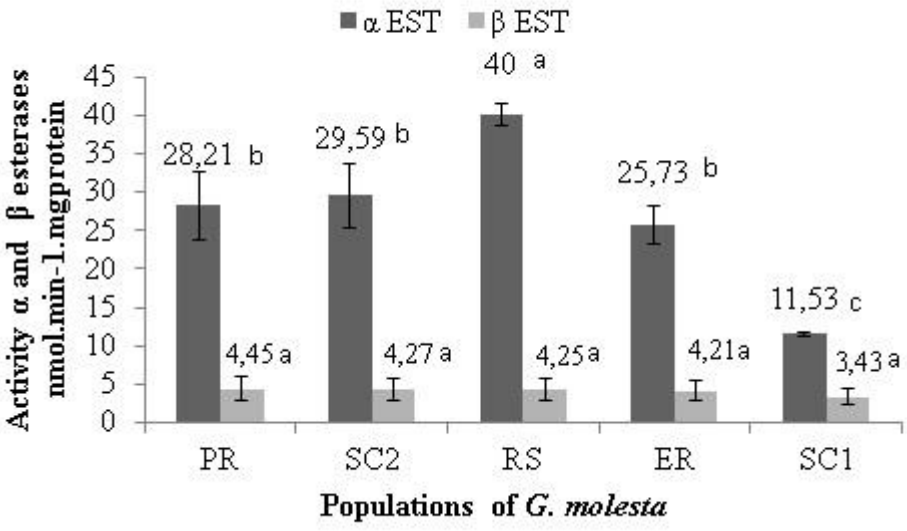

Figure3. Enzimatic activity of Alfa and Beta esterases, expressed in nmol.min.mgprotein of field populations *G. molesta* inapple orchards in Southerm Brasil.

CAPÍTULO II

CARACTERIZAÇÃO DE *Grapholita molesta* (Busk, 1916) (LEPIDOPTERA: TORTRICIDAE) POR ESPECTROSCOPIA NO INFRAVERMELHO PRÓXIMO (NIRS)

Artigo encaminhado à Scientia Agricola journal

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Espectroscopia no infravermelho próximo (NIRS) para caracterização de *Grapholita molesta* (Lepidoptera:Tortricidae)

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Espectroscopia no infravermelho próximo (NIRS) para caracterização de *Grapholita molesta* (Busk, 1916) (Lepidoptera:Tortricidae)

Resumo

A espectroscopia no infravermelho próximo (NIRS*)* está sendo muito utilizada na entomologia na identificação de espécies de diferentes grupos taxonômicos, A utilização na identificação de linhagens resistentes, associado com outras técnicas já estabelecidas, pode ser uma alternativa que complementa os métodos disponiveis. Objetivo deste estudo foi verificar a eficiência da técnica de espectroscopia no infravermelho próximo, em caracterizar populações de *G. molesta* resistentes a inseticidas. O trabalho foi realizado com populações coletadas no campo (RS, SC, PR), comparadas a um controle negativo (população resistente (RT)) e um controle positivo (população susceptivel (PS)). Foram realizados a avaliação no NIRS e, posterior avaliação enzimatica (Acetilcolinesterase (AchE), Glutationa S-transferase (GST) e α e β esterases (α EST e β EST)). Os resultados mostraram que o NIRS separou as populações de *G. molesta,* diferenciando populações que apresentaram alta frequência de resistência das populações que mostraram ser susceptivel, comprovada pelos testes bioquimicos que mostrou um aumento na atividade de GST e α Est para RS e RT, enquanto que PS e SC apresentaram as menores atividades. AchE não foi inibida em PR, RS e RT. Conclui-se que a analise de NIRS é eficiente na caracterização de populações de *G. molesta*, e que pesquisas adicionais são necessárias para estabelecer modelos de calibração para outras espécies pragas.

Palavras-chave: Caracterização de populações; Tolerância; Fenótipo químico; Resistência metabólica.

Abstract

The near infrared spectroscopy (NIRS) is being widely used in entomology to identify different species of taxonomic groups , the use in the identification of resistant species , associated with other established techniques , may be an alternative that complements the available methods The aim of this study was to verify the efficiency of the technique of near infrared spectroscopy in characterizing populations of *G. molesta* resistant to insecticides. The work was carried out with populations collected in the field (RS, PR, SC), compared to a negative control (population tolerant (RT)) and a positive control (susceptible population (PS)). Were performed to evaluate the NIRS and subsequent evaluation enzymatic (acetylcholinesterase , glutathione S - transferase and α and β esterase). The results showed that the NIRS separated *G. molesta* susceptible of resistant, as evidenced by biochemical tests showed that an increase in the activity of GST and α Est for RS and RT, while PS and SC had the lowest activities. AChE not inibited in PR, RS and RT. It is concluded that the NIRS analysis is effective in characterizing populations of *G. molesta* and that additional research is needed to establish calibration models for each species.

Key Words: Characterization of populations; Resistance; Phenotype chemical, metabolic resistance.

Introdução

Resistência é o desenvolvimento de uma habilidade em uma linhagem de organismos em tolerar concentrações de tóxicos que seria letais para a maioria da população. A detecção da resistência é baseada na concentração-mortalidade em relação a uma população susceptível, porém, é dificil de ser detectada, devido a variação natural entre as populações e quando a frequência de resistência é baixa (Jones et al, 2012). Assim, os bioensaios toxicológicos são os mais utilizados em pesquisas visando a detecção da resistência, entretanto, elevados níveis de sobrevivência não deve ser interpretado como evidência de resistência (Roush e Miller, 1986; Robertson et al, 2007; Jones et al, 2011). Devido essas variações ocorridas em cada técnica, o agrupamento de vários procedimentos visando quantificar e qualificar a frequência de resistencia seria uma alternativa.

A técnica de espectroscopia no infravermelho próximo (NIRS) está sendo adaptada à Entomologia, pois permite separar grupos de indivíduos pela expressão química presente em sua estrutura cuticular (Steiner et al, 2002; Klarica et al, 2011). As reações caracteristicas dos grupos funcionais produzidos e absorvidos pela luz infravermelha constituem uma ferramenta eficaz de identificação (Wokman e Weyer, 2008), o que, viabilizariam a identificação de grupos taxonômicos (Aldrich et al, 2007, Mayagaya et al, 2009, Fischnaller et al, 2012, Tramalazza et al, 2013). Assim, a utilização do NIRS para a identificação de linhagens com alta frequência de resistência poderia ser uma alternativa que complementa os métodos disponiveis, tais como, bioensaios toxicológicos e enzimáticos.

Grapholita molesta (Busck, 1916) (Lepidoptera:Tortricidae) é uma praga polífaga, que causa perdas econômicas substanciais na fruticultura de clima temperado. O controle é

realizado com aplicações de inseticidas organofosforados, piretróides e reguladores de crescimento (Monteiro et al, 2009), porém, existem relatos de ineficiência no controle (LBM, comunicação pessoal). O aumento da frequência de aplicação de inseticidas seleciona indivíduos resistentes (Reyes et al, 2007), como foi observado na América do Norte (Kanga et al, 1997; 2003) e Europa (Reyes e Sauphanor, 2008). Parte do principio que no Brasil temos populações com alta frequência de resistência, a possibilidade de definir por um método mais rápido e fácil significaria um avanço nos estudos de resistência na entomologia agrícola, visto que, esta praga é carpófaga, causa danos diretos, além de ser de difícil controle.

O Objetivo deste estudo foi verificar se a técnica de espectroscopia no infravermelho próximo pode ser utilizada para diferenciar populações de *G.molesta* com frequência de resistência.

Material e Métodos

Insetos

Os indivíduos foram coletados em pomares comerciais de macieiras, localizados nos municípios de Vacaria (RS) (28º 30' 44" S, 50º 56' 02" O, 971 m) chamada população RS, em Porto Amazonas (PR) (25º32'8"S, 49º53'33'' O, 854 m) denominada população PR, em São Joaquim (SC) (28º15'S e 49º54' W, 1.360 m) população SC. As larvas foram coletadas em frutos no ciclo produtivo de 2009/2010 e mantidas em dieta artificial para multiplicação (Guennelon et al, 1981), sendo que as populações foram re-infestadas com larvas nas safras de 2010/11 e 2011/12. Duas populações de laboratório foram utilizadas como referência: susceptível (PS), mantida em dieta artificial há 18 anos sem introdução de material genético, e resistente (RT), coletada em pomar de macieira em 2008 e mantida sobre pressão de seleção com inseticida tebufenozide por 18 gerações.

Todos os pomares adotaram o manejo fitossanitário convencional para o controle de *G. molesta*, com aplicações de inseticidas fosforados, carbamatos e diacilhidrazina nos últimos cinco anos. O manejo de pragas do pomar Vacaria apresenta o mais intenso uso de inseticidas, com aproximadamente, 15 pulverizações por safra, principalmente organofosforados. No pomar de Porto Amazonas houve redução no número de pulverizações em função do uso de uma formulação de confusão sexual para *G. molesta*. O pomar de São Joaquim foi o que teve menor número de pulverizações, visto que a densidade da praga foi influenciada pela temperatura.

Análise bioquímica

As larvas utilizadas para os bioensaios enzimáticos foram coletadas em frutos e imediatamente homogeneizadas e mantidas a -80ºC. Para quantificação da atividade das enzimas acetilcolinesterase (AchE), glutationa S-transferase (GST) e esterases (α e β Est) foram feitos *pools*, contendo cinco larvas de 3º ao 5º instar (n= 50 para cada população). Cada *pool* foi homogeneizado na proporção 1:10 (peso: volume) em tampão fosfato de potássio 0,1 M (pH 7,0) com micro homogeneizador. O homogeneizado foi centrifugado por 20 minutos a 10.000 x g a 4°C, sendo o sobrenadante usado para medir a atividade enzimática.

A atividade da AchE foi mensurada a 405 nm através do método de Ellman et al. (1961), modificado para microplacas por Silva de Assis, (1998). Em cada poço foram adicionados 50 µL de amostra, 200 µL de DTNB (5,5 – Ditio-bis-2-nitrobenzoato) a 0,5 mM e 50 µL do substrato acetiltiocolina a 10 mM. A atividade da GST foi mensurada a 340 nm de acordo com o método proposto por Keen et al. (1976). Em cada poço foi adicionado 50 µL de amostra e imediatamente antes das leituras foram acrescidos 100 µL de solução reação (GSH a 0,6 mM, CDNB a 0,5 mM). A atividade das esterases foi mensurada em *end point* a 570 nm de acordo com o método proposto pelo Brasil (2006). Para α Est, foi adicionado 10 µL de amostra diluída 1:30 em cada poço, acrescentado de 200 µL alfa-naftil acetato/Na fosfato,

mantido em repouso em temperatura ambiente por 15 minutos, em seguida foi adicionado 50 µL de Fast Blue após cinco minutos, realizado a leitura. O branco foi elaborado somente com água e o controle positivo foi adicionado 10 µl de solução de alfa-naftol a 0,5 µg/µL. Para β Est, as concentrações de reagentes foram às mesmas utilizadas para α, entretanto foi utilizado como base da reação o β-naftil acetato/Na fosfato, e o controle positivo feito com β-naftol a 0,5 µg/µL.

A concentração de proteína foi determinada pelo método de Bradford, (1976) com albumina soro bovina como padrão. As leituras foram realizadas no espectrofotômetro de microplaca TECAN A 5082 e os resultados da atividade enzimática de AchE, GST, α e β Est, foram expressos em $nmol.min^{-1}.mgproteina^{-1}$.

Os dados foram expressos em média de atividade enzimática (± erro padrão). Análise da variância (ANOVA) foi utilizada para determinar as diferenças entre as populações. Os resultados foram submetidos a Teste de Tukey e considerados significantes quando os valores ($p < 0.05$).

Infravermelho

Os espectros foram obtidos por um espectrofotômetro Excalibur Bio-Rad FTS 3500 GX (Bio-Rad laboratório, Cambrigde, MA, USA) na faixa espectral de 7500 para 4000 cm^{-1} (1428-2500 nm) com a resolução de 1 cm^{-1}. Foram feitas 32 varreduras para cada individuo e medidos 50 larvas de terceiro a quinto instar para cada população. Uma leitura “branco” foi a base de referência para as amostras. Foram realizadas duas análises em: i) duas populações de laboratório, sendo uma resistente (RT) e a outra susceptível (PS); ii) três populações coletadas em pomar de macieira comparada com as populações de laboratório.

Para caracterização das populações resistentes e susceptíveis foi utilizado a Análise Discriminante com Quadrados Mínimos Parciais (AD-PLS). Os espectros foram

transformados usando a primeira derivada de Savistky-Golay (janela de 21 pontos e polinômio de segunda ordem) (Hruschka, 1987).

A validação externa dos modelos foi feita com 10 indivíduos de cada grupo. Os modelos de calibração multivariada foram desenvolvidos com uso do programa (Unscrambler ™ versão 9.7, Camo software AS, Oslo, Norway). Para a caracterização das populações de campo, comparada com as populações de laboratório, foi utilizada a avaliação de componentes principais pelo mesmo programa estatístico.

Resultados

Atividade enzimática

A atividade enzimática de GST foi maior na população RS, sendo 4,1 vezes maior do que a população suscetível (PS) ($F= 8.27$, $df= 6$, $p< 0.0001$) e 2,3 vezes maior que RT (Figura 4a) ($F= 9.25$, $df= 6$, $p< 0.0001$). A maior atividade de α Est foi observada nas RS e RT, não havendo diferenças entre elas (Figura 4b), sendo que, SC se diferenciou das demais ($F= 10.57$, $df= 6$, $p< 0001$). A atividade da β Est não foi significativa entre as populações de campo e de referência (Figura 4b). A atividade de acetilcolinesterase não foi inibida nas populações de campo (PR e RS) e de laboratório (RT) (Figura 4c), enquanto que PS e SC teve inibição de AchE, não se diferenciando entre si.

Infravermelho

A análise no infravermelho próximo separou as populações de laboratorio de larvas resistentes e susceptiveis (Figura 1). O agrupamento dos indivíduos mostra uma pequena variabilidade da RT. O modelo de validação dos dados para RT e PS tiveram correlação média de 0,97 e 0,96 respectivamente com erros de calibração e validação baixos (Tabela 1).

A análise do NIRS com as populações coletadas em pomar, comparadas com as de laboratório, permitiu três agrupamentos das populações, sendo o primeiro RS e RT encontrado no plano x o segundo PR e SC e, o terceiro com a PS, ambos os grupos compartilhem o mesmo plano (z) (Figura 2). O eixo x é aquele explicado por 76%, sendo que o y responde 20% e o eixo z por 4%. Os dados de calibração e validação do modelo AD-PLS apresentaram uma correlação média de 0.87 e 0.81com erro de 0,07 e 0,09, com maior concentração das informações entre 9998,928 cm^{-1} a 7996,828 cm^{-1}.

Discussão

Os resultados enzimáticos mostraram que as populações RT e RS apresentaram as mais elevadas atividades de AchE, GST e α Est, todas associadas ao processo de detoxificação de inseticidas. Isso sugere que as populações RT e RS apresentaram indivíduos tolerantes, quando comparadas com PS, confirmando os resultados de Zhu et al. (2011), os quais mostraram que o uso frequente de inseticidas ascelera o metabolismo dos insetos. A RT foi multiplicada em laboratório sob pressão de seleção com tebufenozide por 18 gerações, enquanto que os indivíduos de RS receberam frequentes pulverizações de inseticidas do grupo organofosforados e carbamatos nos últimos cinco anos. Zhu et al. (2004) mostrou que processo de seleção é contínuo a medida em que a pressão é estável.

A ferramenta do NIRS permitiu traçar um perfil para as populações, agrupando as populações, de acordo com as suas características químicas semelhantes. Dessa forma, um grupo pode estar condicionado a uma maior frequência de resistência, visto que, agrupa a população que recebeu concentrações de inseticidas ao longo de seu desenvolvimento em laboratório, enquanto que o outro acondiciona uma população que está em laboratório a 18 anos, que pode ser considerada susceptível, este agrupamento também foi identificado no aumento da atividade enzimática para estas populações.

A possibilidade de identificar o “Status de tolerância” de indivíduos coletados em campo, comparados com um padrão de susceptibilidade e resistência com utilização do NIRS, traz novas espectativas de aprimoramento nas pesquisas de manejo de resistência. Pois esta identificação apresenta vantagem de ser rápida e simples, sem exigir preparação da amostra, levando em torno de um minuto para gerar cada espectro. Para esta realização, se faz necessário a análise do desempenho dos modelos utilizados, devem apresentar baixo erro de validação (RMSEV) e calibração (RMSEC) assim como, alto valor de coeficiente (R^2), indicando a capacidade de discriminar os grupos avaliados, variando de 90-100% (Jia et al, 2007; Tralamazza et al, 2013). Neste modelo avaliado o RMSEC e RMSEV mostrado na tabela 1, foram baixos variando de 0,004 a 0,01, com variação de 96 a 97% de capacidade de discriminar os dois grupos. A validação externa é essencial para confirmar a qualidade do modelo, mostrando classificação em 100% e baixo RMSEP confirmando assim um modelo robusto para classificar estas populações. Porém outras metodologias instrumentais poderiam ser testadas, como a utilização de um sistema de imagens acoplado ao espectrofotômetro, permitindo um posicionamento preciso do espectro e uma melhor resolução, como metodologia descrita por Klarica et al. (2011).

Os espectros representam absorção da luz infravermelha pela amostra obtida através da interação entre a luz e a vibração, cada molécula com suas características únicas, apresentam absorção e reflexão diferentes, o que sugere que o manejo fitossanitário adotado em cada pomar, exerce pressão seletiva sobre alguns fenótipos que podem ser detectados e caracterizados pela reflectância de energia de suas moléculas, caracterizando um perfil espectral para cada população. Rodríguez- Fernández et al. (2011) sugerem que o perfil espectral possa estar associado a impressão digital molecular das populações e ser eficiente na determinação de muitas características que diferenciam os insetos

A avaliação com a ferramenta NIRS permitiu separar as populações, porém, esta separação pode não estar condicionada a resistência ou susceptibilidade das populações, por outro lado a figura dois mostrou um gradiente que aumentou em relação à população de referencia de susceptibilidade, o que pode ser um indicativo desta separação ser devido a resistência das populações e, pode futuramente ser utilizado com uma primeira análise antes de serem abordados métodos mais elaborados, demorados e dispendiosos.

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Figuras da análise de infravermelho

Tabela1. Calibração e validação de dados para duas populações resistente e suscetível de *G. molesta* avaliada pelo método PLS**.** R^2C calibração; R^2V validação; RMSEC, erro calibração; RMSEV, erro validação.

População	R^2C	RMSEC	R^2V	RMSEV
RT resistente	0.97	0,0004	0.97	0,05
ER susceptivel	0.96	0,06	0.96	0,01

.

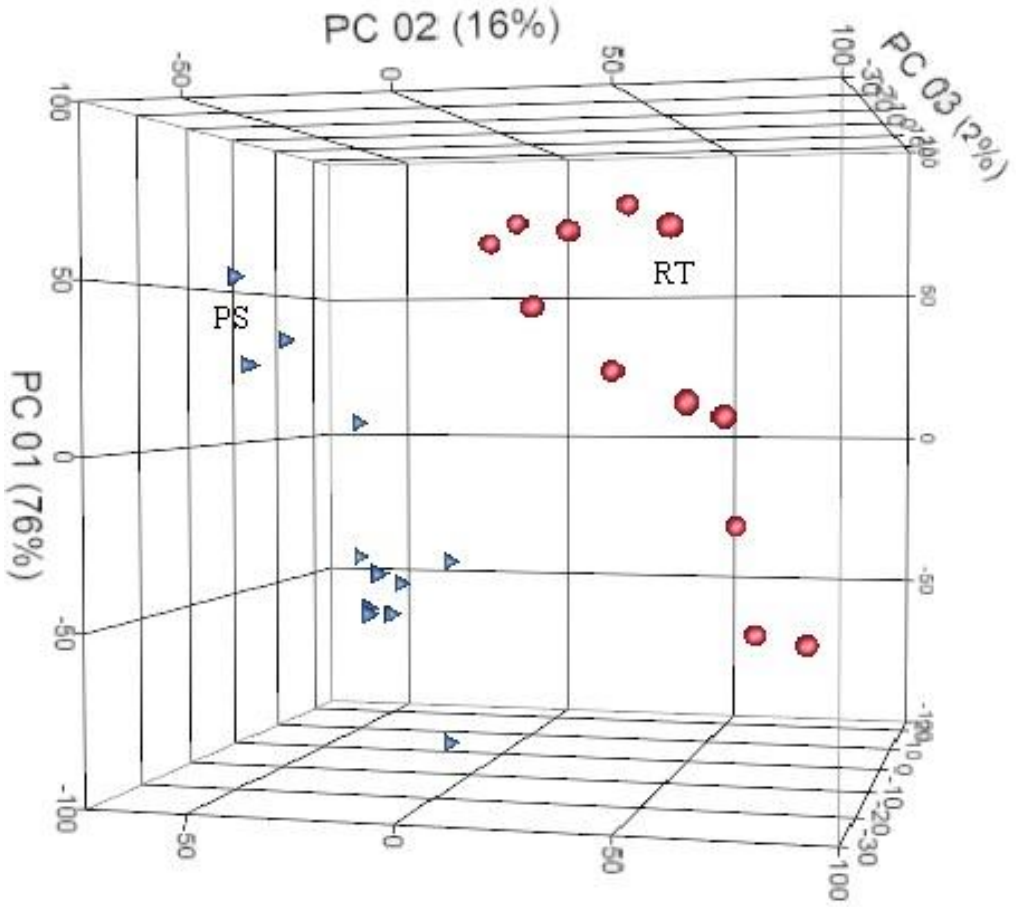


Figure 1. Distribuição de duas populações de *G. molesta* baseados em análise de quadrados mínimos parciais (PLS). População susceptível (PS=▲) e população tolerante (RT=●). Sendo x 76%; y 16%; y 2%.

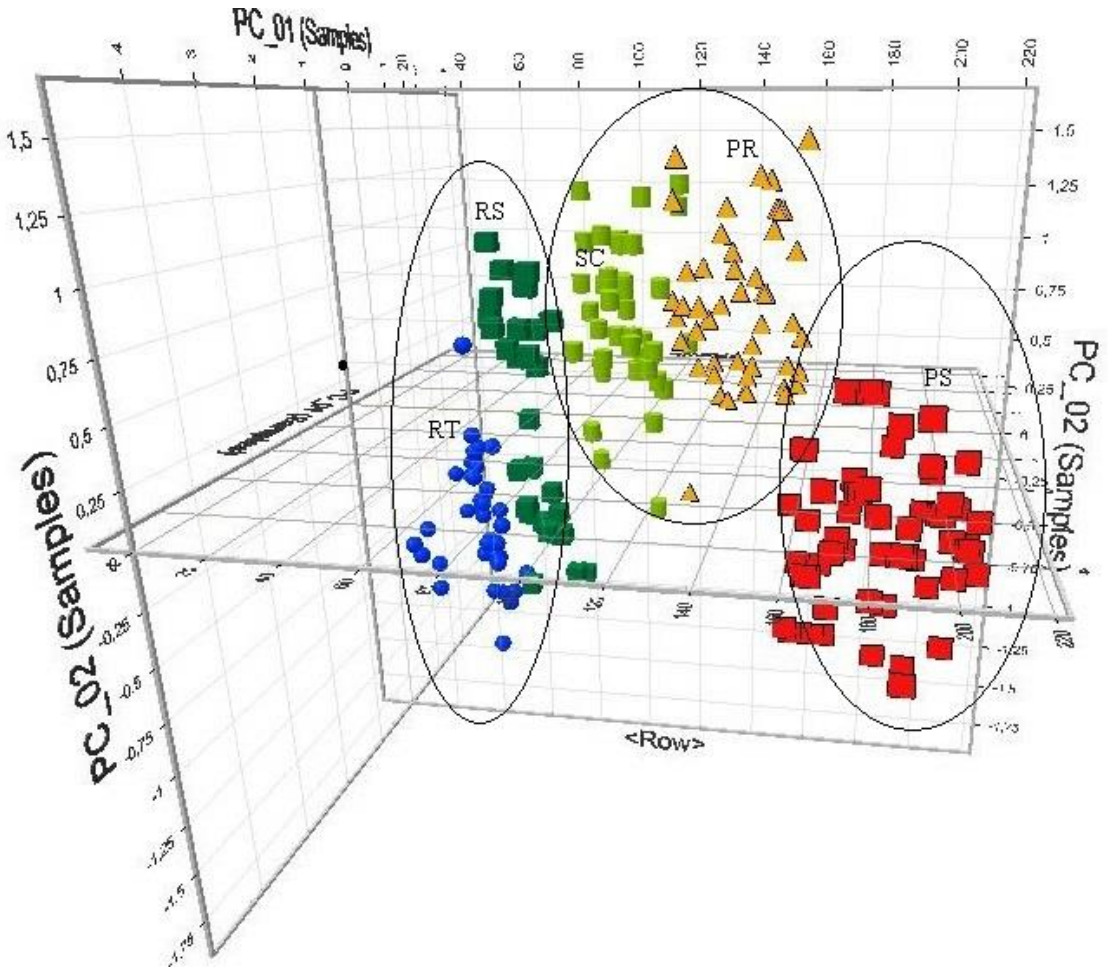
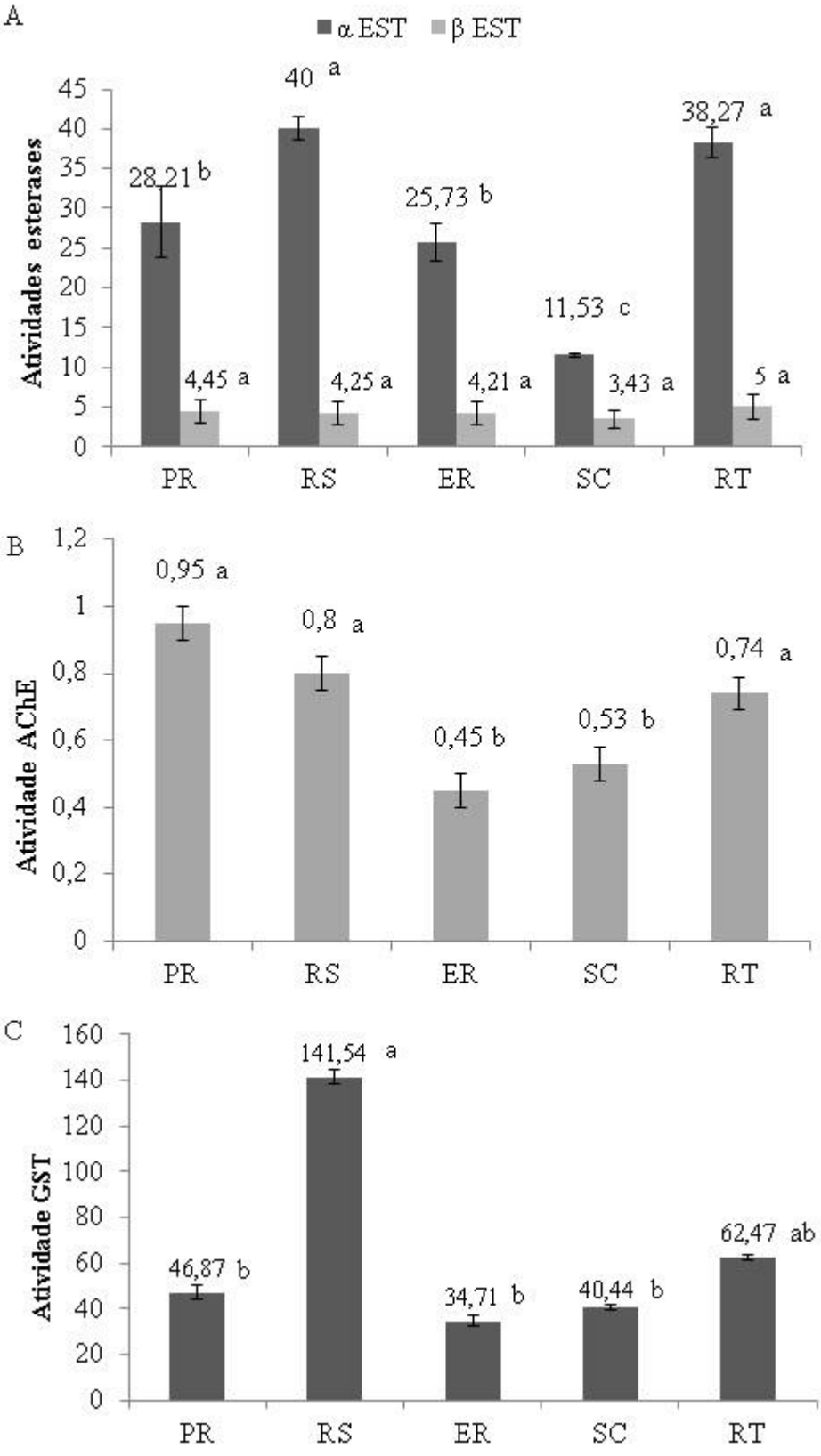


Figure 2. Distribuição de populações de *G. molesta* baseado em Análise de componentes principais (PCA). ■ = (RS), ▲=(PR) e ■= (SC) comparada com ●= RT (induzida a processo de seleção) e ■=PS (população a 15 anos sem introdução de novo material genético).

Avaliação enzimática

Figura 3. (A) Atividade mensurada da enzima Atividade de α e β esterases de populações brasileiras (PR, RS, SC) de *G. molesta*, em relação a populações susceptível (PS) e resistente (RT). (B) atividade de acetilcolinesterase; (C) atividade glutationa S-transferase.


A
α EST
β EST
Atividades esterases
45
40
35
30
25
20
15
10
5
0
28,21 b
4,45 a
40 a
4,25 a
25,73 b
4,21 a
11,53 c
3,43 a
38,27 a
5 a
PR
RS
ER
SC
RT
B
Atividade AChE
1,2
1
0,8
0,6
0,4
0,2
0
0,95 a
0,8 a
0,45 b
0,53 b
0,74 a
PR
RS
ER
SC
RT
C
Atividade GST
160
140
120
100
80
60
40
20
0
46,87 b
141,54 a
34,71 b
40,44 b
62,47 ab
PR
RS
ER
SC
RT

CAPITULO III

SUSCETIBILIDADE DE POPULAÇÕES DE CAMPO E LABORATÓRIO DE *Grapholita molesta* (LEPIDOPTERA: TORTRICIDAE) A INSETICIDAS, BASEADA EM CARACTERÍSTICAS TOXICOLÓGICAS, BIOQUÍMICAS E FÍSICO-QUIMICAS

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Susceptibility of field and laboratory *Grapholita molesta* **(Lepidoptera:Tortricidae) populations with the insecticide, based in toxicological, biochemical, and physicochemical characteristics**

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Comportamento de populações de campo e laboratório de *Grapholita molesta* comparado por características toxicológicas, bioquímicas e físico-químicas.

RESUMO- A utilização de insetos de laboratório para estudos fisiológicos, genéticos e toxicológicos tornou-se muito frequente, porém, a criação contínua em laboratório por várias gerações, sem inserção de material genético silvestre, pode alterar as características fisiológicas e comportamentais da população quando comparada com a população do campo. O objetivo deste trabalho foi comparar a susceptibilidade das populações de *G. molesta* criadas em laboratório com a silvestre, por meio de bioensaios toxicológicos, caracterização por infravermelho próximo e avaliação enzimática. Foram realizados bioensaios com quatro grupos químicos de inseticidas, sendo eles clorpirifós, carbaril, deltametrina e tebufenozide, com sete concentrações definidas após ensaios piloto. Posteriormente, foi realizada avaliação enzimática das enzimas acetilcolinesterase (AchE) e Glutationa-S-Transferase (GST) e também a comparação por espectroscopia de infravermelho próximo (NIRS). Foi possível verificar diferenças entre as populações para dois grupos inseticidas, carbaril e clorpirifós, além de, apresentar alta atividade da enzima GST, com a análise de infravermelho as populações se mostraram distintas entre si. Como conclusão as populações, silvestre e de laboratório são diferentes entre si quanto à susceptibilidade a inseticidas.

PALAVRAS-CHAVE: Criação Massal, Atividade enzimática, NIRS, Toxicologia, Mariposa oriental

ABSTRACT-The use of laboratory insects for physiological studies, both genetic and toxicological, has become very common, but the continuous strains available in the laboratory for several generations without the insertion of genetic material can change the wild physiologic and behavioral characteristics of the population compared with the field population. The aim this study was to verify susceptility of field and laboratory de *G. molesta* populations with the insecticide, based in toxicological, biochemical, and physicochemical characteristics. Experiments were conducted with four groups of chemical insecticides serving as chlorpyrifos, carbaryl, deltamethrin, and tebufenozide, with seven concentrations defined after pilot testing. Thereafter, the activity of acetylcholinesterase (AchE), glutathione S-transferase (GST), and NIRS was evaluated. It was possible to detect differences between populations with regard to carbaryl and chlorpyrifos insecticides. The infrared analysis showed that the populations were distinct from each other, and they exhibit high activity of GST and AchE. The populations from both the field and the laboratory are different in their susceptibility to insecticides.

KEYWORDS: Mass rearing, Enzymatic activity, NIRS, Toxicology, Oriental Fruit Moth

The arthropods rearing techniques introduced advances in entomology, as in physiological studies and genetic toxicology (Nava & Parra, 2005). The knowledge that was generated contributed to an understanding of the behavior and implementation of strategies for pest management, especially in biological control programs (Parra, 2000). However, the continued use of arthropods in the laboratory for several generations can modify the phenotypic and behavioral characteristics as compared with the wild population (Leppla *et al*., 1983). Thus different individuals in their biochemical and physiological characteristics (Hoffman *et al*., 2001).

The use of bioassays in laboratory populations is a common practice among researchers, primarily to evaluate new insecticides to be released in the market. Many of them that did not measure the number of generations of each population and during the period in which the population remained in the laboratory have been introduced new genetic material. As an example, a study was conducted with *Grapholita molesta* (Shearer & Usmani, 2001) and species and *Sitophilus*, *Rhyzopertha*, *Oryzaephilus* (Ceruti & Lazzari, 2003). These implications are often questioned when insects are used to replicate in the behavior laboratory studies (Bravo & Neto, 2004). The analyses that use the term toxicological laboratory populations cannot express the field populations, as, in the evolutionary process of species; it is an adaptation for the population to continue its development in a modified environment (Onstad, 2008).

Populations in the developing field are constantly changing, adapting to the conditions imposed by abiotic and biotic factors (Souza, 2011). Moreover, insects are influenced by the agronomic practices (Ricci *et al*., 2009) and the dispersion of individuals from cultures with different cycles, as occurs between pests of peach and apple (Allen & Brunson, 1943).

The genetic alterations caused by environmental parameters can be assessed by biological (Dres & Malet, 2002), molecular (Thaler *et al*., 2008), and biochemical (Burd &

Potter, 2006) parameters, among others. Susceptibility studies characterize differences among populations, such as were performed for the differentiation of biotypes *Bemisia tabaci* (Hemiptera: Aleyrodidae) (Horowitz *et al*., 2005). The relationships between biotypes due to insecticides are shown in several studies (Ma *et al*., 2007; Sotelo *et al*., 2009).

The mechanism related to inhibition of the insecticides in the important Tortricidae in fruit has been studied in different regions (Kanga, *et al*., 2003; Reyes & Sauphanor, 2008; Siegwart *et al*., 2011). In addition to the quantitative methods used, the qualitative difference in geographically close populations can be carried out by near infrared (NIRS), as demonstrated for *Grapholita molesta* (Lepidoptera: Tortricidae) (Teixeira *et al*., unpublished data).

G. molesta is a major pest on apple and peach trees. In Brazil, its presence has been recorded on peach trees since 1929 (Gonzales, 1986) and on apple trees since the 1980s (Lorenzato, 1988). In the 1980s and 1990s, the program control relied on the use of the pyrethroids and organophosphates, and in the late 1990s, insecticides and growth regulators were registered, among them, belonging to the group diacilhidrazin. The hypothesis this paper, the pressure exerted by the intensive use of insecticides can select resistant populations. These populations are different in terms of toxicology; due to fitness cost suffered by the population of the laboratory in this way cannot be used to represent the field population.

The objective of this study was to verify susceptility of field population *G. molesta* influenced by insecticides based in toxicological, biochemical, and physicochemical characteristics in relation of laboratory population.

Materials and Methods

Insect

Individual *Grapholita molesta* specimens were sampled in apple orchards in the Porto Amazonas region in Parana State (PR), Brazil (latitude 25º32'8'S, longitude 49º53'33''O, and altitude 854m). The first sampling was done in February 2008 and was mantained in the laboratory on an artificial diet (Guennelon et al., 1981) for 36 generations without the introduction of new genetic material called *population 2008* (P2008). This population was used in the study undercard by Siegwart et al (2011). The second sample was done in February 2011 in the same portion of the orchard and was maintained under the same conditions of diet for three generations, called *population 2011* (P2011). These orchards during 3years (2005 to 2007) were mainly protected using on average 5.3 spraying with organophosphate insecticides (chlorpyrifos, fenitrothion, and malathion); 0.3 spraying with diacilhidrazin (tebufenozide); and 1.0 with carbamate (carbaryl). After 2008, they were sprayed with organophosphate insecticides 6.7, 0.7, and 1.0 carbamate insecticide diacilhidrazida.

Bioassays

Bioassays that assess the toxicity of insecticides were performed in ELISA microplate (96 wells) wells filled with 150µl of an artificial diet (Soybean-Wheat Germ Insect Diet, Stonefly Industries, TX, EUA). A volume of 6µL of each concentration of insecticide solution was applied to the diet`s surface according to Reyes and Sauphanor (2008).The insecticides tested were Chlorpyrifos (Lorsban® 480 BR, Dow AgroSciences), deltamethrin (Decis®, Bayer CropScience), carbaryl (Sevin® SL, Bayer CropScience), and tebufenozide (Mimic® 240 SC, Dow AgroSciences), in seven concentrations, defined from the pilot tests. Three

replicates were performed. After drying for 20' at 22 ± 2° C, a larva was placed in each well, with a total of 24 individuals per concentration. Neonates were handled with a fine-tip brush. The microplate wells were sealed with parafilm to prevent leakage of the caterpillar and dehydration of the diet. The mortality was observed after seven days, and the dead ones were those who did not respond to the touch of the brush. The corrected mortality was calculated by the highest concentration Abbott (Abbott, 1925), and a statistical analysis was performed by the probit test program POLO PLUS. The lethal concentration ratio was estimated for each insecticide.

Enzymatic activities

The acetylcholinesterase (AchE) and glutathione S-transferase (GST) analyses were carred out with *pools* (n = 10), with five larvae, from the 3º to 5º instar (n = 50). The pools were homogenized in the ratio of 1:10 (weight:volume) in potassium phosphate buffer 0.1 M (pH 7.0) with a microhomogenizer and centrifuged for 20 min (10.000 x g at 4°C). The supernatant was used for the determination of enzymatic activities.

The activities in the glutathione S-transferase were measured based on the Keen *et al.* (1976) method at 340 nm. Overall, 20 µL of the extract in the microplate and 180 µL of a solution containing (0.6 mM GSH a, 0.5 mM CDNB a) were added. The activities of the acetylcholinesterase were measured at 405 nm by the Ellman *et al.* (1961) method that was modified to the microplate by Silva de Assis (1998). In the microplate, 50 µL of the extract and 200 µL of DTNB were added (5.5 – ditio-bis-2-nitrobenzoate 0.75 mM) and 50 µL substrate acetiltiocoline 10 mM.

The protein concentration was determined by the Bradford method (1976) by using bovine serum albumin as a standard. A microplate spectrophotometer TECAN A 5082 was used for measurements, and the results of the enzymatic activities were expressed in $nmol.min^{-1}.mg\ protein^{-1.}$.

Data were expressed as mean ± standard error of the mean. Analysis of variance (ANOVA) was utilized by determining the differences of the results in the groups. Values of $p < 0.05$ were considered significant by the Tukey test.

Infrared

The spectra were obtained using a Bio-Rad Excalibur FTS 3500 GX spectrophotometer (Bio-Rad Laboratories, Cambridge, MA, USA) operated in the 800- to 2500-nm region. The spectral range was 7500 to 4000 cm^{-1} (1428 to 2500 nm), with a resolution of 1 cm^{-1}. Overall, 32 scans were performed for each individual insect. In each population, 50 pupae were analyzed. Was performed with a white mirror unit to be used as reference samples.The spectra were transformed using the first derivative of Savitzky−Golay (21-point window and second-order polynomial). The ability to differentiate between the two populations at each stage was evaluated using discriminant analysis with least partial squares (PLS-DA). Each specimen from each population was coded as 0 or 1, for P20011 or P2008, respectively.

The Savistky−Golay model obtained for two separate populations was validated externally by means of external samples, and the reference values were 0 (P2008) and 1,0 (P2011). The values not exceeding 0.5 of the deviation from the reference set were considered appropriate.

The multivariate calibration models were developed using the Unscrambler™ program, Version 9.7 (Camo Software AS, Oslo, Norway).

Results

Bioassays

The results showed the probit model adjusted to the concentration response data for all bioassays. There were no statistical differences between the three replicates, and the data were grouped into a single analysis with a total of 504 individuals of each population.

The response to CL_{50} larvae originated from relatives of the population collected in 2011 (P2011) and was significantly different in relation to P2008 for the insecticides chlorpyrifos and carbaryl (Table 1). Analyzing the χ^2, the hypothesis of equality between populations in relation to the effects of insecticides was rejected, showing that the P2008 was more susceptible than in 2011.

The rate of lethal concentration (TCL) of P2011 was around 8- 11 times higher, respectively, for chlorpyrifos and carbaryl. The responses of the larvae of P2011 for the concentrations of deltamethrin and tebufenozide were similar among the populations. The response of the larvae in CL_{95} was not different between P2011 and P2008 for all treatments (data not reported). The corrected mortality of the larvae of P2011 was greater than that found in P2008 for chlorpyrifos and carbaryl. There was a positive correlation (r= 0.99) between corrected mortality and LC_{50} for chlorpyrifos and carbaryl, showing a variation for these products that was directly proportional and inverse to the other insecticides (r = 0.99). Despite the significance of the LC_{50} values for chlorpyrifos, the corrected mortality of larvae between the two populations was small, which was around 3.5%. The same did not occur with carbaryl, which was plus high, around 24%, between populations.

Biochemistry

The enzymatic activity of acetylcholinesterase larvae multiplied in the laboratory for 36 generations (P2008) was lower than the larvae of P2011 (F = 2. 73, df = 1, and P = 0.1450), which was around 47.5% (Figure 1). The GST activity was significantly high for larvae P2011 (F= 11.45, df = 1, and P =0.0147).

Near infrared

The technique of NIRS was able to distinguish between the laboratory and wild populations. The model calibration and validation was a correlation mean, respectively, 0.96 and 0.94, considering the two populations (Table 2). The partial least squares model was adequate, as the external validation obtained values for the reference (Table 2).

The results of NIRS showed that P2008 and P2011 are different (Figure 2).The beams spectres in the larvae of *G. molesta* focus on separate quadrants on the y-axis whose response explained 90%. The y-axis was responsible for the separation of the populations related to the x and z axis.

Analyzing the raw spectra before applying the statistical model, the two populations meet at a certain point (6997.706 nm), while differentiating at the beginning of the spectrum (Figure 3).

The levels of absorbance and reflectance showed values which can indicate that their organic compositions may be similar or common at these points, reporting that in addition to chemical characteristics which differ across the spectrum, there are some genetic characteristics that are similar.

Discussion

The study showed that population *G. molesta* strains in the laboratory (P2008) presented biochemical and toxicological characteristics which suggest that they are more likely similar to those that suffered phytosanitary pressure in the field (P2011). Although, P2008, which was used in this study, was the same as that used in studies of resistance conducted in 2008 (Siegwart *et al*., 2011), manifested resistance to chlorpyrifos in relation to a reference population (multiplied in the laboratory for more than 10 years), and showed a higher activity of AchE and GST. When the P2008 was mantained in the laboratory for 36

generations, under the influence of the artificial medium, many physiological and behavioral characteristics were affected, similar to those occurring in *Ceratitis capitata* (Diptera: Tephritidae) (Bravo & Neto, 2004) and *Grapholita molesta* (Jones et al., 2011). The P2011 suffers selection pressure by organophosphates insecticides, due to the increase of pesticide applications in relation to the period before their collection. The toxicological analyses showed that the larvae responded to the concentrations of the insecticides chlorpyrifos and carbaryl, but they were more tolerant than the larvae grown in an artificial diet.

Biochemical bioassays showed that P2008 was more susceptible to carbamates and organophosphates relative to P2011. The inhibition of the enzyme is related to the find mechanism of resistance in flies (Berticat *et al*., 2008) and moths (Yu *et al*. 2003, Kanga *et al*., 1997; 2001). GST plays a role in different routes of metabolism, protecting cells against chemical toxicity and stress (Chelvanayagam *et al*., 2001).

The presence of this enzyme is often related to resistance to the insecticide Tortricidae (Reyes *et al*., 2007; 2008). The high number of applications of organophosphate insecticides in the Brazilian orchards (Monteiro *et al*., 2009, Siegwart *et al*. 2011) exerts selection pressure and may feature a contrived example of natural selection (Ffrench-Constant *et al*., 2004).

In the absence of selection pressure by insecticides is an instability of resistance alleles in the population (Kanga *et al*., 2003), and over time, they become susceptible (Orr, 1998); the genotype may have an adaptive disadvantage (Crow 1957). Populations are maintained in the laboratory under pressure of selection of individuals by other factors; in this case, the selection is based on the adaptation of the colony to artificial diets, oviposition, the high density of moths, and multiple mating, in addition to abiotic factors that are always constant in laboratories (Cayol, 2000; Franck *et al*., 2011). Once adapted to the artificial conditions,

factors' extrinsic mortality is almost zero, and the amount of food is not a limiting factor. The selection probably favors individuals with high growth rates (Vargas & Carey, 1989).

Qualitative differences between P2008 and P2011 were found with the technique of near infrared spectroscopy (NIRS). This distinction is found to be related to the structural composition of individuals of each population, such as a chemical signature within each species. In this regard, the infrared analysis allows preliminary diagnosis of populations; more complex analysis before identifying these populations not used as reference laboratory toxicological and biochemical tests in the case of representation of a field population. Further studies allowed the use of NIRS, the analysis of the composition of cuticular hydrocarbons ants (Antonielli *et al*., 2008), and the identification of Zootermopsis species and subspecies of (Aldrisch *et al*., 2007) Eucalyptus species and insect-resistant species (Floyd-foley, 2001). Thus, the NIRS probably considered the influence of the environments in which they are multiplied.

G. molesta populations field was selected by the management techniques used, with less susceptibility to the population maintained in the laboratory.

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Tables and figure captions

Table I. Evaluation of LD_{50} of four chemical insecticides for populations multiplied in the laboratory (P2008) and the field population (P2011) *Grapholita molesta* sampled in apple orchards in the same location.

Population	***Corrected mortality	*χ^2/GL	CL50µg/g (95%)	**TCL_{50}	Slope ±SE
		Chlorpyrifos			
P2011	87.00	0.18/2*	6.84 (5.89-7.83)	8.10	2.17±0.21
P2008	83.30	4.60/4*	0.84 (0.12-5.14)		0.51±0.05
		Tebufenozide			
P2011	75.50	0.10/2	8.27 (6.66-10.05)	0.65	1.45±0.02
P2008	62.50	0.07/2	12.66 (9.95-17.41)		1.15±0.20
		Carbaryl			
P2011	83.30	0.23/2*	10.91 (8.40-14.23)	11.28	1.06±0.01
P2008	59.00	4.74/4*	0.96 (0.12-6.49)		0.04±0.05
		Deltametrin			
P2011	93.50	1.32/2	5.12 (3.09-8.33)	0.74	1.06±0.00
P2008	88.50	1.65/2	6.87 (3.16-15.45)		0.72±0.08

* Indicated significantly lack of fit at p=0.05. Polo plus, uses o factor of heterogeneity factor to calculate of estimate of slope Cl50 and CL95.

** Lethal concentration ratios Cl50 and CL95 between P2008 e P2011

*** Insecticide concentration in ppm in diet based in heighted dosage

Table II. Calibration and validation data for two *G. molesta* populations assessed via the PLS method for NIRS. R^2C calibration; R^2V validation; RMSEC, error calibration; RMSEV, error validation.

Population	R^2C	RMSEC	R^2V	RMSEV	Val Ext.[1]
P2008	0.98	0,0006	96	0,05	0,15
P2011	0.94	0,02	92	0,01	1,04

[1] Extemal validation values. Evaluation was based on 0.1 reference values for P2008 and P2011.

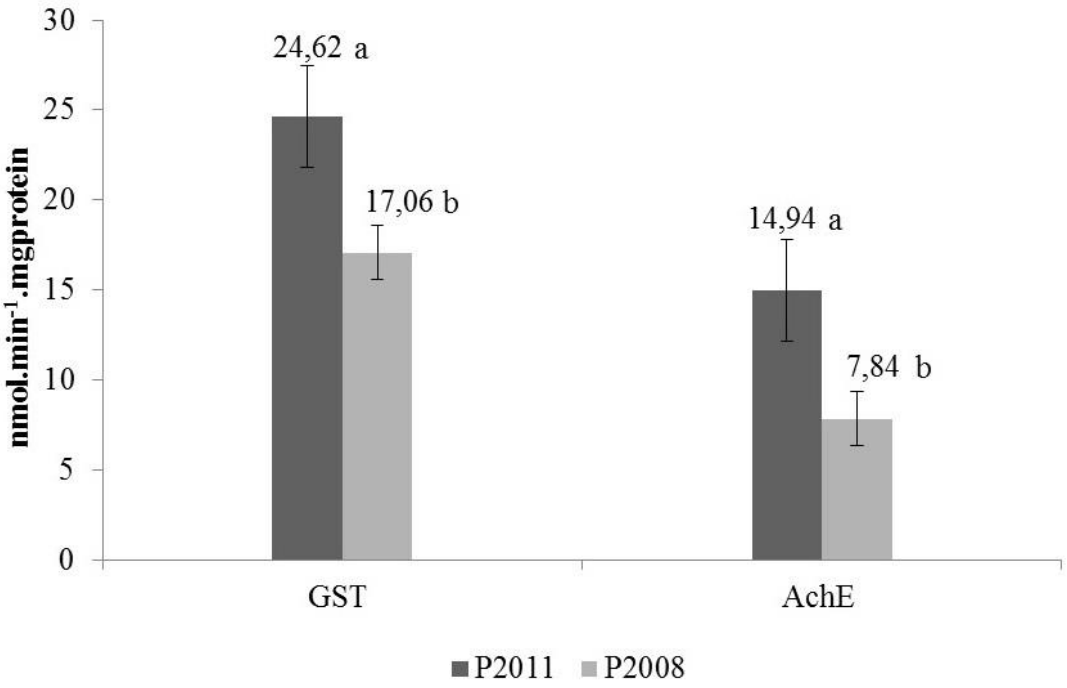

Figure1. Enzymatic activities of Glutathione S-Transferase and Acetylcholinesterase of two populations of *Grapholita molesta*, submitted to a laboratory condition and a wild population.

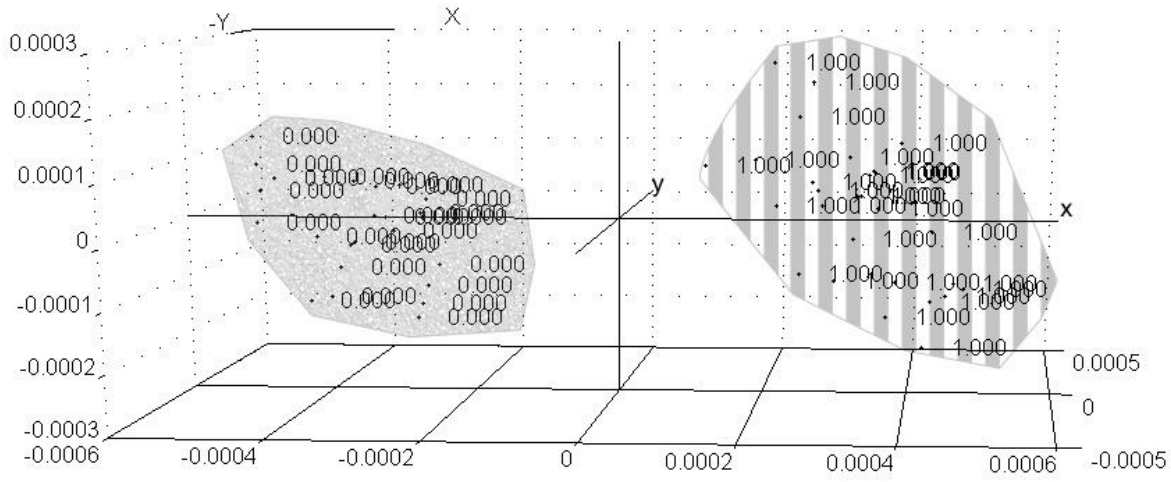

Figure 2. Evaluation of differentiation between two populations of *G. molesta* collected in the same environment, but on different dates. = 0 field population and laboratory population = 1.0. The axis values of the picture displayed for two main components (y = 73%, x = 17 %, z = 10%).

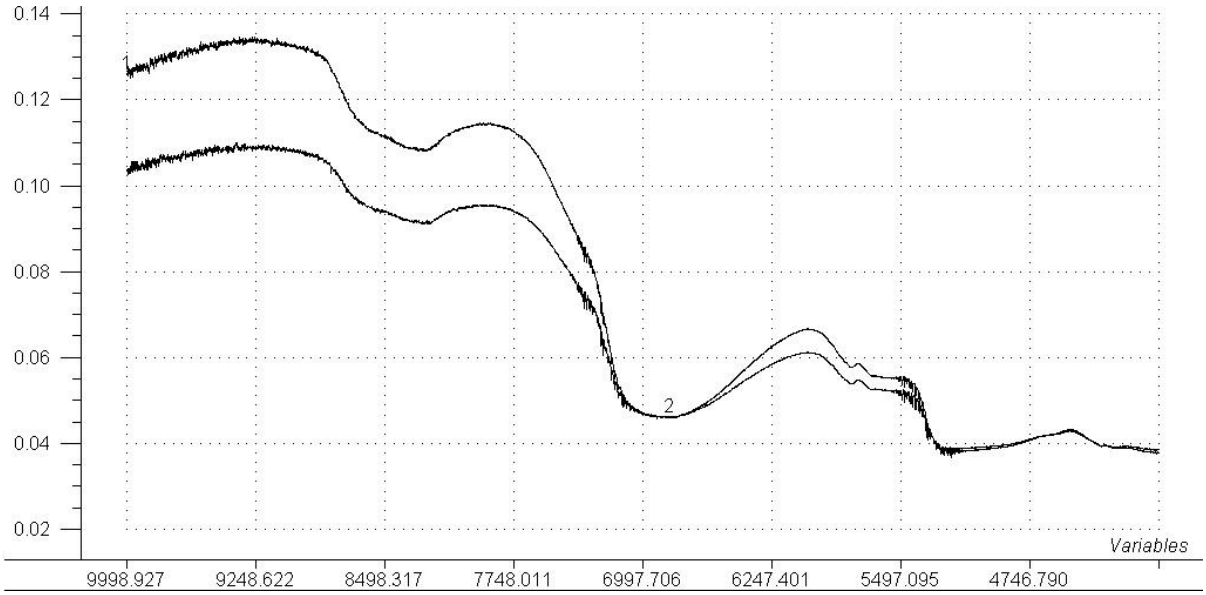

Figure 3. Raw spectra of *G. molesta* populations: 1 = field, 2 = laboratory.

CONSIDERAÇÕES FINAIS

O controle de *G. molesta* em pomares de macieira é baseado em pulverizações de inseticidas em cobertura, principalmente com organofosforado, carbamatos e piretróides, apesar da disponibilidade de outros grupos químicos. Outras pulverizações com metade dos grupos químicos são realizadas para o controle de *Anastrepha fraterculus*. Essa quantidade de ingredientes ativos dos mesmos grupos químicos seleciona populações resistentes a inseticidas.

A resistência a inseticidas já foi detectada em outros estudos, para a mesma praga. No Brasil existem poucos trabalhos realizados que visem à identificação de seleção de resistência comparados a uma população suscetível. Neste caso pode se utilizar a dose diagnóstica, entretanto, esta pode apresentar dados não precisos, devido à variabilidade genética entre os indivíduos coletados influenciados por fatores edafo-climáticos e manejo fitossanitário.

O levantamento fitossanitário permitiu traçar um perfil de alguns pomares de macieira do Sul do Brasil, demostrando que o controle elaborado baseado em calendário, favorece o surgimento de populações tolerantes, como observado neste trabalho no pomar localizado em Vacaria, e que a inclusão de técnicas como a confusão sexual e o monitoramento como base para a aplicação de inseticidas diminui esta seleção de populações em longo prazo, como observado no pomar localizado em Porto Amazonas. As analises toxicológicas e enzimáticas permitiram caracterizar as populações resistentes e estas foram diferenciadas pela técnica de espectroscopia no infravermelho próximo (NIRS). Esta técnica é pouco conhecida na Entomologia Aplicada, e busca-se com ela a identificação de populações de maneira mais rápida e eficaz que técnicas mais laboriosas como análise de DNA. Na identificação de espécie este procedimento já mostrou resultados promissores, em vários taxas. Porém necessita de mais estudos e aperfeiçoamento na sua utilização na Entomologia Aplicada, para a identificação de compostos relacionados à absorção da radiação infravermelha de cada espécie.

Além disso, são necessários estudos complementares envolvendo todas as técnicas de manejo utilizadas no pomar, para um diagnóstico preliminar do perfil dos pomares, visando identificar os aspectos que contribuem para a resistência de espécies de pragas encontradas nestes ambientes, bem como para avaliar a substituição de

produtos químicos tradicionais por produtos ou técnicas com menor impacto ambiental, reduzindo a possibilidade de seleção de indivíduos tolerantes. Aspectos ainda pouco estudados, tais como os estudos genéticos das populações brasileiras, das relações ecológicas e do agroecossistema no geral, necessitam de pesquisas mais aprofundadas. Estes estudos forneceriam subsídios importantes para aperfeiçoar os métodos de controle de *Grapholita molesta*, já disponíveis.

Trabalhos que mostram a quantidade de produtos químicos aplicados no campo são uteis para uma mudança de hábito da população, que com o conhecimento das estratégias utilizadas nos pomares, podem fazer a escolha de consumir um produto com maior qualidade, com baixa aplicação de inseticida ou simplesmente fazer a escolha do produto mais barato no mercado, sem se preocupar com o sistema de produção.