**FRANCYS MARA FERREIRA VILELLA**

# CARACTERIZAÇÃO DE POPULAÇÕES DE *Elasmopalpus lignosellus* E *Spodoptera frugiperda* POR MARCADORES MOLECULARES E SUSCEPTIBILIDADE DESSAS ESPÉCIES ÀS TOXINAS BT E MILHO TRANSGÊNICO

**Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia, para obtenção do título de *Doctor Scientiae.***

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**Defendida em 31 de a gosto de 2001.**

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| **José Henrique Schoereder** | **José Cola Zanucio** |
| **Jorge Abdala Dergan dos Santos (Conselheiro)** | **José Magid Waquil (Conselheiro)** |

Evaldo Ferreira Vilela
(Orientador)

Ao meu Lineu
E a minha mãe

Dedico.

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## BIOGRAFIA

Francys Mara Ferreira Vilella, formou-se em Ciências Biológicas pela Universidade Federal de Viçosa, Viçosa, MG em fevereiro de 1995. Em março do mesmo ano iniciou o curso de Mestrado em Microbiologia Agrícola, na mesma Instituição, concluindo-o em agosto de 1997 e, imediatamente, iniciou o curso de Doutorado em Entomologia na mesma Instituição, participando do Programa de Doutorado com Estágio no Exterior (PDEE/CAPES) na University of Nebraska-Lincoln, Nebraska, USA.

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## RESUMO

VILELLA, Francys Mara Ferreira. D.S., Universidade Federal de Viçosa, agosto de 2001. **Caracterização de populações de *Elasmopalpus lignosellus* e *Spodoptera frugiperda* por marcadores moleculares e susceptibilidade dessas espécies às toxinas Bt e milho transgênico.** Orientador: Evaldo Ferreira Vilela. Conselheiros: José Magid Waquil e Jorge Abdala Dergam Dos Santos.

A lagarta elasmo, *Elasmopalpus lignosellus* (Lepidoptera: Pyralidae), e a lagarta do cartucho, *Spodoptera frugiperda* (Lepidoptera: Noctuidae), são pragas relevantes, particularmente da cultura de milho. O presente estudo objetivou estudar regiões do DNA de *E. lignosellus* e avaliar milhos transgênicos, expressando as toxinas Cry 1 A(b), Cry 9C e Cry 1F, como potencial de controle desta lagarta, além de estudos de tolerância às toxinas de Cry 1 A(b) em *S. frugiperda* avaliando a herdabilidade desta tolerância e diferenciação, por marcadores AFLP, das populações de *S. frugiperda* que apresentaram tolerância e susceptibilidade à toxina avaliada. É possível diferenciar as populações brasileira e americana de *E. lignosellus* com PCR-RFLP e sequenciamento do gene mitocondrial COI. Milhos Bt, expressando as toxinas Cry 1 A(b), Cry 9C e Cry 1F, estão protegidos contra o ataque da lagarta elasmo. *S. frugiperda* mostrou aumento na tolerância à toxina Cry 1A(b) após seleção por quatro gerações e registrou-se um componente hereditário

nesse fenômeno. Os dados de AFLP mostram haver diferença entre as populações tolerantes e susceptíveis.

## ABSTRACT

Vilella, Francys Mara Ferreira. D.S. Universidade Federal de Viçosa, 2001, August. **Population studies and evaluation of Bt transgenic maize for LCB resistance, and selection of FAW for survival on the Cry 1A(b) toxin.** Advisor: Evaldo Ferreira Vilela. Committee Members: José Magid Waquil e Jorge Abdala Dergan dos Santos.

The lesser cornstalk borer (LCB), *Elasmopalpus lignosellus* (Lepidoptera: Pyralidae) and the fall armyworm (FAW), *Spodoptera frugiperda* (Lepidoptera: Noctuidae) are important insect pest. To contribute to managing programs of these insects we propose to study mtDNA region in LCB allowing differentiation on populations from Brazil and USA, and evaluate the efficiency of Bt corn to control this pest. Also, to determine the FAW tolerance to Cry1A(b) toxin and to study the genetic polymorphism of survival of fall armyworm using AFLP technique. The results show that is possible to distinguish LCB populations from Brazil and USA using COI mitochondrial gene by PCR-RFLP and sequencing, and that Bt transgenic maize were able to protect the plants against LCSB damage. Increased tolerance to Cry 1A(b) protein was found in populations of fall armyworm after selection for four generations with Cry 1A(b)toxin. Tolerance of FAW to Cry1 A(b) had a heritage component in the studied generations. The following work, also, describes efforts to detect genetic polymorphisms between survivors.

# INTRODUÇÃO

A lagarta-elasmo, *Elasmopalpus lignosellus* (Zeller) (Lepidoptera: Pyralidae) e a lagarta-do-cartucho, *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae), são pragas importantes, particularmente da cultura de milho. Populações de *E. lignosellus* com resposta contrastantes de machos para armadilhas de feromônio e de *S. frugiperda* para resistência à toxina CRY 1A(b) do *Bacillus thuringiensis* foram submetidas a análise de DNA visando obter marcadores moleculares. A resistência de híbridos de milho transgênico (Bt) à lagarta-elasmo, foi, também, avaliada.

## Prospecção de marcadores moleculares para populações com respostas contrastantes de *Elasmopalpus lignosellus* ao feromônio sexual

Além das gramíneas, a lagarta-elasmo ataca, também, cereais e leguminosas na região Neotropical, tanto na América Latina quanto nos Estados Unidos (Viana, 1981). Está distribuída em todo o território nacional, além de ser também encontrada em áreas dos Estados Unidos, Cuba, Porto Rico, Ilhas Virgens, Trinidad e Tobago, Jamaica, México, Guatemala, Panamá, Venezuela, Guiana Francesa, Paraguai, Uruguai, Argentina e Chile (Neave, 1940 em Tippins, 1982). Seus hospedeiros incluem mais de 60 espécies de plantas cultivadas, incluindo: sorgo,

milho, amendoim, gramíneas em geral, citrus, beterraba, algodão, pêssego, arroz, morango, tomate, trigo e aveia (Tippins, 1982).

Nas gramíneas, o ataque da lagarta elasmo ocorre no interior do colmo, através da abertura de galerias que provocam a morte da planta jovem (Tippins, 1982). Entretanto, tais sinais não são observados em amendoim ou soja, onde os danos são causados, respectivamente, no período de frutificação ou durante toda a fase de crescimento (Tippins, 1982). Esta praga pode, ainda, se alimentar de tecidos meristemáticos e acarretar a destruição da região de crescimento, ou a destruição total ou parcial dos tecidos vasculares responsáveis pela condução de água e nutrientes (Viana, 1981). Segundo Bennett (1962) e Schaaf (1973) os danos da lagarta elasmo são mais intensos em solos arenosos e secos. Apesar das infestações serem esporádicas, elas podem aparecer subitamente e causar danos severos (All et al., 1982).

O controle químico de *E. lignosellus* é difícil em virtude das características de seu comportamento, pois ficam protegidas no interior do colmo da planta durante a alimentação. Por isto, o controle químico é possível, somente, com aplicações preventivas (Pires et al., 1992). Outras formas de controle são através do tratamento de sementes, que nem sempre é eficaz (Waquil & Viana, 1994), e, mais recentemente, pode-se aventar a utilização de plantas transgênicas.

O estudo do comportamento de pragas no campo é importante para programas efetivos de manejo integrado, e a utilização de feromônios é uma estratégia relevante (Vilela & Della Lucia, 2001). Os feromônios são infoquímicos mediadores de interações entre organismos da mesma espécie (ação intraespecífica), produzindo uma resposta comportamental ou fisiológica adaptativamente favorável ao receptor, ao emissor ou a ambos os organismos na interação (Vilela & Della Lucia, 2001). Os feromônios sexuais, principalmente de lepidópteros, têm sido empregados em armadilhas adesivas como atraentes para machos, com o monitoramento de populações de espécies-praga (Pires et al., 1992; Delmore, 1999). O feromônio sexual de *E. lignosellus* foi estudado, sintetizado e aplicado com sucesso para monitoramento dessa praga nos estados norte-americanos da Georgia (Lynch et al., 1984) e da Flórida (Funderburk, 1985), além do México (Loera & Lynch, 1987). No Brasil, Pires et al. (1992) conduziram testes com a composição

americana do feromônio, em populações brasileiras e os resultados mostraram ineficiência na atração de machos de *E. lignosellus* na região de Sete Lagoas (MG). Esta variação de resposta pode estar relacionada a características genéticas da espécie ao longo da sua distribuição. A variação geográfica descreve diferenças fenotípicas que ocorrem ao longo da área de distribuição de uma espécie (Pough et al., 1993). Entretanto, o conceito de espécie é, ainda, controvertido: Futuyma (1992) e Pough et al. (1993) definem espécie, de acordo com a definição de espécie biológica proposta por Ernst Mayr, como sendo os membros em um agregado de um grupo de populações que se intercruzam ou potencialmente intercruzam uns com os outros sob condições naturais (espécies biológicas). O conceito de espécie é, também, considerado uma categoria taxonômica básica à qual espécimes individuais são designados. Amorim (1997) comenta essa última definição de espécie como representando o nível basal na hierarquia de categorias o qual deve receber um binome. Porém, se considerarmos espécie como o último nível basal na categoria taxonômica, como poderíamos considerar subespécie? Subespécie seria uma raça geográfica, um conjunto de populações de uma mesma espécie com uma ou mais características distintas ocupando uma área geográfica diferente de outras subespécies (Futuyma, 1992). Na prática, a determinação taxonômica da espécie e subespécie é difícil, necessitando-se sempre de um especialista que, quase sempre, considera apenas os caracteres fenotípicos morfológicos para a identificação. Amostras brasileira e americana de *E. lignosellus* foram submetidas a Dr. Alma Solis (USDA-ARS, Systematic Entomology Laboratory Beltsville, Maryland, USA - comunicação pessoal), que afirmou que os insetos pertencem a mesma espécie. Marcadores fenotípicos são determinados em parte pelo ambiente e esta influência ambiental é vista como prejudicial pois os caracteres podem ser categorizados incorretamente (Meyer, 1997). Os marcadores morfológicos de *E. lignosellus* não foram eficientes para distinguir as populações americana e brasileira. Então, como diferenciar essas populações? Os melhores caracteres a serem considerados em uma classificação seriam os marcadores moleculares que não são suscetíveis a efeitos ambientais e, portanto, são mais seguros para análise filogenética (Meyer, 1997). A diferenciação populacional de *Ostrinia nubilalis* com marcadores RAPD (*Random Amplified Polymorphism DNA*) mostram ser possível estabelecer padrões

moleculares para diferenciar populações deste inseto nos EUA, tornando possível aplicar-se estratégias específicas de controle para cada população ( Pornkulwat et al. 1998). Estudos sobre o comportamento de insetos-praga, auxiliados por ferramentas de biologia molecular, podem aumentar o conhecimento sobre cada espécie de inseto, auxiliando na geração de novas e efetivas tecnologias de controle de pragas, de modo a contribuir para o avanço necessário à uma agricultura com menos poluição.

As técnicas de biologia molecular são ferramentas poderosas para o estudo da biologia, ecologia e genética de populações de insetos. O marcador pode ser de natureza bioquímica, como isoenzimas, ou de natureza molecular, representado por um segmento de DNA (correspondente a regiões expressas ou não do genoma). A análise de DNA pode fornecer melhor definição quando se quer estudar biótipos, colonização, dispersão e variabilidade intrínseca e extrínseca (Hoy, 1994). A sua aplicação em biologia de populações e ecologia permite a resolução de questões fundamentais com possíveis aplicações no manejo integrado de pragas (Hoy, 1994). O estudo de seqüências de DNA mitocondrial foi utilizado por Brower (1996) para formular hipóteses filogenéticas capazes de explicar a formação de raças paralelas e a evolução do mimetismo em borboletas do gênero *Heliconius*. Silva et al. (1999) utilizaram uma região do rDNA, ITS-2, na diferenciação molecular de cinco espécies de *Trichogramma* em Portugal.

O desenvolvimento de novas técnicas moleculares tronou possível o estudo de variações populacionais, observando-se variações nas seqüências de ácidos nucléicos. Alguns métodos como RFLP ("*Restriction Fragment Length Polymorphism*") e sequenciamento são capazes de detectar mutações de um único nucleotídeo (Parker et al., 1998). Marcadores RFLP se baseiam na geração de fragmentos pela digestão de regiões do DNA com enzimas de restrição que cortam o DNA em seqüências específicas de quatro a seis pares de base e que são detectados por hibridização em Southern blot. Marcadores RFLP podem ser gerados pela amplificação com "*primers*" específicos de determinadas regiões do DNA, como as regiões de DNA mitocondrial, ribossomal e nuclear, e submetidas a corte por enzimas de restrição (PCR-RFLP – "*Polymerase Chain Reaction*"). Os marcadores RFLP permitem detectar o indivíduo heterozigoto e são chamados

marcadores co-dominantes. Em estudos de resistência de insetos esta característica é muito importante. Entretanto, a técnica de RFLP tem as desvantagens de ser muito trabalhosa, ter elevado custo e consumir muito tempo. Assim, PCR-RFLP se mostra como uma alternativa viável para o uso desta técnica, pois elimina a necessidade de hibridização e, consequentemente, a necessidade de sondas e desenvolvimento prévio de bibliotecas genômicas no início dos estudos.

Os marcadores RFLP não são os únicos disponíveis, existem, ainda, os marcadores RAPD, AFLP, microsatélites e aqueles baseados em DNA mitocondrial. O DNA mitocondrial é apropriado para estudos de estrutura de populações e relações entre subespécies, espécies e outras unidades taxonômicas superiores, entretanto, não é recomendando para estudos de exclusão parental uma vez que é, quase sempre, herdado da mãe (Parker et al., 1998). O DNA mitocondrial é uma molécula circular composta por 13 genes que codificam para proteínas, 2 genes ribossomais, 22 tRNA genes e uma região reguladora (Avise, 1994; Crozier & Crozier, 1993; Simon et al., 1994). Os tRNA genes na mitocôndria são conhecidos em uma variedade de ordens de insetos, como Diptera e Hymenoptera (Crozier & Crozier, 1993), e a seqüência completa do genoma mitocondrial é bem conhecida em *Drosophila yabuka* (Diptera: Drosophilidae) (Clary & Wolstenholme 1985 in Hoy 1994) e em *Apis mellifera* (Hymenoptera: Apidae) (Crozier & Crozier, 1993). Uma completa revisão sobre DNA mitocondrial foi sumarizada por Avise (1994) e Simon et al. (1994).

O DNA mitocondrial é uma ferramenta valiosa em estudos de análise filogenética por serem facilmente estudados, herdados como clone, cópia única, não sofrem recombinação e são abundantes (Brown 1985 in Simon et al., 1994; Hillis & Moritz, 1990). O rRNA mitocondrial tem poucos sítios que variam entre espécies muito próximas sendo menos úteis nestes estudo, mas pode ser usado para estudo de divergência em níveis mais profundos (Simon et al., 1994). Os genes mitocondriais mais usados em estudos de filogenia são os genes da citocromo oxidase I (COI), COII e 16S rDNA (Caterino et al., 2000) .

Diferentes regiões do genoma podem estar expostas a diferentes pressões seletivas, dependendo do produto genético formado e/ou tendência do DNA sofrer alterações na sua seqüência gênica, os "*hotspots*" (dePamphilis & Palmer, 1990;

Lewis, 2000). Assim, a escolha de um segmento de DNA para estudo depende não só do grau de relação entre os indivíduos amostrados, mas também do nível de seleção imposta nas diferentes regiões do genoma (Parker et al., 1998).

**Seleção de lagarta-do-cartucho, *Spodoptera frugiperda (Smith),* sobreviventes em toxina do *Bt* (CRY 1A(b))**

A lagarta-do-cartucho, *S. frugiperda* causa danos em diversas culturas, sendo considerada como uma das principais pragas do milho, arroz, sorgo e pastagens (Mitchell et al,. 1985). O potencial reprodutivo de 900-1000 ovos por fêmea, o ciclo de vida em torno de 30 dias e uma grande capacidade de dispersão e migração (Mitchell et al., 1985), tornou *S. frugiperda* uma boa espécie colonizadora. As lagartas alimentam-se das folhas, inicialmente raspando pequenos pontos onde formam janelas translúcidas. A partir deste estágio, a lagarta penetra no cartucho, furando-o em diversos pontos durante a alimentação. Os prejuízos provocados são irremediáveis e, via de regra, seu ataque no início da infestação é localizado na cultura, mas a medida que aumenta sua população, podem ocorrer surtos migratórios com ataque às culturas vizinhas. Em arroz, as lagartas alimentam-se das plantas desde a fase de germinação, consumindo-as até o nível do solo e causando a morte destas.

As táticas de manejo da lagarta-do-cartucho incluem o uso de plantas transgênicas expressando toxinas da bactéria *Bacillus thuringiensis* (Bt). Essa bactéria é comum em solos e produz um cristal contendo proteínas tóxicas à certos insetos, mas inócuas à maioria dos organismos incluindo humanos, animais silvestres e insetos benéficos (Schnepf et al., 1998). Intensos estudos têm sido conduzidos no sentido de demonstrar a inespecificidade desta toxina a animais superiores, incluindo estudos de toxicidade e nenhum caso de doença em humanos ou vertebrados foi reportado nos últimos 15 anos, mostrando que essas toxinas não apresentam efeito tóxico ou patogênico (Rechcigl et al., 2000).

O mecanismo de ação do *B. thuringiensis* envolve a solubilização de cristais protéicos, proteólise destas por proteases transformando-as em toxinas e ligação destas toxinas a receptores nas membranas celulares do intestino médio do inseto criando assim, canais de trocas iônicas nestas membranas. Estes cristais são compostos por pró-toxinas, as quais são ativas apenas após ingestão pelos insetos. Na maioria dos lepidópteros, essas pró-toxinas são solubilizadas em condições alcalinas no intestino médio do inseto (Luthy & Ebersold, 1981; Hofmann et al,. 1988; Tabashnik et al,. 1990: Van Rie et at., 1990; Vachon et al, 1995; Lu & Adang, 1996). Diferenças na taxa de solubilização podem explicar diferenças no grau de toxicidade entre diferentes toxinas (Aronson et al., 1991; Du et al., 1994).

O milho transgênico (Bt) é considerado um importante avanço na tecnologia de controle de lepidópteros em milho. Alguns híbridos de milho expressando a toxina Cry1A(b) têm apresentado níveis de controle satisfatório (Williams et al., 1997, 1998; Buntin et al., 2001), mas, "sobreviventes" de *S. frugiperda* foram observados nestes híbridos (Lynch et al., 1999). Adamczyk & Sumerford (2001) verificaram a existência de aumento da tolerância à toxinas Cry 1A(C) quando esses insetos foram alimentados com algodão transgênico.

Um dos fatores mais importantes no estabelecimento de sistemas de manejo baseados no uso de plantas transgênicas é o estudo do nível de suscetibilidade e tolerência de populações de *S. frugiperda* as toxinas do Bt. Isto possibilita a detecção e o monitoramento da suscetibilidade/resistência de populações de *S. frugiperda* à essas toxinas, possibilitando o uso de táticas adequadas ao manejo da variabilidade das populações deste inseto-praga.

A evolução da resistência dos insetos às toxinas do Bt é uma das mais sérias características a serem consideradas no uso destas toxinas como métodos de controle de pragas. Estudos de seleção em laboratório têm produzido lepidópteros resistentes a essas toxinas, e no campo, o primeiro inseto a apresentar resistência à toxina do Bt foi *Plutella xylostella* (Tabashnik, 1994). Apesar da importância econômica e ecológica dos lepidópteros-praga, pouco se conhece sobre a genética da maioria dos lepidópteros (Heckel et al., 1999), especialmente a genética dos mecanismos relacionado a resistência às toxinas do Bt. Heckel *et al.* (1999)

desenvolveram um mapa genético, com marcadores AFLP, em *P. xylostella* para resistência à toxinas do *B. thuringiensis.*

A técnica de AFLP (*Restriction Fragment Length Polymorphism*) foi descrita por Vos et al. (1995) e se baseia na detecção de fragmentos de restrição por amplificação por PCR. Esta técnica envolve três passos básicos: I) restrição do DNA e ligação dos nucleotídeos adaptadores, II) amplificação seletiva e, III) análise em gel de poliacrilamida dos fragmentos amplificados. A amplificação por PCR dos fragmentos de restrição é conseguida com os adaptadores e os sítios de restrição como alvos para o anelamento dos *primers*. A amplificação seletiva é conseguida com *primers* que prolongam os fragmentos de restrição, amplificando apenas aqueles fragmentos nos quais a extensão do *primer* coincide com os nucleotídeos que flanqueiam os sítios de restrição.

As vantagens dessa técnica são: I) fragmentos de restrição podem ser visualizados por amplificação por PCR sem conhecimento prévio da seqüência nucleotídica, II) o número de fragmentos amplificados que podem ser analisados simultaneamente é alto, aproximadamente 50 a 100 fragmentos são amplificados e detectados no gel de poliacrilamida (Vos et al., 1995). Esta técnica é, ainda, pouco utilizada em entomologia, mas estudos de desenvolvimento de mapa genético para o estudo de resistência de *P. xylostella* à toxinas do Bt toxins (Heckel et al., 1999), de diferenciação populacional em *S. frugiperda* (McMichael & Prowell, 1999) e em *Lymantria dispar* (Reineker et al., 1999) foram realizados com essa técnica

Assim, este trabalho teve como objetivos: I) estudar regiões do DNA de *E. lignosellus* para auxiliar na diferenciação deste inseto e contribuir para o conhecimento das relações das diversas populações de *E. lignosellus;* II) avaliar a resistência de milhos tansgênicos expressando as toxinas Cry 1 A(b), Cry 9C e Cry 1F a essa lagarta: III) realizar estudos de tolerância às toxinas de Cry 1 A(b) em *S. frugiperda* e avaliar a herdabilidade desta tolerância, IV) diferenciar, através de marcadores moleculares AFLP, as populações de *S. frugiperda* com tolerância e susceptibilidade à toxina avaliada no item III.

Os capítulos seguintes representam os resultados destes trabalhos seguindo as normas gerais de publicação da revista Florida Entomologist.

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# MITOCHONDRIAL DNA VARIATION WITHIN LESSER CORNSTALK BORER (LEPIDOPTERA: PYRALIDAE) POPULATIONS

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ABSTRACT

The lesser cornstalk borer (LCB) *Elasmopalpus lignosellus* (Zeller) (Lepidoptera: Pyralidae) is a major insect pest of maize seedlings in the tropics. This insect is also very important to other cereal and leguminous crops in Latin and South America and United States. Considering the limitation to management of this specie with traditional methods, the use of pheromone traps is one alternative to improve insect management. Field trapping LCB with synthetic sex pheromone was succesfully used in the USA, but this success was lower in Brazil. The objective of this study was to investigated the genetic differences between brazilian and USA LCB populations because no morphological differences were reported. LCB populations collected in Brazil and USA were analysed using a portion of the mtDNA region, cytochrome oxidase (COI). Analysis indicated that populations from Minas Gerais, Brasil and Georgia, USA are genetically different.

Key word: *Elasmopalpus lignosellus*, phylogeny, mtDNA, sex pheromone.

**RESUMO**

A lagarta elasmo, *Elasmopalpus lignosellus* (Zeller) (Lepidoptera: Pyralidae) é o principal inseto-praga de plântulas de milho nos trópicos, e também causa danos em outros cereais e leguminosas na América Latina e nos Estados Unidos. As limitações do manejo dessa espécie, torna importante o uso de feromônios como estratégia para melhorar esse manejo. A coleta massal de machos, dessa espécie no campo, com feromônio sexual é feito com sucesso no estado da Georgia, USA, porém, esta eficiência foi baixa no Brasil. Assim, a variabilidade genética de populações de *E. lignosellus* do Brasil e dos USA foi estudada utilizando-se o

gene citocromo oxidase (COI) na região do DNA mitocondrial. Análises mostraram diferenças entre as populações brasileira e americana.

Palavra-chave: *Elasmopalpus lignosellus*, filogenia, lagarta-elasmo, mtDNA, feromônio sexual.

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The lesser cornstalk borer (LCB), *Elasmopalpus lignosellus* (Zeller) (Lepidoptera: Pyralidae), is a major pest of maize, sorghum, rice, wheat and sugarcane seedlings in the tropics, especially on upland areas where, under dry conditions, damage can be total right after plant emergence, but such damage can be reduced by irrigation (Viana & Costa, 1995). This insect is also very important pest in peanuts where it causes the highest damage during the fruiting period, and in soybeans it represents a serious threat throughout the growing season (Tippins, 1982). The LCB is widely distributed in Brazil, USA, Bermuda, Cuba, Puerto Rico, the Virgin Islands, Trinidad and Tobago, Jamaica, México, Guatemala, Panama, Venezuela, French Guiana, Paraguai, Uruguai, Argentina and Chile (Neave 1940 in Tippins, 1982). At least 60 crop species in 14 families are reported as hosts of the LCB, including: alfafa, beets, beans, citrus, corn, oats, peach, sorghum, strawberries, wheat and others (Tippins, 1982).

LCB damage occurs at about the four leaf (seedling) stage, when larvae tunnel into stems causing the classical symptom known as “dead heart”. Generally, seed treatments are used to control this species, but this treatment is not always effective (Waquil & Viana 1994) and insecticide is also necessary (Pires et al. 1992). Seed corn companies offer a variety of corn hybrids with capability of producing the insecticide protein *Cry-protein,* of the soil bacterium, *Bacillus thuringiensis* (Bt) (Wright, 2000). Thus, transgenic

plants provide a new approach to control this insect pests (Vilella et al. 2001).

Studies of insect behavior in field conditions is important for Integrated Pest Management programs, which includes pheromones (Vilela & Della Lucia, 2001). Lynch et al. (1984) isolated and identified the LCB sex pheromone components and conducted studies of field trapping in Georgia, with successful collection of males. Similar results were obtained in Florida (Funderburk, 1985) and Mexico (Loera & Lynch, 1987), but field trapping of LCB males using this synthetic pheromone, was not succesful carried out in Minas Gerais State, Brazil (Pires et al., 1992). This can be due to population genetic variation. Pornkulwat et al. (1998) studying different populations of *Ostrinia nubilalis* showed different populations, these variations can explain why this insect has developed strategies to resist to various control strategies in the USA.

The development of new molecular techniques allows to study variation in nucleic acid sequences. Methods such as RFLP and sequencing are capable to detect single nucleotide mutations. A common use of RFLP in entomology is the PCR-RFLP associated with mtDNA, rDNA or genomic DNA where specific regions are amplified and digested with restriction enzymes (Simon et al., 1994; Brower & DeSalle, 1994).

Systematic studies have been done mostly using mitochondrial DNA because they are easy to manipulate, are clonally inherited, single-copy, nonrecombining and are abundant (Brown 1985 in Simon et al., 1994, Hillis & Moritz, 1990). Additionally, universal primers have been developed from conserved mtDNA regions that amplify specific sections of mtDNA from multiple insect taxa providing templates for comparative study (Simon et al. 1994). The main goal of this study was to look for genetic differences in LCB populations from Brazil and the USA using mtPCR-RFLP and sequencing.

## MATERIAL AND METHODS

The study was conducted at the Department of Entomology and Biotecnology Center Applied to Agriculture (BIOAGRO), at Federal University of Viçosa, MG, Brazil and at the Department of Entomology, University of Nebraska, Lincoln, NE, USA.

Origin of Specimens:

Specimens studied were obtained from different regions in Brazil and the USA. In Brazil, samples were collected in the States of Minas Gerais, Rio Grande do Sul, São Paulo e Góias. These insects were kindly collected and supplied by Dr. José Roberto Salvadori, Embrapa Trigo, Passo Fundo, RS, Br; Dr. Evane Ferreira e José Alexandre Freitas Barrigossi, Embrapa Arroz e Feijão, Goiânia, GO., Br.; Prof. Dr. Fernando Mesquita Lara, Departamento de Entomologia e Nematologia - UNESP-Campus de Jaboticabal, Jaboticabal, SP, Br.; Dr. Paulo Viana, Núcleo de Manejo de Fatores Bióticos, Embrapa Milho e Sorgo, Sete Lagoas, MG, Br.; and Dr. Robert E. Lynch, USDA-ARS-CPMRU, in Tifton GA, USA.

LCB DNA extraction:

Insect DNA extraction was performed following the protocol described by Black and Duteau (1997) with minor modification.

PCR Amplification:

A portion of the mitochondrial cytochrome oxidase subunit I (COI) was amplified using universal primers from Simon et al.(1994) TL2-J-3037 (5´ATGGCAGATTAGTGCAATGG3´) and TK-N-3785 (5´GTTTAAGAGACCAGTACTTG3´). Gene amplification was performed in 25 µl reaction volume containing 10.75 µl sterile dd$H_2O$, 10Mm Tris-HCl (pH 8.3), 0.25 µl of AmpliTaq®DNA Polymerase (10 U/µl), 0.8 µM of each primer, 3

µl of 10 mM dNTPs (2.5 mM de cada dATP, dTTP, dCTP, dGTP), 3.5 µl of 25 mM $MgCl_2$, 3.0 µl of template DNA.

PCR reactions were amplified using Gene Amp® PCR System 2400 (Perkin-Elmer, Branchburg, NJ) with the following temperature profile: 94°C for 2 min.; 35 cycles of 94°C for 1 min., 52°C for 30 seg. e 72 °C for 1:30 min; and a final extension step for 7 min. All amplifications had a negative control containing no DNA template. PCR products (3 µl) were loaded onto 1.0% agarose TBE (0.089 M Tris, 0.089 M boric acid, 0.5 M EDTA – pH 8.0)] gels. After eletrophoresis (50 V for 2 h.), PCR amplicons were visualized over a UV transilluminator and scanned into the Advanced Quantifier Gel Documentation Program (Genomic Solutions, Ann Arbor, MI).

Before sequencing a previous screening was performed using four 6-pb recognition site restriction endonucleases (AlaI, MseI, MspI, DpnII) and six 6-bp recognition site restriction endonucleases (BanII, Ecl 136II, PstI, PvuI, PvuII) following the manufacturers protocol (New England Biolabs, Beverly, MA). The reaction mixture was then incubated at 37°C for 5 hours in PCR reaction tubes on a Gene Amp® PCR System 2400 (Perkin-Elmer, Branchburg, NJ). Digested PCR amplicons were fractionated on 10% polyacrylamide (Sambrock et al. 1989) and stained with ethidium bromide.

Sequencing

PCR products were cleaned from agarose using QIA quick™ Spin Kit for PCR Purification. The DNA was sequenced using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) in the ABI PRISM 310 Genetic Analyzer (Applied Biosystems). PCR reaction products were amplified using Gene Amp® PCR System 2400 (Perkin-Elmer, Branchburg, NJ) with the following temperature profile: a hold of 96°C for 2 min.; 30 cycles of 96°C for 30 sec., 52°C por 20 seconds and 60°C for four minutes). The sequencing reaction was done in 20µl with 8 µl of “mix” (ABI PRISM

BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, 10 pmoles of each primer, 200 a 500 ng of DNA. The PCR products were purified and precipitated using isopropanol 75% and let dryed at room temperature. The pellet was resuspended in bromophenol blue: formamide (5:1) denatured at 95°C for 5 min. The extension products were run into 5% polyacrylamide electrophoresis gel in ABI PRISM 277 Genetic Analyzer (Applied Biosystems). The data were automatically turned into deoxynucleotides. The sequences were aligned using DNASTAR program (DNASTAR Inc.).

## Results And Discussion

It is possible to distinguish LCB populations from Brazil and the USA using COI mitochondrial gene by PCR-RFLP and sequencing (figure 1). Positive results using with this genomic region were found by Clark (2000) in *Diabrotica* spp. This genomic region is the final portion of cytochrome oxidase subunit I (COI), and in LCB is composed by approximately 650 bp, this region is larger in *Ostrinia nubilalis*, with 1600 bp (Gene Bank) and in *Diabrotica* sp., 1308 bp (Clark, 2000). No difference in amplification was found for LCB adult or larvae.

In the last three decades with much advances in PCR techniques, development of automated sequencing and greatly increased computer power, areas in biology including entomology have reached better levels of understanding questions. In entomology areas such as phylogeny and population studies, molecular techniques have been frequently used (Avise, 1994; Black IV & Duteau, 1997; Caterino et al., 2000; Hillis & Moritz, 1990; Hoy, 1994; Parker et al. 1998; Simon et al., 1994; Whitten, 1986.) The database of insect sequences is increasing rapidly, GenBank has about 100,000 insect sequences. Although 80,000 of these are *Drosophila* sequences, a substantial fraction of them have been used in phylogenetic studies(Caterino et al. 2000). But, there is no

record related to LCB in GenBank. Even though a lot has been done, scientists have still a long way to go in areas such as ecology, insect resistance and physiology, in special, gene expression.

In ten mtDNA primers combinations tested in this study only three combinations worked for LCB, but all of them amplified successfully *Spodoptera frugiperda (*Lepidoptera: Noctuidae) fragments. No amplification in the COI region in LCB samples from GO (Figure 2) was recorded which may be explained by a mutation on the priming region of this population. This shows that it is necessary to study another mitochondrial primer combinations which can differentiate these biotypes.

The COI region was the only one who presented polymorphic bands when the PCR product was submitted to endonucleases restriction. This assay was done in 30 insect samples from Sete Lagoas and Georgia, polymorphic bands were obtained from the PCR product cut with the DpnII and Ecl 136II restriction enzymes. After, PCR product from insects collected in SP, MG, RS and USA were sequenced (Figure 2). The sequencing pattern allowed the construction of a dendogram to separate LCB biotypes (Figure 1). The sequence differences were restricted to the $1^{st}$ or $3^{rd}$ base in codon positions and do not compromise protein activity. LCB populations from MG and USA are the more distant group (3.7 % difference) while the USA and SP populations are relatively more similar (3.3% difference) (Table 1).

It is difficult to state if selective pressure could explain such differences in LCB populations and the absence of amplified band in GO population. But host plant effect may play a part in this differentiation. Population divergence studies related to the colonized host have been carried out by McMichael & Prowell (1999), using AFLP markers these authors established differences between *S. frugiperda* populations collected from corn and rice cultures. The MG and USA LCB populations were collected in laboratory colonies

originally founded by individuals collected in maize and peanuts. The SP populations were collected in maize and RS populations in soybean while GO populations was undetermined. LCB in the USA is mainly collected in peanuts, although this specie feed on other crops. Besides corn and soybean, peanuts is still cultivated in SP where it reached its production peak in the 60's for oil production for human use, but today it is mainly used by sweet factories and grain trade. This pattern of production may be one of the factors that contribute to maintain high levels of similarity between populations from SP and USA. Our result clustered Brazilian populations as the most similar, while the American populations would be the most deviant, which is consistent with their geographic distribution. This pattern of divergence is also consistent with dissimilar results of success in field trapping collection, which was efficient in USA, but failed in MG. Based on the almost entirely Neotropical pattern of distribution we may hypothesize that if South America is the geographic origin of LCB, this continent would harbor the highest genetic variation of the species, while the group that occurs in USA represents an extreme of the distribution range which it may by chance preserve higher similarity with insects from São Paulo. It is unclear whether the USA population have established via migration or expanded the species range as a result of human activities. Those hypotheses may be further tested by more encompassing analyses including historic records and with the use of other markers.

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Table 1: Pair Distances of LCB populations from Rio Grande do Sul (RS), São Paulo (SP), United States of America (USA) and Minas Gerais (MG) compared to *Ostrinia nubilalis*. Percent similarity in upper triangle. Percent divergence in lower triangle

| | RS | SP | USA | MG | *Ostrinia nubilalis* | |
|---|---|---|---|---|---|---|
| RS | *** | 98.3 | 95.9 | 83.6 | 85.2 | RS |
| SP | 0.7 | *** | 97.5 | 82.8 | 85.4 | SP |
| USA | 0.7 | 0.3 | *** | 84.2 | 85.4 | USA |
| MG | 3.3 | 3.3 | 3.7 | *** | 73.8 | SL |
| ON | 13.0 | 12.7 | 12.4 | 15.1 | *** | *Ostrinia nubilalis* |
| | RS | SP | USA | MG | *Ostrinia nubilalis* | |

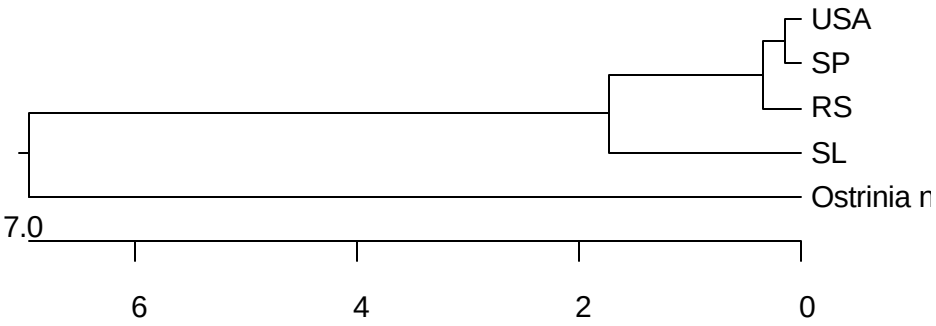


Figure 1: Dendogram separating LCB biotypes from United States (USA), São Paulo (SP), Rio Grande do Sul (RS), Minas Gerais (MG) and *Ostrinia nubilalis* (out group)

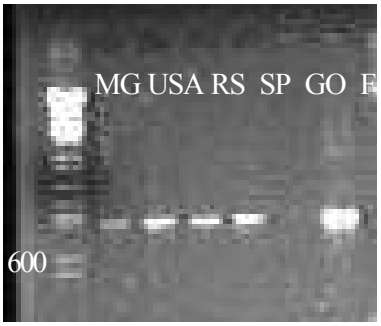


Figura 2: Gel showing mtDNA product from Minas Gerais (MG), United States (USA), Rio Grande do Sul (RS), São Paulo (SP), *Spodoptera frugiperda* (FAW), and absent band in Góias (GO) populations, using the COI primer.

```
          1                                                          60
RS      -AAACCCC-TTTATAAAGGATTTATCCTTTTTTTAGAAATTGCAACATGATCAAATTTAA
SP      -AAACCCC-TTTATAAAGGATTTATCCTTTTTTTAGAAATTGCAACATGATCAAATTTAA
USA     CAAACCCCATTTATAAAGGATTTATCCTTTTTTTAGAAATTGCAACATGATCAAATTTAA
MG      -AAACCCC-TTTATAAAGGATTTGTCCTTTTTCTAGAAATKGCAACATGATCAAATTTAA
O.Nu    -AAACCCCATTTATAAAGGATTTATCCTTTTTTTAGAAATAGCAACTTGATCTAATTTTA

         61                                                        120
RS      ATCTTCAAAATAGAGCTTCTCCTTTAATAGAACAAATTATTTTTTTCCATGACCATACTT
SP      ATCTTCAAAATAGAGCTTCTCCTTTAATAGAACAAATTATTTTTTTCCATGACCATACTT
USA     ATCTTCAAAATAGAGCTTCTCCTTTAATAGAACAAATTATTTTTTTCCATGACCATACTT
MG      ATCTTCAAAATAGAGCTTCTCCTTTAATAGAACAAATTATTTTTTTCCATGACCATACTT
O.Nu    GTCTACAAAATAGAGCCTCTCCATTAATAGAACAAATTATTTTTTTTTTATGATCATACAT

        121                                                        180
RS      TAGTTATTCTAATTATAATTACTATTTTAGTAGGATATTTAATAATTAATTTATTTTTTA
SP      TAGTTATTCTAATTATAATTACTATTTTAGTAGGATATTTAATAATTAATTTATTTTTTA
USA     TAGTTATTCTAATTATAATTACTATTTTAGTAGGATATTTAATAATTAATTTATTTTTTA
MG      TAGTTATTCTAATTATAATTACTATTTTAGTAGGATATTTAATAATTAATTTATTTTTTA
O.Nu    TAATTATTTTAGTTATAATTACAATTTTAGTAGGATATTTAATAATAAGATTATTTTTTA

        181                                                        240
RS      ATAAATATATTAATCGATTTTTATTAGAAGGACAAATAATTGAACTAATTTGAACTATCC
SP      ATAAATATATTAATCGATTTTTATTAGAAGGACAAATAATTGAACTAATTTGAACTATCC
USA     ATAAATATATTAATCGATTTTTATTAGAAGGACAAATAATTGAACTAATTTGAACTATCC
MG      ATAAATATATTAATCGATTTTTATTAGAAGGACAAATAATTGAACTAATTTGAACTATCC
O.Nu    ATAAATATATTAATCGATTTCTACTTGAAGGTCAAATAATTGAATTAATTTGAACAATTA

        241                                                        300
RS      TTCCAGCTATTACTTTAATTTTCATTGCTCTTCCATCTTTACGATTATTATATTTATTAG
SP      TTCCAGCTATTACTTTAATTTTCATTGCTCTTCCATCTTTACGATTATTATATTTATTAG
USA     TTCCAGCTATTACTTTAATTTTCATTGCTCTTCCATCTTTACGATTATTATATTTATTAG
MG      TTCCAGCTATTACTTTAATTTTCATTGCTCTTCCATCTTTACGATTATTATATTTATTAG
O.Nu    TTCCAGCTATCACATTAATCTTTATTGCTTTACCATCACTTCGATTATTATATTTACTAG

        301                                                        360
RS      ATGAATTAAATAATCCTTTAATTACCTTTAAAATCAATTGGACATCAATGATATTGAAGT
SP      ATGAATTAAATAATCCTTTAATTACCTTTAAAATCAATTGGACATCAATGATATTGAAGT
USA     ATGAATTAAATAATCCTTTAATTAC-TTTAAAATCAATTGGACATCAATGATATTGAAGT
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MG      ATGAATTAAATAATCCTTTAATTAC-TTTAAAATCAATTGGACATCAATGATATTGAAGT
O.Nu    ATGAATTAAATAAACCATTAATTACATT-AAAATCAATTGGACATCAATGATATTGAAGT

        361                                                       420
RS      TATGAATATTCTGATTTTAATAA-TATTGAATTTGACTCTTATATAATTCCTAT-AAATG
SP      TATGAATATTCTGATTTTAATAA-TATTGAATTTGACTCTTATATAATTCCTAT-AAATG
USA     TATGAATATTCTGATTTTAATAAATATTGAATTTGACTCTTATATAATTCCTAT-AAATG
MG      TATGAATATTCTGATTTTAATAATATTGGAATTTGACTCTTATATAATTCCTATTAAATG
O.Nu    TATGAATATTCAGATTTTAATAA-CATTGAATTTGATTCTTATATAACCCCTAT-AAATG

        421                                                       480
RS      AAATATCCCCAAATAATTTTCGTCTTTTAGATGTTGATAATCGAATTATTTTACCTATAA
SP      AAATATCCCCAAATAATTTTCGTCTTTTAGATGTTGATAATCGAATTATTTTACCTATAA
USA     AAATATCTCCAAATAATTTTCGTCTTTTAGATGTTGATAATCGAATTATTTTACCTATAA
MG      AAATATCTCCAAATAATTTTCGTCTTTTAGAAGTTGATAATCGAATTATTTTACCCATAA
O.Nu    AAATAAATAAAAATAATTTTCGTCTTTTAGAAGTAGATAATCGAATTATTTTACCAATAA

        481                                                       540
RS      ATAATCAAATTCGAATTATAGTTACAGCAACAGATGTCATCCATTCTTGAACTATTCCTT
SP      ATAATCAAATTCGAATTATAGTTACAGCAACAGATGTCATCCATTCTTGAACTATTCCTT
USA     ATAATCAAATTCGAATTATAGTTACAGCAACAGATGTCATCCATTCTTGAACTATTCCTT
MG      ATAATCAAATTCGAATTATAGTTAC-GCAACAGATGT-AT-CATTC-TGAACTYTTYCGT
O.Nu    ATAACCAAATTCAAATTATAGTTACCGCAACAGATGTTATTCATTCATGAACTATCCCAT

        541                                                       600
RS      CTTTAGGAGTTAAAGTTGATGCTAACCCCGGTCGATTAAATCAAACTAGTTTTTATATGA
SP      CTTTAGGAGTTAAAGTTGATGCTAACCCCGGTCGATTAAATCAAACTAGTTTTTATATGA
USA     CTTTAGGAGTTAAAGTTGATGCTAACCCCGGTCGATTAAATCAAACTAGTTTTTATATGA
MG      CTTTAGGAGTTAATATTGGKGCTAACCCCGGTCGATTAAATCAAACTAGTTTTTATATAA
O.Nu    CATTAGGAGTAAAAGTAGATGCTAATCCAGGTCGCCTAAATCAAACTAATTTTTTTATTA
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Figure 3: COI region sequenced in LCB samples of Rio Grande do Sul (RS), São Paulo (SP), United States(USA), Minas Gerais(MG) and *O. nubilalis* (out group)

# EVALUATION OF BT TRANSGENIC MAIZE FOR THE LESSER CORNSTALK BORER (LEPIDOPTERA: PYRALIDAE) RESISTANCE

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## ABSTRACT

Two experiments were conducted to determine if maize seedlings from transgenic Bt hybrids are protected against lesser cornstalk borer (LCB). In the first experiment, the maize hybrids P33G27, P34R07, P34G82, P33A14 and P34D34 expressing Bt toxin Cry 1 A(b), G8539 expressing Cry 9C toxin and G8539 (non-Bt) as a control were tested at the third and fourth leaf growth stages. In the second experiment, using only one plant growth stage (3 leaf stage), the Bt hybrids Mycogen (Cry 1F), P 33G27 (Cry 1 Ab) and two Garst 8539 isolines (Cry 9C and non-Bt) were tested at four temperatures during the day time (20; 24; 28 and 32 ± 0.7$^{o}$C) and just one night temperature (20 ± 0.7$^{o}$C). Larval survival, larval weight, damage score and number of dead plants showed a high resistance level of the Bt transgenic maize. This resistance was not affected by daytime temperature. Bt transgenic maize were able to protect the plants against LCB damage regardless of plant age and temperature.



Key word: *Elasmopalpus lignosellus*, pest management, insect resistance, temperature, *Zea mays*



## RESUMO

Dois experimentos foram conduzidos para determinar se plântulas de milho transgênico estão protegidos contra a lagarta-elasmo. No primeiro, os híbridos de milho Bt P33G27, P34R07, G85.39, P34G82, P33A14, P34D34 e as duas versões do híbrido G8539 (Cry 9C e não-Bt) foram testados em dois estágios de desenvolvimento (3 e 4 folhas). No segundo, os híbridos Bt Mycogen (Cry 1F), P 33G27 (Cry 1Ab) e as duas versões do híbrido Garst 8539 foram avaliados sob quatro temperaturas (20; 24; 28 e 32 ± 0.7 $^{o}$C). Cada plântula foi artificialmente infestada com duas larvas de elasmo (2$^{o}$/3$^{o}$ instar). Para cada experimento, as plantas foram avaliadas

uma semana após a infestação contando-se o número de plantas mortas. No primeiro experimento, foi determinado, ainda, o número e peso das larvas sobreviventes e estimou-se os danos através de notas. Os resultados não mostraram diferença significativa entre os híbridos transgênicos para todas as variáveis observadas. Entretanto, houve diferença significativa entre os híbridos transgênicos e o controle (não-Bt). A idade das plantas e a temperatura não afetaram os parâmetros observados.

Palavras-chave: *Elasmopalpus lignosellus*, manejo integrado de pragas, Lagarta-elasmo, resistência, plantas transgênicas, temperature, *Zea mays*

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The lesser cornstalk borer (LCSB), *Elasmopalpus lignosellus* (Zeller) (Lepidoptera: Pyralidae), is a major pest of maize seedlings in the tropics, especially in dry upland areas but its damage can be reduced by irrigation (Viana & Costa, 1995). This insect is also very important to cereal and leguminous crops in Latin and South America and the United States. LCB damage begins when corn plants reach four leaf (seedling) stage, when its larvae tunnel into stems causing what is classically known as “dead heart”. Generally, seed treatments are used to control this species, but this treatment is not always effective (Waquil & Viana 1994).

Seed corn companies offer a variety of corn hybrids transformed to produce the insecticidal protein (Cry-protein) from the naturally occurring soil bacterium, *Bacillus thuringiensis* (Bt) (Wright, 2000). Thus, transgenic plants provide a new approach for controlling lepidopteran insect pests.

The objective of this study was to determine if B*t* genes expressing protein toxins in maize plants would protect seedlings against LCSB damage and whether different daytime temperatures affect insect-plant interactions.

## MATERIAL & METHODS

This study was conducted at the Department of Entomology, University of Nebraska, Lincoln, State of Nebraska, USA. Seed samples of the Bt maize hybrids P33G27, P34R07, P34G82, P33A14, and P34D34 expressing the toxin Cry 1A(b) were obtained from the market and the hybrid Garst 8539 expressing Cry 9C and the control hybrid Garst 8539 (non-Bt) were obtained directly from the seed producer. Insects used to artificially infest maize seedlings were obtained from the LCB colony maintained by the USDA-ARS-CPMRU, in Tifton Georgia. Infested seedlings were held in environmental chambers and two experiments were developed.

Bt maize resistance to LCB:

This study consisted of seven maize hybrids tested at two plant growth stages (third and fourth leaf) at a temperature of 27 ± 0.7$^{o}$C and photophase of 14 h light: 10 h darkness. The experimental unit consisted of nine cells (one seedling/cell) filled with an artificial growth medium (seedling starter), with each treatment replicated three times in a completely randomized design. Soil in each pot was covered with a layer of vermiculite after plant emergence and before hand infestation with insects to facilitate larval establishment. Each seedling was infested with two larvae (second/third instar) per plant. One week after infestation, when susceptible maize lines were showing more than 80% “dead heart” symptoms, the experiment was evaluated by taking the weight and number of surviving larvae, number of undamaged plants, and by scoring the plants using a progressive visual damage score (Table 1).

Temperature effect on resistance of Bt maize:

Six seeds of each three Bt maize hybrids, Mycogen (Cry 1F), P 33G27 (Cry 1Ab) and Garst 8539 (Cry 9C) were sown in 500 ml pots filled with an artificial growth medium. The the hybrid Garst 8539 (non-Bt version) was used as control. After germination, the plants were thinned to five plants/pot. Each treatment was replicated three times. Plants were maintained in growth chambers regulated at constant temperatures (20$^{o}$C, 24$^{o}$C, 28$^{o}$C and 32$^{o}$C). Infestation and evaluation methods were the used in the previous experiment, except the larval variables which were not observed.

Data were subjected to analysis of variance by MSTAT program and means were compared using Duncan's Multiple Range Test (DMRT). Number of dead plants was analyzed for both experiments. Weight and number of surviving larvae per 10 plants and plant visual damage score were also analyzed in the first experiment.

## Results And Discussion

No significant differences ($P \leq 0.05$) among the Bt transgenic hybrids in the number of the lesser cornstalk borer (LCB) larvae per 10 plants, larval weight, visual damage score or number of dead plants were observed. However, significant differences ($P \leq 0.05$) between the Bt and the non-Bt maize hybrids for all observed variables were found.

### Bt maize resistance to LCB:

The analysis of variance showed a effect (P  0.05) of hybrids, but no effect (P  0.05) of plant age or plant age/hybrid interaction for all variables. Also, no differences (P  0.05) among transgenic hybrids were found, but difference (P  0.05) between the Bt maize and the non-Bt control were observed. The number and weight of surviving LCB larva are (Fig.1) showed a high correlation (r= 0.991).

Although few larvae survived on Bt maize, they weight less than those of non-Bt maize. However, no difference (P  0.05) on weight of larvae among Bt maize hybrids was found. A significant reduction on number of survivors, caused by Bt toxin, was reported to LCB on transgenic sugarcane (Fitch et al. 1996). This was also observed for the European corn borer (Marçon et al. 2000), fall armyworm (FAW), the southwestern corn borer (SWCB) (Williams et al. 1997), and the corn earworm, FAW and SWCB (Williams et al. 1998).

Degree of plant damage and number of dead plants per plot were highly correlated (r=0.998). These variables separated treatments into two groups (Fig 2.): no difference (P  0.05) among the Bt hybrids, and difference (P  0.05) between the control and the Bt hybrids. Damage rating has the advantage of being more sensitive and it allows the detection of differences in low levels of damage, i.e., at plant damage levels that do not kill the plant. Thus, plant damage rating can discriminate degrees of resistance. Based only on the number of dead plants no damage was detected on the Bt maize, except on the hybrid P33G27. However, the damage rating method registered some damage on all hybrids. Thus, the surviving Bt plants were due to the resistance of the plant, and not due to escape. The only mechanism of the Bt maize resistance is the antibiosis due to the expression of the toxin. However, reduced feeding and low larval survival on Bt maize compared to non-Bt maize suggests a significant feeding inhibition. Thus, nonpreference may also serve as an important component of resistance of Bt maize to LCB. Field experiments evaluating the resistance of Bt maize to FAW, also reveled less damage and lower weight of FAW on Bt maize than those on non-Bt maize (Williams et al. 1997). This shows the necessity of developing additional studies to establish interactions between the Bt maize and insects that feed ont this transgenic plants. It is important to understanding the Bt plant/insect interaction to manage Bt plants, reducing the

risk of this plants become susceptible to the insects after exposure under field conditions.

Temperature effect on the resistance of Bt maize:

The results of the experiment evaluating the hybrids Mycogen (Cry 1F), P33g27 (Cry 1Ab), Garst 8539Bt (Cry 9C) and Garst 8539 (non-Bt) under four daytime temperature regimes are presented in Figure 3. The analysis of variance showed difference ($P \leq 0.05$) on the number of dead plants in transgenic and non-transgenic maize. However, a significant interaction ($P \leq 0.05$) between temperature and genotype for this variable was found, which shows that temperature affected plant development, specially between 20 and 24$^{o}$C (Fig. 3), also, affected damage level which was significantly lower on control plants at 20$^{o}$C. No effect ($P \leq 0.05$) among Bt maize hybrids expressing the Cry 1F, Cry 1Ab or Cry 9C toxin was found in the four temperatures tested. Therefore, Bt transgenic maize can be considered an effective approach to manage LCB damage some larvae survived on the Bt transgenic maize, but they were small and of low weight and, cause no significant plant damage at all temperatures tested. LCB damage is often severe under dry conditions which shows that both temperature and water stress should be considered is the interaction between resistance of Bt maize and LCB.

Transgenic Bt plants have been used to control many pests, including LCB. Fitch et al. (1996) found 80-100% mortality of LCB on transgenic sugarcane. Singsit et al. (1997) reported various levels of Bt transgenic peanut resistance to LCB, ranging from complete mortality to a 66% reduction in larval weight. In addition, no study has documented effectiveness of Bt transgenic maize against FAW, CEW and SWCB (Lynch et al. 1999a, b). Thus, the use of transgenic maize hybrids can be an effective strategy to

manage many insect pests in IPM programs, and this approach can reduce pest damage and product costs.

## Acknowledgments

The authors thank the Brazilian Agencies “Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)” and the “Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)” for Scholarships and financial support. Also, Ariovaldo Luchiari/Embrapa Labex/USDA-ARS SWCRU for providing some transgenic maize seeds and Dr. José Cola Zanuncio and Dr. José Henrique Schoereder /UFV, Frederick P. Baxendale and E. Heinrichs /UN-L for reviewing the originals.

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Table 1. Damage rating used to estimate progressive level of damage by LCB on maize seedlings.

| Damage Rating | **Description of damage symptom** |
|---|---|
| 0 | No visible damage |
| 1 | Larval feeding scars at the base of the first leaf sheath |
| 2 | First leave sheath bored at the base |
| 3 | Second leave sheath with feeding scars at the bore site |
| 4 | Second leave sheath bored |
| 5 | Third leave sheath with feeding scars at the bore site |
| 6 | Third leave sheath bored |
| 7 | Central leaf wilted |
| 8 | Central leaf dead |
| 9 | Entire plant wilted |
| 10 | Entire plant dead |

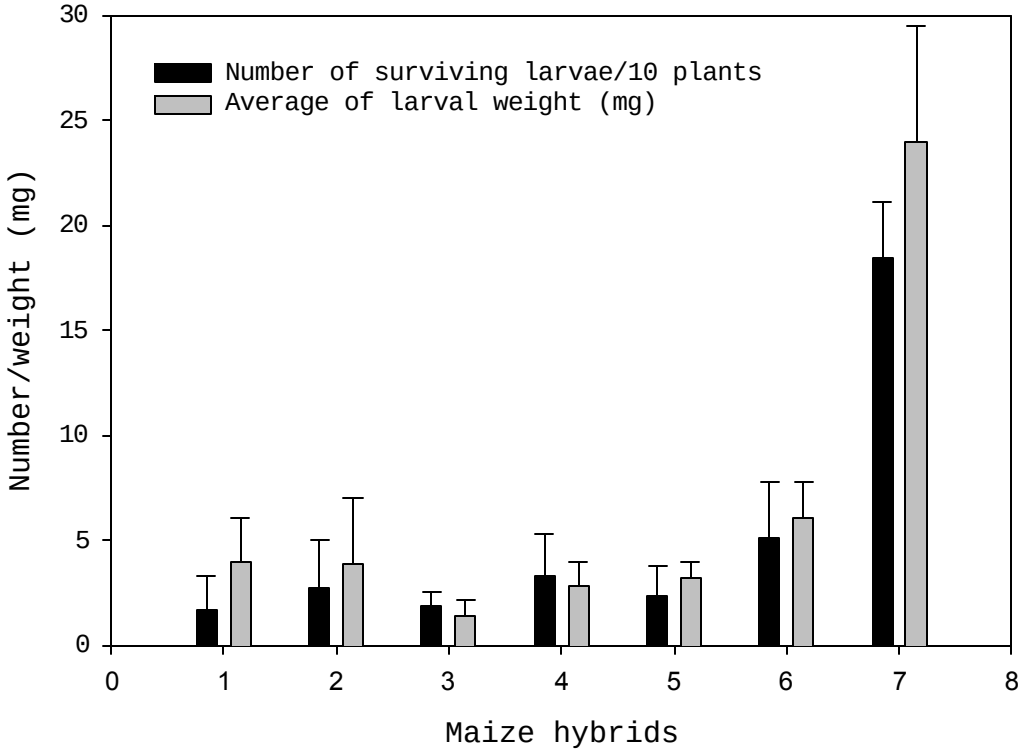


Fig. 1. Number (standard error) and larvae weight (standard error) of surviving *Elasmopalpus lignosellus* on Bt maize hybrids (1 - P34R07; 2 – P33G27; 3 – P34G82; 4 – P33A14; 5 – P34D34; 6 – G8539) and Non-Bt (G8539) maize under 27$^{o}$C and 14 h light:10 h dark photophase.

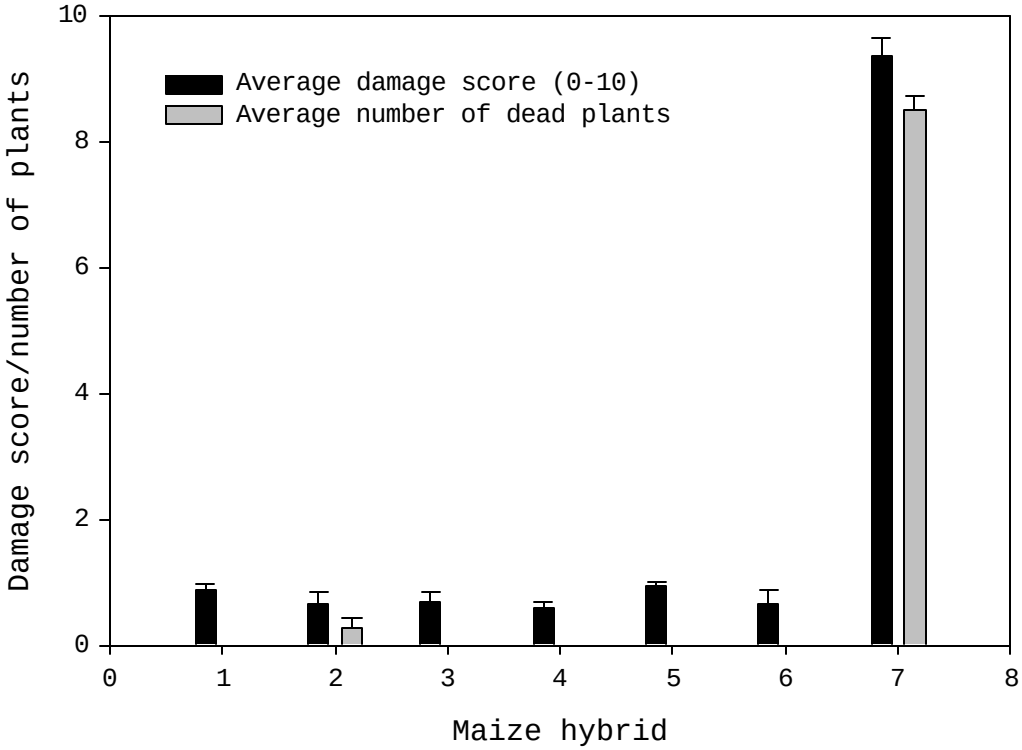


Fig. 2. Mean number (standard error) of dead maize plants for Bt (1 - P34R07; 2 – P33G27; 3 – P34G82; 4 – P33A14; 5 – P34D34; 6 – G8539) and non-Bt (G8539) maize under LCB artificial infestation in a growth chamber at 27$^{o}$C and 14 h light:10 h dark photophase.

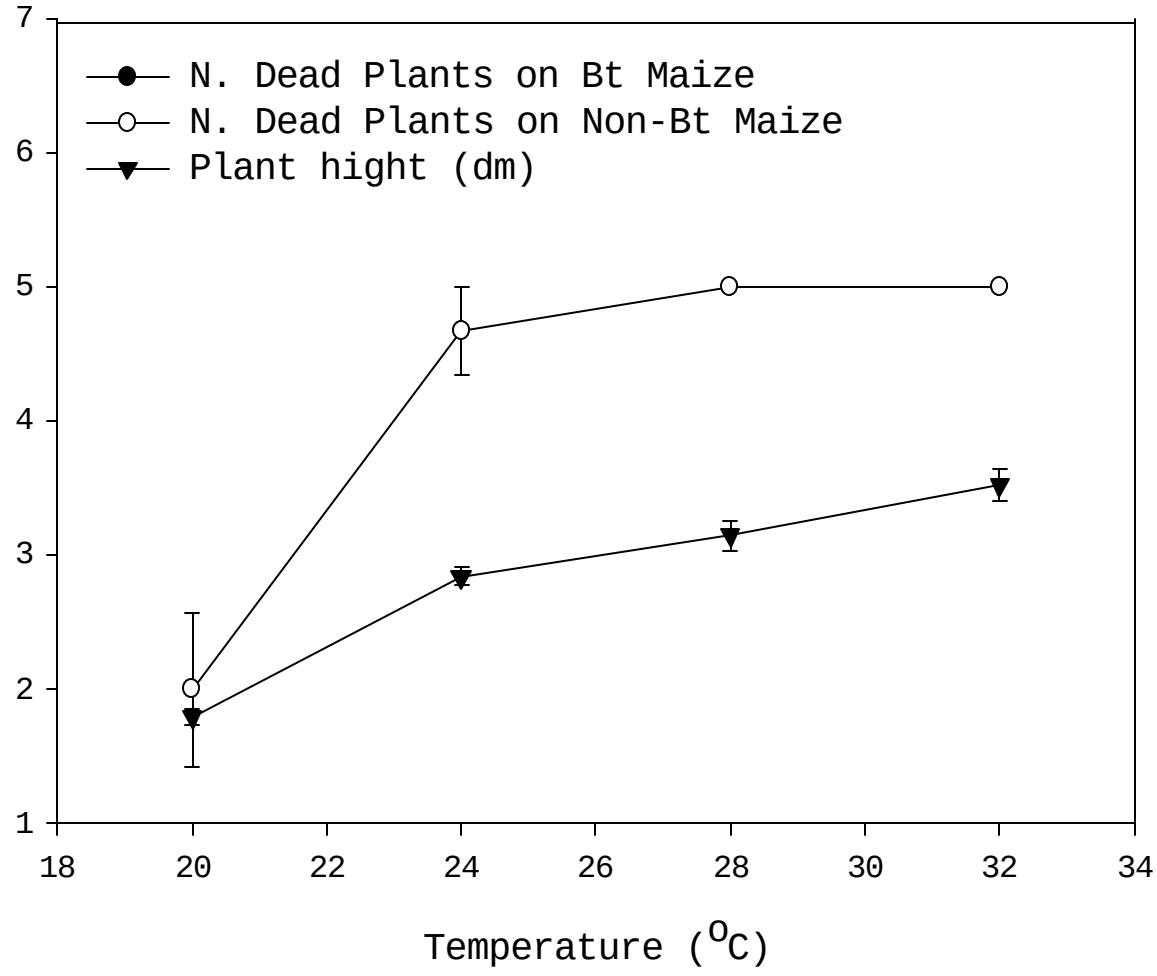


Fig.3. Effect of temperature on numbers of dead plants and plant height (standard error) of Bt (Mycogen/Cry 1F, P33G27/Cry 1Ab, G8539/Cry 9C) and non-Bt (G8539 maize artificially infested by LCB in growth chambers at photophase of 14 h.

# Selection of the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae) for survival on Cry 1A(b) Bt toxin

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This paper will be submitted to Florida Entomologist

## ABSTRACT

Transgenic maize hybrids expressing *Bacillus thuringiensis* (Bt) toxin are considered an important technology to control lepidopteran pests of maize. Bt maize hybrids expressing the Cry 1A(b) toxin have been shown a significant level to the fall armyworm (FAW), *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae) resistance, although larvae survival of this pest has been observed on these hybrids. Increased tolerance to Cry 1A(b) toxin was found on populations of fall armyworm after selection for four generations. Tolerance of FAW to Cry1 A(b) had a heritage component but no information is available on whether survivors are genetically different from those that did not survive exposure to these plants. The following work, also, describes efforts to detect genetic polymorphisms which allow survival of the fall armyworm to Cry 1A(b) toxin.



Key-word: Insecta, resistance, transgenic plants, molecular markers, AFLP.



## RESUMO

Plantas transgênicas que expressam toxinas da bactéria *Bacillus thuringiensis* (Bt) representam um importante avanço para o controle de lepidópteros pragas. Híbridos de milho Bt expressando a toxina Cry 1A(b) têm mostrado significativo nível de resistência a *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae). Entretanto, larvas dessa praga tem sido observadas em campo e em laboratório. Seleção de insetos sobreviventes em bioensaios mostra um aumento do nível de tolerância à toxina Cry 1A(b) em populações de *S. frugiperda* após quatro gerações, indicando que essa tolerância é herdada.

Palavras-chave: Insecta, resistência, plantas transgênicas, marcador molecular, AFLP.

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Maize is one of the most important crops in the world agribusiness and it is cultivated all over the countries. As a forage or grain, corn represents about 70% of all food material for poultry, swine, beef cattle and others small animals. Pest control is one of the most important fact to consider in maize crops and insect resistance to pesticides is a major problem in modern agriculture (Gould et al. 1997).

The common soil bacterium *Bacillus thuringiensis* (Bt) produces crystals containing proteins that are toxic to certain insects, but are harmless to most other organisms including people, wildlife, and most beneficial insects (Schnepf 1998). Bt toxins has been shown to be completely safe to user and the environment. The extensive laboratory studies including mammalian toxicity studies, coupled with no reported cases of human or animal disease after more than 15 years of widespread use demonstrate that the tested isolates are not toxic or pathogenic (Rechcigl et al. 2000). So, Genes encoding Bt toxins have been incorporated into and expressed by crop plants, thus providing environmentally benign control of insect pests. On the other hand, some maize pest species such as the fall armyworm, *Spodoptera frugiperda* (FAW) have presented a challenge to the IPM technology. Recently, some Bt maize hybrids expressing the Cry 1A(b) toxin have been shown to provide a significant level of control, although FAW survivors have been observed on these hybrids (Williams et al. 1997 and 1998).

Evolution of resistance by pests is the most serious threat to the continued efficacy of Bt toxins (Heckel et al.

1999). Although the diamondback moth (*Plutella xylostella)* is the only reported insect with resistance to Bt toxins in open-field populations, laboratory selection has produced resistance in several other species of Lepidoptera (Tabashnik 1994). Most models to predict Bt resistance in insects assume that resistance is attributable to a mutation at a single locus. In contrast, quantitative genetic models make no assumption regarding the number of genes involved, and the expression of a trait is assumed to depend on environmental as well as genetic factors (Firko & Hayes 1990). As a tool for resistance assessment, quantitative genetics enables predictions to be made regarding the speed and magnitude of genetics change associated with resistance (Alinia et al. 2000). Despite their enormous economic and ecological importance, surprisingly little is know about the genetics of most Lepidoptera (Heckel et al. 1999), especially the process related to the resistance against bt toxins. However no information is available whether the survivors are genetically different. Thus, selection of laboratory colonies for genetic resistance provides a model to study the potential evolution of resistance. In this work, we propose to study the genetic polymorphism of survival of fall armyworm screened on the Cry 1Ab toxin and to begin a first screening using AFLP technique to look for differences among the survivors. This technique has already been use by Heckel et al. (1999) in insect resistance study where they found a marker related to resistance genes.

## Material & Methods

The study was conducted at the Department of Entomology, University of Nebraska, Lincoln, NE, USA.

### Insect population

FAW eggs were obtained from a laboratory colony maintained by DeKalb Agricultural Research in Union City, TN.

The insects were reared on artificial diet and maintained at 28° C.

Bioassays

Bt bioassays of neonate larvae were performed according to Marçon et al., 2000. The bioassay was performed using trays with 128-wells each. Approximately 1 ml of the diet was dispensed into each well and allowed to solidify. Each well containing diet was treated with 30 µl of a Cry 1A(b) toxin concentration prepared in 0.1% Triton-X100 nonionic detergent. Control treatment was 0.1% Triton-X100 only. All the treatments were allowed to dry and one neonate FAW larva was transferred for each individual well. Ten days after infestation, the treatments were analyzed by counting the number of FAW survivors and recording the weight of each surviving larva.

According to the above methodology, the LC50 of Cry 1A(b) to neonate FAW was estimated using the following Cry 1A(b) toxins concentrations: 15.62, 31.25, 62.5, 125, 250, 500 and 1000 ng/cm$^2$. The percentage mortality was corrected by Abbot's formula and then submitted to Probit analysis and the LC50 of the original lab population of FAW was estimated. The $F_o$ population was screened on maize plants expressing Cry 1A(b) toxin, obtaining families $F_1$. Progenies from single pair mating were exposed to the calculated LC50 and families with surviving FAW larvae ≤ 60% of control larval weight were considered tolerant to the toxin. Also, larvae weighing ≤10% of the control weight were considered susceptible.

This procedure was performed to establish susceptible and resistant populations based on survivorship as well as weight gain. The proportion of surviving insects for each selected family was compared to the expected 50% of mortality by Z test.

FAW DNA extraction:

For preservation identified specimens were placed in 95% ethanol. The insect DNA extraction was performed following the protocol described by Black and Duteau (1997) with minor modifications.

AFLP

For this step individuals from tolerant and susceptible populations were chosen. The AFLP reactions were performed according to the Li-Cor® Biotechnology Division protocol:

**I) Restriction Digestion**

Genomic DNA was digested with two restriction endonucleases simultaneously EcoR I (6-pb recognition site) and Mse I (4-pb recognition site). The reaction conditions were: 1.25 µl One Phorall Buffer 1X [50 mM Tris-HCl (pH 7.5), 50 mM Mg-acetate e 250mM K-acetate], 0.32 µl Mse I enzyme and 0.08 µl EcoR I enzyme [1.2 units/µl each in 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mg/ml de BSA, 50% (v/v glycerol), 0.125 µl BSA, 3.725 µl distilled water and 7 µl DNA template. Incubated at 37°C for 2 ½ hs. After, the enzymes were inactivated by incubation at 72°C for 15 min. Final volume 12.5 µl.

**II) Adapters ligation**

The genomic DNA fragments were ligated to EcoR I and Mse I adapters to generate template DNA for amplification. The reaction conditions were: 0.5 µl of each Mse I and EcoR I adapters [0.4 mM ATP, 10 mM Tris-HCl (pH 7.5), 10 mM Mg-acetate and 50mM K-acetate], 0.5 µl $T_4$ DNA ligase 1X buffer, 0.125 $T_4$ DNA ligase (1 unit/µl in 10 mM Tris-HCl (pH 7.5), 1 mM DTT, 50 mM KCl, 50% v/v glycerol) and 3.350 µl double distilled water. Final Volume 5 µl.

This mixture were added to the 12.5 µl restriction reaction, incubated at room temperature for 3 h. After, TE buffer dilution 1:10 was performed.

**III) Amplification Reactions**

PCR was performed in two consecutive reactions: pre – amplification and amplification. In pre-amplification step the genomic DNA fragments were amplified in 15.35 µl volumes containing 10 µl pre-amp pimer mix II, 1.25 µl PCR 1 X buffer with MgCl (15 mM), 0.25 µl AmpliTaq®DNA Polymerase (5 U/µl) and 1.25 µl of DNA. Then, the PCR products were diluted (1:20) and used as templates of amplification step. PCR Pre-amplification reactions were performed in Gene Amp® PCR System 2400 Perkin-Elmer. Program: 20 cycles at 94°C for 30 sec., 56°C for 1 min. and 72 °C for 1 min.

In amplification step the genomic DNA fragments were amplified in 8.56 µl. volumes containing 4.8 µl dd $H_2O$, 1.2 µl PCR 1 X buffer, 0.06 µl AmpliTaq®DNA Polymerase (5 U/µl), 2.0 µl Mse I primer (6.7 ng/µl, dNTP), 0.5 µl EcoR I labeled primer (27.8 ng/µl) and 2.0 µl diluted template (1:20)

PCR amplification reactions were performed in Gene Amp® PCR System 2400 Perkin-Elmer. Program: 1 cycle at 94°C for 30 sec., 65°C for 30 sec. and 72 °C for 1 min. Touch down: 94°C for 30 sec., 65°C until 56°C for 30 sec., decreasing 0.7°C at each cycle, and 72 °C for 1 min. 23 cycles at 94°C for 30 sec., 56°C for 30 sec. and 72 °C for 1 min. The PCR products were run in denaturing polyacrylamide 5% gel.

The amplification primer combinations were: Eco R I AGG and Mse I CAC, CAA, CTG, CAG and Eco R I ACT and Mse I CAG, CAC, CAT and CTA.

## RESULTS AND DISCUSSION

Nineteen families were selected in $F_1$, eight in $F_2$ and six in $F_3$ generation (table 1). based on the LC50 of 690

ng/cm$^2$ with results of Probit analysis for the original population ($F_0$) (Fig.1).

Expected mortality for each family in generations $F_1$, $F_2$, and $F_3$ was 50%, but in the F1 generation seventeen families showed mortality under 50% and two families above 50% (Table 1). Just one family was considered susceptible in $F_2$ generation (Table 1) and one in $F_3$ generation (Table 1). After four generations, the F4 family 527C4II5III6 (not showed) was used to estimate a new LC50 with the same procedure, but with higher concentrations: 125, 250, 500. 1000, 2000, 4000 and 8000 ng/cm2. The LC50 was 3,770.98 ng/cm$^2$ with a 5 fold increase in relation to the original population (Figure 1).

FAW larvae surviving to exposure to Cry 1A(b) are more tolerant and it has a heritable component. This shows a potential to increase proportion of survivors of FAW on Bt-toxins Cry 1A(b) through generations with the low dose strategy. However, extrapolation of such results to field conditions should be carefully considered because Bt maize employs a higher dose strategy and due to a significant reduction on fitness of insects based on a significant reduction in the weight of surviving larvae. Also in field conditions the possible tolerant individual to Bt could dilute this conditions through mating with non-susceptible individual. this is more limited in laboratory conditions because population used is samller. Adanczyk & Sumerford (2001) found increased tolerance to Cry 1A(c) transgenic Bt cotton but no evidence of fitness or vigor differences from FAW progeny previously fed on conventional or transgenic Bt cotton.

Also, it was evaluated the weight of all insects. There was observed no correlation between percentage of mortality and percentage of growth inhibition (weight base) suggesting independent traits.

The AFLP data shows a possible difference between tolerant and susceptible populations (Figure 2). The gel

shows presence of bands in tolerant populations that is absent in susceptible populations and vice-versa. Nevertheless, this is just a screening test for primer that can be used to other future studies supporting the hypothesis of genetic difference between tolerant and susceptible FAW populations.

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Table 1: Family generation, control weight, weight of survivor, percentage of mortality, number of insects evaluated and status of each family of FAW survivor on Bt toxin for 3 generations.

| Generation | Family | Control weight | Treatment weight | % dead | n | Status |
|---|---|---|---|---|---|---|
| F1 | 507 | 387.31 | 81.51 | 10.42 | 64 | R |
| F1 | 514 | 269.48 | 29.01 | 45.87* | 64 | S |
| F1 | 516 | 254.75 | 92.99 | 9 | 126 | R |
| F1 | 519 | 336.47 | 85.74 | 13.97 | 128 | R |
| F1 | 522 | 294.78 | 160.25 | 0 | 64 | R |
| F1 | 524 | 213.47 | 31.89 | 10.42 | 127 | R |
| F1 | 527 | 332.77 | 112.18 | 6.25 | 127 | R |
| F1 | 530 | 141.54 | 55.22 | 16.37 | 125 | R |
| F1 | 602 | 145.17 | 97.49 | 0 | 39 | R |
| F1 | 625 | 213.21 | 54.11 | 0 | 128 | R |
| F1 | 626 | 304.74 | 101.12 | 4.17 | 128 | R |
| F1 | 628 | 220.73 | 22.47 | 27.08 | 128 | R |
| F1 | 629 | 255.35 | 89.65 | 0 | 128 | R |
| F1 | 630 | 259.3 | 70.89 | 0 | 128 | R |
| F1 | 631 | 191.42 | 43.63 | 13.57 | 128 | R |
| F1 | 632 | 224.2 | 52.52 | 0 | 128 | R |
| F1 | 647 | 150.39 | 0.99 | 78.57* | 128 | SS |
| F1 | 653 | 271.17 | 32.27 | 7.29 | 128 | R |
| F1 | 679 | 278.35 | 20.29 | 8.33 | 128 | R |
| | | | | | | |
| F2 | 507C1 | 310.32 | 49.94 | 13.59 | 244 | R |
| F2 | 514C2 | 211.76 | 22.7 | 13.66 | 251 | R |
| F2 | 514C3 | 162.17 | 18.22 | 63.84* | 256 | S |
| F2 | 527C4 | 131.21 | 47.36 | 12.88 | 382 | R |
| F2 | 527C9 | 242.59 | 49.51 | 8.16 | 256 | R |
| F2 | 527C12 | 222.34 | 41.55 | 21.46 | 256 | R |
| F2 | 530C4 | 184.34 | 29.53 | 12.69 | 128 | R |
| F2 | 632C1 | 220.56 | 27.95 | 34.32 | 768 | R |

| | | | | | | |
|---|---|---|---|---|---|---|
| F3 | 507C1II1 | 239.75 | 32.04 | 25.73 | 116 | R |
| F3 | 514C2II3 | 149.05 | 27.34 | 0 | 128 | R |
| F3 | 527C4II1 | 283.33 | 15.44 | 24.11 | 256 | R |
| F3 | 527C4II5 | 257.97 | 6.88 | 65.08* | 256 | S |
| F3 | 527C4AII10 | 248.05 | 26.87 | 6.73 | 255 | R |
| F3 | 527C4BII5 | 303.53 | 20.64 | 17.5 | 76 | R |

Percentages of dead insects with * showed no significant differences from 50 %. The symbol S stands for susceptibility, SS for high level of susceptibility and R for resistance.

Figure 1: Probit analysis for $F_0$ and $F_4$ generations showing different dose response

Figure 2: AFLP screening, primers EAGG-MCTG and EAGG-MCAG, showing band pattern in susceptible and tolerant FAW populations.