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Análise do potencial de *Bacillus thuringiensis* como agente de controle de *Spdoptera frugiperda* (Lepidoptera, Noctuidae) e *Ostrinia nubilalis* (Lepidoptera, Pyralidae)

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Área de Concentração: Diversidade e Manejo da Vida Silvestre

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A tese intitulada "Análise do potencial de *Bacillus thuringiensis* como agente de controle de *Spdoptera frugiperda* (Lepidoptera, Noctuidae) e Ostrinia *nubilalis* (Lepidoptera, Pyralidae)", elaborada por Gabriela Cristina Alies, foi julgada adequada e aprovada por todos os membros da Banca Examinadora, para obtenção do título de DOUTORA EM BIOLOGIA, com área de concentração: Diversidade e Manejo de Vida Silvestre.

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RESUMO

A bactéria *Bacillus thuringiensis* exibe alta atividade tóxica específica para insetos devido à sintese de delta-endotoxinas, codificadas por genes *cry*. A presente pesquisa visou avaliar a atividade tóxica de cepas de *Bacillus thuringiensis* oriundas de regiões orizícolas do Estado do Rio Grande do Sul, como agente de controle de insetos-praga da cultura do arroz irrigado e milho *Spodoptera frugiperda* e *Ostrinia nubilalis,* a fim de selecionar cepas potenciais para o controle das mesmas e identificar se as atividades enzimáticas dos receptores alcalino fosfatase (ALP) e aminopeptidase (APN) podem ter um importante papel na resistência de *B. thuringiensis.* Neste trabalho foram utilizados testes fenotípicos e técnicas de PCR para a identificação das subclasses do gene *cry*1; perfil de proteínas para a observação da composição do complexo esporo-cristal, rep-PCR para a observação da variabilidade genética, DNA plasmidial, quantificação das enzimas ALP e APN para se observer perfil de resistência das cepas e a atividade tóxica frente aos insetos-praga. Os ensaios de quantificação de proteínas de ALP e APN apresentaram níveis reduzidos, sendo um biomarcador potencial para a resistência de toxinas Cry. Os resultados dos estudos de rep-PCR demonstraram um elevado grau de similaridade entre as regiões orizícolas, provavelmente associadas à especiação ecológica. Na caracterização do perfil protéico, os resultados revelaram diferenças entre as cepas em estudo, sendo algumas semelhantes àquelas utilizadas como padrão da análise (*Bt. thuringiensis* 4412; *Bti* IPS 82 e *Bt.* sorovar HD1). Nos ensaios do perfil plasmidial, as cepas formaram três padrões distintos. Para os dados de toxicidades avaliados pelos bioensaios com as lagartas de primeiro ínstar de *O. nubilalis,* todas as cepas testadas apresentaram mortalidade superior a 75%, com destaque à cepa *Bt.*1893-15 que causou 95%. Nos dados dos ensaios biológicos realizados contra as lagartas de *S. frugiperda*, a cepa *Bt.* 3420-11 destacou-se com mortalidade superior a 88%.

Palavras-chave: *Bacillus thuringiensis.* Lepidoptera. Bioensaios. Ensaios *in vitro.* PCR.

ABSTRACT

The bacterium *Bacillus thuringiensis* presents a high specific activity against insects due to delta-endotoxin syntheses, codified by *cry* genes. This study aimed to evaluate the toxic activity of strains of *B. thuringiensis* derived from rice fields of Rio Grande do Sul, as an agent of control of insect pest of rice and corn *Spodoptera frugiperda* and *Ostrinia nubilalis*, in order to select potential strains to control and identify whether the enzymatic activity of alkaline-phosphatase (ALP) and aminopeptidase (APN) receptors may play an important role in the resistance of *B. thuringiensis*. In this study we used phenotypic tests and PCR techniques to identify subclasses of gene *cry*1; proteins profile to observe the composition of the spore-crystal complex, rep-PCR for the observation of genetic variability, plasmid DNA, quantification of the enzymes ALP and APN to observe resistance profile of strains and bioassay capacity against insect pests. Assays for quantification of proteins ALP and APN showed reduced levels being a potential biomarker for resistance to Cry toxins. The results of studies of rep-PCR demonstrated a high degree of similarity between the rice regions, probably associated with ecological speciation. Regarding the protein profile characterization, the results revealed differences between the strains, some being similar to those used as standard analysis (*Bt thuringiensis* 4412, *Bt* IPS 82 and Bt HD1). In trials of plasmid profile, the strains formed three distinct patterns. In data and toxicity assessed by bioassays against first instar *O. nubilalis* larvae, all tested strains showed mortality exceeding 75%, highlighting the *Bt* strain 1893-15, which caused 95% mortality. In the data of the biological assays conducted against *S. frugiperda* larvae, the *Bt* strain 3420-11 stood out showing mortality greater than 88%.

Keywords: *Bacillus thuringiensis.* Lepidoptera. Bioassays *in vitro*. PCR.

SUMÁRIO

1 INTRODUÇÃO GERAL

A produção mundial de arroz e milho na safra 2011/2012 foi de aproximadamente 460,5 e 867,5 milhões de toneladas, respectivamente (FAO, 2011). No panorama internacional, o Brasil ganha destaque, pois é um dos maiores produtores de arroz, tendo produzido em 2011 cerca de 14,8 milhões de toneladas deste cereal (IBGE, 2011), sendo o Estado do Rio Grande do Sul o líder nacional de produção de arroz.

Apesar dos altos índices ocorrem reduções acentuadas de produtividade em lavouras devido ao ataque de insetos-praga, doenças, alterações nas condições climáticas e competições de plantas daninhas. As pragas: insetos, pássaros e moluscos podem causar perdas entre 10 e 35% de produtividade. Grande parte dessa problemática deve-se às mudanças tecnológicas exercidas nas práticas de manejo, muitas vezes necessárias devido às características de cada região e sistema de cultivo, mas que interferem no equilíbrio do ecossistema (EMBRAPA, 2011).

Entre as espécies de insetos, na cultura do arroz irrigado, na região Sul do Brasil destacam-se: *Spodoptera frugiperda*, *Oryzophagus oryzae*, *Tibraca limbativentris* e *Oebalus poecilus* (SOSBAI, 2007; OLIVEIRA et al., 2010). Por outro lado, na cultura do milho, nos Estados Unidos da América, destaca-se a *Ostrinia nubilalis*.

No manejo dos insetos-praga, como estratégia do controle populacional podem ser adotadas algumas práticas culturais, como: eliminação das plantas daninhas, adubação do solo, áreas armadilhas, entre outras (VIEIRA et al., 1999). Por outro lado, nas monoculturas de relevância econômica, atualmente predomina a utilização dos inseticidas químicos.

Considerando os problemas relacionados a ação desses inseticidas sobre os ecossistemas (SILVA, et al., 2002), métodos alternativos de controle são necessários, como o controle microbiano de insetos, por meio de entomopatógenos, com ênfase na aplicação de bactérias, virus e fungos (ALVES, 1998; POLANCZYK e ALVES, 2005, ALVES 2008).

Entre os organismos aplicados no controle biológico de pragas, o *Bacillus thuringiensis* destaca-se por ser um organismo que durante sua fase de esporulação

produz inclusões cristalinas (proteínas Cry), as quais são responsáveis pela atividade entomopatogênica das cepas bacterianas. As proteínas Cry ou δ-endotoxinas são codificadas por genes também denominados *cry,* que compilam toxinas patogênicas a diversas ordens de insetos (LIANG et al., 2008).

A utilização de *B. thuringiensis* no controle biológico, de forma mais eficaz às diversas espécies de pragas de importância agrícola, tem sido enfatizado visando minimizar ou até mesmo substituir o controle químico. A bactéria entomopatgênica destaca-se como atóxica aos humanos, à fauna, à flora, consequentemente não prejudicial aos ecossistemas devido à especificidade das proteínas cry (PARDO-LOPEZ et al., 2006).

Essas características impulsionam os estudos de identificação dos genes *cry* e a caracterização de novas cepas de *B. thuringiensis,* pois a patogenicidade e a especificidade das mesmas são determinadas pelos tipos de genes *cry* funcionais, aliado ao fato desses genótipos evidenciarem alto grau de variabilidade genética para a referida espécie bacteriana (LIANG et al., 2008).

Atualmente existem várias classes e subclasses de genes *cry* caracterizados e catalogados que codificam proteínas Cry com atividade inseticida a diferentes ordens de insetos, tais como: Lepidoptera, Coleoptera, Diptera, Hymenoptera, Hemiptera, Isoptera, Orthoptera, Siphonaptera e Thysanoptera (FEITELSON et al., 1992; ARANDA et al., 1996; CAVADOS et al., 2001; CASTILHOS-FORTES et al., 2002; DE MAAGD et al., 2003; PINTO et al., 2003) além de nematóides (JOUZANI et al., 2008; MARROQUIM et al., 2000). Até o momento, já foram descritos mais de 500 genes *cry*, os quais estão distribuídos em 68 classes (CRICKMORE, 2011).

As proteínas Cry ou δ-endotoxinas são produzidas de forma inativa e ao serem ingeridas pelos insetos suscetíveis, sofrem ação do pH intestinal e de proteases, as quais solubilizam o cristal e ativam as toxinas. Estas, por sua vez, se ligam aos receptores presentes no tecido epitelial do intestino médio das formas imaturas dos insetos, desencadeando uma sinalização nas células epiteliais que ocasiona a quebra do equilíbrio osmótico da célula, o rompimento e o extravasamento do conteúdo intestinal para a hemocele do inseto. Em consequência, a larva interrompe a alimentação, entra em paralisia geral e morre por inanição ou septicemia (BRAVO e SOBERON, 2008; FIUZA, 2009).

A especificidade das proteínas Cry deve-se principalmente a sua interação com receptores localizados nas microvilosidades do intestino médio dos insetos. Muitos receptores têm sido caracterizados, como a aminopeptidase N (APN) e as caderinas presentes nas formas imaturas dos lepidópteros (lagartas). Para outros grupos de pragas, como os nematóides, os glicolipídios são uma importante classe de receptores às proteínas Cry. Porém outros receptores incluem a fosfatase alcalina (ALP) (PIGOTT e ELLAR, 2007).

Nesse contexto, a presente pesquisa foi desenvolvida no Laboratório de Microbiologia e Toxicologia da Universidade do Vale do Rio dos Sinos, em São Leopoldo (Brasil), e parte como estágio Doutoral (CAPES-PDEE) no Laboratório de Entomologia da University of Nebraska (UNL) em Lincoln, Nebraska (Estados Unidos), sendo os dados obtidos apresentados na forma de artigos científicos e a tese estruturada nos seguintes capítulos:

- **Capítulo 1:** Revisão Bibliográfica.

- **Capítulo 2:** Marcadores moleculares para estudos da diversidade do gênero Bacillus

- **Capítulo 3:** Phenotypic characterization and the application of the rep-PCR technique in a study of new strains of *Bacillus thuringiensis* in the South of Brazil.

- **Capítulo 4:** Screening of new Brazilian *Bacillus thuringiensis* strains against *Spodoptera frugiperda* (Lepidoptera: Noctuidae).

- **Capítulo 5:** Biological characterization of *Bacillus thuringiensis* strains against *Ostrinia nubilalis* (Lepidoptera, Pyralidae)

- **Capítulo 6:** Levels of membrane alkaline phosphatase and aminopeptidase to lepidopteran strains resistant to Cry toxins from *Bacillus thuringiensis*.

CAPÍTULO 1

REVISÃO BIBLIOGRÁFICA

2.1 REVISÃO DE LITERATURA

2.1.1 *Spodoptera frugiperda*

Na agricultura, a lagarta-do-cartucho *S. frugiperda* (Lepidoptera: Noctuidae) foi reconhecida pela primeira vez em 1797, na Geórgia (EUA) por J. E. Smith. Este inseto possui ampla distribuição geográfica, ocorrendo desde a região central dos EUA até a Argentina e em algumas ilhas a oeste da Índia (BERTELS, 1970; PEDIGO, 1989).

S. frugiperda é um inseto de metamorfose completa e os adultos, de hábito noturno, acasalam-se três dias após a emergência. As fêmeas podem colocar cerca de 1000 a 2000 ovos e as lagartas eclodem entre dois e três dias após a postura dos ovos (FERRAZ, 1982). Durante a fase larval, o inseto pode apresentar de quatro a sete ínstares e a duração desta fase varia de 12 a 30 dias (PARRA; HADDAD, 1989). Ao completar o desenvolvimento, a lagarta penetra no solo, onde se transforma em crisálida, cuja fase tem duração média de 11 dias (VALICENTE; CRUZ, 1991) e no laboratório a duração desta fase está entre 8 e 11 dias (SILVEIRA et al., 1997). Os adultos emergem a noite e apresentam acentuado dimorfismo sexual, com as asas anteriores de cor parda escura, e as posteriores, branco-acinzentada (CRUZ et al., 1997; FERRAZ, 1982).

2.1.2 *Ostrinia nubilalis*

O. nubilalis (Lepidoptera, Pyralidae), denominada *European Corn Borer*, é uma lagarta que chegou aos Estados Unidos, provavelmente, em um carregamento marítimo de linho, em 1914, sendo reportada como inseto-praga na cultura de milho em 1917, em Massachusetts por S.C. Vinal. Em poucos anos se espalhou pela Costa Leste e Canadá. Diferentes pontos de introdução e dispersão pelo transporte agrícola foram determinantes para o rápido estabelecimento na Costa Oeste. Relatos identificam sérios danos e queda de produtividade de milho em Ontário, em 1927. Em 1936, atinge o Centro-Norte, encontra-se amplamente disseminada no "*Corn Belt*", e atinge outras regiões como o Sul dos EUA. Em 1950, já estava na Região das Montanhas Rochosas, Estado do Colorado (DICKE, 1977; BARRY;

DARRAH, 1994).

Atualmente, *O. nubilalis* é a principal praga do milho nos Estados Unidos e Canadá. Perdas resultantes dos danos desta lagarta e os custos para seu controle ultrapassam US$ 1 bilhão por ano. Por exemplo, a perda durante um único surto em 1995, no Estado de Minnesota, ultrapassou U$ 285 milhões. Em milho cultivado para grão nos EUA, os relatos de perdas de rendimento devido aos danos de *O. nubilalis* de primeira e de segunda geração são principalmente perdas fisiológicas, ao invés de danos em espigas. Híbridos com resistência à primeira geração (resistentes a desfolha) podem reduzir tais perdas.

Esse inseto-praga pode causar danos durante todas as fases de desenvolvimento do milho e podem ser extremamente elevadas as perdas durante os picos das infestações, especialmente se o dano inicia antes do enchimento da espiga (IOWA STATE UNIVERSITY, 2010).

2.1.3 Controle biológico

O controle biológico é definido como a ação de inimigos naturais sobre uma população de praga, resultando numa posição geral de equilíbrio mais baixa do que prevaleceria na ausência daqueles (GRAVENA, 1992). O controle microbiano é um ramo do controle biológico que objetiva principalmente a redução ou manutenção da população de pragas em nível inferior ao dano econômico, dentro do contexto do manejo integrado de pragas (PANIZZI; PARRA, 1991; ALVES, 1998; GALLO et al., 2002).

Como alternativa ao emprego de inseticidas químicos no controle de lepidópteros-praga, existe um grande complexo de inimigos naturais com potencial para constituir novas táticas de controle de que venham minimizar ou até mesmo substituir a utilização dos inseticidas convencionais (ASHLEY, 1979; FERRAZ, 1998; GALLO et al., 2002; GARDNER et al., 1984).

Entre os entomopatógenos, os baculovírus englobam o grupo de vírus mais estudados e utilizados como bioinseticida. Nos lepidópteros, como *Spodoptera frugiperda,* os vírus têm como principal rota de infecção a ingestão de alimento contaminado, com a subsequente liberação das partículas virais nas células epiteliais do intestino médio, causando a morte do inseto em poucos dias (RIBEIRO et al., 1999). A fase larval é a mais suscetível à infecção e o aparecimento dos

sintomas e a morte do inseto dependem de diversos fatores, como: idade do hospedeiro, virulência do isolado, condições climáticas e outras (VALICENTE; CRUZ, 1991).

No caso do fungo *Beauveria bassiana*, de ampla distribuição geográfica, é mais frequente em insetos e em amostras de solo, sendo encontrado no campo em coleópteros, lepidópteros, hemípteros, dípteros, himenópteros e ortópteros. A infecção pode ocorrer por via oral, pelo tegumento ou pelos espiráculos, sendo que 12 horas após o contato com o inseto, ocorre a germinação dos conídios. Decorridas 72 horas, os insetos podem ser totalmente colonizados, levando à morte em função da falta de nutrientes e do acúmulo de substâncias tóxicas (JOUZANI et al., 2008).

Quanto à bactéria *Bacillus thuringiensis*, a atividade inseticida está predominantemente associada às proteínas Cry que podem controlar diferentes espécies de pragas pertencentes as ordens: Lepidoptera, Coleoptera, Diptera, Hymenoptera, Hemiptera, Isoptera, Orthoptera, Siphonaptera e Thysanoptera (FEITELSON et al., 1992; ARANDA et al., 1996; CAVADOS et al., 2001; CASTILHOS-FORTES et al., 2002; DE MAAGD et al., 2003; PINTO et al., 2003) além de ácaros, nematóides e fitopatógenos (JOUZANI et al., 2008; MARROQUIN et al., 2000).

2.1.4 *Bacillus thuringiensis*

A microflora bacteriana dos insetos, confinada no intestino, é rica, diversa e compreende bactérias Gram positivas e negativas. Muitas delas auxiliam na digestão dos alimentos, porém algumas são patogênicas e recebem grande atenção dos pesquisadores devido ao seu potencial para o controle de pragas agrícolas e urbanas (PRIEST, 2000). Entre estes patógenos destaca-se *Bacillus thuringiensis* (Bacillaceae), bactéria em forma de bastonete, formadora de esporos e capaz de produzir inclusões cristalinas durante a esporulação, que são responsáveis pela atividade tóxica desta espécie (BOBROWSKI et al., 2003; GLARE; O'CALLAGHAM, 2000), conforme ilustra a Figura 1.

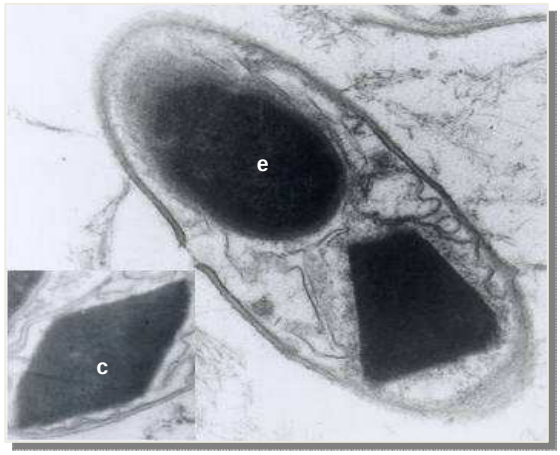

Figura 1 - *Bacillus thuringiensis* em MET; (e) Esporo; (c) Cristal (BOBROWSKI et al., 2003).

B. thuringiensis foi pela primeira vez descrito por Berliner, em 1911, quando este pesquisador isolou o bacilo de *Anagasta kuehniella*. Posteriormente, ele o nomeou *B. thuringiensis* em homenagem à província de Thuringia (Alemanha), onde o primeiro inseto infectado foi encontrado. Em 1908, Iwabuchi a denominou como *B. sotto ishiwata*, que posteriormente foi considerado nome inválido e o nome mais recente (*Bacillus thuringiensis*) foi mantido (GLARE; O'CALLAGHAN, 2000).

Geralmente a denominação *Bacillus thuringiensis* aplica-se a uma única espécie, porém essa bactéria pertence a um complexo de várias espécies: *B. anthracis, B. cereus, B. mycoides, B. pseudomycoides B. thuringiensis e B. weihenstephanensis*. As espécies *B. thuringiensis* e *B. cereus*, por exemplo, mostram características fenotípicas e bioquímicas comuns, mas por definição, *B. thuringiensis* pode ser diferenciado pela presença dos cristais (LUTHY; WOLFERSBERGER, 2000), visíveis em microscopia de contraste de fase (LYSENKO, 1983; LOGAN et al, 2009).

2.1.5 Atividade tóxica de *Bacillus thuringiensis*

B. thuringiensis desenvolve-se, em condições aeróbicas, em meios de cultura simplificados como Ágar Nutriente. Sob certas restrições, como ausência de nutrientes ou acúmulo de metabólitos indesejáveis, esta bactéria entra em processo de esporulação durante a fase estacionária. No início da esporulação *B. thuringiensis* sintetiza uma grande quantidade de proteínas com atividade inseticida. As proteínas acumuladas formam um corpo de inclusão cristalina, razão pela quais

elas são denominadas Cry (YAMAMOTO; DEAN, 2000). Estas toxinas são codificadas por genes *cry* e sua toxicidade está ligada à região N-terminal das cadeias polipeptidicas, enquanto a porção C-terminal determina a forma da estrutura do cristal (LI et al., 1991).

Os genes cry podem estar localizados tanto no cromossomo como em grandes plasmídeos (40-200 MDa) (GONZÁLES et al., 1982; SANCHIS et al., 1998).

A inclusão cristalina pode ser responsável por mais de 25% do peso seco das células. A quantidade de proteína produzida em laboratório (cerca de 0,5 mg/mL) e o tamanho dos cristais indicam que cada célula tem que sintetizar de 10^6 a $2x10^6$ moléculas de δ-endotoxina para formar o cristal (AGAISSE; LERECLUS, 1995).

Após os insetos ingerirem as suspensões, de esporos e cristais, inicia o processo de solubilização em pH alcalino, liberando as protoxinas que em presença de enzimas digestivas (proteinases) são convertidas em um ou até seis polipeptídeos tóxicos (δ-endotoxinas). Essas toxinas hidrolizadas atravessam a membrana peritrófica, ligam-se aos receptores específicos do intestino médio, interferindo no balanço osmótico e gradiente iônico da membrana apical formando poros que aumentam a permeabilidade da membrana. O aumento na absorção de água causa lise celular e eventual ruptura e desintegração das células do intestino médio. Logo após a ingestão, os insetos cessam a alimentação já reduzindo os danos causados nas plantas hospedeiras (COPPING; MENN, 2000, BRAVO et al., 2008).

As proteinases, juntamente com as peptidases e dipetidases, formam o grupo das proteases que são enzimas que hidrolisam as ligações peptídicas. As próteses clivam ligações peptídicas internas, as peptidases atacam as ligações de oligopeptídeos a partir do resíduo N-terminal (aminopeptidases) ou C-terminal (carboxipeptidases) e as dipeptidases hidrolisam dipeptídeos (PANIZZI; PARRA, 1991).

Embora os produtos comerciais disponíveis têm como alvo predominantes as espécies pertencentes às ordens dos lepidópteros, dípteros e coleópteros, Glare e O'callaghan (2000) citam que mais de 1.000 espécies de insetos, pertencentes a diversas ordens, são suscetíveis a este patógeno. Além da patogenicidade e virulência contra insetos-praga, outros aspectos como os efeitos subletais sobre os indivíduos sobreviventes, embora difíceis de detectar, certamente ocorrem e

representam um importante parâmetro, que auxilia na avaliação de sua atividade tóxica. Também é pouco estudada a interação com outros entomopatógenos, pois além de incrementar a eficácia desta tática de controle biológico, também fornece indícios sobre o impacto ambiental de *B. thuringiensis*. Embora existam muitos estudos sobre parasitóides, predadores e patógenos, poucos são aqueles que tratam da interação entre os mesmos, especialmente os patógenos que atuam em espécies-praga de difícil controle (POLANCZYK, 2004).

2.1.6 Utilização de *Bacillus thuringiensis* na agricultura

A eficácia e especificidade das cepas de *B. thuringiensis* e suas toxinas no controle de insetos-praga favoreceu a formulação de biopesticidas à base deste patógeno e, desde o primeiro produto lançado na França, em 1938, mais de 100 formulações foram colocadas no mercado mundial, sendo atualmente responsáveis por mais de 90% do faturamento com bioinseticidas. O continente americano é responsável por 50% deste mercado, principalmente os Estados Unidos e o Canadá, sendo que a América Latina representa apenas 8 a 10% do total (TAMEZ-GUERRA et al., 2001). O produto à base de *B. thuringiensis* com maior alcance no mercado mundial é o Dipel (*Bt. kurstaki* HD1). Este produto, pouco tóxico para ácaros, coleópteros, dípteros e hemípteros é altamente eficiente para 170 lepidópteros-praga (BEEGLE; YAMAMOTO, 1992; GLARE; O'CALLAGHAN, 2000).

Além do *B. thuringiensis* ser à base da formulação dos bioinseticidas com maior sucesso comercial no mundo, marca o início da substituição dos inseticidas químicos em vários agroecossistemas. Na década de 80, o surgimento de novas técnicas, especialmente aquelas voltadas para a tecnologia do DNA recombinante, e manifestações públicas a respeito do uso abusivo dos inseticidas convencionais, ampliaram o interesse dos órgãos de pesquisa e indústrias sobre a utilização do *Bt.* tanto na agricultura quanto na saúde pública (SOBERON et al., 2009).

2.2 REFERÊNCIAS

AGAISSE, H.; LERECLUS, D. 1995. How does *Bacillus thuringiensis* produce so much insecticidal crystal protein? *Journal of Bacteriology*. 177: 6027-6032.

ALVES, S. B. 1998. Patologia e controle microbiano: vantagens e desvantagens. In: ALVES, S. B. (Ed.). Controle microbiano de insetos. Piracicaba: FEALQ, 1, p. 21-38.

ARANDA, E.; PEFEROEN, M.; GUURECA, L.; BRAVO, A. 1996. Interactions of *Bacillus thuringiensis* crystal proteins whith the midgut epithelial cell of *Spodoptera frugiperda* (Lepidoptera: Noctuidae). Journal of Invertebrate Pathology 68: 203-212.

ASHLEY, T. R. 1979. Classification and distribution of fall armyworm parasites. *Florida Entomologist*. 62: 114-123.

BARRY, B. D.; DARRAH, L. L. 1994. Impact of mechanisms of resistance on European corn Borer resistance in selected maize hybrids. In: International symposium held at the international maize and wheat improvement center. Proceedings. México: Cimmyt. p. 21-28.

BEEGLE, C. B.; YAMAMOTO, T. 1992. Invitation paper (C.P. Alexander Fund): History of *Bacillus thuringiensis* Berliner research and development. *The Canadian Entomologist*. 124: 587-616.

BERTELS, A. 1970. Estudos da influência da umidade sobre a dinâmica de flutuação de lepidópteros, pragas do milho. *Pesquisa Agropecuária Brasileira*. 5: 67-79, 1970.

BOBROWSKI, V. L.; FIUZA, L. M.; PASQUALI, G.; BODANESE-ZANETTINI, M. H. 2003. Genes de Bacillus thuringiensis: uma estratégia para conferir resistência a insetos em plantas. Ciência Rural, 33, n. 5, set-out, 2003.

BRAVO, A.; SOBERON, M. 2008. How to cope with resistance to *Bt* toxins? *Trends Biotechnol*. 26: 573-579.

CASTILHOS-FORTES R.; MATSUMURA A.T.S.; DIEHL E.; FIUZA L.M. 2002. Susceptibility of *Nasutitermes ehrhardti* (Isoptera: Termitidae) to *Bacillus thuringiensis* subspecies. Brazilian Journal of Microbiology. 33(3): 219-222.

CAVADOS, C. F. G.; FONSECA, R. N.; CHAVES, J. Q.; RABINOVITCH, L.; ARAÚJO-COUTINHO, C. J. P. C. 2001. Identification of Entomopathogenic *Bacillus* Isolated from *Simulium* (Diptera, Simuliidae) Larvae and Adults. Memórias do Instituto Oswaldo Cruz 96(7): 1017-1021.

COPPING, L. G.; MENN, J.J. 2000. Review biopesticides: a review of their action, applications and efficacy. *Pest Management Science*. 56: 651-676.

CRUZ, I.; VALICENTE, F. H.; SANTOS, J. P.; WAQUIL, J. M.; VIANA, P. A. 1997. Manual de identificação de pragas da cultura do milho. Sete Lagoas: *EMBRAPA/CNPMS*. 67 p.

DE MAAGD, R. A.; BRAVO, A.; BERRY, C.; CRICKMORE, N.; SCHNEPF, H. E. 2003. Structure, diversity and evolution of protein toxins from spore-forming entomopathogenic bacteria. Annual Review of Genetics 37: 409-433.

DICKE, F.F. 1977. The most important corn insects. In: SPARGUE, G. F. (Ed.) Corn and corn improvement. *American Society of Agronomy*. 501-590.

FEITELSON, J. S.; PAYNE, J.; KIM, L. 1992. *Bacillus thuringiensis* insects and beyond. Biotecnology 10: 271-275.

FERRAZ, L. C. C. B. 1998. Nematóides entomopatogênicos. In: ALVES, S.B. (Ed.). Controle microbiano de insetos. 2.ed. Piracicaba: FEALQ. p. 541-570.

FERRAZ, M.C.V.D. 1982. Determinação das exigências térmicas de *Spodoptera frugiperda* (J.E. Smith, 1797) (Lepidoptera, Noctuidae) em cultura de milho. Piracicaba. 81p. Tese (Mestrado) – Escola Superior de Agricultura “Luiz de Queiroz”, Universidade de São Paulo.

GALLO, D.; HAKANO, O.; SILVEIRA NETO, S.; CARVALHO, R.P.L.; BAPTISTA, G.C.; BERTI FILHO, E.; PARRA, J.R.P.; ZUCCHI, R. A.; ALVES, S.B.; VENDRAMIM, J.D.; MARCHINI, L.C.; LOPES, J.R.S.; OMOTO, C. 2002. Entomologia Econômica. Piracicaba: *FEALQ*, 2002. 920 p.

GARDNER, W.A.; NOBLET, R.; SCHWEHR, R. 1984. The potential of microbial agents in managing populations of the fall armyworm (Lepidoptera: Noctuidae). *Florida Entomologist*. 67: 325-332.

GLARE, T.R.; O’CALLAGHAN, M. 2000. *Bacillus thuringiensis*: biology, ecology and safety. *Chichester: John Wiley*. 350p.

GONZÁLES, J.M.J.; BROWN, B.S.; CARLTON, B.C. 1982. Transfer of *Bacillus thuringiensis* to *Bacillus cereus*. *Proceedings of the National Academic of Science*. 79: 6951-6955.

GRAVENA, S. 1992. Controle biológico no manejo integrado de pragas. *Pesquisa Agropecuária Brasileira.* 27: 281-300.

IOWA STATE UNIVERSITY. How corn is damaged by the european corn borer. Ames, Iowa: Entomology Department, 2010. Disponível em: <http://www.ent.iastate.edu/pest/cornborer.

JOUZANI, G. S.; SEIFINEJAD, A.; SAEEDIZADEH, A.; NAZARIAN, A.; YOUSEFLOO, M.; SOHEILIVAND, S.; MOUSIVAND, M.; JAHANGIRI, R.; YAZDANI, M.; AMIRI, R. M.; AKBARI, S. 2008. Molecular detection of nematicidal crystalliferous *Bacillus thuringiensis* strains of Iran and evaluation of their toxicity on free-living and plant-parasitic nematodes. Canadian Journal of Microbiology, 54:812-822.

LI, J.; CARREL, J.; ELLAR, D.J. 1991. Cristal structure of insecticide delta-endotoxina from *Bacillus thuringiensis* at 2,5 A resolution. *Nature*. 353: 815-821.

LOGAN, N. A.; BERGE, O.; BISHOP, A.H.; BUSSE, H.J.; DE VOS, P.; FRITZE, D.; HEYNGRICKX, M.; KÄMPFER, P.; RABINOVITCH, L.; SALKINOJA-SALONEM,

M.S.; SELDIN, L.; VENTOSA, A. 2009. Proposed minimal standards for describing new taxa of aerobic, endorpore-forming bactéria. International Journal of Systematic and Evolutionary Microbiology. 59: 2114-2121.

LUTHY, P.; WOLFERSBERGER, M.G. 2000. Pathogenisis of *Bacillus thuringiensis* toxins. In: CHARLES, J.F.; DELÉCLUSE, A.; NIELSEN-LE ROUX, C. (Ed.). Entomopathogenic bacteria: from laboratory to field application. Netherlands: *Kluwer Academic Publishers*. p. 167-180.

LYSENKO, O. 1983. *Bacillus thuringiensis*: evolution of a taxonomic conception. *Journal of Invertebrate Pathology*. 41: 295-298.

MARROQUIN, L.D.; ELYASNIA, D.; GRIFFITTS, J.S.; FEITELSON, J.S.; AROIAN, R.V. 2000. *Bacillus thuringiensis*: toxin susceptibility and isolation of resistance mutants in the nematode *Caenorhabditis elegans*. Genetics 155: 1693-1699.

OLIVEIRA, L.C; VENDRAMIM. J.D.; ROSSETTO, C.J. 1997. Efeito de genótipos de milho no desenvolvimento de *Spodoptera frugiperda* (J.E. Smith). Anais da Sociedade Entomológica do Brasil. 26: 291-298.

PANIZZI, A.R.; PARRA, J.R.P. 1991. Ecologia nutricional de insetos e suas implicações no Manejo de Pragas. São Paulo: Manole. 359 p.

PARDO-LOPEZ, L.; GOMEZ, I.; RAUSELL, C.; SANCHEZ, J.; SOBERON, M. 2006. Structural changes of the Cry1Ac oligomeric pre-pore from *Bacillus thuringiensis* induced by N-acetylgalactosamine facilitates toxin membrane insertion. Biochemistry 45: 10329-10336.

PARRA, J.R.P.; HADDAD, M.L. 1989. Determinação do número de ínstares de insetos. Piracicaba: FEALQ. 49p.

PEDIGO, L.P. 1989. Entomology and pest management. New York: MacMillan. 646 p.

PINTO, L. M. N.; AZAMBUJA, A. O.; DIEHL, E.; FIUZA, L.M. 2003. Pathogenicity of *Bacillus thuringiensis* isolated from two species of *Acromyrmex* (Hymenoptera, Formicidae). Brazilian Journal of Biology. 63(2): 301-306.

POLANCZYK, R. A. 2004. Estudos de *Bacillus thuringiensis* Berliner visando ao controle de *Spodoptera frugiperda* (J. E. Smith). 144 p. Tese (Doutorado) - Escola Superior de Agricultura Luiz de Queiroz, Piracicaba.

PRIEST, F.G. 2000. Biodiversity of the entomopathogenic, endospore-forming bacteria. In: CHARLES, J.F.; DELÉCLUSE, A.; NIELSEN-LE ROUX, C. (Ed.). Entomopathogenic bacteria: from laboratory to field application. Netherlands: Kluwer Academic Publishers, 2000. p. 1-22.

RIBEIRO, C.; DUVIC, B.; OLIVEIRA, P. 1999. Insect immunity – effects of factors produced by a nematobacterial complex In immunocompetent cells. *Journal of Insect Physiology*. 45: 677-685.

SANCHIS, V.; LERECLUS D.; MENOU, G.; CHAUFAUX, J.; LECADET, M. M. 1998.

Multiplicity of endotoxinas genes with different insecticidal specificities in *Bacillus thuringiensis aizawai* 7.29. *Molecular Microbiology*. 2: 393-404.

SOBERON, M., GILL, S.S.; BRAVO, A. 2009. Signaling versus punching hole: How do *Bacillus thuringiensis* toxins kill insect midgut cells? *Cell. Mol. Life Sci*. 66: 1337-1349.

TAMEZ-GUERRA, P.; GALÁN-WONG, L.J.; MEDRANO- ROLDÁN, H.; GARCIA-GUTIÉRREZ, C.; RODRIGUEZ-PADILLA, C.; GOMEZ-FLORES, R.A.; TAMEZ-GUERRA, R.S. 2001. Bioinseticidas: su empleo, produción y comercialización en México. Ciencia UANL. 4: 143-152.

VALICENTE, F.H.; CRUZ, I. 1991. Controle biológico da lagarta-do-cartucho, *Spodoptera frugiperda*, com o baculovírus. Sete Lagoas: Embrapa. 23p. (EMBRAPA-CNPMS, Circular Técnica, 15).

YAMAMOTO, T.; DEAN, D.H. 2000. Insecticidal proteins produced by bacteria pathogenic to agriculturas pests. In: CHARLES, J.F.; DELÉCLUSE, A.; NIELSEN-LE ROUX, C. (Ed.). Entomopathogenic bacteria: from laboratory to field application. Netherlands: Kluwer Academic Publishers. p. 81-100.

3 CAPÍTULO 2 – Artigo de revisão

MARCADORES MOLECULARES PARA ESTUDOS DA DIVERSIDADE DO GÊNERO *BACILLUS*

MARCADORES MOLECULARES PARA ESTUDOS DA DIVERSIDADE DO GÊNERO *BACILLUS*

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RESUMO

Diversas metodologias são propostas para estudos que envolvem ecologia microbiana. Os recentes avanços em ferramentas moleculares para o estudo da diversidade e função destas comunidades estão impulsionando e contribuindo para uma melhor compreensão da ecologia microbiana, onde pesquisadores aplicam esse conhecimento para gerenciar e entender os processos biotecnológicos. Biologia molecular juntamente com tecnologias genômicas estão em constante desenvolvimento contribuindo para uma explosão de metodologias promissoras. Este artigo irá descrever brevemente um resumo atualizado dessas técnicas, destacadas por sua ampla utilização, apresentando o seu potencial na caracterização de bactérias, especialmente *Bacillus* spp.

Palavras-chave: *Bacillus* spp., marcadores moleculares, RAPD-DNA, rep-PCR.

ABSTRACT

Several methods are proposed for studies involving microbial ecology. Recent advances in molecular tools to study the diversity and function of these communities are driving and contributing to a better understanding of microbial ecology, where researchers apply this knowledge to manage and understand the biotechnological processes. Molecular and genomic technologies are under constant development by contributing to an explosion of promising methodologies. This article will briefly describe a current summary of these techniques, highlighted by their extensive use, showing its potential in characterization of bactéria, specially *Bacillus* spp.

Keywords: *Bacillus* spp., molecular markers, RAPD-DNA, rep-PCR.

1 INTRODUÇÃO

O Gênero *Bacillus* é extremamente heterogêneo, tanto geneticamente quanto fenotipicamente, formando um extenso grupo taxonômico bacteriano, que possui variadas características fisiológicas, bioquímicas, citológicas e genéticas (Crighton et al., 2012). Essa diversidade ficou evidente, com a clássica caracterização fenotípica baseada principalmente em morfologia, nutrição, características de crescimento, utilização de diferentes substratos e avaliações fisiológicas (Slepecky & Hemphill, 1992; Chung et al., 2010;). Embora reações fisiológicas são geralmente utilizadas para determinar as espécies do gênero, as inconsistências nos resultados dos testes pode tornar difícil a identificação (Ash *et al*. 1991). Ash e colaboradores, em 1991, utilizando análise sequencial do RNA 16s de 51 espécies, classificaram e caracterizaram em 5 grupos filogenéticos. A organização foi iniciada em 1992 para a criação do gênero *Alicyclobacillus* que agrupou três espécies termófilas e acidófilas. Posteriormente foram propostos e validados outros gêneros incluindo: *Paenibacillus* (1994), *Aneurinibacillus* (1996), *Brevibacillus* (1996), *Salibacillus* (1999), *Gracilibacillus* (1999), *Geobacillus* (2001), *Ureibacillus* (2001), *Virginibacillus* (1998) e *Lysinibacillus* (2007).

Inúmeras espécies deste gênero e de correlatos possuem importância médica com uma ampla variedade de possibilidades: na produção de antibióticos (particularmente a bacitracina do *B. licheniformis* e a polimixina do *P. polymyxa*) ou vitaminas (como vitamina B12 e B2 do *B. megaterium* e biotina e riboflavina do *B. subtilis*); como base para testes de antibióticos (*B. cereus*, *B. circulans*, *B. megaterium*, *B. pumilus*, *B. subtilis* e *Geobacillus stearothermophilus*); na validação de desinfetantes (*B. cereus*) e no monitoramento de fumigação (*B. subtilis*); nos processos de esterilização (*G. stearothermophilus*) e de radiação (*B. pumilus*); e em diversos exames clínicos (como teste para ácido úrico utilizando o *B. fastidiosus*, um teste para detecção de *Chlamydia* utilizando uma variante da subtilisina do *B. subtilis* e um teste para triagem de sangue para fenilcetonúria utilizando também o *B. subtilis*). As endonucleases de restrição e DNA polimerases de várias espécies de *Bacillus* são consideradas importantes ferramentas de pesquisa para melhor esclarecimento de doenças e no aprimoramento de diagnósticos (Logan & Turnbull 2003).

Também integram o grupo de *Bacillus* espécies entomopatogênicas, as quais, por sintetizarem protoxinas de natureza glicoprotéica, as delta-endotoxinas, ou protoxinas cristalinas, são capazes de causar intoxicação em um considerável número de insetos. A espécie *B. thuringiensis* é capaz de produzir intoxicação e, ou, septicemia nos gêneros: *Diptera*, *Coleoptera* e *Lepidoptera*, e muitas estirpes, isoladas, têm exibido atividade contra *Hymenoptera*, *Homoptera*, *Orthoptera*, *Mallophaga* e *Isoptera* (Castilhos-Fortes *et al*. 2002; Pinto *et al*. 2003), assim como contra nematóides, ácaros e protozoários (Iudina 1996; Schnepf *et al*. 1998). As delta-endotoxinas, são corpos sólidos cristalinos imersos no citoplasma, situando-se adjacentes aos esporos. Possuem massas moleculares que variam de 25 kDa até 140 kDa sintetizados durante a esporogênese, e quando observados sob microscopia de contraste de fase (1.000 aumentos ou mais), exibem as formas mais variadas. Assim, numa investigação sobre 2.793 cepas da espécie *B. thuringiensis* isoladas de diferentes regiões geográficas do planeta, 45,9% das delta-endotoxinas eram do tipo bi-piramidal, 14,2% esféricas, 4,4% retangulares ou cubóides, 16,4% irregularmente esféricas e 19,1% do tipo puntiforme-irregulares (Bernhard *et al*. 1997).

Abordagens moleculares estão sendo cada vez mais utilizadas para a rápida identificação de espécies e linhagens (Goto et al. 2000). Desde meados da década de 1990 são utilizados dois métodos distintos para a identificação de bactérias: fenotipagem e genotipagem (Tenover et al., 1999). No método de fenotipagem a identificação das cepas é baseada em caracteres fenotípicos, incluindo morfologia das colônias em diferentes meios de cultura, testes bioquímicos, sorológicos, patogenicidade e susceptibilidade a antibióticos (Li and Fournier, 2009; Jackson et al., 2006). Estes caracteres estão intimamente relacionados com a sua diversidade genética. Em meados de 1980 vários métodos genéticos se desenvolveram para a genotipagem de bactérias e tornaram-se frequentemente utilizados devido a sua alta resolução (Cai et al., 2008). O perfil genético identificado de todas as bactérias por um método de genotipagem pode ser tão específico como uma única impressão digital (Li and Fournier, 2009).

Os atuais métodos de genotipagem bacterianos podem ser classificados em três categorias: padrões de bandas de DNA; sequenciamento de DNA baseado no

polimorfismo das sequências e hibridização do DNA (Li and Fournier, 2009; Versalovic et al.,1991).

No primeiro método, os padões de bandas podem ser gerados diretamente por: digestão de endonucleases de restrição (ER), amplificações de regiões do genoma ou por uma combinação de amplificações com enzimas de restrição (Li et al., 2009). No método de seqüenciamento de DNA, a discriminação entre as cepas bacterianas é realizada após a determinação e comparação de uma sequência genética conhecida. Nos métodos de hibridação, a discriminação é realizada por meio da análise da hibridação de sondas específicas. Sistemas de macro e microarranjos de DNA têm sido desenvolvidos para obter resultados precisos e mais rápidos para a descrição bacteriana (Versalovic et al., 2009).

Sabe-se que a diversidade das bactérias é maior que a de qualquer outro grupo de organismos, no entanto, os meios de cultivo são, em maior ou menor extensão, seletivos a grupos particulares. Até mesmo quando se quer utilizar um meio seletivo para determinado organismo-alvo, algumas estirpes não são cultiváveis e provavelmente serão excluídas das análises (Coutinho et al., 1999). Amann et al. (1995) sugerem que apenas 1 a 10% das espécies bacterianas do planeta tenham sido identificadas, deixando vasta porção dessa biota desconhecida e não estudada.

Considerando a importância e a contribuição dos métodos moleculares para taxonomia, ecologia e genética que facilitem a identificação de um padrão molecular de isolados, esta revisão apresenta as principais técnicas utilizadas na microbiologia molecular.

2 MÉTODOS BASEADOS NO PADRÃO DE BANDAS DO DNA

Métodos baseados em padrões de bandas de DNA classificam as bactérias de acordo com o tamanho dos fragmentos gerados por amplificação em PCR ou digestão do DNA genômico por endonucleases de restrição ou uma combinação de ambos (Versalovic et al., 1994). A identificação de bandas geradas pode ser determinada por métodos convencionais através de eletroforese em gel de agarose ou sistemas automatizados de eletroforese (Li et al., 2009).

2.1 PCR - Reação em Cadeia da Polimerase

Os métodos moleculares receberam grande impulso com o desenvolvimento da técnica conhecida como PCR. Essa técnica descrita por Saiki et al. (1985), permite amplificar pequenos e específicos segmentos do genoma, possibilitando a obtenção*, in vitro*, de várias cópias de determinada região do DNA. Como a reação de PCR é específica, pode-se obter a amplificação de seqüências de nucleotídeos-alvo mesmo em uma amostra com grande diversidade de seqüências, permitindo a detecção de organismos específicos em misturas heterogêneas. Seqüências do DNA de determinados microrganismos podem ser amplificadas, utilizando-se *primers* (seqüências iniciadoras) complementares àquelas localizadas em locais específicos do genoma. O que ocorre é a extensão do fragmento de DNA a partir dos *primers*, pela ação da DNA polimerase termoestável, a Taq DNA polimerase (isolada originalmente do microrganismo *Thermus aquaticus*). Antes da extensão, o DNA é desnaturado e o *primer* anelado, sendo o ciclo (desnaturação - anelamento - extensão) repetido várias vezes, permitindo a amplificação exponencial daquela seqüência específica (Saiki et al.,1985).

A detecção de organismos específicos em extratos de planta ou de solo pode ser determinada utilizando-se essa técnica. Nesse tipo de estudo, os *primers* específicos são usados na PCR com o DNA extraído dos extratos como molde. A detecção de determinado organismo é indicada pela formação de um produto de amplificação de tamanho definido. Para a confirmação da especificidade dessa amplificação, pode-se fazer uma hibridização ou o seqüenciamento do produto de PCR (Kirchhof et al., 1997).

A sensibilidade e a especificidade da PCR podem ser aprimoradas com um método conhecido por *nested* PCR, em que as amplificações iniciais são realizadas com um par de *primers* sem grande especificidade como os universais. Com o produto amplificado, uma segunda rodada de amplificações é conduzida, dessa vez com os *primers* específicos. Essa técnica foi utilizada, com sucesso por Rosado et al. (1998) para aumentar a probabilidade de amplificação específica do DNA do *nif*H de *Paenibacillus azotofixans* a partir de amostras de solo. Grande parte das técnicas moleculares atualmente utilizadas apropriam-se da PCR ou de suas variações para o estudo da diversidade de bactérias nos mais diferentes ambientes.

2.2 RAPD - DNA polimórfico amplificado ao acaso

Esta técnica também conhecida como AP-PCR (PCR com primers arbritários) utiliza *primers* curtos com seqüências de nucleotídeos arbitrários na PCR para amplificar o DNA genômico de forma aleatória. Fragmentos polimórficos de RAPD são potencialmente úteis para identificar diferenças seqüenciais, níveis taxonômicos e poder discriminatório em níveis intra-específicos (Fungaro & Vieira, 1998). Ao contrário da clássica análise de PCR, a região genômica que será amplificada não é conhecida e a amplificação depende das posições que são complementares as sequências de *primers* (Bart et al., 1998). Os amplicons de diferentes tamanhos produzidos em múltiplos loci por RAPD-PCR podem ser observados em diferentes padrões de bandas em gel de agarose e as bactérias podem ser genotipados em função desses padrões.

As análises de RAPD têm se aplicado na diferenciação de isolados de cepas de *B. thuringiensis*, bem como na diferenciação de genótipos de *B. anthracis* (Levy & Feldman 2004), *B. subtilis* (Matarante et al. 2004), e bacilos termofílicos (Rückert et al. 2004).

Mohammad et. al (2004) utilizaram a técnica amplificando o DNA genômico total de 16 isolados de *B. thuringiensis* de diferentes habitats da Jordânia e três estirpes de referência comprovando a incidência de polimorfismo. Sadder et al. (2006) também comprovaram alto polimorfismo entre os isolados de *B. thuringiensis* na Jordânia. Pattanayak et al. (2001) utilizaram 19 *primers* aleatórios para avaliar o polimorfismo em 21 estirpes de *B. thuringiensis* de diferentes sorotipos. Um total de 172 fragmentos polimórficos (variando em tamanho de 161-2789 pb) foram amplificados a partir de 13 dos 19 *primers*, revelando valores muito baixos de similaridade genética (3-68%) entre os sorovares, indicando a divergência elevada.

2.3 rep-PCR-Sequências palindrômicas extragênicas repetidas

A rep-PCR faz uso de sequências oligonucleotídicas iniciadoras complementares de sequências de DNA repetitivas conservadas e presentes em numerosas cópias no genoma da maioria das bactérias (Versalovic et al., 1994). As funções destes elementos intercalados repetitivas de DNA ainda são desconhecidos (Versalovic et al., 1991). Três famílias de seqüencias rep foram identificadas: REP

(*Repetitive Extragenic Palindromic elements)* com 35-40 pares de bases, as quais são conservadas em várias espécies bacterianas; ERIC (*Enterobacterial Repetitive Intergenic Consensus elements*) de 124-127 pares de bases, as quais contêm um elemento repetitivo central invertido altamente conservado e que estão localizados nas regiões extragênicas do genôma bacteriano e o elemento BOX com 154 pares de bases. A reprodutibilidade da REP-PCR é muito maior do que RAPD-PCR com os iniciadores utilizados para amplificação específica (Li et al., 1999).

As sequências REP e ERIC foram inicialmente identificadas no seqüenciamento dos genomas de *Escherichia coli* e *Salmonella typhimurium* (Hulton et al., 1991), mas existem numerosas descrições da sua existência em outras espécies bacterianas. A sua amplificação por PCR pode ser feita com um único par ou vários pares de iniciadores.

O uso de *primers* amplifica seletivamente regiões distintas no genoma. Os *primers* BOX são geralmente recomendados por gerar impressões digitais mais robustas e produzir um padrão de fragmentos mais complexo. Os *primers* para REP geram menor complexidade, mas ainda produzem impressões digitais reproduzíveis e que permitem a diferenciação dos isolados. O conjunto de *primers* para o ERIC é mais sensível a condições sub ótimas da PCR, como presença de contaminantes na amostra de DNA, porém gera padrões de bandeamento altamente discriminatórios (Rademaker & De Bruijn, 1997).

Os elementos repetitivos podem estar presentes em ambas as orientações e os iniciadores estão concebidos de forma a permitirem a síntese de DNA a partir da repetição invertida, nos REP e ERIC-PCR, e da subunidade boxA, nos BOX-PCR, amplificando regiões genômicas distintas localizadas entre os elementos repetitivos (Versalovic et al, 1991; Martin et al, 1994).

Regiões repetitivas apresentam maior variabilidade do que outras regiões genômicas e podem ser utilizadas para analisar a relação genética entre estirpes (Van Belkum et al. 1997). De acordo com Brumlik et al. (2001) a referida técnica permitiu a distinção de 105 cepas de *B. anthracis* e outras cepas do grupo *B. cereus*. Kim et al. (2001) utilizaram BOX A1R baseada em rep-PCR para estabelecer a relação genética entre 17 cepas do grupo *B. cereus*. Concluiram também que *B.*

anthracis pode ser separado a partir das espécies estreitamente relacionadas, mas a relação genética entre as outras espécies do grupo *B. cereus* não foi bem descrita por causa de um pequeno número de estirpes.

A utilização simultânea destas técnicas aumenta o poder discriminatório da tipagem. Os resultados apresentam ainda uma boa correlação com os resultados de RFLP-PFGE, tendo, no entanto, menor poder discriminatório (Ibrahim et al., 1994).

2.4 RFLP - Polimorfismo do tamanho de fragmentos de restrição

Esta técnica se baseia na digestão por enzimas de restricão do DNA ou de seqüências específicas, seguida por separação eletroforética em gel dos fragmentos resultantes. As enzimas de restrição reconhecem seqüências específicas de bases na dupla hélice do DNA e clivam ambos os fragmentos duplex em pontos específicos (Stryer, 1996). O RFLP é baseado em padrões de restrição com o uso de enzimas selecionadas com base na sua habilidade de revelar polimorfismo nos fragmentos de DNA analisados. Devido a dificuldades nas análises de muitas bandas, hibridação com DNA são frequentemente usados nesta técnica. Por exemplo, utilizando sondas derivadas de IS6110, o elemento de inserção é um método padrão ouro para a digitação do complex *Mycobactedeuterio tuberculosis* (Stryer, 1996).

Quando são realizados estudos de RFLP com o DNA total bacteriano, gera-se uma grande quantidade de fragmentos. Portanto, as diferenças nos tamanhos dos fragmentos não podem ser visualizadas diretamente no gel, posto que os inumeráveis fragmentos resultantes do tratamento com a enzima de restrição produzem, nesse gel, um efeito de arraste contínuo. Para se fazer a detecção dos marcadores RFLP, os fragmentos do gel são transferidos para uma membrana de nylon (ou nitrocelulose) por um processo denominado *Southern Blot*. Por fim, a visualização de fragmentos polimórficos é possível por meio da hibridização contra sondas de DNA que apresentam sequências homólogas as do DNA imobilizado na membrana (Ferreira & Grattapaglia, 1998).

Gündish et al. (1993) analisaram os resultados de RFLP obtidos depois da digestão do DNA total de estirpes de *Azospirillum* por enzimas de corte raro. Esses

autores utilizaram uma técnica especial de eletroforese, conhecida como Eletroforese de Gel com Campo Pulsátil (PFGE). Devido a mudanças periódicas no campo elétrico, essa técnica permite separar fragmentos de alto peso molecular, resultando em um padrão de restrição bastante claro do genoma bacteriano. Ao contrário da PGFE, permite produzir centenas de fragmentos de restrição curtos devido à digestão do DNA genômico com frequência de corte (Li et al., 1999).

Laguerre et al. (2001) utilizaram essa metodologia para avaliar a diversidade de estirpes de rizóbio. As análises de RFLP dos genes simbióticos nodC e nifH mostraram, de maneira geral, sua correlação com o espectro hospedeiro e independência do *status* taxonômico, já que os resultados não foram concordantes com aqueles baseados nas seqüências do 16S rDNA. Segundo os autores, esses resultados suportam a tese de que a transferência lateral entre espécies de rizóbio desempenha importante papel na diversidade e na estrutura de suas populações naturais.

2.5 PCR-RFLP - Fragmentos de DNA obtidos por Enzimas de Restrição

Este método baseia-se na digestão e separação dos fragmentos de eletroforese em gel de agarose após a amplificação de um locus específico com *primers* específicos (Tenover et al., 1999). A diferença do método de RFLP é a limitação da região de DNA de interesse e tem sido amplamente utilizada para a tipagem e discriminação de uma variedade de bactérias (Li et al., 2009).

2.6 MLVA- Análise do Número Variável de Repetições em Tandem

MLVA é uma ferramenta de genotipagem que fornece dados com base no número de sequências repetitivas encontradas nos cromossomos bacterianos (Ramazanzadeh & McNerney, 2007). Números variáveis de repetições em tandem (VNTR) são as repetições consecutivas que estão dispersas em múltiplas cópias no genoma bacteriano. VNTRs podem ser encontradas nas regiões não-codificadoras, bem como, em genes e o número de repetições em série podem variar entre as cepas (Li et al., 2009; Ramazanzadeh & McNerney, 2007). O número de repetições pode ser determinado utilizando iniciadores que são complementares a regiões conservadas flanqueando as repetições em tandem. Esta discriminação é realizada comparando os produtos de PCR para determinar o grau relativo de bactérias

(Ramazanzadeh & McNerney, 2007). O MLVA mostrou-se como uma boa ferramenta para estudos epidemiológicos e filogenéticos em espécies bacterianas por revelar quantitativamente as similaridades e diferenças entre isolados (Roring et al., 2002) Dentre as diferentes espécies bacterianas analisadas com o MLVA destacam-se: *Yersinia pestis* (Adair et al., 2000; Le Fléche et al., 2001; Klevystska et al., 2001; Pourcel et al., 2004), *Y. enterocolitica* (De Benito et al., 2004), *B. anthracis* (Keim et al., 2000), *Haemophilus influenzae* (Van Belkum et al., 1999), *Enterococcus faecalis* (Titze-de-Almeida, 2004), *Mycoplasma genitalium* (Roring et al., 2002) e *Francisella tularensis* (Farlow et al., 2001).

3 MÉTODOS BASEADOS EM SEQUENCIAMENTO DE DNA

O sequenciamento de DNA é o processo de leitura das bases de nucleotídeos de uma molécula (Sanger et al., 1977). Ele inclui qualquer método ou a tecnologia que é usada para determinar a ordem das quatro bases (adenina, guanina, citosina e timina) em uma cadeia de DNA (Sanger et al., 1977).

3.1 MLST - Multilocus Sequence Typing

Métodos baseados em seqüência de DNA estão tornando-se o “padrão-ouro” para o estudo de genética de populações microbianas. Este método baseia-se no sequenciamento de regiões específicas de sete genes (*housekeeping*) e utiliza a reação de PCR para amplificar os genes a sequenciar (Feil et al, 2004). Para cada situação particular, pode ser necessário determinar empiricamente qual o gene cuja sequencia é mais indicada. MLST envolve o seqüenciamento de fragmentos de 450-600 pb de genes específicos e a designação de perfis de alelos, que resultam em tipos de sequências - ST (Olvera et al., 2007) e têm sido utilizada com sucesso para a determinação de “complexos clonais” (CC) de vários patógenos humanos, animais e microbiológicos (Cooper & Feil, 2004).

O principal objetivo do MLST é o de proporcionar um sistema de tipagem portátil, preciso e altamente discriminativo, sendo que essa abordagem tem sido utilizada com sucesso em diferentes tipos de bactérias e outros microrganismos (Maiden et al., 1998).

3.2 Método Sanger

O método de Sanger também é conhecido por sequenciamento didesoxi de terminação da cadeia, com base na síntese da cadeia de DNA através da utilização de didesoxinucleótidos (ddNTP) em adição aos nucleótidos normais (dNTP) encontradas no DNA que interrompem a etapa de alongamento da amplificão.

Por este método, uma fita simples de DNA que será seqüenciada é hibridizada com um primer de desoxinucleotídeos marcado na extremidade 5' (Speed, 2002). Quatro misturas de reações são preparadas, onde os *primers* utilizados serão alongados por uma DNA polimerase. Cada mistura contém quatro desoxinucleosídeos trifosfato normais e quatro didesoxinucleosídeos trifosfato em uma razão de aproximadamente 1/100. Uma vez que um didesoxinucleotídeo não tem extreminade 3'-OH, não é possível haver alongação a partir do nucleotídeo adicionado, parando a reação. Desta forma, cada mistura de reação produzirá cadeias prematuramente terminadas de acordo com a ocorrência de um didesoxinucleotídeo adicionado. Cada mistura é então separada em um gel de sequenciamento por eletroforese para se detectar a cada um dos nucleotídeos presentes na sequência de DNA lida (Sanger et al, 1977).

3.3 Pirosequenciamento

O método de pirosequenciamento consiste em uma nova abordagem molecular e se beneficia de uma técnica capaz de captar a emissão de luz causada pela adição de uma luciferase, acoplada à polimerização do DNA previamente fragmentado e aderido a microesferas, com o uso de sequências adaptadoras (Ahmedian et al., 2006). Ao contrário do método de Sanger, o pirosequenciamento não é um método eletroforético. Este método baseia-se na detecção em tempo real de pirofosfatases libertados durante o alongamento da cadeia e a síntese de DNA ocorre através de um complexo de reações que inclui enzimas (ATP sulfurilase e luciferase) e substratos (adenosina 5' fosfossulfato e luciferina). O grupo pirofosfato, liberado durante a adição de um nucleotídeo, resulta na produção de luz detectável. Portanto, quando um novo nucleotídeo é incorporado em uma cadeia crescente de DNA através da DNA polimerase, pirofosfato é gerado de maneira estequiométrica, resultando na produção de ATP. O ATP produzido leva à conversão enzimática da

luciferina com emissão de fótons. À medida que os componentes da reação diminuem, um novo ciclo de reagentes é introduzido e então a incorporação de nucleotídeos específicos é avaliada de maneira sequencial.

Como a clássica PCR, este também requer iniciadores para o alongamento da cadeia (ATP sulfurilase, Apirase, Adenosina Luciferase e fosfosulfate) (Margulies et al, 2005).

4 MÉTODOS BASEADOS EM HIBRIDIZAÇÃO DO DNA

O uso de sondas moleculares é um método versátil que permite não só o estudo de organismos isolados e cultivados como também o desses organismos em amostras ambientais. Uma sonda consiste em fragmento marcado de DNA ou RNA complementar à seqüência de um gene-alvo de interesse. Em condições controladas, ocorre a hibridização entre a sonda e sua seqüência-alvo (Coutinho et al., 1999). Sondas de ácidos nucléicos podem ser construídas para detecção de genes específicos e então serem usadas para detecção de organismos com o genoma correspondente. A combinação da PCR, com a utilização de sondas, aumenta significativamente a sensibilidade dos protocolos de detecção (Rosado et al., 1999).

4.1 RNA Ribossômico como Marcador Filogenético

Clonagem e sequenciamento do 16S rDNA e 16S rRNA tem sido cada vez mais utilizados em estudos de ecologia molecular microbiana. As primeiras aplicações de técnicas baseadas em ácidos nucléicos foram referentes a relações filogenéticas entre microrganismos, determinada pela análise da seqüência do 16S rDNA (Macrae, 2000). Pace et al. (1986), também avaliararam a composição táxon de uma comunidade microbiana por meio de análises de moléculas de RNA ribossômico.

Os ácidos ribonucléicos ribossomais (rRNA) são considerados os biopolímeros mais adequados para estudos de diversidade microbiana (Muyzer, 1993). Seus genes, rDNAs, são universalmente distribuídos, sendo uma das moléculas com o maior grau de conservação. Sua variabilidade pode apresentar-se em maior ou menor extensão em diferentes regiões da molécula (Lane at al. 1985).

16S rRNA (1500 nucleotídeos) geram uma grande quantidade de informações úteis para inferências filogenéticas. Apesar do 23S rRNA conter duas vezes mais informações e, portanto, gerar maior acurácia nas inferências filogenéticas, a molécula menor (16S rRNA), devido à facilidade de seqüenciamento, tornou-se referência (Stahl, 1997).

Com a utilização da reação em cadeia da polymerase (PCR), fragmentos do gene do 16S rRNA, em amostras complexas, podem ser seletivamente amplificados e bibliotecas genômicas derivadas das amplificações dessas amostras são produzidas pelo método de clonagem. Estes clones contêm fragmentos definidos que podem ser rapidamente seqüenciados. Outra rota para esse tipo de caracterização molecular é a clonagem e o seqüenciamento do cDNA transcrito a partir do 16S rRNA com utilização da enzima transcriptase reversa (Amann et al., 1995).

Clones são desenvolvidos a partir da extração de DNA genômico através de uma amostra seguido de amplificação e clonagem dos genes 16S rRNA. Esta etapa de clonagem é necessária para separar as diferentes cópias do modelo misto de 16S rRNA presentes no DNA genômico extraído de comunidades microbianas (Sanz & Kochling 2007). Os clones também permitem a detecção ou quantificação de um determinado grupo de microrganismos, ou genes funcionais, como descrito por Zhu et al. (2007). Uma vez clonado, o gene 16S rRNA pode ser rastreado por uma variedade de métodos, como a hibridização de colônias com sondas de genes específicos, colônias PCR com primers para a inserção dos produtos PCR clonados, e seqüenciamento de DNA seguido de avaliações filogenéticas sobre a diversidade microbiana da amostra original (Olsen et al. 1991, Cole et al. 2003).

Atualmente, outras técnicas vêm sendo desenvolvidas, com base na utilização desses marcadores, fazendo com que sejam as ferramentas moleculares mais utilizadas para a exploração da diversidade e da análise da estrutura de comunidades microbianas (Kozdroj & Elsas, 2000).

4.2 Microarray ou Microchips de DNA

A tecnologia de microchips de DNA representa uns dos últimos avanços dos

métodos moleculares, com grande potencial para a elucidação de uma gama de aspectos da ecologia microbiana, incluindo identificação de variações na estrutura de comunidades entre diferentes amostras (Ogram, 2000), identificação de grupos filogenéticos que podem estar ativos ou sem atividade durante determinado período ou sob determinado tratamento (Guschin et al., 1997) e identificação de diferenças entre estirpes isoladas de diferentes ambientes (Murray et al., 1999).

Nessa metodologia, os microchips são preparados com sondas de oligonucleotídeos imobilizadas em uma matriz de gel de poliacrilamida ligada à superfície de uma placa de vidro (Yershov et al., 1996). Os oligonucleotídeos sintetizados para serem utilizados nos microchips devem ser purificados por eletroforese em gel ou por cromatografia líquida de alta performance. Essa exigência aumenta o controle sobre a qualidade da estringência, assegurando grande especificidade. As amostras de DNA ou RNA são marcadas com fluorocromos ou outros compostos que permitem sua quantificação. Centenas ou mesmo milhares de oligonucleotídeos diferentes podem ser imobilizados em um único microchip, permitindo a detecção simultânea de grande variedade de microrganismos em uma amostra. Além disso, um microchip pode ser utilizado por até 30 vezes, sem deterioração do sinal de hibridização, bastando ser lavado com água destilada (Guschin et al., 1997).

Small et al. (2001) utilizaram o método de microarray para detecção do 16S rRNA intacto extraído de amostras de solo. RNAs totais de *Geobacter chapellei* e *Desulfovibrio desulfuricans* (redutores de ferro e sulfato, respectivamente) foram detectados com a hibridização com sondas universais e espécie-específicas. Com base nos resultados mostrou-se a possibilidade de aplicação da técnica de microarray na detecção direta de microrganismos em amostras ambientais, sem a necessidade de utilização da PCR.

4.3 FISH- Hibridização *in situ* fluorescente

FISH é uma técnica molecular específica para RNA ou DNA e atua na detecção de sequências de células microbianas dentro dos seus ambientes naturais, permitindo a visualização direta de microrganismos não-cultivados (Amann et al. 2001). Esse método utiliza sondas fluorescentes que são geralmente sequências

curtas de DNA (16-20 nucleótidos) marcadas com corante fluorescente que reconhece as sequências de RNA e DNA em células fixas com a qual hibridiza *in situ*, sendo possível detectar representantes específicos de nível taxonômico diferente (Sanz & Kochling 2007).

5 CONSIDERAÇÕES FINAIS

Tecnologias genômicas juntamente com ferramentas moleculares estão em constante desenvolvimento e aperfeiçoamento, marcados pela introdução de novas tecnologias na ecologia microbiana (Kahvejian et al. 2008).

Várias técnicas de tipagem molecular foram desenvolvidas durante a última década para a identificação e classificação de bactérias a nível de estirpe (McCartney, 2002). Os métodos atuais de perfis moleculares, por exemplo, fragmentos de DNA obtidos por enzimas de restrição (RFLP) e análise do gene 16S, representam ferramentas poderosas para o estudo da diversidade de bactérias e fungos em ambientes complexos (Kahvejian et al. 2008). Por outro lado, as comunidades microbianas permanecem entre as mais difíceis de se caracterizar devido a sua grande diversidade. Os perfis moleculares destas comunidades microbianas obtidas por *primers* universais são, assim, muitas vezes complexos, e as espécies menos abundantes, mas potencialmente muito importantes podem escapar da detecção (Copley, 2000).

As técnicas moleculares possuem algumas limitações, mesmo assim as informações obtidas com sua aplicação melhoram o conhecimento sobre o mundo microbiano (Dunbar et al 1999). Mesmo com as limitações conhecidas e observadas para todas as ferramentas moleculares, as vantagens proporcionadas pelas técnicas abordadas anteriormente são mais representativas do que as desvantagens, uma vez que os dados gerados melhoram o conhecimento sobre o mundo microbiano (Dunbar et al., 1999).

À medida que a eficiência das técnicas moleculares possibilita análises comparativas de comunidades microbianas, atenção deve ser dada à padronização dos parâmetros ligados ao processamento das amostras, pois qualquer diferença detectada entre os padrões dessas comunidades é atribuída a diferenças na

estrutura gênica das comunidades e não a diferenças na preparação das amostras (Marsh et al., 2000).

Outro fator importante que deve ser considerado é que apesar das ferramentas moleculares fornecerem novas perspectivas às avaliações da diversidade microbiana dos solos, elas não possibilitam que organismos, com potencial valor biotecnológico, sejam cultivados e trabalhados, mostrando a necessidade de se desenvolver técnicas de cultivo que permitam o estudo e a utilização desses organismos para propósitos biotecnológicos.

6 REFÊRENCIAS BIBLIOGRÁFICAS

ADAIR, D.M.; WORSHAM, P.L.; HILL, K.K.; KLEVYTSKA, A.M.; JACKSON, P.J.; FRIEDLANDER, A.M. 2000. Diversity in a Variable-Number Tadem Repeat from *Yersinia pestis. Journal of Clinical Microbiology*. 38: 1516-1519.

AHMEDIAN, A.; EHN, M.; HOBER, S. 2006. Pyrosequencing: history, biochemistry and future. *Clinica Chimica Acta*. 363:83-94.

AMANN, R. I.; LUDWIG, W.; SHLEIFER, K-H. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiology and Molecular Biology Review 59, 143-169.*

AMANN, R.; FUCHS, B.M.; BEHRENS, S. 2001. The identification of microorganisms by fluorescence *in situ* hybridization. *Current Opinion in Biotechnology*. 3:231-236.

ASH, C.; FARROW, L.A.E.; WALLBANKS, S.; COLLINS, M.D.; 1991. Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit-ribosomal RNA sequences. *Letters in Applied Microbiology.* 13, 202-206.

BART, A.; SCHUURMAN, I.G.; ACHTMAN, M.; CAUGANT, D.A.; DANKER, J.; VAN DER ENDE, A. 1998. Randomly amplified polymorphic DNA genotyping of serogroup A meningococci yields results similar to those obtained by multilocus enzyme electrophoresis and reveals new genotypes. *J Clin Microbiol*. 36:1746-1749.

BERNHARD, K.; JARRET, P.; MEADOWS, M.; BUTT, J.; ELLIS, D.J.; ROBERTS, G.M.; PAULI, S.; RODGERS, P.; BURGES, D. 1997. Natural, isolates of *Bacillus thuringiensis* worldwide distribution, characterization, and activity against insects pests. *Journal of Invertebrate Pathology.* 70:59- 68.

BRUMLIK, M.J.; SZYMAJDA, U.; ZAKOWSKA, D.; LIANG, X.; REDKAR, R.J.; DEL-VECCHIO, V.G. 2001. Use of long-range repetitive element polymorphism-PCR to differentiate *Bacillus anthracis* strains. *Applied and Environmental Microbiology.* 67: 3021–3028.

CAI, C.G.; CHEN, JI-SHUANG.; QI, JIONG-JIONG.; YUN, Y.; ZHENG, X. 2008. Purification and characterization of keratinase from a new *Bacillus subtilis* strain. *J*

Zhejiang Univ Sci B. 9: 713–720.

CASTILHOS-FORTES, R.; MATSUMURA, A.T.S.; DIEHL, E.; FIUZA, L.M. 2002. Susceptibility of Nasutitermes ehrhardti (Isoptera: Termitidae) to *Bacillus thuringiensis* subspecies. *Brazilian Journal of Microbiology* 33: 219-222.

CHUNG, E.; KWEON, H.; YIACOUMI, S.; LEE, I.; JOY, D.C.; PALUMBO, A.V.; TSOURIS, C. 2010. Adhesion of spores of *Bacillus thuringiensis* on a planar surface. *Environmental science e technology*. 44:290-296.

COLE, J.R.; CHAI, B.; MARSH, T.L.; FARRIS, R.J.; WANG, Q.; KULAM, S.A.; CHANDRA, S.; McGARREL, D.M.; SCHMIDT, T.M.; GARRITY, G.M.; TIEDJE, J.M. 2003. The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucleic Acids research.* 31: 442–443.

COOPER, J.E.; FEIL, E.J. 2004. Multilocus sequence typing—what is resolved? *Trends Microbiol*. 12:373–377

COPLEY, J. 2000. Ecology goes underground. *Nature*. 406:452-454.

COUTINHO, H. L. C.; OLIVEIRA, V. M.; MANFIO, G. P.; ROSADO, A. S. 1999. Evaluating the microbial diversity of soil samples: Methodological innovations. *Anais da Academia Brasileira de Ciências*, Rio de Janeiro. 71:491-503.

CRIGHTON, T.; HOILE, R.; COLEMAN, N.V. 2012. Comparison of quantitative PCR and culture-based methods for evaluating dispersal of *Bacillus thuringiensis* endospores at a bioterrorism hoax crime scene. *Forensic Science International*. 219:88-95.

DE BENITO, I.; HANAGE, W.P.; DAVIS, J. 2004. A polymorphic tandem repeat potentially useful for typing in the chromosome of *Yersinia enterocolitica. Journal of General Microbiology*. 150:199-204.

DUNBAR, J.; TAKALA, S. M.; BARNS, S. M.; DAVIS, J. A.; KUSKE, C. R. 1999. Levels of community diversity in four arid soils compared by cultivation and 16S rRNA gene cloning. *Applied and Environmental Microbiology*. 65:1662-1669.

FARLOW, J.; KIMOTHY, L.; SMITH, L.; JANE, W. 2001. *Francisella tularensis* strain typing using multiple-locus, variable-number tandem repeats analysis. *Journal of Clinical Microbiology*. 39:3186-3192.

FEIL, E.J.; LI, B.C.; AANENSEN, D.M.; HANAGE, W.P.; SPRATT, B.G. 2004. eBURST: Inferring Patterns of Evolutionary Descent among Clusters of Related Bacterial Genotypes from Multilocus Sequence Typing Data. *Journal of Bacteriology*. 186:1518-1530.

FERREIRA, M.E.; GRATTAPAGLIA, D. 1998. Introduccion al uso de marcadores moleculares en el analisis genetico. Brasilia: Embrapa-Cenargen, 220 p.

FUNGARO, M.H.P.; VIEIRA, M.L.C. 1998. Aplicações da PCR em ecologia molecular. In: MELO, I. S. de; AZEVEDO, J.L. de, (Ed.). Ecologia microbiana. Jaguariúna: Embrapa-CNPMA. p. 205- 227.

GOTO, K.; OMURA, T.; HARA, Y.; SADAIE, Y. 2000. Application of the partial 16S rDNA sequence as an index for rapid identification of species in the genus *Bacillus*. *J Gen Appl Microbiol*. 46:1-8

GÜNDISH, C.; KIRCHHOF, G.; BAUR, M.; BODE, W.; HARTMANN, A. 1993. Identification of *Azospirillum* species by RFLP and pulsed-field gel electrophoresis. *Microbe Releases.* 2: 41-45.

GUSCHIN, D. Y.; MOBARRY, B. K.; PROUDINIKOV, D.; STAHL, D. A.; RITTMANN, B.; IZARBEKOV, A. D. 1997. Oligonucleotide microchips as genosensors for determinative and environmental studies in microbiology. *Applied and Environmental Microbiology*. 63:2397- 2402.

HULTON, C.S.J., HIGGINS, C.F., SHARP, P.M. 1991. ERIC sequences: a novel family of repetitive genomes of *Escherichia coli*, *Salmonella typhimurium* and other bacteria. *Molecular Microbiology* 5:825-834.

IBRAHIM, S.I.L.; PERRY, R.N.; BURROWS, P.R.; HOOPER, D.J. 1994. Differentiation of species and popula- tions of Aphelenchoidesand of Ditylenchus angustus using a fragment of ribosomal DNA. *Journal of Nematology*. 26:412-421.

IUDINA, T.G. 1996. A comparison of the antibacterial activity of the parasporal bodies of different bacilli. Izv. *Akad Nauk Ser Biol./Proc. Russ. Acad. Sci. (Biol),* 5:535–541.

JACKSON, S.G., GOODBRAND, R.B., AHMED, R.; KASATIYA, S. 2006. *Bacillus cereus* and *Bacillus thuringiensis* isolated in a gastroenteritis outbreak investigation*. Lett Appl Microbiol.* 21:103–105.

KAHVEJIAN, A.; QUACKENBUSH, J.; THOMPSON, J.F. 2008. What would you do if you could sequence everything? *Nat Biotechnol.* 26:1125–1133.

KEIM, P.; PRICE, L.B.; KLEVYTSKA, A.M.; SMITH, K. 2000. Multiple-locus variable-number tandem repeat analysis reveals genetic relationships within *Bacillus anthracis. Journal of Bacteriology***.** 182: 2928-2936.

KIM, W.; HONG, Y.P.; YOO, J.H.; LEE, W.H.; CHOI, C.S.; CHUNG, S-I. 2001. Genetic relationships of *Bacillus anthracis* and closely related species based on variable-number tandem repeat analysis and BOX-PCR genomic fingerprinting. *FEMS Microbiology Letters*. 207: 21-27.

KIRCHHOF, G.; REIS, V. M.; BALDANI, J. I.; ECKERT, B.; DÖBEREINER, J.; HARTMANN, A. 1997. Ocurrence, physiological and molecular analysis of endophytic diazotrophic bacteria in gramineous energy plants. *Plant & Soil, The Hague*. 194: 44-55.

KLEVYTSKA, A. M.; LANCE, B.; PRICE, L.; JAMES, M.; SCHUPP, L. 2001. Identification and characterization of variable-number tandem repeats in the *Yersinia pestis* genome. *Journal of Clinical Microbiology.* 39: 3179-3185.

KOZDRÓJ, J.; ELSAS, J.D. 2000. Response of the bacterial community to root exudates in soli polluted with heavy metals assessed by molecular and cultural approaches. *Soil Biology and Biochemistry*. 32:1405-1417.

LAGUERRE, G.; NOUR, S. M.; MACHERET, V.; SANJUAN, J.; DROUIN, P.; AMARGER, N. 2001. Classification of rhizobia based nodC and nifH gene analysis reveals a close phylogenetic relationship among *Phaseolus vulgaris* symbionts. *Microbiology Reading.* 174:981-993.

LANE, D. L.; PACE, B.; OLSEN, G. J.; STAHL, D. A.; SOGIN, M. L.; PACE, N. R. 1985. Rapid determination of 16S ribossomal RNA sequences for phylogenetic analyses*. Proceedings of the National Academy of Sciences.* 82:6955-6959.

LE FLÈCHE, et al. 2001. A tandem repeats database for bacterial genomes: application to the genotyping of *Yersinia pestis* and *Bacillus Anthracis. BMC microbiology.* 1:15-21.

LEVY, A.A.; FELDMAN, M. 2004. Genetic and epigenetic reprogramming of the wheat genome upon allopolyploidization. *Biological Journal of the Linnean Society*. 82: 607–613.

LI, W.; RAOULT, D.; FOURNIER, P. 2009. Bacterial strain typing in the genomic era. *FEMS Microbiol Rev.* 33:892-916.

LI, Z.X.; LI, X.H.; KINNY, P.D.; WANG, J.; 1999. The breakup of *Rodinia*: did it start with a mantle plume beneath South China? *Earth Planet. Sci. Lett*. 173:171–181.

LOGAN, N.A.; TURNBULL, P.C. 2003. *Bacillus* and related genera. Pp. 445-460. In P. MURRAY, R. & BARON, E.J. *Ed. Manual of Clinical Microbiology*, 8th ed., vol. 1. *American Society for Microbiology.*

MACRAE, A. 2000. The use of 16S rDNA methods in soil microbial ecology. *A Brazilian Journal of Microbiology.* 31:77–82.

MAIDEN, M.C.J.; BYGRAVES, J.A.; FEIL, E.; MORELLI, G.; RUSSELL, J.E.; URWIN, R.; ZHANG, Q.; ZHOU, J.; ZURTH, K.; CAUGANT, D.A. 1998. Multilocus sequence typing: A portable approach to the identification of clones within populations of pathogenic microorganisms. *Proceedings of the National Academy of Sciences*. 95:140-3145.

MARGULIES, M., EGHOLM, M.; ALTMAN, W.E.; ATTIYA, S.; BADER, J.S.; BEMBEN, L.A. 2005. Genome Sequencing in Open Micro fabricated High Density Picoliter Reactors. *Nature.* 437:376-380.

MARSH, T. L.; SAXMAN, P.; COLE, J.; TIEDJE, J. 2000. Terminal Restriction Fragment Lenght Polymorphism Analysis program, a web-based research tool for

microbial comunity analysis. *Applied and Environmental Microbiology*. 66: 3616-3620.

MARTIN B.; HUMBERT O.; CAMARA M.; GUENZI E.; WALKER J.; MITCHELL T.; ANDREW P.; PRUDHOMME M.; ALLOING G.; HAKENBECK R.; MORRISON D.A.; BOULNOIS G.J.; CLAVERYS J.P. 1994. A highly conserved repeated DNA element located in the chromosome of *Streptococcus pneumoniae. Nucleic Acids Research.* 20:3479–3483.

MATARANTE, A.; BARUZZI, F.; COCCONCELLI, P.S.; MMOREA, M. 2004. Genotyping and toxigenic potential of *Bacillus subtilis* and *Bacillus pumilus* strains occurring in industrial and artisanal cured sausages. *Applied and Environmental Microbiology.* 70:5168–5176.

MCCARTNEY A. 2002. Application of molecular biological methods for studying probiotics and the gut flora. *Br J Nutr*. 88:29–37.

MOHAMED, J.A.; HUANG, W.; NALLAPAREDDY, S.R. 2004. Influence of origin of isolates, especially endocarditis isolates, and various genes on biofilm formation by Enterococcus faecalis. *Infect Immun*. 72:3658–63.

MURRAY, A.E.; LI, G.S.; LIES, D.; NEALSON, K.H.; ZHOU, J.Z.; TIEDJE, J.M. 1999. 1999. Applications of DNA microarray technology to investigating gene expression in *Shewanella oneidensis* MR-1 and functional diversity among related species. In: *Conference of Small Genomes*, Arlington, Virginia, USA.

MUYZER, G.; DE WALL, E.C.; UITTERLINDEN, A.G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology.* 59:695-700.

OGRAM, A. 2000. Soil molecular microbial ecology at age 20: methodological challenges for the future. *Soil Biology and Biochemistry*. 32:1499-1504.

OLSEN, G.J.; OVERBEEK, R.; LARSEN, N.; Woese, C.R. 1991. The Ribosomal Database Project: updated description *Nucleic Acids Research.* 19 4817. p.

OLVERA A.; CERDA-CUELLAR, M.; NOFRARIAS, M.; REVILLA, E.; SEGALE, J.; ARAGON, V. 2007. Dynamics of *Haemophilus parasuis* genotypes in a farm recovered from an outbreak of Glässer's disease. *Veterinary Microbiology*.123: 230-237.

PACE, N.R.; OLSEN, G.J.; WOESEC, R. 1986. Ribosomal RNA phylogeny and the primary lines of evolutionary descent. *Cell Press Journals.* 45:325-326.

PATTANAYAK, D.S.K.; CHAKRABARTI, P.A.; Kumar, P.; Naik, S. 2001. Characterization of genetic diversity of some serovars of *Bacillus thuringiensis* by RAPD*. Indian journal of experimental biology.* 39:897-901.

PINTO, L.M.N.; AZAMBUJA, A.O.; DIHEL, E.; FIUZA, L.M. 2003. Pathogenicity of *Bacillus thuringiensis* isolates from two species of *Acromirmex* (Hyme- noptera: Formicidae). *Brazilian Journal Biology.* 63: 301-306.

POURCEL, C.; ANDRE-MAZEAUD, F.; NEUBAUER, H.; RAMISSE, F.; VERGNAUD, G. 2004. Tandem repeats analysis for the high resolution phylogenetic analysis of *Yersinia pestis***.** *BMC Microbiology*. 4:22-26.

RADEMAKER, J.L.W.; DE BRUIJN, F.J. 1997. Characterization and classification of microbes by REP-PCR genomic fingerprinting and computer-assisted pattern analysis. In Caetano-Anollés G, Gresshoff PM. (Eds.), DNA markers: protocols, applications, and overviews. *J. Wiley and Sons*. 15:151-171.

RAMAZANZADEH, R.; MCNERNEY, R. 2007. Variable Number of Tandem Repeats (VNTR) and its Application in Bacterial Epidemiology. *Pakistan Journal of Biological Sciences*. 10:2612-2621.

RORING, S.; Chapman, I.; David, L. 2002. Development of variable-number tandem repeat typing of *Mycobacterium bovis:* Comparison of results with those obtained by using existing exact tandem repeats and spoligotyping*. Journal of Clinical Microbiology.* 40:2126-2133.

ROSADO, A. S.; DUARTE, G. F.; MENDONÇA-HAGLER, L. C. 1999. A moderna microbiologia do solo: Aplicação de técnicas de biologia molecular. In: SIQUEIRA, J. O.; MOREIRA, F. M. S; LOPES, A. S.; GUILHERME, L. R. G.; FAQUIN, V.;

FRUTINI-NETO, A. E.; CARVALHO, J. G. (Ed.). Inter-relação fertilidade, biologia do solo e nutrição de plantas. Viçosa: SBCS, Lavras: UFLA/ DCS, 1999. p. 429-448.

ROSADO, A. S.; DUARTE, G. F.; SELDIN, L.; ELSAS, J. D. van. 1998. Genetic diversity of nifH gene sequences in Paenibacillus azotofixans strains and soil samples analysed by denaturin gradient gel electrophoresis of PCR-amplified gene fragments. *Applied and Environmental Microbiology*. 64:2770-2779.

RUCKERT, A.; RONIMUS, R.S.; MORGAN, H.W. 2004. A RAPD-based survey of thermophilic bacilli in milk powders from different countries. *International Journal of Food Microbiology.* 96:263–272.

SADDER, M.T.; KHYAMI-HORANI, H.; AL-BANNA, L. 2006. Application of RAPD technique to study polymorphism among *Bacillus thuringiensis* isolates from Jordan. *World Journal of Microbiology & Biotechnology.* 22:1307–1312.

SAIKI, R.K.; SHARF, S.; FALOONA, F.; MULLIS, K.B.; HORN, G.T.; ERLICH, H.A.; ARNHEIM, N. 1985, `Enzymic amplification of ß-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science*. 230:1350-1354.

SANGER, F.; NICKLEN S.; COULSO; A.R. 1977. DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America*.74:5463-5467.

SANZ, J. L.; KOCHLING, T. 2007. Molecular biology techniques used in wastewater treatment: An overview*. Process Biochemistry.* 42:119–133.

SCHNEPF, H.E.; CRICMORE, N.; VANRIE, J.; LERECLUS, D.; BAUM, J.; FEITELSON, J.; ZFIDER, D.R.; EAN, D.H. 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiology and Molecular Biology Reviews.* 62:775-806.

SLEPECKY, R.A.; HEMPHILL, H.E. 1992. The Genus *Bacillus* Nonmedical in “The Prokaryotes”. Vol II. Balows A, Trüper HG, Drowkin M, Tno WH, Schleifer KH (Ed). Springer-Verlag, New-York, Berlin, Heidelberg, London, Paris, Tokyo, Hong-Kong, Barcelona, Budapest. 48, 1697-1745.

SMALL, J.; CALL, D. R.; BROCKMAN, F. J.; STRAUB, T. M.; CHANDLER, D. P.

Direct detection of 16S rRNA in soil extracts by usisng oligonucleotide microarrays. Applied and Environmental Microbiology, Washington, DC, v. 67, p. 4708-4716, 2001.

SPEED, T. Sanger's Method. 2002. www.berkeley.edu/users/terry/Classes /s260.1998/ Week8b/week8b/node9.html (May, 2012).

STAHL, D. A. 1997. Molecular approaches for the measurement of density, diversity, and phylogeny. In: HURST, C. J.; KNUDSEN, G. R.; McINERNEY, M. J.; STETZENBACH, L. D.; WATER, M. W. *Manual of environmental microbiology.* Pp.102-114.

STRYER, L. 1996. Explorando os genes. In: Bioquímica (Quarta Edição). New York, FREEMAN, W.H. & Company.

TENOVER, F.C.; ARBEIT, R.D.; GOERING, R.V. 1999. How to select and in- terpret molecular strain typing methods for epidemiological studies of bacterial infections: a review for healthcare epidemiologists. *Infect Control Hosp Epidemiol.* 18:426-439.

TITZE-DE-ALMEIDA, R.; ARBEIT, R.; GOERING, R. 2004. Multilocus variable-number tandem-repeats polymorphism among brazilian *Enterococcus faecalis* strains. *Journal of Clinical Microbiology*. 42:4879-4881.

VAN BELKUM, A.; SCHERER, S.; VAN LEEUWEN, W.; WILLEMSE, D.; VAN ALPHEN, L.; VERBRUGH, H. 1997. Variable number of tandem repeats in clinical strains of *Haemophilus influenzae*. *Infect Immun.* 65:5017–5027.

VAN BELKUM, ARBEIT, R.D. 1999. Occurrence and structure-function relationship of pentameric short sequence repeats in microbial genomes. *Research in Microbiology*. 150:617-626.

VERSALOVIC J.; SCNEIDER, M.; De BRUJIN, F.J.; LUPSKI, J.R. 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction Meth. *Molecular and Cellular Biology* 5:25-40.

VERSALOVIC, J.; KOEUTH, T.; LUPSKI, J.R. 2009. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes.

Nucleic Acids Res. 24:6823–6831.

VERSALOVIC, J.; KOEUTH, T.; LUPSKI, J.R.; 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Research* 19: 6823–6831.

YERSHOV, G.; BARSKY, V.; BELGOVSKIY, A.; KIRILLOV, E.; KREIDILIN, E.; IVANOV, I.; PARINOV, S.; GUSCHIN, D.; DROBISHEV, A.; DUBILEY, S.; MIRZABEKOV, A. 1996. DNA analysis and diagnostics on oligonucleotide microships. *Proceedings of the National Academy of Sciences*. 93:4913-4918.

ZHU, J.; KAPOOR, A.; SRIDHAR, V.V.; AGIUS, F.; ZHU, J.K. 2007. The DNA glycosylase/lyase ROS1 functions in pruning DNA methylation patterns in Arabidopsis. *Current Biology*. 17:54–59.

4 CAPÍTULO 3

Phenotypic characterization and the application of the rep-PCR technique in a study of new strains of *Bacillus thuringiensis* in the South of Brazil

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Phenotypic characterization and the application of the rep-PCR technique in a study of new strains of *Bacillus thuringiensis* in the South of Brazil

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ABSTRACT

Worldwide, *Bacillus thuringiensis* (*Bt),* is now the most widely used bio-pesticide. Its toxicity derives from the production of specific proteins (delta-endotoxins) during sporulation. In this study, 26 *Bt* strains from South Brazil were analyzed by phenotype and molecular testing of sequential amplifications of repeated sequences (rep-PCR) to evaluate the intra-specific similarities between the strains, and to determine the internal homogeneity. The assays demonstrated many similarities with the new strains of *Bt* obtained of the soil in the South of Brazil indicating a high degree of clonability between the rice culture regions, probably associated with asexual reproduction and/or ecological speciation.

KEYWORDS: *Bacillus thuringiensis*, rep-PCR, SDS-PAGE.

RESUMO

Em escala mundial, *Bacillus thuringiensis* (*Bt*), é o biopesticida mais utilizado. A sua toxicidade deriva da produção de proteínas específicas (delta-endotoxinas) durante a esporulação. Neste estudo, 26 cepas de *Bt* do Sul do Brasil foram analisados por testes fenotípicos e testes moleculares de amplificação seqüencial de seqüências repetidas (rep-PCR) para avaliar as semelhanças intra-específicas entre as cepas e para determinar a homogeneidade interna. Os ensaios demonstraram muitas semelhanças com as novas variedades de *Bt* obtidos do solo no Sul do Brasil, indicando um alto grau de clonabilidade entre as regiões orizícolas, provavelmente associadas à reprodução assexuada e/ou especiação ecológica.

PALAVRAS-CHAVE: *Bacillus thuringiensis*, rep-PCR, SDS-PAGE.

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1 INTRODUCTION

Bacillus thuringiensis is a bacterium, which produces crystal inclusions (delta-endotoxin) during its sporulation, which show specificity for different insect orders such as Diptera, Coleoptera, Lepidoptera, Hymenoptera, Homoptera, Orthoptera, Isoptera and Mallophaga [1,2,3,4], as well as nematodes, mites and protozoa [5]. The delta-endotoxins are classified into two multigene families - Cry and Cyt - based on glycoprotein structure and mechanism of action [5].

It is important to look for new strains of *B. thuringiensis* and currently many studies are done in research centers and one of the most recent advancesin this area ist he use of PCR [6]. This technique has been used to identify strains without the needs of other techniques, allowing the identification of new but fast Cry proteins [7]. The use of repeated DNA sequences such as REP and ERIC such as rep-PCR for bacterial classification is becoming frequentthat allows for comparison of possible genetic similarities between different bacterial genomes [8, 9, 10].

Amplicons obtained from rep sequences generate different electrophoretic patterns among different strains. Similar approaches using other repetitive sequences such as ERIC and BOX sequences have been developed for *Escherichia coli* and *Salmonella typhimurium* [11] and *Streptococcus pneumoniae*, respectively (12). Analysis of strains based on Rep-PCR proved to be simple, rapid and reproducible in a wide variety of organisms [13, 14].

Since it became important to overcome the growing need to avoid resistance by insect pests, the objective of this study is to analyze by molecular methods 26 strains of *B. thuringiensis* derived from 5 producing regions of rice Rio Grande do Sul (RS), to assess the degree of similarity between intra-specific strains of *B. thuringiensis* is to clarify the existence of homogeneity among these strains, isolated from different and distinct sites.

2 MATERIALS AND METHODS

2.1 Bacterial strains

The 26 strains of *B. thuringiensis* of this study came from producing regions of rice in Rio Grande do Sul, *Fronteira Oeste* (*FO*), *Campanha* (*CAM*), *Depressão Central* (*DC*), *Litoral Sul* (*LS*) e *Litoral Norte* (*LN*). The isolates are stored at the Colletion of Entomopathogenic Bacteria – Unisinos.

2.2 Phenotypic assays

B. thuringiensis strains were evaluated by biochemical, physiological and citomorphological specific tests according to the protocols of [15, 16, 17, 18].

2.3 Organic compounds

All *B. thuringiensis* strains were assessed for tolerance to ethanol (150 and 250µL), creolin (40 and 60µL), phenol (40 and 60µL) and xylol (150 and 250µL) by adding the corresponding amount of each organic compound in 10mL of simple glucose growing environment (MUG) for 48 hours at 33℃, Growth of strains were determined after 5 days at 30° C [19].

2.4 Chemical pesticides

In the tests with chemical pesticides-Fipronil (4.0µL), Pirazosulfuronetil (4.0µL), Quincloraque (0.020 g), Propanil (255.0µL) and Azoxistrobina (26.0µL), the lineages were grown in Petri dishes with MUG for 48 hours at 33℃ and the recommended amounts of each pesticide were deposited at points 2cm apart, in B.O.D at 33℃. After incubation for 24 hours, we me asured the areas where the growth had been inhibited.

2.5 Protein profiles

The protein profile of crystal components of *B. thuringiensis* strains was determined through sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [20] at 12%. Strains were grown in broth MUG the 33℃ + 1° C under 175 rpm agitation over 48 h or until cellular lysis with release of spores and crystals. Bacterial suspensions were centrifuged (10,000 rpm, 20 min) and the pellet was ressuspended in 1mL of distilled water. Next, 75µL of these suspensions were mixed with 25µL of sample buffer (Tris–HCl 0.5M pH 6.8; SDS 10%; ß-mercaptoethanol 4%; glycerol 8%; bromophenol blue 0.1%) and boiled at 100° C over 10 min as previously described by [21]. Samples were immediately evaluated under electrophoresis. With previous knowledge of molecular weight, markers such as myosin-205kDa; ß-galactosidase-116kDa; phosphorylase B—97.4kDa; bovine

albumin— 66kDa; egg white albumin-45kDa and carbonic anhydrase-29kDa were used to determine the size of proteins [21].

2.6 rep-PCR

DNA of *B. thuringiensis* strains was extracted according to the methodology described by [22]. Amplification reactions using the primers REP1 (5'-ATTAAAGTTTCACTTTAT-3 '), BOX1AR (5'-CTAC GGCAAGGCGACGCT-3') and ERIC (5'-AAGTAA GTGACTGGGGTGAGCG -3 ') were performed as described by [23]. In the positive control process was used the *B. thuringiensis thuringiensis* 4412 strain and the DNA negative-free control. The amplification was done under the following conditions: denaturation (4 min at 94ºC; 35 cycles at 94ºC for 30sec), ringing (30sec at 45ºC), extensions (1min at 72ºC) and 10 min of extensions at 72ºC. To analysis of the amplification products, 5µL of amplicons were electrophoresed in 2% agarose gels in Tris–borate EDTA (TBE 1X) (Tris–borate 89mM; EDTA 2mM pH 8.0) buffer at 75 V over 3 h. The visualization was under UV light, after treatment with 0.5µg/mL ethidium bromide solution, during 15 min.

2.7 Numerical analysis

The tests were transformed into binary matrixes and evaluated with a numerical analysis program NTSYS-pc (2.1 version). Profile analysis was performed based on the calculation of the matching similarity coefficient, used to establish the similarity matrix. This matrix was transformed into a phenogram using the Unweighted Pair Group Method (UPGMA), according to [24]. To assess the clustering fit, a cophenetic value matrix was calculated and compared to the original one. The correlation between both matrices was assessed using the correlation coefficient r.

3 RESULTS

The isolates of *B. thuringiensis* analyzed based on phenotypic characterization showed, in most cases, the characteristic parameters of this species. Small variations were found in the length of the cells, observed by optical microscopy, and the position of the spore in the sporangium.

Phenograms generated for the responses of the strains in the presence of chemical pesticides and organic compounds (not shown) are similar to those generated for SDS-PAGE. In two tests formed five groups, which contain lines from

the same geographic region, indicating that for chemicals and pesticides strains respond similarly. The levels of similarity between the groups vary approximately between 85 and 60%.

Growth performance of the front lines to chemical pesticides indicated that all strains were sensitive to the herbicide Stam®. The strains of group I were those that grew in the presence of greater number of pesticides (four). The two strains of the group grew in the presence of 2 (Facet® and Priori®), those belonging to group III, grew in the presence of 2 (Priori® and Sirius®), group IV in the presence of 2 (Klap® and Facet®) and group V in the presence of 3 (Klap®, Facet® and Sirius®).

The results of the growth profile of the isolates against toxic organic compounds in different concentrations (Ethanol, Xylol, Phenol and Creolin) showed that the strains of group I grew only in the presence of Creolin and Phenol in the concentration of 60µL; strains of group IV grew in the presence of Phenol Concentration (60µL), Phenol (concentration 40µL) and Xylol while the group V strains grew only in the presence of two concentrations of phenol. The non-toxic strains showed higher tolerance to different organic products used in the experiment. The isolates were 100% resistant to cadmium and had variable growth in Mercury, Aluminum and Zinc. (Figure 1).

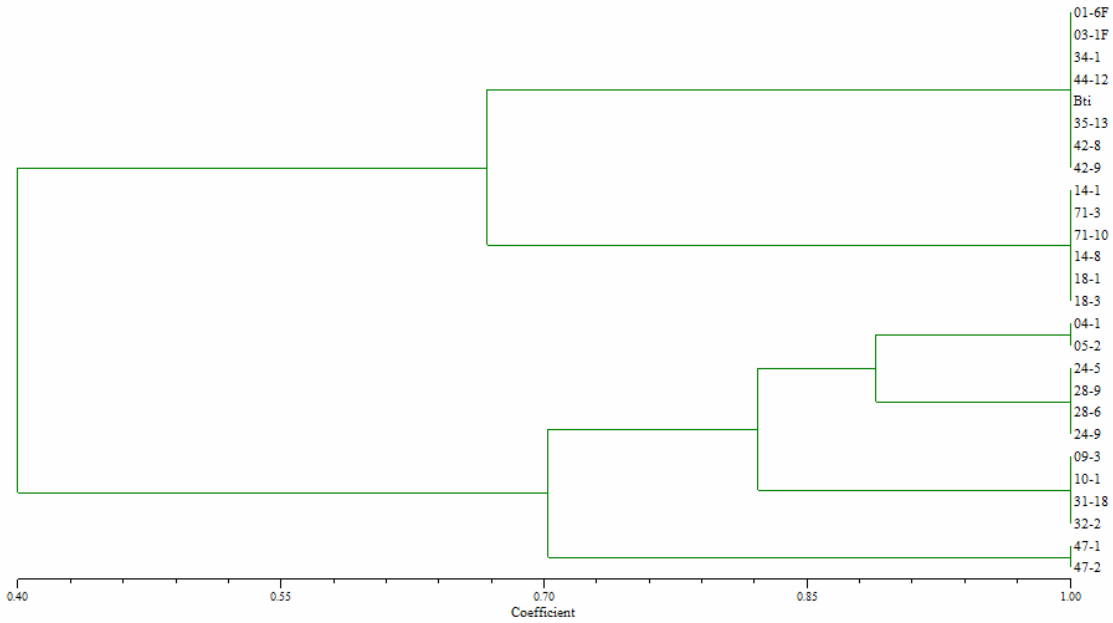

Figure 1: Phenogram resulting from the numerical analysis based on data obtained from the growth in the presence of organic comounds strains of *Bacillus thuringiensis*.

The characterization of isolates by SDS-PAGE revealed different protein profiles enabling the identification of 5 classes of proteins. Figure 2 (A) shows the electrophoretic patterns found in the strains studied with their molecular weights. For definition of groups in the dendogram use as reference similarity of about 80% (Figure 1-B). In Group I, including strains that shared the same protein profile of strain *B. thuringiensis* 44-12 (standard (Cry 1), groups II and III were included in the non-toxic strains with bands indicating molecular weight of 25, 40, 55 and 100 kDa and 40, 50, 80 and 110 kDa, respectively. The weights found for the lines IV and V were respectively 25, 50, 80 and 110 kDa and 25, 50, 55, 80 and 145 kDa. The cophenetic correlation coefficient generated for the dendrogram was r = 0.91.

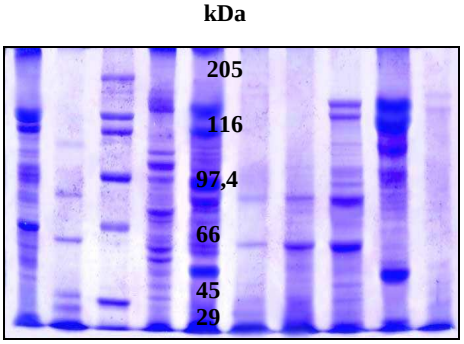

Fig 2 (A): Protein profile of the crystals of strains *B.thuringiensis.* Line: (1) IRGA 31-18; **(**2) IRGA 42-8; (3) IRGA 32-2 ; (4) IRGA 29-8; (5) Molecular weight marker (kDa); **(**6) IRGA 06-1; (7) IRGA 03-1; (8) IRGA 35-13; (9) IRGA 28-9.

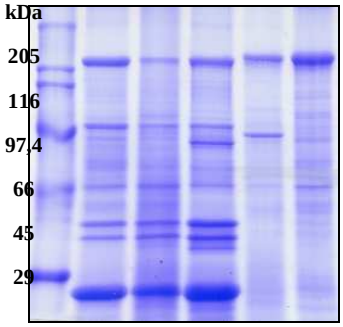

Fig 2: (B) Protein profile of the crystals of strains *B.thuringiensis* Line: (1) Molecular weight marker kDa); (2) IRGA 24-5; (3) IRGA 24-9; (4) IRGA28-6; (5) IRGA 47-1; (6) IRGA 47-2.

The results of PCR analysis performed using a single primer for REP, BOX and ERIC are shown in Figure 3. The three primers showed polymorphism, ie the number of bands found for the set of primers ranged from 3 to 9 for each sample region and band size was between 0.4 and 3.4 kilobase. The cluster analysis performed using the banding pattern generated by primer set formed groups

containing strains from the same geographic region. To form the groups was considered minimum similarity of 80% applies, so the cutoff point at a difference of 20% being formed five separate groups. The topology of the dendrogram generated by PCR is similar to that generated by SDS-PAGE analysis for the responses to chemical and pesticides. The cophenetic correlation coefficient for PCR analysis and SDS-PAGE was r ≥ 0.9, only the data of chemical compounds, the cophenetic correlation coefficient was below 0.8 (0.79).

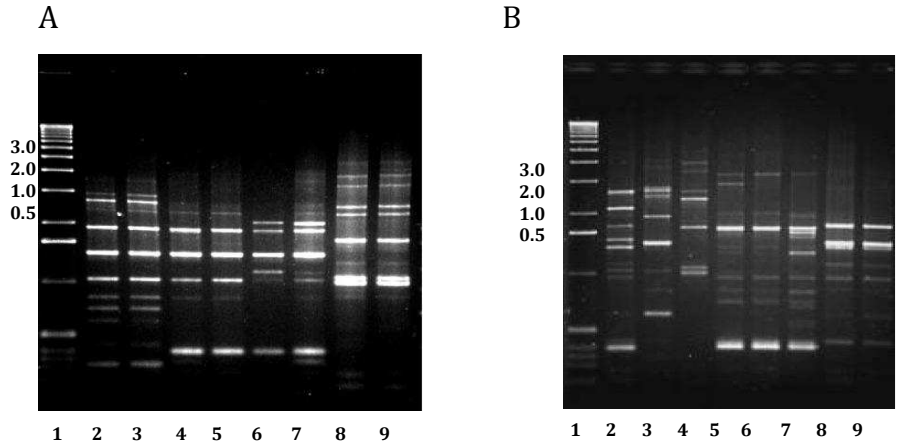

Figure 3: Analysis of *Bacillus thuringiensis* strains by Rep-PCR. (A) REP1. Line: (1) Marked Molecular Weight (1kb DNA ladder, Invitrogen); **(**2) IRGA 06-1; (3) IRGA 34-1 ; (4) IRGA 03-1; (5)IRGA 35-13; **(**6) IRGA 42-8; (7) IRGA 42-9; (8) IRGA 47-1; (9) IRGA 47-2. (B) BOX. Line: (1) Marked Molecular Weight (1kb DNA ladder, Invitrogen); **(**2) IRGA 71-3; (3) IRGA 71-9; (4) IRGA 31-18; (5) IRGA 28-9; **(**6) IRGA 28-6; (7) IRGA 10-1; (8) IRGA 24-9; (9) IRGA 24-5.

4 DISCUSSION

In this study we used genotypic and phenotypic techniques to analyze the levels of differentiation among 26 isolates of *B. thuringiensis* from soils in 5 rice production regions of the State of Rio Grande do Sul.

The technique of SDS-PAGE is important to identify the pattern of proteins and provides an overview of the potential of insecticide strains analyzed, especially the presence of bands of 130 and 65 kDa, a characteristic of strains active against Lepidoptera and Coleoptera [25].

The protein patterns were generated by the strains of groups IV and V regions of the Frontier West and South Coast, respectively may indicate the production of toxins potentially different insecticide, which should be further evaluated.

The methods of PCR (BOX, REP and ERIC) have been used frequently for characterizing and identifying strains in many organisms [26, 27, 28], particularly in

scale populations. Several studies have shown that it is possible to separate isolated using these techniques according to region of origin and also with the host [29, 23]. Our results, like those produced in other studies, demonstrate the applicability of PCR methods for identification of distinct strains of microrganisms, with the advantage of being easy to perform, having lower cost and generate results quickly. In the presence of standard PCR for banks of isolates of *B. thuringiensis* in the laboratory, these methods facilitate the identification of new strains, or strains with significant genetic differences from those already known [23, 27].

The groups in our study produced by the technique of PCR were always lines toxic and non-toxic.

The phenotypic analysis, specifically the ability to grow in the presence of pesticides and organic compounds, are important for evaluating the possible use of these strains on a system of integrated management of rice. In this case, we should note the fact that strains with pattern similar to *B. thuringiensis* subsp. israelensis grow in the presence of most pesticides used.

Characterization studies using PCR should be expanded in search of a better definition of the heterogeneity or homogeneity within each lineage in search of a more specific association between pattern of SDS-PAGE and PCR. This association will be a great advantage in finding new lines.

The results for all tests carried out show a very similar response to strains from the same region, demonstrating a high degree of clonality associated with, probably, the asexual reproduction and / or ecological specialization [30, 31, 32] On the other hand strains from different regions show a high degree of difference, because the maximum similarity between them was in the range of 60%. Currently, the increasing use of molecular techniques, a large number of species and strains of bacteria have been relocated in an attempt to obtain a more objective classification that reflects the real relationships among the various groups [33, 34, 35].

According [36], the use of conserved repetitive sequences in the genome, contributes to differentiation of intra-specific genomic groups. This may mean that if or when a different strain adapts to an ecological environment, the selection of certain features in the genome, characterizing its biodiversity, can occur.

5 REFERENCES

[1] Iudina TG (1996) A comparison of the antibacterial activity of the parasporal bodies of different bacilli. Izv. Akad Nauk Ser Biol./Proc. Russ. Acad. Sci. (Biol) 5, 535–541.

[2] Crickmore N (2000) The diversity of *Bacillus thuringiensis* d-endotoxins. In: Charles, J.-F., Dele´cluse, A., Nielsen-LeRoux, C. (Eds.), Entomopathogenic Bacteria: From laboratory to Field Application, first ed. Kluwer Academic Publishers, Dordrecht, pp. 65–79.

[3] Castilhos-Fortes R, Matsumura ATS, Diehl E, Fiuza LM (2002) Susceptibility of Nasutitermes ehrhardti (Isoptera: Termidae) to *Bacillus thuringiensis* subspecies. Braz J Microbiol 33:219–222

[4] Pinto LMN, Azambuja AO, Diehl E, Fiuza LM (2003) Pathogenicity of *Bacillus thuringiensis* isolated from two species of Acromyrmex (Hymenoptera, Formicidae). Braz J Biol 63:301–306

[5] Schnepf E, Crickmore N, Van-Rie J, Lereclus D, Baum J, Feitelson J, Zeigler DR, Dean DH (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol Mol Biol Rev 62:775–806

[6] Bravo A, Haendrickx K, Jansens S, Peferoen M (1992) Immunocytochemical analysis of specific binding of *Bacillus thuringiensis* insecticidal crystal proteins to lepidopteran and coleopteran mid-gut membranes. Journal Invertebrate Pathology 60:247-253.

[7] Carozzi NB, Kramer VC, Warren GW, Evola S and Koziel M (1991) Prediction of insecticidal activity *Bacillus thuringiensis* strain by polymerase chain reaction product profiles. Appl Environ Microbiol 57:353-356

[8] Versalovic J, Koeuth T and Lupski JR (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Research 19:6823-6831

[9] Louws FJ, Fulbright DW, Stephens CT, De-Bruijn FJ (1994) Specific genomic fingerprint of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequence and PCR. Appl Environ Microbiol 60:2286-2295

[10] Selenska-Pobell S, Gigova L, Petrova N (1995) Strain-specific fingerprints of *Rhizobium galegae* generated by PCR with arbitrary and repetitive primers. Journal of Applied Bacteriology 79:425-431

[11] Hulton CSJ, Higgins CF, Sharp PM (1991) ERIC sequences: a novel family of repetitive elements in the genomes of *Escherichia coli*, *Salmonella typhimurium* and other enterobacteria. **Molecular Microbiology** 5: 825-762.

[12] Martin C, Sibold C, Hakenbeck R (1992) Relatedness of penicillin-binding protein 1a genes from different clones of penicillin-resistant Streptococcus pneumoniae isolated in South Africa and Spain. Embo Journal 11: 3831–3836

[13] De Brujin FJ (1992) Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl Environ Microbiol 58:2180-2187

[14] Lima ASG, Guidelli AM, Abreu IL, Lemos MVF (2002) Identification of new isolates of *Bacillus thuringiensis* using rep-PCR products and δ-endotoxin electron microscopy. *Genetics and Molecular Biology* 25:225-229

[15] Gordon, R.E., Haynes, W.C., Pang, C.N., 1973. The Genus *Bacillus*. Agric. Handbook n_ 427 US Department of Agriculture, Washington, DC.

[16] Cowan, S.T., Steel, K.J., 1974. In: Cowan and Steel's Manual for Identification of Medical Bacteria. Cambridge University Press, London.

[17] Claus D, Berkeley RCW (1986) Genus *Bacillus*. Cohn 1872. In: Sneath, P.H.A., Mair, N.S., Sharpe, M.E., Holt, J.G. (Eds.), Bergey's Manual of Systematic Bacteriology, 2nd ed. Williams & Wilkins, Baltimore, pp. 1105–1141.

[18] Vasconcelos FJM, Rabinovitch L (1994) A new formula for an alternative culture medium, without antibiotics, for isolation and presumptive quantification of *Bacillus cereus* food. J Food Microbiol 58:235–238

[19] Silva KR, Rabinovitch L, Seldin L (1999) Phenotipic and genetic diversity among *Bacillus sphaericus* strains isolated in Brazil, potentially useful as biological control agents against mosquito larvae. Res Microbiol 150:153-160

[20] Laemmli UK, Favre M. 1973. Maturation of the Head of Bacteriophage T_4. I. DNA Packaging Events. J Mol Biol 80:575-599

[21] Lecadet MM, Chaufaux J, Ribier J, Lereclus D (1992) Construction of novel *Bacillus thuringiensis* strains with different insecticidal activities by transduction and transformation. Appl Environ Microbiol 58:840-849

[22] Sambrook J, Fritch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. 2nd edition. *Cold Spring Harbor*. New York. **3nd** Volume.

[23] Reyes-Ramirez A, Ibarra JE (2005) Fingerprinting of Bacillus thuringiensis Type Strains and Isolates by Using Bacillus cereus Group-Specific Repetitive Extragenic Palindromic Sequence-Based PCR Analysis. Appl Environ Microbiol 71:1346-1355

[24] Rohlf, F.J. 2002. NTSYS-PC: Numerical Taxonomy for System for the IBM PC Microcomputer, Setauket, New York: *Applied Biostatisics*.

[25] Crickmore, N. 2006. Beyond the spore – past and future developments of *Bacillus thuringiensis* as a biopesticide. *Journal of Applied Microbiology*, **101**:616-619

[26] Oliveira E. J., Rabinovitch, L., Monnerat, R.G., Passos, L.K.J., Zahner, V. 2004. Molecular Characterization of *Brevibacillus laterosporus* and Its Potential Use in Biological Control. Applied and Environmental Microbiology, November 2004, p. 6657-6664, Vol. 70, No. 1

[27] Alves, A., Phillips, A.J.L., Henriques http://www.sciencedirect.com/science/?_ob=ArticleURL&_udi=B6VN3-4MM7WRFc3852b95cac74 - aff1#aff1 I., Correia A. 2007. Rapid differentiation of species of *Botryosphaeriaceae* by PCR fingerprinting. Research in Microbiology. V. 158, n.2, p.112-121.

[28] Rincón, F. Arenal, I. González, E. Manrique, M.M. Lucas and J.J. Pueyo, Diversity of rhizobial bacteria isolated from nodules of the gypsophyte *Ononis tridentata* L. growing in spanish soils, *Microbial Ecology* **56** (2008), pp. 223–233

[29] MEHTA, Y.R., MEHTA, A. & ROSATO, Y.B. 2002. ERIC and REP-PCR banding patterns and sequence analysis of the internal transcribed spacer of rDNA of *Stemphylium solani* isolates from cotton. Current Microbiology, New York, USA, 44: 323-328.

[30] Trevors, J.T. Review: Bacterial population genetics. 1998. World Journal of Microbiology & Biotechnology. v. 14, p. 1-5

[31] Cohan FM (2002). What are bacterial species? *Annu Rev Microbiol* **56**, 457–487

[32] Koeppel, A., Perry, E.B. Sikorski, J., Krizanc, D., Warner, A., Ward,D.M., Rooney, A.P., Brambilla, E., Connor, N., Ratcliff, R.M., Nevo, E., Cohan F.M. 2008.

Identifying the fundamental units of bacterial diversity: A paradigm shift to incorporate ecology into bacterial systematics. Proc Natl Acad Sci U S A > v.105(7).

[33] Ash C, Farrow JAE, Wallbanks S, Collins MD (1991) Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small subunit ribosomal RNA sequences. Lett Appl Microbiol 13:202-206

[34] Ash C, Priest FG, Collins MD (1993) Molecular identification of rRNA group 3 bacillus using a PCR probe test. Antonie Loss Wenhoek 64:253-260.

[35] Shida O, Takagii H, Kadowaki K, Komagata K (1996) Proposal for two genera, *Brevibacillus* gen. nov. and *Aneurinibacillus* gen. nov. Int J Syst Bacteriol 46: 939-946

[36] Abriouel H, Omar NO, Lopez RL, Martinez Canamero M, Ortega E, Galvez A (2007) Differentiation and characterization by molecular techniques of *Bacillus cereus* group isolates from Poto Poto and deguè, two traditional cereal-based fermented foods of Burkina Faso and Republic of Congo. J Food Prot **70**:1165–1173

5 CAPÍTULO 4

Artigo de Pesquisa

SCREENING OF NEW BRAZILIAN *BACILLUS THURINGIENSIS* STRAINS AGAINST *SPODOPTERA FRUGIPERDA* (LEPIDOPTERA: NOCTUIDAE).

SCREENING OF NEW BRAZILIAN *BACILLUS THURINGIENSIS* STRAINS AGAINST *SPODOPTERA FRUGIPERDA* (LEPIDOPTERA: NOCTUIDAE).

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ABSTRACT

Bacillus thuringiensis is an entomopathogenic bacterium that, during sporulation, produces crystal proteins, coded by *cry* genes, which can act on crop pests such as *Spodoptera frugiperda,* abolishing its destructive effect. In this study 20 strains of B. thuringiensis isolates from different regions of rice from Rio Grande do Sul, Brazil, were characterized. The strains of B. thuringiensis for biochemical, physiological and cytomorphologic specific taxonomic studies of bacteria showed characteristic parameters for the species. The electrophoretic protein profiles of crystal components were studied and showed the presence of three distinct groups and one strain showed insecticide activity and protein profiles similar to the reference strain *B. thuringiensis thuringiensis* 4412. Based on the results obtained in the present study, the combination of classical and molecular methods helps us to group the isolates and give more detail than a single method for classification and characterization of native strains with activity against pests.

KEY-WORDS: *Bacillus thuringiensis*, insetos-praga, bioensaios.

RESUMO

Bacillus thuringiensis é uma bactéria entomopatogênica que, durante a esporulação, produz proteínas cristal, codificadas pelos genes *cry,* podendo atingir pragas de diversas ordens. Esta bactéria é utilizada no controle de pragas como *Spodoptera frugiperda,* causadora de sérios prejuízos na agricultura. Neste estudo 20 cepas de *B. thuringiensis* isolados de diferentes regiões de arroz do Rio Grande do Sul, Brasil, foram caracterizados. As cepas de *B. thuringiensis* para os testes bioquímicos, fisiológicos e citomorfológicas específicos para estudos taxonómicos de bactérias apresentaram parâmetros característicos para a espécie. Os perfis de proteínas electroforéticas dos componentes de cristal foram estudados e apresentaram de três grupos distintos e uma cepa apresentou atividade insecticida

e perfis de proteína similares a cepa referência *B. thuringiensis thuringiensis* 4412. Com base nos resultados obtidos no presente estudo, a combinação de métodos clássicos e moleculares nos ajuda a agrupar os isolados e dar mais detalhes do que um método único de classificação e caracterização de estirpes nativas com atividade contra pragas.
PALAVRAS-CHAVE: *Bacillus thuringiensis*, insetos-praga, bioensaios.

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1 INTRODUCTION

Economic losses in agriculture owing to pests are huge. It is estimated that in the world 15% of these are due to insect attacks on crops (James, 2011). The residual effects produced by insecticides, environmental pollution, toxicity, and induced resistance in insects, provoked the use of microorganisms as an alternative and efficient means of control (Bravo and Quintero, 1993).

Among the groups of organisms that are being studied because of their potential as biocontrol agents, are bacteria belonging to the genus *Bacillus*. *Bacillus thuringiensis* strains have showed a wide range of specificity for insects like Lepidoptera, Coleoptera, Hymenoptera, Homoptera, Mallophaga and Diptera (Feitelson et al., 1992; De Maagd et al., 2001; Cavados et al., 2001; Pinto et al., 2003; De Maagd et al., 2003). Intensive screening programs have isolated and characterized *B. thuringiensis* from soil samples, plant surface, dead insects, stored grains and aquatic habitats (Bravo et al. 1998, Reyes- Ramirez & Ibarra 2005, Reyes-Ramirez & Ibarra 2008). Indeed, commercial insecticides derived from this bacterium have a long history of successful use in the biocontrol of insect pests (Beegle and Yamamoto, 1992; Bravo et al., 2011; Federici, 2005; Sanahuja et al., 2011; Sanchis, 2011), in agriculture (Navon, 2000; Sanchis and Bourguet, 2008; Sauka and Benintende, 2008) and forestry (van Frankenhuyzen, 2000), and disease vectors (Becker, 2000; Lacey and Undeen, 1986; Thiéry et al., 1996). For the last decade and a half, various transgenic crops that express insecticidal Bt toxins have been grown over a rapidly increasing area (Cannon, 2000; Ferré et al., 2008; James, 2010; Shelton et al., 2002).

The entomopathogenic activity of this bacterium is principally due to the

presence of proteinaceous inclusions known as insecticidal crystal proteins (Cry proteins). Cry genes harbored in megaplasmid code these Cry proteins and they exhibit a variety of biological actions, including cytolitic, hemolytic action, in addition to entomocidal activities (Lima et al. 2002). The first classification of protein crystals insecticides also called Cry proteins, called the spectrum of toxicity in insects and invertebrates (Hofte and Whiteley 1989). With subsequent isolation of more genes for these proteins, this classification has become impractical, since emerged homologous to known genes, but with different specificity, as well as genes combined with specificities (Bravo 1997, Crickmore et.al. 1998).

The cry genes are located in chromosome and plasmids that some strains being present only these genes into plasmids. The *cry*1Ac gene for example, on strain *kustaki* HD37 is located on a plasmid of 50MDa (Lereclus et.al., 1993) while other strains as subspecies *aizawai* contains five genes, four located on chromosome or megaplasmid a fifth one plasmid of 45MDa (Sanchis et.al., 1998).

The toxicity and specificity strains are determined by functional types cry genes, together with genes evidencing a high degree of genetic variability for this species. Characterization of cry genes is often the chosen form for performing for new isolates searches (Mullis and Fallona, 1987). The PCR technique has become essential in the characterization of little known isolates and indicates the possibility of each gene to several orders of insect pests (Carozzi et.al., 1991; Bravo et.al., 1998; Pocar & Juarez-Perez 2003).

In Brazil, B. thuringiensis has been used as a bioinsecticide for the control of many pests, especially the *S. frugiperda* caterpillar that reaches various crops, mainly corn and rice. Most products of *B. thuringiensis* used to control pests lepidopteran is based on spore-crystal mixture produced by the strain HD-1, *B. thuringiensis* subsp*. kurstaki,* which has a wide spectrum of larvicidal activity within the Lepidoptera Order (Navon, 1993). However, insects belonging to the family Noctuidae, in which included various Spodoptera species are insensitive to these products (Moar t al. 1990; Inagaki et al. 1992; Navon, 1993; Lambert et al. 1996). In addition, there are reports of this pest resistance evolution and to the chemical insecticide, showing that this sit-control presented serious practical limitations (Yu, 1992; Diez-Rodrigues and Omoto, 2001). The detection and identification of wild

Bacillus spp. isolates may be of great value due to highly specific action and with no effect on other non-target insects, plants and domestic animals (Lima et al. 2002). Recently, the need to develop safe pesticides has accelerated the search for new strains of *B. thuringiensis* with different modes of action (Bernhard and Utz, 1993). These new strains may increase the list of commercial products available in the battle against insect larvae belonging to the orders Lepidoptera, Diptera and Coleoptera. Data on *B. thuringiensis* are limited in Rio Grande do Sul, the aim of this study was the characterization of strains of *B. thuringiensis* isolated from rice regions with high toxicity to *Spodoptera frugiperda*, biologic activity determined by qualitative bioassays and protein profiles of the protoxin crystals by SDS-PAGE and Cry profile to provide additional taxonomic information.

2 MATERIALS AND METHODS

2.1 Bacterial strains and their origins

The isolates of *B. thuringiensis* from soil samples collected in irrigated rice areas of Rio Grande do Sul (RS) were used in this study (Figure 1). These 20 isolates belong to the bacterial entomopathogenic collection of the UNISINOS Toxicology and Microbiology Laboratory. The *B. thuringiensis thuringiensis* 4412 used as positive control.

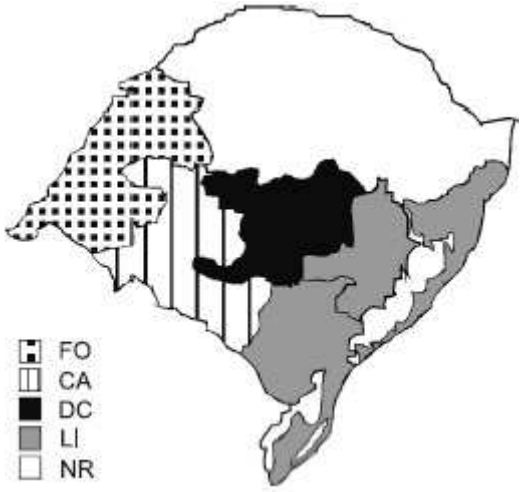

Figure 1. Representative map of Rio Grande do Sul rice fields.

Legenda: FO: Fronteira Oeste; CA: Campanha; DC: Depressão Central; LI:

Litoral; NR: No Rice fields area.

2.2 Phenotypic tests

Strains of *B. thuringiensis* were evaluated by biochemical, physiologic and cytomorphologic specific tests for taxonomic studies of bacteria belonging to the Genus Bacillus, according to the protocols of Gordon et al. (1973), Cowan and Steel (1974), Claus and Berkeley (1986) and observed in interferential phase contrast microscope ZEISS Software Axion Vizion SF64.

2.3 Antibiograms susceptibility

The antibiograms resistance and susceptibility profile was performed according to the technique of antibiotic diffusion from discs impregnated with known amounts, according to Bauer et al. (1966) and Committee for Clinical Laboratory Standards (NCCLS, 2003). The antimicrobials used were as follows: Cefoxitin, 30µl (CT); Cloranphenicol, 30µl (CO); Eritromicin, 15µl (ET);Estreptomicin, 10µl (ET); Penicilin G, 10µg (PN); Tetraciclin, 30µl (TE) and Vancomycin, 5µl (VA). For the interpretation of results the measurements of the halos of resistance were compared with standard measures for each antimicrobial tested (Table 1).

Table 1: Parameters for interpretation of the diameters of inhibition zones in antibiograms

Antibiotics *	Abbreviation	Discs Concentration	Resistance (mm)	Sensitivity (mm)
Cefoxitin	CT	30µl	$\leq$ 17	$\geq$ 18
Cloranphenicol	CO	10µl	$\leq$ 17	$\geq$ 18
Estreptomicin	ET	10µl	$\leq$ 14	$\geq$ 15
Eritomicin	EL	15µl	$\leq$ 17	$\geq$ 18
Penicilin G	PN	10µl	$\leq$ 21	$\geq$ 22
Tetraciclin	TT	30µl	$\leq$ 18	$\geq$ 19
Vancomycin	VA	5µl	$\leq$ 17	$\geq$ 18

2.4 Bioassays against *S. frugiperda*

2nd instar larvae of *S. frugiperda* were obtained from colonies grown in the

insect's chamber- UNISINOS, maintained at 25 ± 2ºC, 80% Relative Humidity (RH) and for a 12-hour photoperiod. The bioassays were conducted in Biological Oxygen Demand (B.O.D.) chambers, at 25 ± 2ºC, 80% RH and for a 12-hour photoperiod. The bacterial isolates were grown in Usual Glicosed Medium (De Barjac and Lecadet, 1976) at 28ºC and 180 rpm for 48 h. Cultures were centrifuged at 4.500 rpm for 15 min, the supernatant was discarded and the pellet was washed in phosphate buffer. The pellet was suspended in sterile distilled water. The bacterial concentration was determined with a Neubauer chamber and an optical microscope. All bioassay data were corrected by the Abbott's formula (Abbott, 1925). The culture corresponding to $1x10^{10}$ cells/mL was added to the Poitout diet (Poitout and Bues, 1974), previously organized in mini-plates (30 mm of diameter), where 20 larvae of 2nd instar were individualized for each isolate. In the control group, the culture was substituted by sterile distilled water. Mortality was observed until 7th day after treatment application.

Figure 2: Bioassays with *Spodoptera frugiperda*

2.5 Protein profiles of the protoxin crystals

The protein profiles of the crystal components of *B. thuringiensis* strains were determined through sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli and Favre, 1973) with 10% gels. The isolates were grown in in Usual Glicosed Medium (De Barjac and Lecadet, 1976) at 33℃ ± 1℃ under 175 rpm agitation for 48 hour until cellular lysis with release spores and endotoxin crystals. Bacterial suspensions were centrifuged (10,000 rpm for 20 min) and the pellet was suspended in 1 mL of distilled water. Next, 75 mL of these suspensions

was mixed with 25 mL of sample buffer (Tris–HCl 0.5 M pH 6.8; SDS 10%; b-mercaptoethanol 4%; glycerol 8%; bromophenol blue 0.1%) and boiled at 100℃ for 10 min as previously described by Lecadet et al. (1992). Samples were immediately evaluated with electrophoresis. Known molecular weight markers such as myosin—205 kDa; b-galactosidase—116 kDa; phosphorylase B—97.4 kDa; bovine albumin—66 kDa; egg white albumin—45 kDa and carbonic anhydrase—29 kDa, were used to determine the size of proteins.

2.6 DNA templates and PCR analysis

Templates were prepared from 12 to 18-h in nutrient agar. Further DNA extraction was performed as described by (Hansen & Hendriksen, 2001). Five pairs of universal primers designed by Ben-Dov et al (1997, 1999) *cry1Ab*, *cry1F*, *cry1B*, *Cry1C* and *cry1D.*

Amplification was carried out in a DNA Masterycler Personal (Eppendorf) for 35 reaction cycles using TBE buffer. The electric current migration was approximately 76 V for 1h. Reactions were routinely carried out in 25µL; 5µL of template DNA was mixed with reaction buffer, 0.1mM (each) dNTP, 0.3 to 0.5mM (each) primer and 0.3 U *Taq* DNA polymerase (GIBCO-BRL). Template DNA was denatured (1 min at 95℃) and annealed to primers (40 to 50s at 57℃) and extensions of PCR products were achieved at (50 to 90s at 72℃). Each experiment was associated with negative (without DNA template). The fragments were analyzed on 1.5% agarose gel and visualized by UV light incidence after treatment for 15 min with 0.5µg/mL ethidium bromide solution. To check the reproducibility of the method, after standardized three amplifications were performed in each sample.

3 RESULTS

The *B. thuringiensis* strains analyzed were chosen because they belong from soils of rice-fields of Rio Grande do Sul. Phenotypic features strains showed parameters characteristic for the species (Table 2 and Figure 3). Small variations were found in characters such as: the cell length in light microscopy, growth in Nutrient Broth containing 5% NaCl, strain and the location of spores in the sporangia, where the sub-terminal position predominated.

Table 2. Sources and region of isolation for *Bacillus thuringiensis* strains used in the study

Strains	Origin	Corrected	Detected Cry					Colonies morphology			
BBE	(Region[a])	Mortality	*1Ab*	*1F*	*1B*	*1C*	*1D*	Form*	Edge	Size	Structure
***Bt* 4412**	ref	100%	-	-	+	-	-				
2835-4	LI	11,1%	-	-	-	+	-	C	smooth	G	dense
1547-1	LI	5,6%	-	-	-	+	-	C	smooth	G	dense
3467-4	FO	21%	-	-	-	-	-	C	serrated	G	dense
3280-1	CA	16%	-	+	-	+	-	C	smooth	M	dense
3420-5	LI	15%	-	-	-	-	-	C	smooth	G	dense
3419-1	CA	11%	-	-	-	+	-	C	smooth	G	dense
3420-6	CA	21%	-	-	-	+	-	C	smooth	G	dense
3420-10	CA	16%	-	+	-	+	-	C	smooth	G	dense
3420-11	CA	88,8%	-	+	-	+	-	C	smooth	G	dense
1893-7P	LI	5%	-	-	-	-	-	C	wavy	P	granulated
1893-13	LI	16,6%	-	-	-	+	-	C	wavy	M	granulated
1893-14	LI	16%	-	-	-	-	-	C	smooth	P	dense
1893-16	LI	5%	-	-	-	-	-	C	smooth	P	dense
1893-24	LI	10%	-	-	-	+	-	C	wavy	P	granulated
1458-1	FO	20%	-	+	-	-	-	C	wavy	P	granulated
1458-2	FO	15,7%	-	+	-	-	-	C	wavy	P	granulated
1458-4	FO	25%	-	+	-	+	-	C	wavy	G	granulated
1490-5	FO	5,5%	-	+	-	+	-	C	wavy	G	granulated
3133-5	DC	10%	-	+	-	-	-	C	smooth	M	granulated
1829-2	DC	0%	-	+	-	+	-	C	smooth	G	smooth

[a] LI: Litoral; FO: Fronteira Oeste; CA: Campanha; DC: Depressão Central; + gene presence; - gene abscene;* C=circular

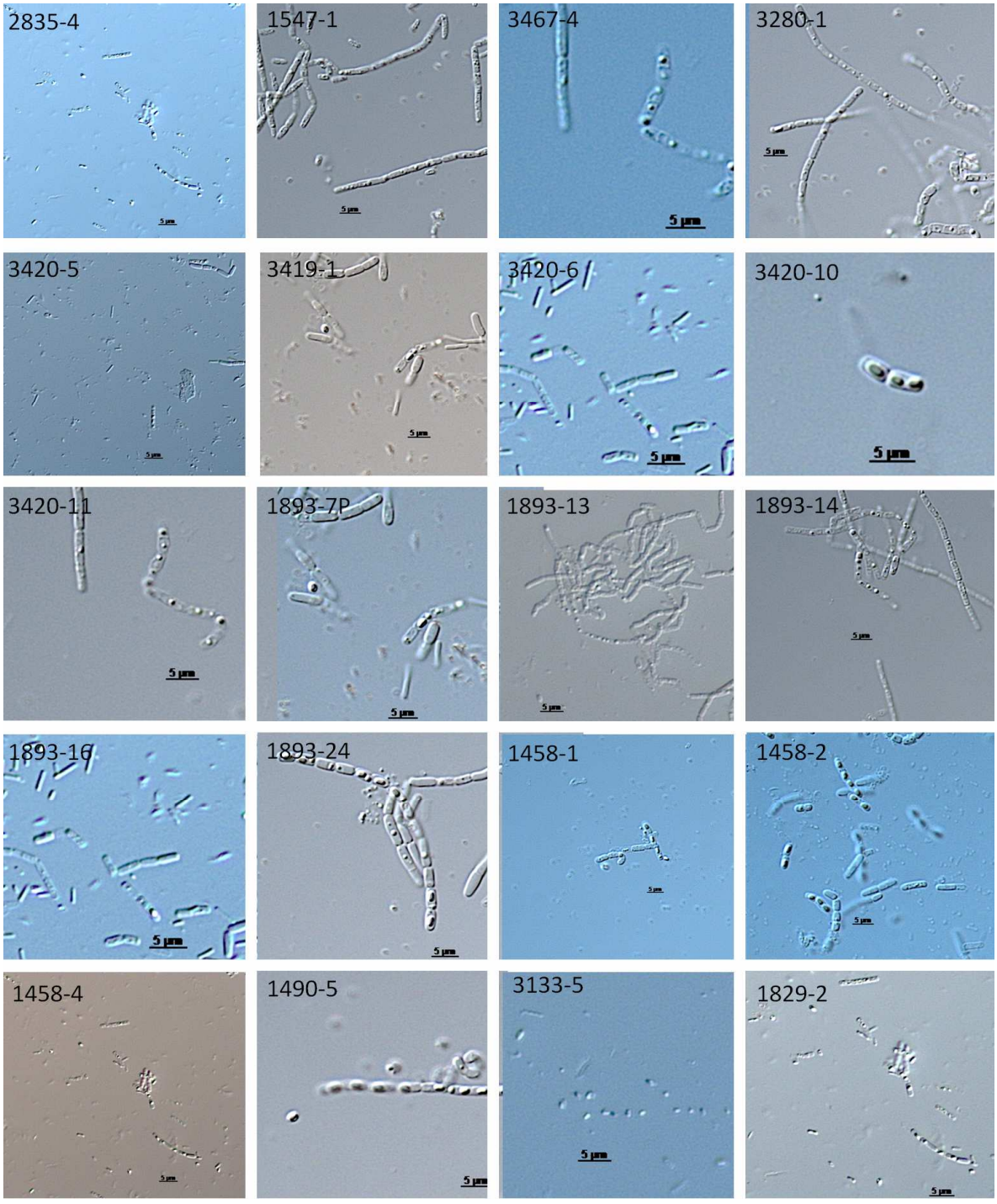

Figure 3: Complex spore-crystal of *Bacillus thuringiensis* strains observed in interferential phase contrast microscope.

Among the strains of *B. thuringiensis* subjected to bioassays in this study to *S. frugiperda*, one strain (3420-11) showed mortality superior to 85% as the reference strain *B. thuringiensis thuringiensis* 4412. Four strains (1458-1, 1458-4, 3420-6, 3467-4) caused mortality equal or superior to 20% (Figura 4).

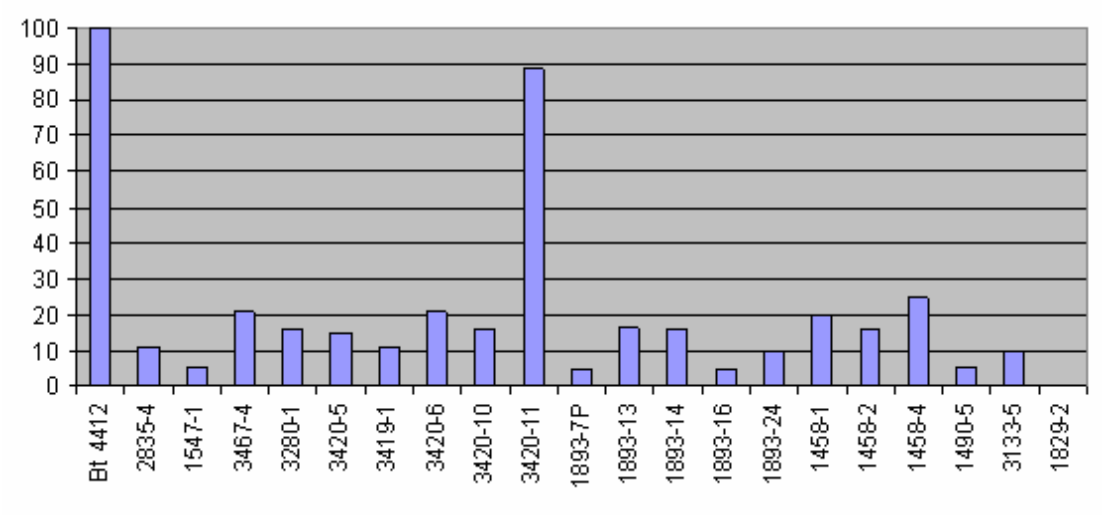

Figure 4. Corrected mortality (%) of *Bacillus thuringiensis* strains submitted to bioassays with *Spodoptera frugiperda*

With respect to antibiograms resistance profile, *B. thuringiensis* strains were 100% resistant to two, and the Penicillin G (PN) and Vancomycin (VA) (Table 3). In the sensitivity profile strains showed 100% inhibition at one antibiotic, Cloraphenicol (CO) (Table 3). The remaining strains showed multiple profiles resistance and sensitivity but reported a significant heterogeneity in accordance with the source region (Figure 5). Table 3 presents the results of the percentage of resistance and sensitivity of the strains to antibiotics.

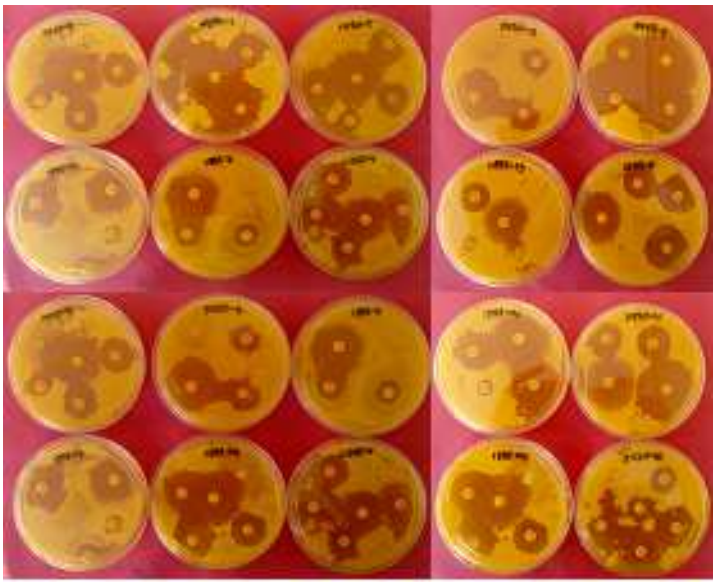

Figure 5: Multiple profiles resistance and sensitivity to antibiotics.

Table 3. Profile of resistance and sensitivity of strains as well the percentage of the response sensitivity and the resistance to strains of *Bacillus thuringiensis.*

Strains	ANTIBIOTICS						
	CT	CO	ET	EL	PN	TE	VA
2835-4	R	S	R	R	R	S	R
1547-1	R	S	R	R	R	S	R
3467-4	S	S	S	S	R	S	R
3280-1	S	S	R	R	R	R	R
3420-5	R	S	R	R	R	S	R
3419-1	S	S	R	R	R	S	R
3420-6	S	S	R	R	R	S	R
3420-10	S	S	R	R	R	S	R
3420-11	S	S	R	R	R	S	R
1893-7P	R	S	R	R	R	R	R
1893-13	R	S	R	R	R	R	R
1893-14	R	S	R	R	R	R	R
1893-16	R	S	R	R	R	R	R
1893-24	R	S	R	R	R	R	R
1458-1	S	S	S	S	R	S	R
1458-2	S	S	S	S	R	S	R
1458-4	S	S	S	S	R	S	R
1490-5	S	S	S	S	R	S	R
3133-5	R	S	S	R	R	R	R
1829-2	R	S	S	R	R	R	R
% R^2	50	0	65	75	100	40	100
% S^2	50	100	35	25	0	60	0

Legend:
1 - Cefoxitin, 30□g (CT); Cloranphenicol, 30□g (CO); Estreptomicin, 10□g (ET); Eritromicin, 15□g (EL); Penicilin G, 10un. (PN); Tetraciclin, 30□g (TE) and Vancomycin, 5□g (VA).
(R) = Resistance (S) sensitivity
2 - % R = Response in the percentage of resistance for each of the antibiograms.
3 - % S = Response in the percentage of sensitivity for each of the antibiograms.

Results from protein analysis by SDS–PAGE for the strains showed the presence of four distinct groups (Figure 1). Group 1 comprised one toxic *B. thuringiensis* strain of Campanha region (3420-11) with the similar larvicidal activity and protein pattern in SDS–PAGE as B. *thuringiensis* thuringiensis4412. Group 2 comprised strains of Fronteira Oeste region 3467-4, 1458-1, 1458-2, 1458-4, 1458-5.

Group 3 comprised strains of Litoral and Group 4 strains of Depressão Central Region.

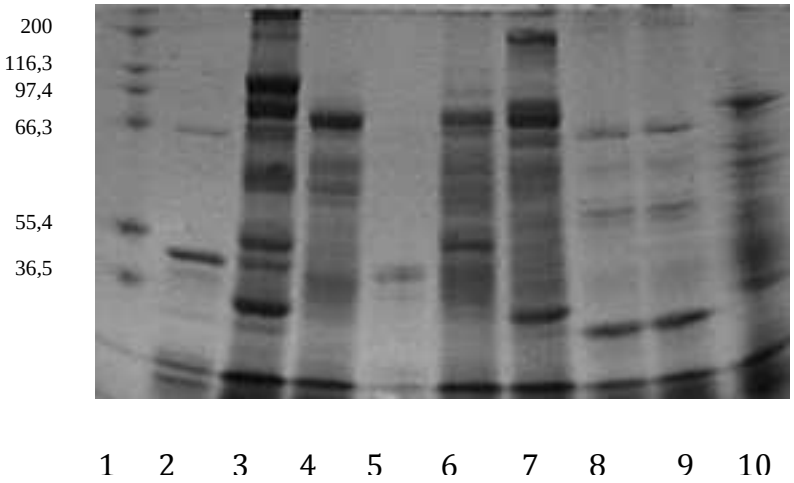

Figura 2. Protein profile of *Bacillus thuringiensis* strains d-endotoxins. Lane 1: protein standards (myosin, 205 kDa; b-galactosidase, 116 kDa; phosphorylase B, 97.4 kDa; bovine albumin, 66 kDa; egg white albumin, 45 kDa; carbonic anhydrase, 29 kDa); Lane 2, *B. thuringiensis thuringiensis* 4412; Lane 3, 3420-11; Lane 4, 1892-2; Lane 5, 3467-4; Lane 6, 3420-6; Lane 7, 3280-1; Lane 8, 2835-4; Lane 9, 1547-1; Lane, 10: 1893-13.

The PCR amplifications analysis of the isolates revealed the presence of amplified fragments characteristic of cry1C and *cry1F* genes (figure 3 and 4). The genes *cry1Ab*, *cry1B* and *cry1D* did not amplify DNA fragments corresponding. Results in Fig 3 and 4 showed the expected sizes of PCR products of *cry*1F and *cry*1C genes. Another *cry* genes investigated (*cry1Ab*, *cry1B*, and *cry1D)* were not found in our isolates.

Among the 20 new *B. thuringiensis* strains (Table 1) from soil samples of rice field areas in Rio Grande do Sul *cry*1C genes were the most frequent ones, followed by cry1F genes. The *cry1F* gene was found in three rice field area in Rio Grande do Sul (Fronteira Oeste, Campanha and Depressão Central) and is located mostly in the Fronteira Oeste region. Results from *cry*1F gene showed the presence in all rice field areas and mostly in the Litoral region.

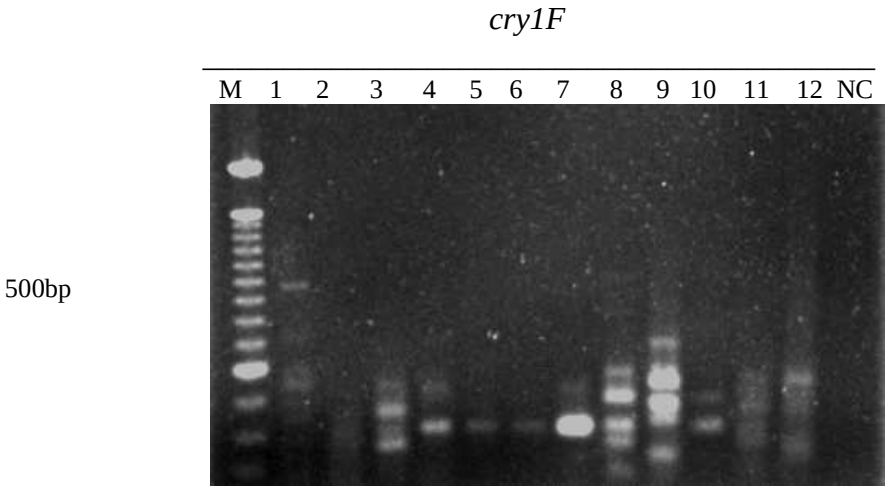

Figure 3. Presence of *cry*1F in *B. thuringiensis* isolates. (M) Molecular Weight Marker (100pb, Gibco BRL); (1) 1893-16; (2) 2835-4; (3)1893-24; (4) 1490-5; (5) 3420-10; (6) 3133-3; (7) 3280-1; (8) 1458-4; (9) 1458-1; (10) 1458-2; (11) 1829-2; (12)1893-7; (NC) Negative Control.

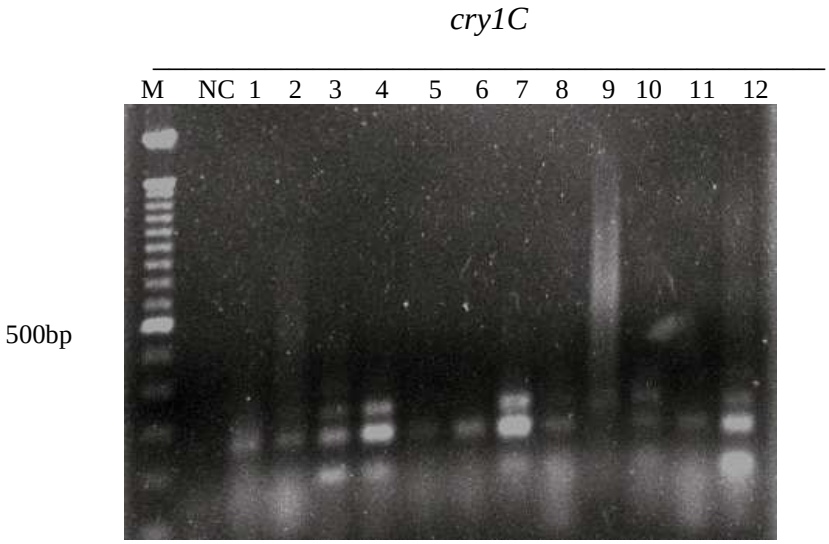

Figure 4. Presence of *cry*1C in *B. thuringiensis* isolates. (M) Molecular Weight Marker (100pb, Promega – G210a); (NC) Negative Control; (1) 2835-4; (2) 1893-24; (3) 1547-1; (4) 1490-5; (5) 3420-10; (6) 3419-1; (7) 3280-1; (8) 1458-4; (9) 3420-5; (10) 1829-2; (11) 3420-6; (12) 1893-13.

4 DISCUSSION

The characterization of microorganisms is essential for microbial ecology studies. Classification, identification and differentiation of bacteria have traditionally relied on test based on phenotypic characteristics (Porcar et al. 1999). The phenotypic methods used for classifying *B. thuringiensis* and biochemical characters, have both contributed to the establishment of an useful classification (Iriarte et al. 2000, Khyami-Horani et al. 2003). Here we report the characterized 20 *B. thuringiensis* strains isolated from soil samples collected in irrigated rice areas of Rio Grande do Sul (RS). The interest for the identification of these strains is the search that makes them potential targets for studies that aim at the development of new bacterial insecticides.

The biochemical, physiological, morphological and staining features of strains comprise a classical method, simple and convenient, for strain grouping and confirmation regarding known characteristics used as criteria for inclusion in the species. However, this method is based on phenotypic characteristics that can be influenced by environmental factors such as laboratory growth conditions. On the other hand, the genotypic methods used in the present study demonstrated the possibility of distinguishing the strains since they are methods based on bacterial genome analysis (Iriarte et al., 2000).

The results obtained in pathogenic strains also revealed that 1 showed high toxicity (larval mortality> 50%) against *S. frugiperda* being the most promising for the control as HD-1 strain, which has broad spectrum of larvicidal activity within the Lepidoptera Order (Navon, 1993). However, *Spodoptera* species belonging to the family Noctuidae are insensitive to these agents (MOAR et al. 1990; Inagaki et al. 1992; Navon, 1993; Lambert et al. 1996). Bioassays showed the pathogenic potential of the strain in control of larvae of *S. frugiperda.* According to Lee et al. (1996), Schnepf et al. (1998), subclasses cry1 gene correlations with bioassays indicate that the toxicity of some strains to target insects can occur because of synergistic interactions among the toxins found, or even by the interaction of these with the spores, making it necessary to perform and analyze and bioassays with individual proteins together to confirm the toxin responsible for mortality.

To enlarge the strains characterization were evaluated in seven antibiotics behavior, since the presence of resistance and sensitivity is a further knowledge of the spread of strains of rice fields. Resistance and sensitivity characteristics can be associated with plasmids, which can be transferred between bacteria of the same species or related species different taxonomic groups. *B. thuringiensis* and *B. cereus* species genetically related, it is known that the transfer plasmids containing genes encoding the crystal proteins (Gonzalez et al, 1982). It's noteworthy that the issue of plasmids conjugated to other genes are also transferred, including genes for resistance to antimicrobial agents (if they are present in plasmids) contributing to significant genetic diversity in these species.

This event suggested the characterization of strains compared to seven related antibiotics (Table 1). Table 3 illustrates the responses of resistance and sensitivity, is observed at which a significant heterogeneity among strains from different origins. It is noteworthy that this heterogeneity and profile of multi-resistance strains of *B. thuringiensis* have already been demonstrated in Silva (2002), the study of antimicrobial resistance of strains of 34 serovars *B. thuringiensis* of and *B. cereus* isolates from different materials.

Because some strains of *B. thuringiensis* are not pathogenic, there is no standardized characterization of antibiotics as the concentrations that determine susceptibility or resistance. The use of antibiotics in combination with other techniques, such as PCR (Knight et al., 2005) analysis and optical microscopy are used to characterize and identify new isolates of *B. thuringiensis* and differentiate them from other bacteria (Monnerat et al., 2001).

The presence of three groups of strains with different protein profiles was demonstrated through SDS–PAGE. Group 1 comprised one toxic *B. thuringiensis* strain (3420-11), with the same larvicidal activity and the same protein pattern in SDS–PAGE as B. *thuringiensis thuringiensis* 4412. Group 2 comprised strains of Fronteira Oeste region, Group 3 comprised strains of Litoral and Group 4 strains of Depressão Central Region. These results were similar to those reported by Ragni et al. (1996) compared with toxic activity that demonstrated that *B. thuringiensis* strains have toxic activity to larvae of *Aedes aegypti* and *Culex quinquefasciatus* and similarity with crystal proteins of *B. thuringiensis* serovar *israelensis.*

Genetic variability between *B. thuringiensis* strains was tested through the use of techniques that are based on the PCR. These techniques have applications such as the determination of persistence of bacteria in the environment, which can be evidenced by the presence of *cry* genes (Bourque et al., 1993). In this report the PCR amplifications analysis of the isolates revealed the presence of amplified fragments characteristic of *cry1C* and *cry1F* genes. According Bohovora et al., (1997) the *cry1D*, *cry1E* and *cry1F* are more efficient to *S. frugiperda*. Praça et al. (2004) worked with *B. thuringiensis* strains for controlling the *S. frugiperda* and *Anticarsia gemmatalis* showed that probably mortality obtained in these species is due to gene *cry1B*.

Based on the results obtained in the present study the combination of classical and molecular methods helps us to group the isolates and give more details than a single classification method and, therefore, makes an important contribution to our knowledge of intra-specific differences for this important and useful bacterial species. The characterization of native strains with activity against pests, gives us new tools to be introduced management program. This will decrease the disturbance of ecosystem balance through reducing the release of chemical pesticides or other exogenous biological products.

5 REFERENCES

Abbott, W.S. 1925. A method of computing the effectiveness insecticide. *J. Econ. Entomol.* 18:265-267.

Bauer, A.W., Kirby, W.M.M., Sheris, J.C., Turck, M. 1966. Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol.* 45:493-496.

Ben-Dov, N., Shefer, G., Irintchev, A., Wernig, A., Oron, U., Halevy, O. 1999. Low-energy laser irradiation affects satellite cell proliferation and differentiation in vitro. *Biochim. Biophys. Acta* 1448:372-380.

BEN-DOV, E., Shefer, G., Irintchev, A.1997. Extend screening by PCR for seven *cry-group* genes from field-collected strains of *Bacillus thuringiensis. Applied and Environmental Microbiology***.** 63:4883-4890.

Bravo, A., Quintero, R. 1993. Importancia potencial de *Bacillus thuringiensis* en el control de plagas. *Redbio, Santiago, Chile.*

Beegle, C.C., Yamamoto, T., 1992. History of Bacillus thuringiensis Berliner research and development. *Can. Entomol*. 124:587–616.

Becker, N. 2000. Bacterial control of vector-mosquitoes and blackflies. In: Charles, J.F., Delécluse, A., Nielsen-LeRoux, C. (Eds.), Entomopathogenic Bacteria: from Laboratory to Field Application. *Kluwer Associate Publishing, Norwell*, MA, USA.36:383–396.

Bernhard, K., Utz, R. 1993. Production of Bacillus thuringiensis insecticides for experimental and commercial uses, pp. 255 - 267. In Entwistle, P.F., Cory, J.S.,

Bourque, S.N., J.R. Valéro, J. Mercier. M.C. Lavoie, and R.C. Levesque. 1993. Multiplex polymerase chain reaction for detection and differentiation of the microbial insecticide *Bacillus thuringiensis*. Appl. Environ. Microbiol. 59:523-527.

Bravo, A. 1997. Phylogenetic Relationships of *Bacillus thuringiensis* d-Endotoxin Family Pro- teins and Their Functional Domains. Journal of Bacteriology, 179:2793–2801.

Bravo, A., Sarabia, S., López, L., Ontiveros, H., Abarca, C., Ortiz, A., Ortiz, M., Lina, L., Villalobos, F., Peña, Nuñez-Valdez, G.M., Soberon, M., Quintero, R. 1998. Characterization of *cry* genes in Mexican *B. thuringiensis* strain collection. *Appl. Environ. Microbiol*. 64:4965-4972.

Bravo, A., Likitvivatanavong, S., Gill, S.S., Soberón, M. 2011. *Bacillus thuringiensis*:

a story of a successful bioinsecticide. *Insect Biochem. Mol. Biol*. 41: 423–431.

Bohorova, N., Cabrera, M., Abarca, C., Quintero, R., Maciel, A.M., Brito, R.M., Hoisington, D., Bravo, A. 1997. Susceptibility of four tropical lepidopteran maize pests to *Bacillus thuringiensis* CryI type insecticidal toxins. *Journal of Economical Entomology* 90:412-415.

Cannon, R.J.C., 2000. *Bt* transgenic crops: risks and benefits*. Integr. Pest Manage*. Rev: 5:151–173.

Carozzi, N.B., Kramaer, V.C., Warreen, G.W., Evola, S., Kozid, M.G. 1991. Prediction of Insecticidal activity of *Bacillus thuringiensis* strains by polymerase chain reaction product profile. *Appl. Environ. Microbiol*. 57:3057-3061.

Cavados, C.F.G., Fonseca, R.N., Chaves, J.Q., Rabinovitch, L., Araújo-Coutinho, C.J.P.C. 2001. Identification of Entomopathogenic *Bacillus* Isolated from Simulium (Diptera, Simuliidae) Larvae and Adults. *Memórias do Instituto Oswaldo Cruz* 96:1017-1021.

Claus, D., Berkeley, R.C.W. 1986. Genus *Bacillus* Cohn 1872. In: Sneath, P.H.A., Mair, N.S., Sharpe, M.E., Holt, J.G. (Eds.), Bergey's Manual of Systematic Bacteriology, 2nd ed. Williams & Wilkins, Baltimore, pp. 1105–1141.

Cowan, S.T., Steel, K.J. 1974. In: Cowan and Steel's Manual for Identification of Medical Bacteria. Cambridge University Press, London.

Crickmore, N., Zeigler, D.R., Feitelson, J., Schnepf, E., Van Rie, J., Lerecus, D., Baum, J., Dean, D.H. 1998. Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal protein. *Microbiology and Molecular Biology* Reviews 62: 807-813.

De Barjac, H., Lecadet, M.M. 1976. Dosage biochimique d'exotoxine thermostable *Bacillus thuringiensis* d'après l'inhibition d'RNA-polymerases bacteriennes. *C.R. Acad. Sci.* 282: 2119-2122.

De Maagd, R.A., Bravo, A., Berry, C., Crickmore, N., Schnepf, E. 2003. Structure, diversity and evolution of protein toxins from sporeforming entomopathogenic bacteria. *Annual Review of Genetics*. 37:409 - 433.

De Maagd, R. A., Bravo, A., Crickmore, N. 2001. How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. *Trends in Genetics*. 17:193-199.

diez-rodrigues, G. I., Omoto, C. 2001. Herança da resistência de *Spodoptera*

frugiperda (J.E.Smith) (Lepidoptera: Noctuidae) a lambdacialotrina. *Neotropical Entomology***.** 30:311-316.

Federici, B.A. 2005. Insecticidal bacteria: an overwhelming success for invertebrate pathology. *J. Invertebr. Pathol*. 89:30–38.

Feitelson, S., Payne, L., Kim, L. 1992**.** *Bacillus thuringiensis:* insects and beyond. *Biol Technology.* 10:271-275.

Ferré, J., Van Rie, J., MacIntosh, S.C. 2008. Insecticidal genetically modified crops and insect resistance management (IRM). In: Romeis, J., Shelton, A.M., Kennedy, G.G. (Eds.), Integration of Insect-Resistant Genetically Modified Crops within IPM Programs. *Springer, Netherlands*, pp. 41–85.

Gonzalez, J.M., Brown, B.J., Carlton, B.C. 1982. Transfer of *Bacillus thuringiensis* plasmids coding for delta-endotoxin among strains of *B. thuringiensis* and *B. cereus*. *Proceedings of the National Academy of Sciences of the United States of America*. 79:6951-6955.

Gordon, R.E., Haynes, W.C., Pang, C.N., 1973. The Genus Bacillus. *Agric. Handbook* n°427 US Department of Agriculture, Washington, D C.

Hansen, B.M., Hendriksen, N.B. 2001. Detection of enterotoxin *Bacillus cereus* and *Bacillus thuringiensis* strains by PCR analysis. *Applied and Environmental Microbiology*. 67:185- 189.

Höfte, H., Whiteley, H.R. 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol. Rev*. 53:242-255.

Inagaki, S., Miyasono, M., Ishiguro, T., Takeda, R., Hayashi, Y. 1992. Proteolytic processing of δ-endotoxin of *Bacillus thuringiensis* var. *kurstaki* HD-1 in insensitive insect, *Spodoptera litura:* unusual proteolysis in the presence of sodium dodecy1 sulfate. *Journal of Invertebrate Pathology.* 60: 64-68.

Iriarte, J., Dumanoir, V.C., Bel, Y.M., Porcar, M.D., Ferrandis, M.M. Lecadet, J., Ferre Caballero, P. 2000. Characterization of *Bacillus thuringiensis* ser. *balearica* (Serotype H48) and ser. navarrensis (Serotype H50): two novel serovars isolated in Spain. *Curr. Microbiol.* 40:17-22.

James, C. 2011. Global Status of Commercialized Biotech/GM Crops. International Service for the Acquisition of Agri Biotech Applications Nº 43. *ISAAA*: Ithaca, NY.

James, C. 2010. Global Status of Commercialized Biotech/GM Crops: International

Service for the Acquisition of Agri Biotech.12. *ISAAA*. Ithaca, NY.

Khyami-Horani, H., Hajaij, M., Jean-Francois, C. 2003. Characterization of *Bacillus thuringiensis* ser. *jordanica* (Serotype H71), a novel serovariety isolated in Jordan. *Curr. Microbiol*. 47:26-31.

Knight, A.L., Lacey, L.A., Stockhoff, B.A. and Warner, R.L. (2005) Activity of Cry1 endotoxins of Bacillus thuringiensis for four tree fruit leafroller pest species (Lepidoptera: Tor- tricidae). *J Agric Entomol.* 15:93–103.

Lacey, L.A., Undeen, A.H. 1986. Microbial control of black flies and mosquitoes. *Annu. Rev. Entomol*. 31:265–296.

Laemmli, U.K.; Favre, M. 1973. Maturation of the head of bacteriophage T4. *J. Mol. Biol*. 80:575–599.

Lambert, B., Buysse, L., Decock, C., Jan-Sens, S., Piens, C., Saey, B., Seurinck, J., Van Audenhove, K., Van Rie, J., Van Vliet, A., Peferoen, M. 1996. A *Bacillus thuringiensis* insecticidal crystal protein with a high activity against members of the family Noctuidae. *Applied and Environmental Microbiology.* 62:80-86.

Lecadet, M.M., Chaufaux, J., Ribier, J., Lereclus, D. 1992. Construction of novel Bacillus thuringiensis strains with different insecticidal activities by transduction and transformation. *Appl. Environ. Microbiol*: 58:840–849

Lee, M. K., Curtiss, A., Alcantara, E. A., Dean, D. H. 1996. Synergistic effect of the *Bacillus thuringiensis* toxins CryIAa and CryIAc on the gypsy moth, *Lymantria dispar. Applied and Environmental Microbiology***.** 62:583-586.

Lereclus, D., Delécluse, L., Lecadet, M. M. 1993. Diversity *of Bacillus thuringiensis* toxins and genes. In: P. Entwistle, J.S.; Cory, M.J.; Baley & S. Higgs (ed.), *Bacillus thuringiensis*, an environmental biopesticide: the- ory and pratice, John Wiley & Sons, Ltd., Chichester, U.K. p. 37-69.

Lima, A.S.G., Guidelli, A.M., Abreu, I.L., Lemos, M.V.F. 2002. Identification of new isolates of *Bacillus thuringiensis* using rep-PCR products and electron microscopy. *Gen. Mol. Biol*. 25:225-229.

Moar, W. J., Masson, L., Brousseau, R., Trumble, J. T. 1990. Toxicity to *Spodoptera exigua* and *Trichoplusia ni* of individual P1 protoxins and sporulated cultures of *Bacillus thuringiensis* subsp. Kurstaki HD-1 an NRD-12. **A***pplied and Environmental Microbiology***.** 56:2480-2483.

Monnerat, R.G., Silva, S.F., Wernwck, J.O.S., Dias, J.M.C.S. 2001. Métodos de coleta, isolamento, caracterização e armazenamento de estirpes de *B. sphaericus. Embrapa Recursos Genéticos e Biotecnologia* (Circular Técnica, 9).

Mullis, K. B., Fallona, F.A. 1987. Specific synthesis of DNA in vitro via polymerase chain reaction. *Methods Enzymol*. 155:335-350.

Navon, A., 2000. *Bacillus thuringiensis* insecticides in crop protection reality and prospects*. Crop Prot*. 19:669–676.

Navon, A. 1993. Control of lepidopteran pests with *Bacillus thuringiensis*. In: ENTWISTLE, P.F. et al. (Eds). *Bacillus thuringiensis*, an environmental biopesticide: theory and practice. Chichester: John Wiley & Sons. p.125-146.

Praca, L.B., Batista, A.C.E., Martins, É.S. 2004. Estirpes de *Bacillus thuringiensis* efetivas contra insetos das ordens Lepidoptera, Coleoptera e Diptera. *Pesquisa Agropecuária Brasileira*. 39:11-16.

Pinto, L.M.N.; Azambuja, A.O.; Dihel, E.; Fiuza, L.M. 2003. Pathogenicity of Bacillus thuringiensis isolates from two species of Acromirmex (Hyme- noptera: Formicidae). Brazilian Jour- nal Biology 63(2): 301-306.

Porcar, M.; Juárez-Pérez, V. 2003. PCR-based identification of *Bacillus thuringiensis* pesticidal crystal genes FEMS Microbiology Reviews. 26: 419-432.

Poitout, S., Bues, R. 1974. Élevage des chenilles de vint huit espèces de lépidoptères Noctuidae et deux espèces d'Arctiidae sur milieu artificiel simple. Particularités de l'élevage selon les espèces. *Annales de Zoologie, Ecologie Animale*. 6:431–441.

Porcar, M., Iriarte, J., Cosmao, V., Ferrandis, M.D., Lecadet, M.M., Ferré, J., Caballero. P. 1999. Identification and characterization of the new *B. thuringiensis* serovars *pirenaica* (serotype H57) and *iberica* (serotype H59). *J. Appl. Microbiol*. 87:640-648.

Ragni, A., Cosmao, V. 1996. Characterization of six highly mosquitocidal *Bacillus thuringiensis* strains that do not belong to H-14 se- rotype. *Curr Microbiol,* 32:48-54.

Reyes-Ramires, A., Ibarra, J.E. 2005. Fingerprinting of *Bacillus thuringiensis* type strains and isolates by using *Bacillus cereus* group-specific repetitive extra- genic palindromic sequence-based PCR analysis. *Appl. Environ. Microbiol*. 71:1346-55.

Reyes-Ramírez, A., Ibarra, J.E. 2008. Plasmid patterns of *B. thuringiensis* type

strains. *Appl. Environ. Microbiol*. 74:125-129.

Sanahuja, G., Banakar, R., Twyman, R.M., Capell, T., Christou, P., 2011. *Bacillus thuringiensis*: a century of research, development and commercial applications. *Plant Biotechnol. J*. 9: 283–300.

Sanchis, V. 2011. From microbial sprays to insect resistant transgenic plants: history of the biospesticide *Bacillus thuringiensis*. A review. *Agron. Sustain. Dev*. 31:217–231.

Sanchis, V., Bourguet, D. 2008. *Bacillus thuringiensis*: applications in agriculture and insect resistance management. A review. *Agron. Sustain. Dev*. 28:11–20.

Sanchis, V.; Lereclus D.; Menou, G.; Chaufaux, J.; Lecadet, M. M. 1998. Multiplicity of δ endotoxinas genes with different insecticidal specificities in *Bacillus thuringiensis* aizawai 7.29. *Molecular Microbiology.* 2:393-404.

Sauka, D.H., Benintende, G.B. 2008*. Bacillus thuringiensis*: generalidades. Un acercamiento a su empleo en el biocontrol de insectos lepidópteros que son plagas agrícolas. Rev. *Argent. Microbiol*. 40:24–140.

Schnepf, E., Crickmore, N., Rie, J. Van., Lereclus, D., Baum, J., Feitelson, J., ZeiGler, D.R., Dean, D.H. 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiology and Molecular Biology* Reviews**.** 62:775-806.

Silva, C.C.A. 2002. Aspectos do sistema imunológico dos insetos. *Biotecnol. Cienc. Desenv*. 24:68-72.

Thiéry, I., Back, C., Barbazan, P., Sinègre, G. 1996. Applications de Bacillus thuringiensis et de B. sphaericus dans la démoustication et la lutte contre les vecteurs de maladies tropicales. *Ann. Inst. Pasteur Actualités*. 7:247–260.

Shelton, A.M., Zhao, J.Z., Roush, R.T., 2002. Economic, ecological, food safety, and social consequences of the deployment of Bt transgenic plants. *Annu. Rev. Entomol*. 47:845–881.

Van Frankenhuyzen, K. 2000. Application of *Bacillus thuringiensis* in forestry. In: Charles, J.F., Delécluse, A., Nielsen-LeRoux, C. (Eds.), Entomopathogenic Bacteria: From Laboratory to Field Application. *Kluwer Associate Publishing, Norwell*, MA, USA, pp. 371–376.

YU, S.J. 1992. Detection and biochemical characteri- zation of insecticide resistance in fall armyworm (Lepidoptera: Noctuidae). *Journal of Economic Entomology*. 85:675-682.

6 CAPÍTULO 5

Artigo de pesquisa

BIOLOGICAL CHARACTERIZATION OF BRAZILIAN *BACILLUS THURINGIENSIS* STRAINS AGAINST *OSTRINIA NUBILALIS* (LEP., PYRALIDAE).

BIOLOGICAL CHARACTERIZATION OF BRAZILIAN *BACILLUS THURINGIENSIS* STRAINS AGAINST *OSTRINIA NUBILALIS* (LEP., PYRALIDAE).

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ABSTRACT

Bacillus thuringiensis is an entomopathogenic bacterium that, during sporulation, produces crystal proteins, coded by *cry* genes, which can action on crop pests such as *Ostrinia nubilalis* causing its destructive effect. The objective of this work was to characterize seven *B. thuringiensis* isolated from South of Brazil and characterizer by means of molecular and microbiological techniques and confirme their action by development of biassays, seeking the biological control of *O. nubilalis*. Insecticidal activity, plasmid profiles and antibiograms analyses were performed for identification and determination of the degree of genetic diversity using the subclasses of the gene *cry1A*. The molecular techniques suggest high genetic similarity among the studied isolates and the antibiograms performed show that all the isolates were sensitive to the antibiotics streptomycin, rifampicin, and erythromycin. Two strains were highly toxic against first instar larvae. One strain was found to be even more toxic than the reference strain *Bacillus thuringiensis* var. *kurstaki* 4HD-1. The biassays demonstrated the entomopathogenic potential of the new strains in the control of the *O. nubilalis* larvae.

Keywords: Bioassay, *Ostrinia nubilalis*, *Bacillus thuringiensis*.

RESUMO

Bacillus thuringiensis é uma bactéria entomopatogênico que durante a esporulação, produz proteínas cristalíferas, codificadas por genes cry, que podem atuar sobre as pragas agrícolas, como *Ostrinia nubilalis*. O objetivo deste trabalho foi caracterizar

por meio de técnicas moleculares, microbiológicas e bioensaios, sete novas cepas de *B. thuringiensis* isoladas do Sul do Brasil. A atividade insecticida, os perfis plasmidiais e a análise dos antibiogramas foram realizados para a identificação e a determinação do grau de diversidade genética utilizando as subclasses do gene Cry1A. As técnicas moleculares revelaram alta similaridade genética entre os isolados estudados e os antibiogramas realizados mostram que todos os isolados foram sensíveis a estreptomicina, rifampicina e eritromicina. Duas cepas foram altamente tóxica contra larvas de primeiro ínstar e uma cepa apresentou maior toxicidade em comparação a cepa padrão *B. thuringiensis* var. *kurstaki* HD-1. Os bioensaios demonstraram que os novos isolados têm potencial entomopatogênico para o controle de larvas *de O. nubilalis*.

Palavras-chave: Bioensaios, *Ostrinia nubilalis*, *Bacillus thuringiensis*.

1 INTRODUCTION

Bacillus thuringiensis is a spore-forming bacterium well-known for its insecticidal properties associated with its ability to produce crystal inclusions during sporulation. These inclusions are proteins encoded by cry genes and have shown to be toxic to a variety of insects and other organisms like nematodes and protozoa (Konecka et al. 2007). The primary action of Cry proteins is to lyse midgut epithelial cells through insertion into the target membrane and form pores (Bravo et al. 2007). The activity spectrum of Bt toxins continually increases as the result of the continuing isolation of new strains around the world (Alvarez et al., 2011).

Ostrinia nubilalis (the European corn borer, ECB) is a cosmopolitan pest of maize (*Zea mays* L.) (Figure 1). It first appeared after the introduction of maize to Western Europe, approximately 500 years ago (Rebourg et al. 2003) and was accidentally introduced in the Americas in the early twentieth century (Thompson and Parker 1928).

Figure 1: The european corn borer, *Ostrinia nubilalis* larvae (Lep., Pyralidae).

Its distribution now includes most corn-growing areas of the United States and Canada, causing losses in excess of $1 billion dollars annually (Ostlie et al. 1997). European corn borer females lay eggs on leaves, and early instars migrate to the whorl where they feed on the mesophyll until moving to leaf sheaths and midribs. When larvae reach the third instar, they bore into the stalk, ear, and earshank on reproductive corn interrupting the transport of water and nutrients (Mason et al. 1996). Although transgenic corn has proven to be highly efficient in managing European corn borer (Siegfried et al. 2007), there is a concern that resistance to *Bt* toxins may evolve in European corn borer populations.

Currently, control of this pest relies on chemical insecticides. Nevertheless, the rapid increase in resistance to insecticides together with the potential adverse environmental effects produced by these chemicals have encouraged the development of alternative methods for Lepidoptera control (Beron and Salerno 2006; Monnerat et al. 2007). Among these methods the use of *B. thuringiensis* as a biocontrol agent has shown to be extremely valuable. The diversity of Cry toxins produced by *B. thuringiensis* allows the formulation of a variety of bioinsecticides by using the bacteria themselves or by expressing their toxin genes in transgenic plants. To date, many plant species have been genetically modified with cry genes, resulting in transgenic plants with a high level of resistance to insect pests (Christou et al. 2006; Gassmann et al. 2009). However, it has been reported that several pests have developed resistance against Cry proteins (Sauka and Benintende 2008; Tabashnik et al. 2009; Gassmann et al. 2009). Crucial to this development is the identification of novel and more active strains with respect to insect pests of economically important crops. The present paper molecularly and phenotypically characterizes these strains.

In addition their toxicity against *O. nubilalis* with resistent/susceptible profile to Cry 1Ab and 1F is assessed with an assay.

2 MATERIAL AND METHODS

2.1 Bacterial strains and their origins

The strains used in this study are shown in Table 1 and Figure 2. All cultures were grown in Nutrient Broth or Nutrient Agar Difco (Meat extract—3 g/L; Meat peptone—5 g/L; Agar—15g/L pH 6.8±2) at 33℃±1℃ for 18h as needed. *B. thuringiensis* var. *kurstaki* 4HD1 (Bt 4HD1) was used as reference strain and provided by the *Bacillus* Genetic Stock Center (BGSC), Columbus, Ohio, USA.

Table 1. Sources and region of isolation for *Bacillus thuringiensis* strains used in the study

Strains - BBE	Specie	Sources of Isolation	Location (RS)
1547-2	*Bacillus thuringiensis*	Solo	Mostardas
2840-3	*Bacillus thuringiensis*	Solo	Mostardas
3280-1	*Bacillus thuringiensis*	Solo	Dom Pedrito
3420-5	*Bacillus thuringiensis*	Solo	Dom Pedrito
3420-12	*Bacillus thuringiensis*	Solo	Dom Pedrito
1893-13	*Bacillus thuringiensis*	Solo	Sta. Vitoria do Palmar
1893-15	*Bacillus thuringiensis*	Solo	Sta. Vitoria do Palmar

Figure 2: Sites of obtained the new *Bacillus thuringiensis* strains (Rio Grande do Sul, Brazil).

2.2 Phenotypic analysis

B. thuringiensis strains were characterized by conventional microbiological methods, like Gram staining, shape and position of spores, colony morphology and cell motility. The following characteristics were also studied: catalase production and nitrate reduction (Kaur et al. 2005).

2.3 Antibiograms

The determination of the resistance and susceptibility was performed by serial dilution, applying increasing concentrations of antibiotic to the culture media BHI solid, determining the lowest concentration able to inhibit the growth of bacteria, based on the method de-scribed by Lightfoot et al. (1989).

The antibiotics were used: streptomycin, erythromycin and rifampicin, all kept in stock concentration of 100 mg/mL. Antibiotics were used in concentrations of 60, 80, 100 and 120 µg/mL.

2.4 Bioassay against european corn borer

Rearing procedures for European corn borer were based on those developed at the USDA-ARS Corn Insects Research Unit, Ames, IA (Guthrie et al. 1965). Larvae were reared at 27± 1ºC in a photoperiod of 24 hour and 80% RH on a wheat germ-based diet (Lewis and Lynch 1969). At pupation, insects were moved to mating cages where adults were maintained with 8-hour scotophase at 18± 1ºC and 16-hour photophase at 27± 1ºC with 80%RH. Cages were misted with water twice a day, and adult diet was provided to maximize egg production (Leahy and Andow 1994). Egg masses were collected and incubated within plastic petri dishes containing moistened filter paper until hatching. All bioassays were conducted by exposing neonates (<24 h after hatching) to treated artificial diet. Bioassays involved surface treatment of single wells of artificial diet to minimize the amount of *B. thuringiensis* required. The rearing diet developed for *Heliothis uirescens* (King et al. 1985) and adapted for European corn borer (Bruce Lang, Mycogen Seeds, personal communication) was used. Bioassays were performed in 128-well trays (each well 16mm diameter, 16mm high) (Figure 3). Approximately 1ml of diet was dispensed into each well and allowed to solidify. The strains were grown at 30 ℃ ± 1 ℃ in Nutrient Agar for 48 h when the formation of large amounts of spores (P80%) and d-endotoxins (protein crystals) was observed. Spores and crystals were suspended in 0.1% Triton-X 100 non-ionic detergent to obtain uniform spreading onto the diet.

Each well was treated with 30µl of the appropriate solution. Control treatments consisted of wells treated with 0.1% Triton-X 100 only. Wells were allowed to air dry for 1h, and 1 neonate was transferred into each well. Wells then were covered with vented lids and trays were held at 27ºC, 24-hour scotophase, and 80%RH. Mortality and individual larval weights were recorded 7 days later. When mortality was recorded larvae that had not grown beyond 1st instar and weighed ≤ 0.1mg were considered to be dead. As a result, the criterion for mortality used in this study accounts for both severe growth inhibition and death. Concentrations were replicated three times for each bioassay (total of 48 larvae per concentration per bioassay).

Four colonies of *O. nubilalis* were tested: SKY – resistant to toxin Cry1Ab; 2BE - resistant to toxins Cry1Ab e Cry1F; CLNDO – resistant to toxin Cry1F and Mead: Susceptible to toxins Cry1Ab e Cry1F. Mortality data were analyzed by probit

analysis (Finney, 1971) using POLO-PC (LeOra Software1987).

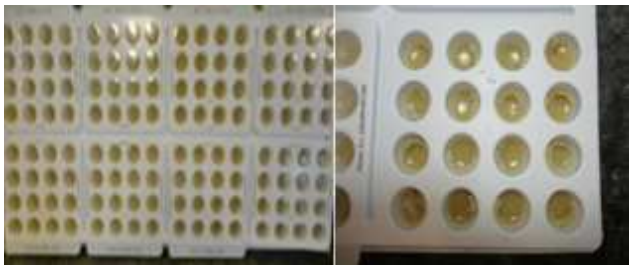

Figure 3: Bioassay against european corn borer using plates, in laboratory.

2.5 Plasmid DNA analysis

Each strain was cultured in 50mL Luria-Bertani (LB) broth (Merck), at 30℃. Vegetative cells were centrifuged at 20,200g for 15 min at 4℃. Each pellet was resuspended in 20mL cold TES buffer (30mM Tris base, 5mM EDTA, 50mM NaCl; pH 8.0) and centrifuged. Cells were resuspended in 2mL lysis buffer (TES buffer containing 20% Sucrose, 2mg/mL, lysozyme, and 1µL/mL of RNAse from a 10mg/mL stock solution) and incubated at 37℃ for 3h. The spheroplast suspension was supplemented with 3mL of 8% SDS in TES buffer and incubated at 68℃ for 10min. Then, 1.5mL of 3M Sodium acetate (pH 4.8) was added, and the suspension was incubated at -20℃ for 30min. The suspension was centrifuged at 20,200g for 20min at 4℃. The supernatant was filtered. Two volumes of cold absolute ethanol were added to the supernatant and incubated overnight at -20℃. Plasmid-enriched DNA was centrifuged at 20 200g for 20min at 4℃. Each pellet was dissolved in 100µL Tris-EDTA (pH 8.0) (10mMTris-HCl, 1mM EDTA) and stored at -20℃ until further use. In order to visualize the plasmid pattern from each strain, 10µL of each plasmid enriched DNA solution was loaded, along with the analytical marker DNA wide range (Promega), in 0.5% agarose gels with 1x Tris-borate-EDTA buffer (45mM Tris-borate, 1mM EDTA), Ethidium bromide at 0.5µg/mL and run at 2V/cm for 20h. All gels included the plasmid preparation from *B. thuringiensis* strain as a reference and strains isolated from biolarvicides Bactivec (Reyes- Ramírez & Ibarra 2008).

3 RESULTS AND DISCUSSION

In this work we analyzed seven *B. thuringiensis* stains from three distinct regions of southern of Brazil. With respect to phenotypic features strains showed parameters characteristic for the species (results not shown).

With respect to antibiotics features strains 1547-2 and 2840-3 were resistant to the antibiotic rifampicin in all concentrations differentiating from others (susceptible). The antibiotic erythromycin was effective to inhibit the growth of all isolates and strain pattern. The streptomycin antibiotic showed variation of resistance of strains 3280-1, 3420-5 and 3420-12 (Region of Don Pedrito) to 100 µg/mL and up to 60 µg/mL other isolates.

Because some strains of *B. thuringiensis* are not pathogenic, there isn't standardized characterization of antibiotics as the concentrations that determine susceptibility or resistance (Knight et al., 2005).

Mortality data (LC_{50}) for *O. nubilalis* populations exposed to purified protein Cry1Ab and Cry1F *B. thuringiensis* are presented in Table 2. All strains showed mortality above 75%. The strain 1893-15 caused 95% mortality to *O. nubilalis* neonates, as the reference strain HD-1. Comparisons of EC_{50} values indicate that the 1547-2 strain exhibited slightly lower potency than the other strains tested.

Bioassays showed the pathogenic potential of strains in control of first instar larvae *O. nubilalis.* According to Lee et al. (1996), Schnepf et al. (1998), subclass genes correlation: cry1Ab and cry1F with bioassays indicate that the toxicity of some strains to target insects can occur because of synergistic interactions among the toxins found, or even by the interaction of these with the spores, making it necessary to perform and analyze and bioassays with individual proteins together to confirm the toxin responsible for mortality. According to Lee et al. (1996), Schnepf et al. (1998), genes subclass correlation: cry1Ab and cry1F with bioassays indicate that the toxicity of some strains to target insects can occur because of synergistic interactions among the toxins found, or even by the interaction of these with the spores, making it necessary to perform and analyze and bioassays with individual proteins together to confirm the toxin responsible for mortality.

Table 2. Probit analysis of mortality of European corn borer neonates exposed to the four proteins from B. *thuringiensis.*

Strains	KS EC^{50}	KS LC^{50}	Mead EC^{50}	Mead LC^{50}	SKY EC^{50}	SKY LC^{50}	CLNDO EC^{50}	CLNDO EC^{50}
1547-2	12.84	42.81	10.52	20.49	12.41	25.73	5.74	27.12
2840-3	14.43	38.39	12.30	25.58	15.40	29.30	7.87	30.13
3280-1	14.40	22.43	19.23	44.12	16.36	39.27	8.40	31.67
3420-5	13.24	19.50	14.22	38.59	12.40	52.25	10.56	31.40
3420-12	12.34	41.56	8.98	32.78	14.54	53.67	14.43	35.67
1893-13	11.23	39.12	9.41	31.29	13.87	49.44	12.32	36.45
1893-15	15.78	46.12	15.55	46.89	14.56	49.78	14.50	39.34

All strains showed significance at $P < 0.05$ (data not shown).

The plasmid profile of seven Brazilian *B. thuringiensis* strains included in this repor is showed in the figure 4. Only strain 1893-13 did not show any plasmid. The analysis of plasmid profiles of isolates showed three different patterns, the strain 1893-15 showed the same profile and the strain isolated *B. thuringiensis* serovar *israelensis* IPS 82. 1547-1, 2840-3, 3420-5 and 3280-1 strains showed were similar patterns. 3420-5 strain showed a single band of 5000pb. Furthermore, strain 1893-13 was a distinct pattern of the plasmid. Vilas-Boas and Lemos (2004) consider this type of study important to differentiate isolates and characterize them. These authors consider that the appearance or absence of bands may be due to the presence of different configurations of the same plasmid, or no recovery of some plasmids during the DNA extraction. However, these losses they may also occur during separation by centrifugation and during the withdrawal process bromide and cesium chloride samples.

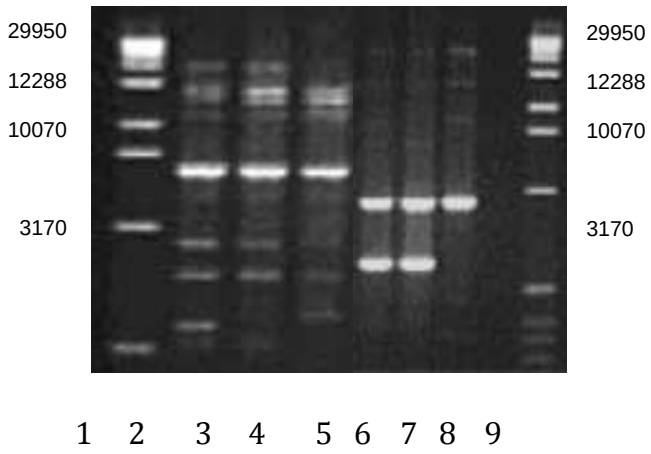

Figure 4. Plasmid patterns from Brazilian *Bacillus thuringiensis* strains*:* (A) Lane 1, Analytical Marker DNA wide Range (Promega) (in pb); Lane 2, 1547-2; Lane 3, 2840-3; Lane 4, 3280-1; Lane 5, 1893-15; Lane 6, *B. thuringiensis* serovar *israelensis* IPS 82 Lane 7, 3420-5; Lane 8, 1893-13; Lane 9, Analytical Marker DNA wide Range (Promega) (in pb).

Based on the results obtained in the present study, the combination of methodologies and bioassays provides an additional alternative for evaluation of new *B. thuringiensis* strains and, therefore, makes an important contribution to our knowledge of intraspecific differences for this important and useful bacterial species.

4 REFERENCES

ALVAREZ, A.; PERA, L.; LOTO, F. 2009. Insecticidal crystal proteins from native Bacillus thuringiensis: numerical analysis and biological activity against *Spodoptera frugiperda*. *Biotech Lett.* 31:77–82.

BERON, C.; SALERNO, G. 2006. Characterization of *Bacillus thuringiensis* isolates from Argentina that are potentially useful in insect pest control. *Biocontrol.* 51:779–794

BRAVO, A.; GILL, S.; SOBERO´N, M. 2007. Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. *Toxicon* 49:423–435

CHRISTOU, P.; CAPELL, T.; KOHLI, A. 2006. Recent developments and future prospects in insect pest control in transgenic crops. *Trends Plant Sci* 11:302–308.

FINNEY, D. J. 1971. Probit analysis. *Cambridge University Press*, Cambridge, England.

GASSMANN, A.; CARRIE`RE, Y.; TABASHNIK, E. 2009. Fitness costs of insect resistance to *Bacillus thuringiensis*. *Annu Rev Entomol.* 54:147–163

GOMES-MONNERAT, R.; CARDOSO-BATISTA, A.; TELLES DE MEDEIROS, P. 2007. Screening of Brazilian *Bacillus thuringiensis* isolates active against *Spodoptera frugiperda*, *Plutella xylostella* and *Anticarsia gemmatalis. Biol Control*. 41:291–295

GUTHRIE, W.D.; RAUN, E.S.; DICK, F.F.; PESHO, G.R.; CARTER, S.W. 1965. Laboratory production of European corn borer egg masses. *Iowa J. Sc*i. 40:9-14.

KAUR, J.; MUNSHI, G.; SINGH, R. 2005. Effect of carbon source on production of lytic enzymes by the sclerotial parasites *Trichoderma atroviride* and *Coniothyrium minitans*. J Phytopathol 153:274–279.

KING, E.G.; HARTLEY, G.G.; MARTIN, D.F.; LASTER, M.L. 1985. Large-scale rearing of a sterile backcross of the tobacco budworm (Lepidoptera: Noctuidae). *J. Econ. Entomol*. 78:1166-1172.

KNIGHT, J.R.; ALLAN, R.J.; FOLLAND, C.K.; VELLINGA, M.; MANN, M.E. 2005. A signature of persistent natural thermohaline circulation cycles in observed climate. Geophysical Research Letter, 32: 1-4.

KONECKA, E.; KAZNOWSKI, A.; ZIEMNICKA, J. 2007. Molecular and phenotypic

characterization of *Bacillus thuringiensis* isolated during epizootics in *Cydia pomonella* L. *J Invertebr Pathol.* 94:56–63.

LEAHY, T.C.; ANDOW, D.A. 1994. Eggweight, fecundity, and longevity are increased by adult feeding in *Ostrinia nubilalis* (Lepidoptera: Pyralidae). *Ann. Entomol. Soc. Am*. 87:342-349.

LEE, M.K.; CURTISS, A.; ALCANTARA, E.A.; DEAN, D.H. 1996. Synergistic effect of the *Bacillus thuringiensis* toxins CryIAa and CryIAc on the gypsy moth, *Lymantria dispar. Applied and Environmental Microbiology.* 62:583-586.

LeOra Software. 1987**.** POLO-PC: A User's Guide to Probit and Logit Analysis, Berkeley, CA.

LEWIS, L.C.; LYNCH, R.E. 1969. Rearing the European corn borer, *Ostrinia nubilalis* (Hübner), on diets containing corn leaf and wheat germ. *Iowa J. Sci.* 44:9-14.

LIGHTFOOT, N.F.; SCOTT, R.J.D.; TURNBUL, P.C.B. 1989. Antimicrobial susceptibility of *Bacillus anthracis. Medical Bulletin*. 68: 95-98.

MASON, C.E.; RICE, M.E.; CALVIN, D.D.; VAN DUYN, J.W.; SHOWERS, W.B. HUTCHISON, W.D.; WITKOWSKI, J.F.; HIGGINS, R.A.; ONSTAD, D.W.; DIVELY, G.P. 1996. European corn borer - Ecology and Management. Iowa State University, Ames, IA.

OSTLIE, K.R.; HUTCHISON, W.D.; HELLMICH, R.L. 1997. *Bt* corn and European corn borer: long-term success through resistance management. *University of Minnesota Extension Service*, St. Paul, MN.

REBOURG, C.; CHASTANET, M.; GOUESNARD, B.; WELCKER, C.; DUBREUIL, P.; CHARCOSSET, A. 2003. Maize introduction into Europe: the history reviewed in the light of molecular data. *Theor. Appl. Genet.* 106:895–903.

REYES-RAMÍREZ, A.; IBARRA, J.E. 2008. Plasmid patterns of *B. thuringiensis* type strains. *Appl. Environ. Microbiol*. 74:125-129.

SAUKA, D.; BENINTENDE, G. 2008. *Bacillus thuringiensis*: generalidades. Un acercamiento a su empleo en el biocontrol de insectos lepidopteros que son plagas agricolas. *Rev Argent Microbiol.* 40:124–140.

SCHNEPF, E.; CRICKMORE, N.; RIE, J. van; LERECLUS, D.; BAUM, J.;

FEITELSON, J.; ZEI- GLER, D. R.; DEAN, D. H. *Bacillus thuringiensis* and its pesticidal crystal proteins*. Microbiology and Molecular Biology Reviews*. 62:775-806.

SIEGFRIED, B. D.; SPENCER, T. A.; CRESPO, A.L.B.; STORER, N.P.; HEAD, G.P.; OWES, E.D.; GUYER, D. 2007. Ten years of *Bt* resistance monitoring in the European corn borer: What we know, what we don't know and what we can do better. *American Entomologist.* 53:208-214.

TABASHNIK, B.; CARRIERE, Y. 2009. Environmental impact of genetically modified crops. In: Ferry N, Gatehouse A (eds) Insect resistance to genetically modified crops. CAB International, pp 74–101.

THOMPSON, W.R.; PARKER, H.L. 1928. The European corn borer and its controlling factors in Europe. *US Dep. Agric. Tech. Bull.* 59: 62.

VILAS-BÔAS, G.T.; LEMOS, M.V. 2004. Diversity of cry genes and genetic characterization of *Bacillus thuringiensis* isolated from Brazil. *Canadian Journal Microbiology.* 50:605-613.

7 CAPÍTULO 6

Artigo de pesquisa

LEVELS OF MEMBRANE ALKALINE PHOSPHATASE AND AMINOPEPTIDASE TO LEPIDOPTERAN STRAINS RESISTANT TO CRY TOXINS FROM *BACILLUS THURINGIENSIS*

LEVELS OF MEMBRANE ALKALINE PHOSPHATASE AND AMINOPEPTIDASE TO LEPIDOPTERAN STRAINS RESISTANT TO CRY TOXINS FROM *BACILLUS THURINGIENSIS*

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ABSTRACT

The evolution of toxins resistance in target pests threatens the durability of crops expressing *Bacillus thuringiensis* proteins. To date, field resistance that resulted in control failures or reduced efficacy of *B. thuringiensis* crops has been documented in several target pest species of corn and cotton. Knowledge of *B. thuringiensis* resistance mechanism is essential in understanding resistance evolution and for developing effective management strategies. The most common mechanism of *B. thuringiensis* resistance in the insect species that have been investigated is cadherin-mediated resistance. Recent studies showed that reduced level of membrane-bound alkaline phosphatase (ALPs) is also associated with two major pests targeted by *Bacillus thuringiensis* crops including *Ostrinia nubilalis* and *Spodoptera frugiperda*. To identify ALP and APN enzymatic activities that may have a potential role in the *B. thuringiensis* toxicity and resistance, in this study we analyzed the expression of four strains - resistant and susceptible to Cry1Ab and Cry1F - enzymes present in *Brush Border Membrane Vesicles* (BBMV) in *O. nubilalis.*

Keywords: ALPs, *Ostrinia nubilalis*, Cry1Ab.

RESUMO

A evolução da resistência das pragas-alvo às toxinas ameaçam a durabilidade das culturas transgênicas que expressam protteinas de *Bacillus thuringiensis*. Os dados de resistência a campo causam falhas no controle ou eficácia reduzida das plantas *Bt* em várias espécies-alvo que são pragas do milho e algodão. Conhecer do mecanismo de resistência de *B. thuringiensis* é essencial para entender a evolução

da mesma e para desenvolver estratégias eficazes de manejo. O mecanismo mais comuns de resistência ao *B. thuringiensis* nas espécies dos insetos investigados tem sido pela resistência à caderina. Estudos mostram que o nível reduzido da enzima alcalina fofatase (ALP) presente na membrana intestinal está também associada com a resistência de *Ostrinia nubilalis* e *Spodoptera frugiperda* à proteina Cry1Ab de *B. thuringiensis.* Para identificar a atividade enzimática de ALP e da Aminopeptidase (APN) que podem ter um importante papel na resistência ao *B. thuringiensis,* neste estudo, analisamos a expressão de quatro linhagens, resistentes e suscetíveis a Cry1Ab e Cry1F, relacionadas as enzimas localizadas na *Brush Border Membrane Vesicles* (BBMV) de larvas de *O. nubilalis*.

Palavras-chave: ALPs, *Ostrinia nubilalis*, Cry1AB.

1 INTRODUCTION

The European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Piralidae), is one of the most destructive pests of corn in the United States. It is a cosmopolitan species originally distributed in Europe and from there introduced into North America, where it has now spread to most of southern Canada and the U.S. east of the Rocky Mountains (Hudon et al., 2011; Mason et al., 1996). Transgenic corn plants that express proteins from *Bacillus thuringiensis* have become an integral component of crop management systems to control this pest, and therefore, *Bacillus thuringiensis* resistance development in *O. nubilalis* would severely limit the economic and environmental benefits of employing transgenic plants or formulated *B. thuringiensis* for pest management (Shelton et al., 2010). Transgenic corn hybrids expressing the Cry1Ab or Cry1F insecticidal proteins from *B. thuringiensis* for control of *O. nubilalis* have been used commercially in the U.S. since 1996 and 2003, respectively. In 2011, after 3 years of the release of stacked *B. thuringiensis* corn for both corn rootworm (*Diabrotica* spp.) and corn borer control, *B. thuringiensis* corn was planted on 57% of U.S. corn acreage, exceeding 65% in some states such as Iowa and Illinois with even higher levels of adoption in some counties (USDA NASS, 2012).

The insecticidal activity of *Bacillus thuringiensis* proteins produced in parasporal crystals during sporulation (Cry toxins) has been widely studied in

lepidopteran insects, and there is relatively good evidence for the way the proteins act once ingested by a susceptible insect. To exert toxicity, activated toxins have to diffuse through the peritrophic membrane and bind to receptors on the apical microvillae of the brush border membrane of midgut epithelial cells (Aronson and Shai 2001). Studies of toxin binding in vitro began after development of a protocol to prepare brush border membrane vesicles (BBMV) for studies in vitro (Wolfersberger et al. 1987). Several studies have used BBMV preparations to verify association between binding and toxicity and to elucidate post-binding events (Hofmann et al. 1988, Van Rie et al. 1989, Ihara et al. 1993, Liang et al. 1995).

The post-binding events that account for pore formation are still under investigation. Binding studies indicate biphasic kinetics of toxin interaction with receptors, which occurs through primary binding of domain III in one receptor, followed by binding of domain II in another (Jenkins et al. 2000). Pore formation also occurs after oligomerization of Cry toxin monomers (Gómez et al. 2002a, Bravo et al. 2007). Oligomerization of monomeric toxins under alkaline conditions may increase flexibility of the pre-pore structure accounting for pore formation (Rausell et al. 2004) although the importance of the oligomerization step for toxicity has been questioned by other authors that suggest univalent binding (Griko et al. 2007).

Previous studies suggest that both cadherin and APN proteins participate in events that lead to toxicity in vivo including oligomerization, toxin membrane insertion, and cell death (Gómez et al. 2006). Immunohistochemistry studies to identify the localization of B. thuringiensis toxins and putative receptors, alkaline phosphatase (ALP), aminopeptidase (APN) and cadherin on three regions of midgut of *Manduca sexta* (Lepidoptera: Crambidae) gave further support for this multiple receptor interaction model (Chen et al. 2005). In all regions of the midgut, they verified that putative receptors for Cry toxins were present on the microvilli of midgut epithelial cells.

Bravo et al. (2007) used oligomerization and the concept of toxin interaction with two receptors to propose a mode of action for *B. thuringiensis* toxins. In this model, crystals are solubilized and protoxins are activated by proteases. The active toxin then binds to cadherin-like protein (Bt-R1) and membrane bound proteases, which eliminate one alpha-helix forming a pre-pore oligomeric structure. This

structure binds to APN which orients the oligomeric complex towards the membrane to form the pore. These authors also affirm that both cadherin and APN are key elements for toxicity but believe that other molecules may also be involved.

B. thuringiensis toxin receptors in epithelial midgut cells of Lepidoptera. In Lepidoptera, several proteins bind Cry1A toxins and may mediate toxicity, including APNs, ALP and cadherins (Knight et al. 1994, Sangadala et al. 1994, Gill et al. 1995, Nagamatsu et al. 1998, Jurat-Fuentes and Adang 2004). APN (~120 to 170 kDa) is the putative receptor for Cry toxin in cotton bollworm, Helicoverpa armigera (Hübner) (Noctuidae), tobacco budworm, Heliothis virescens (Fabricius) (Noctuidae) (Adang et al. 1995, Gill et al. 1995,), *L. dispar* (Lee et al. 1996), *M. sexta* (Ellar 1994, Knight et al. 1994, Sangadala et al. 1994, Luo et al. 1996), and *Plutella xylostella* (Luo et al. 1997a). Although studies with mutant toxins have shown that toxin binding to APN is partially related to toxicity (Jenkins et al. 2000), silencing of midgut APN of in the cluster caterpillar, *Spodoptera litura* (Noctuidae) by double-stranded RNA increased sensitivity to Cry1C (Rajagopal et al. 2002). In addition, increased tolerance to *B. thuringiensis* toxins in later larval stages of some lepidopteran species has been associated with decreased APN activity although reductions in binding across developmental stages were detected only in *M. sexta* (Gilliland et al. 2002).

Proteomic analysis of BBMV proteins has revealed an ALP (62 to 68 kDa) receptor for Cry1Ac toxin in *M. sexta* (McNall and Adang 2003) *and H. virescens* (Jurat-Fuentes and Adang 2004). There is no data establishing association between binding to ALP and cell toxicity of Cry1Ac in *M. sexta* (McNall and Adang 2003), but a resistant strain of *H. virescens* exhibited lower levels of ALP activity than a susceptible strain (Jurat-Fuentes and Adang 2004), indicating a correlation between ALP and susceptibility. Other studies indicate that cadherin-like proteins (~210 kDa) play a major role in *B. thuringiensis* toxin mode of action. This receptor has been reported in studies with *M. sexta* (Vadlamudi et al. 1995, Keeton et al. 1998), *Bombyx mori* (Ihara et al. 1993), *H. virescens* (Banks et al. 2001), and *O. nubilalis* (Hua et al. 2001). Introduction of cadherin genes (BtR175) from *B. mori* and *O. nubilalis* in genome of Sf9cells generated functional susceptibility to Cry toxins (Nagamatsu et al. 1998, Flannagan et al. 2005). In addition, disruption of a cadherin gene by retro transposon-mediated insertion lead to high levels of resistance to Cry1Ac in *H. virescens* (Gahan et al. 2001).

An immunochemical study using cadherin and aminopetidase antibodies to saturate receptors of midgut epithelial cells revealed that toxicity was reduced only when cells were pretreated with cadherin antibody (Hara et al. 2003). In addition, single amino acid mutations in the cadherin receptor from *H. virescens* affect its toxin binding ability to Cry1A toxins (Xie et al. 2005).

Binding analyses of Cry toxins to lepidopteran BBMV have been carried out since 1988 (Hofmann et al. 1988) and have showed the importance of midgut receptors in the mode of action of Cry toxins.

The main goal of the present study was to identify if reduced expression of ALP and APN is a potential biomarker for resistance to Cry toxins to Europe Corn Borer.

2 MATERIALS AND METHODS – University of Nebraska

2.1 Insect and rearing

The resistant *O. nubilalis* strain originated from a field collection of larvae from Kandiyohi Co., MN in 2001 (Siegfried et al. 2007). This strain was divided into two subpopulations, one group was kept in the absence of Cry1Ab and Cry1F toxin and the other group was tested against Cry1Ab and Cry1F in diagnostic assays. The infants who survived diagnostic bioassays (Marçon et al. 2000) were selected in leaf discs of plants, and the survivors were then selected by exposure to Cry1Ab and Cry1F a concentration that was approximately 20-fold excess of the concentration of diagnosis. Surviving the selection were pooled and used to initiate the population SKY (resistant to toxin Cry1Ab); 2BE (resistent to 1Ab and 1F); Mead (susceptible to toxins Cry1Ab and Cry1F) and CLNDO (resistant to toxin Cry1F).

All insects were reared in the laboratory (Figure 1) using artificial diet developed for *Heliothis virescens* (King and Hartley, 1985) and adapted for European corn borer (Bruce Lang, Mycogen Seeds, personal communication). Fifth instar larvae from each strain were dissected, and midguts frozen and kept at 280uC until used to prepare BBMV as described below.

Figure 1: *Ostrinia nubilalis* rearing.

2.2 Preparation of luminal gut proteases

Gut proteinases were obtained from fifth instar larvae (Siqueira et al. 2004). The head and thorax and last three abdominal segments were excised from last instar *O. nubilalis* larvae and the midguts pulled from the carcass. All dissections were conducted at ice temperature. Guts were removed with forceps and immediately submersed in ice-cold buffer A (Tris 200 mM, pH 9.0, calcium chloride 20 mM). Five guts were aliquoted per 100 µl of buffer A, and at least 10 samples of each colony were frozen at -20 ℃ until used in sub sequent activity assays. Samples were quick-thawed by spinning at 15,000g for 3 min to precipitate gut tissue and content, and the supernatant used for activity measurement. Protein concentrations from gut lumen preparations were determined by the Bradford method (Bradford, 1976) with bovine serum albumin as a standard. The protein concentration of each preparation was standardized to 4 mg/mL.

2.3 Brush border membrane vesicles (bbmv) purification

BBMV were isolated by the differential centrifugation method of Wolfersberger et al. 1987). BBMV proteins were quantified by the method of Bradford 1976, using BSA as standard, and kept at 280uC until used. Specific activity of N-aminopeptidase (APN) using leucine-p-nitroanilide as substrate was used as a marker for brush border enzyme enrichment in the BBMV preparations. APN activities in the final BBMV preparations from all insect species were enriched 5–8 fold when compared to initial midgut homogenates.

2.4 Quantification of Alkaline phosphatase and Aminopeptidase activities

Specific ALP and APN enzymatic activities of BBMV proteins (1 mg) from *O.*

nubilalis were measured as described by Jurat-Fuentes and Adang, 2004. Enzymatic activities were monitored for 2–5 min. as changes in OD at 405 nm wavelength at room temperature in a microplate reader (BioTek). Data shown are the mean specific activities from at least three independent BBMV batches from each strain measured in at least three independent experiments.

2.5 Western Blotting

BBMV proteins to be analyzed by one-dimension electrophoresis were solubilized in sample buffer (Laemmli, 1970). Solubilized BBMV proteins (5µg BBMV) were then heat-denatured for 5 min. and loaded on SDS-7,5% PAGE gels. Following electrophoretic separation BBMV proteins were transferred Protein transferring was carried out at 248 mA for 2 hour on ice. Filters were blocked for one hour in PBST (135 mM NaCl, 2 mM KCl, 10 mM Na2HPO4, 1.7 mM KH2PO4, pH 7.5, 0.1% Tween-20) plus 3% BSA. After blocking, all filter incubations and washes were done in PBST plus 0.1% BSA. Blocked filters were probed with antisera against *B. mori* mALP and cadherin on BBMV. Rabbit anti-ALP antibody serum was kindly provided by Dr. Mike Adang from University of Georgia (United States).

Four colonies of *O. nubilalis* were tested: SKY – resistant to toxin Cry1Ab; 2BE - resistant to toxins Cry1Ab e Cry1F; CLNDO – resistant to toxin Cry1F and Mead: Susceptible to toxins Cry1Ab e Cry1F. Mortality data were analyzed by probit analysis (Finney 1971) using POLO-PC (LeOra Software1987).

3 RESULTS

To identify reduced levels ALP and APN enzymatic activities in *O. nubilalis* that may have a potential role in *B. thuringiensis* toxicity and resistance, we analyzed the expression of four strains with resistant and susceptible strains of *O. nubilalis* (Table 1) and protein concentrations from gut lumen preparations (Figure 2 and 3).

The results showed that the SKY populations - resistant toxins Cry1Ab and Cry1F have the lowest indices of ALP and APN, as has been observed for other pest species (Yang et al., 2010).

Population resistant to Cry1F (CLNDO) showed the highest levels of both ANP and ALP. 2BE population (susceptible to Cry1Ab and Cry1F) Mead (susceptive for both) also showed a low level of enzymatic activity.

Table 1. Average specific ALP and APN enzymatic activities of BBMV proteins

APN activity			**ALP activity**		
Strain	BBMV1,2,3	Mean	**Strain**	BBMV3	Mean
MEAD	17		**MEAD**	11	
	20			7	
	16			6	
	17.66666667	17.66666667		8	8
CLNDO	54		**CLNDO**	28	
	48			20	
	50			22	
	50.66666667	50.66666667		23.33333333	23.33333333
2BE	29		**2BE**	13	
	32			11	
	36			14	
	34	34		12.5	12.5
SKY	13		**SKY**	12	
	17			10	
	16			11	

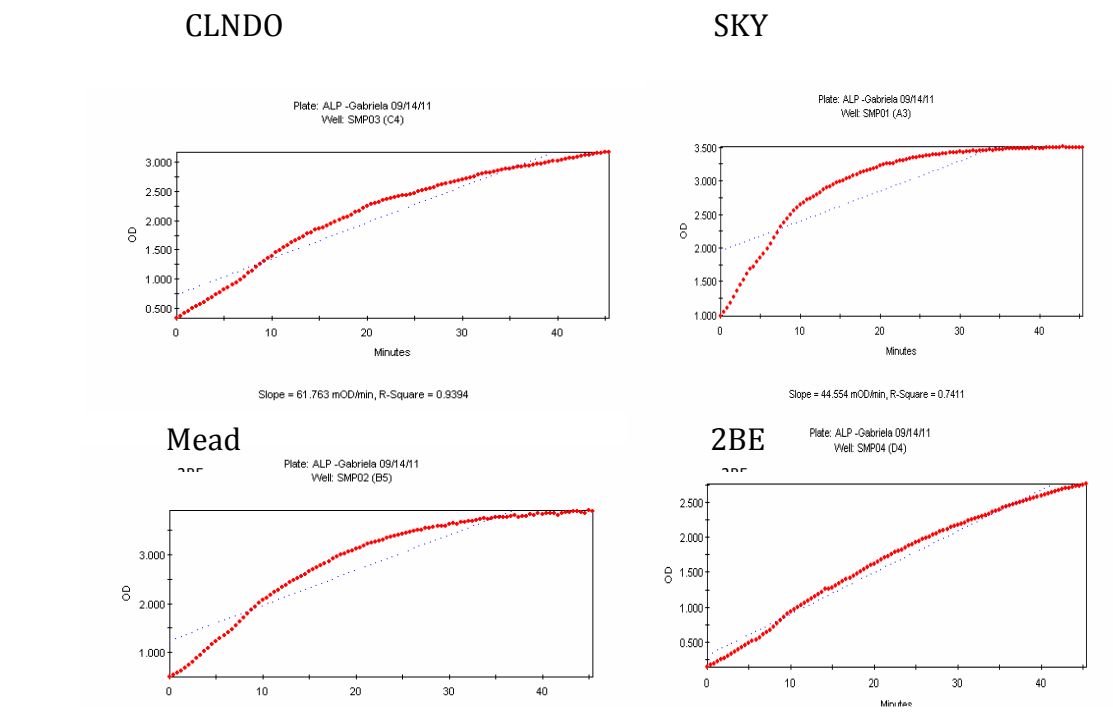

Figure 2. Curve activity alkaline phosphatase protein concentrations from gut lumen preparations in *Ostrinia nubilalis* populations.

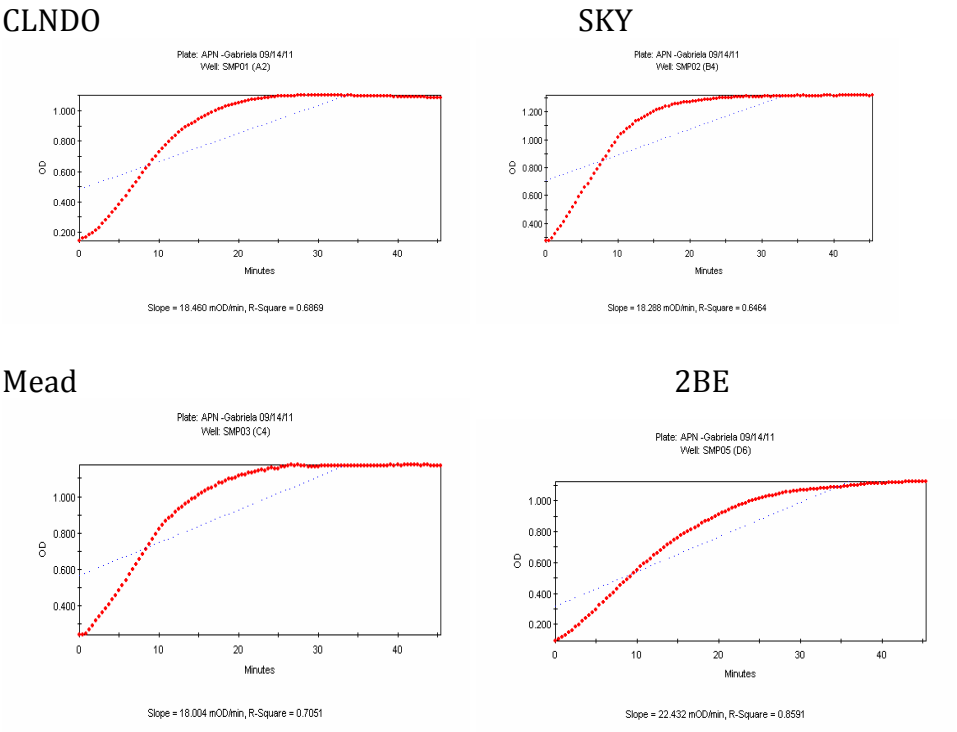

Figure 3. Curve activity aminopeptidase protein concentrations from gut lumen preparations in *Ostrinia nubilalis* populations.

The Western blot was performed to confirm the specific proteins of the given sample BBMV (Figure 4).

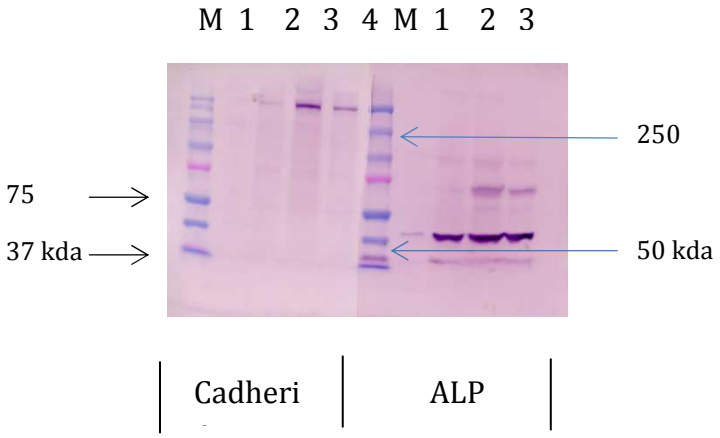

Figure 4. Lane M, 1, 2, 3 and 4 represent Marker, BBMV from ECB midgut of 2BE (S to 1Ab and 1F), CLNDO (R to 1F), Mead (S to 1Ab and 1F) and Sky (R to 1Ab) respectively.

4 DISCUSSION

Studies of BBMVs isolated from Lepidoptera larvae show strong affinity links between proteins and receptors are considered factors in the spectrum insecticide (Hoffman et al., 1988).

We report on the correlation between reduced ALP protein, activity, and APN expression levels in strains of four colonies with diverse resistance and susceptible phenotypes against Cry toxins. Currently *O. nubilalis* is efficiently controlled by transgenic *B. thuringiensis* cotton (Blanco et al., 2009), but numerous reports highlight the potential for the development of field resistance to *B. thuringiensis* crops (Lu et al., 2004; Meng et al., 2004; Caccia et al., 2010).

Previous reports have suggested that direct interaction between *B. thuringiensis* Cry toxins and lepidopteran midgut ALP results in decreased ALP activity (Sangadala et al., 2004; McNall et al., 2003).

Even though no correlation between ALP activity levels and resistance to insecticides has been reported in the literature to date, reduced ALP activity levels in insects have been reported to occur after intoxication with lectins (Kaur et al., 2009), infection with cytoplasmic polyhedrosis virus (CPV) or *B. thuringiensis* in *B. mori* (Miao 2002).

Sangadala et al., 1994 reported that the interaction between the toxin Cry1Ac and ALP resulted in a decreased ALP enzymatic activity in *M. sexta.* Several studies have demonstrated that membrane-bound ALPs in several lepidopteran species can act as Cry toxins binding proteins (Juan-Fuentes and Adang, 2004; Ning et al, 2010; Arenas et al, 2010; McNaill and Adang, 2003). In addition, recent studies have shown that reduced ALP gene expression is associated with *B. thuringiensis* resistance in three major pests targeted by *B. thuringiensis* crops.

Numerous studies have suggested that APNs and ALPs are receptors of *B. thuringiensis* Cry toxins and are involved in *B. thuringiensis* resistance in many insect species (Knight et al, 1994; Denolf et al, 1997, Oltean et al, 1999; Bravo et al, 2004). As observed for the cadherin gene, previous reports study (Yang et al., 2010) also showed that cDNAs of three APN genes were identical between the Cry1Ab-SS strains of *Diatraea saccharalis*, but the expression levels of all the three APN genes were significantly reduced in the resistant strain compared to those of the susceptible strain. Taken together, these results suggest that the reduction in expression of both

the cadherin and APNs is associated with the Cry1Ab resistance in *D. saccharalis*.

Considering that the *O. nubilalis* resistant strains in this study present reduced levels toxins our data suggest that reduced ALP and APN expression is a potential biomarker for resistance to diverse Cry toxins and is independent of the resistance mechanism. Furthermore, our data with Cry resistant Lepidopterans suggests that reduced ALP and APN is a common phenomenon in *B. thuringiensis* resistant lepidopteran larvae.

It is known that receptors such as cadherin, ALP, and APN are recognized by different Cry toxins such as Cry4Ba and Cry11Aa (Pigott and Ellar, 2007; Ferre and Van Rie, 2002; Rajagospal et al., 2002; Bolin et al., 1999).

Based on our data, reduced ALP expression represents a biomarker that would detect resistant insects independently of the resistance mechanism or cross-resistance phenotype. Therefore, a biomarker not based on individual DNA sequence but rather diagnostic of a phenotype associated with resistance to Cry toxins, such as the one identified in this study ALPs and APN, and these were first identified in different lepidopteran insects as Cry1A toxin binding proteins (Shelton et al., 2010; Chaufaux et al, 2001; Khajuria et al., 2009).

5 REFERENCES

ADANG, M.J.; PASKEWITZ, S.M.; GARCZYNSKI, S.F.; SANGADALA, S. 1995. Identification and functional characterization of the *Bacillus thuringiensis* Cry1Ac Delta-endotoxin receptor in *Manduca sexta. Mol Action of Insecticides on Ion Channels.* 591:320-329.

ARENAS. I.; BRAVO, A.; SOBERON, M.; GOMEZ, I. 2010. Role of alkaline phosphatase from *Manduca sexta* in the mechanism of action of *Bacillus thuringiensis* Cry1Ab toxin. *J Biol Chem.* 285:12497–12503.

ARONSON, A.I.; SHAI, Y. 2001. Why *Bacillus thuringiensis* insecticidal toxins are so effective: unique features of their mode of action. *FEMS Microbiol. Lett*. 195:1-8.

BANKS, D.J.; JURAT-FUENTES, J.L.; DEAN, D.H.; ADANG, M.J. 2001. *Bacillus thuringiensis* Cry1Ac and Cry1Fa delta-endotoxin binding to a novel 110 kDa aminopeptidase in *Heliothis virescens* is not N-acetylgalactosamine mediated. *Insect Biochem*. 31:909-918.

BLANCO, C.A.; ANDOW, D.A.; ABEL, C.A.; SUMERFORD, D.V.; HERNANDEZ, G. 2009. *Bacillus thuringiensis* Cry1Ac resistance frequency in tobacco budworm (Lepidoptera: Noctuidae). *J Econ Entomol* 102: 381–387.

BOLIN, P.C.; HUTCHISON, W.D.; ANDOW, D.A. 1999. Longterm selection for resistance to *Bacillus thuringiensis* Cry1Ac endotoxin in a Minnesota population of European corn borer (Lepidoptera: Crambidae). *J Econ Entomol.* 92: 1021–1030.

BRADFORD, M.M. 1976**.** A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. 72: 248-254.

BRAVO, A.; GÓMEZ I.; CONDE, J.; MUNÕZ-GARAY, C.; SÁNCHEZ, J.; MIRANDA, R.; ZHUANG, M.; GILL, S.S.; SOBERON, M.. 2004. Oligomerization triggers binding of a *Bacillus thuringiensis* Cry1Ab pore-forming toxin to aminopeptidase N receptor leading to insertion into membrane microdomains. *Biochim Biophys Acta*. 1667:38–46.

BRAVO, A.; GILL, S.S.; SOBERON, M. 2007. Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. *Toxicon* 49:423–435.

CACCIA, S.; HERNANDEZ-RODRIGUEZ, C.S.; MAHON, R.J.; DOWNES, S.; JAMES, W. 2010. Binding site alteration is responsible for field-isolated resistance to *Bacillus thuringiensis* Cry2A insecticidal proteins in two Helicoverpa species. P*LoS ONE*. 5:9975.

CHAUFAUX, J.; SEGUIN, M.; SWANSON, J.J.; BOURGUET, D.; SIEGFRIED, B.D. 2001. Chronic exposure of the European corn borer (Lepidoptera: Crambidae) to CrylAb *Bacillus thuringiensis* toxin. *J Econ Entomol.* 94: 1564–1570.

CHEN, J. A.; BROWN, M.R.; HUA, G.; ADANG, M.J. 2005. Comparison of the localization of *Bacillus thuringiensis* Cry1A delta-endotoxins and their binding proteins in larval midgut of tobacco hornworm, *Manduca sexta. Cell Tissue Re*s. 321:123-129.

DENOLF, P.; HENDRICKX, K.; VAN DAMME, J.; JANSENS, S.; PEFEROEN, M. 1997. Cloning and characterization of *Manduca sexta* and *Plutella xylostella* midgut aminopeptidase N enzymes related to *Bacillus thuringiensis* toxin-binding proteins. *Eur J Biochem.* 248:748–761.

ELLAR, D.J. 1994**.** Structure and mechanism of action of *Bacillus thuringiensis* endotoxins and their receptors. *Biocontrol Sci. Techn*. 4:445-447.

FERRE, J.; VAN RIE, J. 2002. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. *Annu Rev Entomol.* 47:501–533.

FLANNAGAN, R.D.; YU, C.G.; MATHIS, J.P.; MEYER, T.E.; SHI, X.M.; SIQUEIRA, H.A.A.; SIEGFRIED, B.D. 2005. Identification, cloning and expression of a Cry1Ab cadherin receptor from European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae). Insect Biochem. *Mol. Biol*. 35:33-40.

GAHAN, L.J.; GOULD, F.; HECKEL, D.G. 2001. Identification of a gene associated with *Bt* resistance in *Heliothis virescens*. *Science*. 293:857-860.

GILL, S.S.; COWLES, E. A.; FRANCIS, V. 1995**.** Identification, isolation, and cloning of a *Bacillus thuringiensis* Cry1Ac toxin binding protein from the midgut of the lepidopteran insect *Heliothis virescens*. *J. Biol. Chem.* 270:27277-27282.

GILLILAND, A., C. E. CHAMBERS, E. J. BONE, AND D. J. ELLAR. 2002. Role of *Bacillus thuringiensis* Cry1 {delta} Endotoxin Binding in Determining Potency during Lepidopteran Larval Development. *Appl. Environ. Microbiol*. 68:1509-1515.

GRIKO, N.B., ROSE-YOUNG, L.; XUE, Z.; CARPENTER, L.; CANDAS, M.; IBRAHIM, M.A.; JUNKER, M.; BULLA, L.A. 2007. Univalent binding of the Cry1Ab toxin of *Bacillus thuringiensis* to a conserved structural motif in the cadherin receptor BT- R1. *Biochemistry*. 46:10001-10007.

GÓMEZ, I.; MIRANDA-RIOS, J.; RUDINO-PINERA, E.; OLTEAN, D.I.; GILL, S. S.; BRAVO, A.; SOBERON, M. 2002. Hydropathic complementarity determines interaction of epitope (HITDTNNK876)-H-869 in *Manduca sexta* Bt-R-1 receptor with loop 2 of domain II of *Bacillus thuringiensis* Cry1A toxins. J. Biol. Chem. 277: 30137-30143.

GÓMEZ, I., ARENAS, I.; BENITEZ, I.; MIRANDA-RÍOS, J.; BECERRIL, B.; GRANDE, R.; ALMAGRO, J.C.; BRAVO, A.; SOBERÓN, M. 2006. Specific epitopes of domains II and III of *Bacillus thuringiensis* Cry1Ab toxin involved in the sequential interaction with cadherin and aminopeptidase-N Receptors in *Manduca sexta. J. Biol. Chem*. 281:34032-34039.

HARA, H.; ATSUMI, S.; YAOI, K.; NAKANISHI, K.; HIGURASHI, S.; MIURA, N.; TABUNOKI, H.; SATO, R. 2003. A cadherin-like protein functions as a receptor for *Bacillus thuringiensis* Cry1Aa and Cry1Ac toxins on midgut epithelial cells of *Bombyx mori* larvae. *FEBS Lett*. 538:29-34.

HOFMANN, C.; LUTHY, P. R.; HUTTER, PLISKA, V. 1988. Binding of the delta-endotoxin from *Bacillus thuringiensis* to brush-border membrane vesicles of the cabbage butterfly (Pieris *brassicae). Eur. J. Biochem.* 173:85–91.

HUA, G.; MASSON, L.; JURAT-FUENTES, J.L.; SCHWAB, G.; ADANG, M.J. 2001. Binding analyses of *Bacillus thuringiensis* Cry delta-endotoxins using brush border membrane vesicles of *Ostrinia nubilalis. Appl. Environ. Microbiol*. 67:872-879.

HUDON, M.; LEROUX, E.J.; HARCOURT, D.G. 2011. Seventy years of European corn borer (*Ostrinia nubilalis*) research in North America. *Agric. Zool. Rev*. 3:53–96.

IHARA, H.; KURODA, E.; WADANO, A.; HIMENO, M. 1993. Specific toxicity of delta-endotoxins from *Bacillus thuringiensis* to *Bombyx mori. Bioscience Biotechnology and Biochemistry*. 57: 200-204.

JENKINS, J.L., LEE, M.K. VALAITIS, A.P.; CURTISS, A.; DEAN, D.H. 2000. Bivalent sequential binding model of a *Bacillus thuringiensis* toxin to gypsy moth

aminopeptidase N receptor. J*. Biol. Chem*. 275:14423-14431.

JURAT-FUENTES, J.L.; ADANG, M.J. 2004. Characterization of a Cry1Ac-receptor alkaline phosphatase in susceptible and resistant *Heliothis virescens* larvae. *Eur. J. Biochem.* 271: 3127–3135.

KAUR, M.; SINGH, K.; RUP, P.J.; KAMBOJ, S.S.; SINGH, J. 2009. Anti-insect potential of lectins from Arisaema species towards Bactrocera cucurbitae. *J Environ Biol* 30: 1019–1023.

KEETON, T.P.; FRANCIS, B.R.; MAATY, W.S.A.; BULLA, L.A. 1998. Effects of midgut- protein-preparative and ligand binding procedures on the toxin binding characteristics of BT-R-1, a common high-affinity receptor in *Manduca sexta* for Cry1A *Bacillus thuringiensis* toxins. *Appl. Environ. Microbiol*. 64:2158-2165.

KHAJURIA, C.; ZHU, Y.C.; CHEN, M-S.; BUSCHMAN, L.L.; HIGGINS, R.A. 2009. Expressed sequence tags from larval gut of the European corn borer (*Ostrinia nubilalis*): Exploring the genes potentially involved in *Bacillus thuringiensis* toxicity and resistance. *BMC Genomics.* 10:286.

KING, E.G.; HARTLEY, G.G. 1985. *Heliothis virescens*. Pages 323-328 *In* Singh P, Moore RF (eds.). Handbook of Insect Rearing Vol. II. Elsevier, New York.

KNIGHT, P.J.K.; CRICKMORE, N.; ELLAR, D.J. 1994. The receptor for *Bacillus thuringiensis* Cryla(C) delta endotoxin in the Brush-border membrane of the Lepidopteran *Manduca sexta* is aminopeptidase-N. *Mol. Microbiol.* 11:429-436.

LAEMMLI, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 227: 680–685.

LEE, M.K., YOU, T.H.; YOUNG, B.A.; COTRILL, J.A.; VALAITIS, A.P.; DEAN, D.H. 1996. Aminopeptidase N purified from Gypsy moth brush border membrane vesicles is a specific receptor for *Bacillus thuringiensis* CryIAc toxin. Appl. Environ. Microbiol. 62: 2845-2849.

LeOra Software. 1987. POLO-PC: A User's Guide to Probit and Logit Analysis, Berkeley, CA.

LIANG, Y.Z.; PATEL, S.S.; DEAN, D.H. 1995. Irreversible binding-kinetics of *Bacillus thuringiensis* CryIa delta-endotoxins to Gypsy moth brush border membrane vesicles is directly correlated to toxicity. *J. Biol. Chem*. 270:24719-24724.

LU, M.G.; RUI, C.H.; ZHAO, J.Z, JIAN, G.L.; FAN, X.L. 2004. Selection an heritability of resistance to *Bacillus thuringiensis* subsp *kurstaki* and transgenic cotton in *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Pest Manag Sci.* 60:887–893.

LUO, K., LU, Y.J.; ADANG, M.J. 1996. A 106 kDa form of aminopeptidase is a receptor for *Bacillus thuringiensis* CryIC delta-endotoxin in the brush border membrane of *Manduca sexta. Insect Biochem. Mol. Biol*. 26: 783-791.

LUO, K., TABASHNIK, B.E.; AND M. J. ADANG. 1997A. Binding of *Bacillus thuringiensis* Cry1Ac toxin to aminopeptidase in susceptible and resistant diamondback moths (Plutella *xylostella).* Appl. Environ. Microbiol. 63: 1024-1027.

MARÇON, P.C.R.G.; YOUNG, L.G.; STEFFEY, K.L.; SIEGFRIED, B.D. 2000. Baseline susceptibility of European corn borer (Lepidoptera: Crambidae) to *Bacillus thuringiensis* toxins. *Journal of Economic Entomology.* 92: 279–285.

McNALL, R.J.; ADANG, M.J.; 2003. Identification of novel Bacillus thuringiensis Cry1Ac binding proteins in Manduca sexta midgut through proteomic analysis. Insect *Biochem Molec Biol* 33: 999–1010.

MIAO, Y.G. 2002. Studies on the activity of the alkaline phosphatase in the midgut of infected silkworm*, Bombyx mori* L. *J Appl Ent* 126: 138–142.

MASON, C.E.; RICE, M.E.; CALVIN, D.D.; VAN DUYN, J.W.; SHOWERS, W.B.; HUTCHISON, W.D.; WITKOWSKI, J.F.; HIGGINS, R.A.; ONSTAD, D.W.; DIVELY, G.P. 1996. European corn borer – ecology and management. *Iowa State University*, Ames, IA.

MENG, F.; SHEN, J.; ZHOU, W.; CEN, H. 2004. Long-term selection for resistance to transgenic cotton expressing Bacillus thuringiensis toxin in *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae). *Pest Manag Sci* 60: 167–172.

NAGAMATSU, Y.; TODA, S.; YAMAGUCHI, F.; OGO, M.; KOGURE, M. NAKAMURA, M., SHIBATA, Y.; KATSUMOTO, T. 1998. Identification of *Bombyx mori* midgut receptor for *Bacillus thuringiensis* insecticidal Cry1a toxin. *Bioscience Biotechnology and Biochemistry.* 62:718-726.

NING, C.; WU, K.; LIU, C.; GAO, Y.; JURAT-FUENTES, J.L. 2010. Characterization of a Cry1Ac toxin-binding alkaline phosphatase in the midgut from *Helicoverpa armigera* (Hubner) larvae. *J Insect Physiol.* 56: 666–672.

PIGOTT, C. R.; ELLAR, D.J. 2007. Role of receptors in *Bacillus thuringiensis* crystal toxin activity. Microbiol*. Mol. Biol. Rev*. 71:255–281.

RAJAGOPAL, R.; SIVAKUMAR, S.; AGRAWAL, N.; MALHOTRA, P.; BHATNAGAR, R.K. 2002. Silencing of midgut aminopeptidase-N of *Spodoptera litura* by double-stranded RNA establishes its role as *Bacillus thuringiensis* toxin receptor. *J Biol Chem* 277:46849–46851.

RAUSELL, C.; PARDO-LOPEZ, L.; SANCHEZ, J.; MUNOZ-GARAY, C. MORERA, C.; SOBERON, M.; BRAVO, A. 2004. Unfolding events in the water-soluble monomeric Cry1Ab toxin during transition to oligomeric pre-pore and membrane-inserted pore channel. *J. Biol. Chem*. 279: 55168-55175.

OLTEAN, D.I.; PULLIKUTH, A.K.; LEE, H.K.; GILL, S.S. 1999. Partial purification and characterization of *Bacillus thuringiensis* Cry1A toxin receptor A from *Heliothis virescens* and cloning of the corresponding cDNA. *Appl Environ Microbiol.* 65: 4760–4766.

SANGADALA, S.; WALTERS, F.S.; ENGLISH, L.H.; ADANG, M.J. 1994. A mixture of *Manduca sexta* aminopeptidase and phosphatase enhances Bacillus thuringiensis insecticidal CryIA(c) toxin binding and $^{86}Rb(+)$-K+ efflux in vitro. *J Biol Chem* 269: 10088–10092.

SHELTON, A.M.; ZHAO, J.Z.; ROUSH, R.T. 2010. Economic, ecological, food safety, and social consequences of the deployment of *Bt* transgenic plants. *Annu. Rev. Entomol*. 47: 845–881.

SIEGFRIED, B.D.; SPENCER, T.A.; CRESPO, A.L.B.; STORER, N.P.; HEAD, G. P. E.; OWES, D.; GUYER, D. 2007. Ten years of *Bt* resistance monitoring in the European corn borer: What we know, what we don't know and what we can do better. *American Entomologist*. 53: 208-214.

SIQUEIRA, H.A.A.; NICKERSON, K.W.; MOELLENBECK, D.; SIEGFRIED, B.D. 2004. Activity of gut proteinases from Cry1Ab-selected colonies of the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Crambidae). *Pest. Manag. Sci*. 60: 1189- 1196.

VADLAMUDI, R.K., WEBER, E.; JI, I.; JI, T.H.; BULLA, L.A. 1995. Cloning and expression of a receptor for an insecticidal toxin of *Bacillus thuringiensis. J. Biol. Chem.* 270: 5490 - 5494.

VAN RIE, J., JANSENS, S.; HOFTE, H.; DEGHEELE, D.; MELLAERT, H. 1989. Specificity of *Bacillus thuringiensis* endotoxins. Importance of specific receptors on the brush border membrane of the mid-gut of target insects. *Eur. J. Biochem.* 186: 239-247.

WOLFERSBERGER, M.G.; LUTHY, P.; MAURER, A.; PARENTI, P.; SACCHI, V.F.; HANOZET, G.M. 1987. Preparation and partial characterization of amino acid transporting brush border membrane vesicles from the larval midgut of the *Cabbage butterfly* (Pieris brassicae*). Comp Biochem Physiol* 86A: 301–308.

XIE, R.Y.; ZHUANG, M.B.; ROSS, L.S.; GOMEZ, I.; OLTEAN, D.I.; BRAVO, A.; SOBERON, M. GILL. 2005. Single amino acid mutations in the cadherin receptor from *Heliothis virescens* affect its toxin binding ability to Cry1A toxins. J*. Biol. Chem*. 280:8416-8425.

YANG, Y.; ZHU, Y.C.; OTTEA, J.; HUSSENEDER, C.; LEONARD, B.R. 2010. Molecular characterization and RNA interference of three midgut aminopeptidase N isozymes from *Bacillus thuringiensis* susceptible and resistant strains of sugarcane borer, *Diatraea saccharalis*. *Insect Biochem Mol Biol.* 40: 592–603.

8 CONSIDERAÇÕES GERAIS

- Nos estudos de caracterização fenotípica e aplicação da técnica de rep-PCR, as 26 cepas testadas apresentaram uma resposta muito semelhante às regiões pertencentes, conferindo um alto grau de clonabilidade entre as regiões orizícolas, provavelmente associadas à especiação ecológica.

- Nos ensaios de screening com *S. frugiperda* e as 20 cepas de *B. thuringiensis*, o isolado 3420-11 apresentou mortalidade corrigida superior a 85%. Na avaliação molecular através da técnica de PCR, o gene *cry1C* foi o mais freqüente, seguido pelo gene *cry*1F.

- Para *O. nubilalis*, a cepa de *B. thuringiensis* 1893-15 foi altamente tóxica contra as larvas de primeiro instar, sendo a mais promissora para estudos mais aprofindados no controle dessa espécie de inseto-praga.

- Os níveis de atividade enzimática de ALP e APN em *O. nubilalis* foram analisados em quatro populações de insetos, sendo que esse índice apresentou-se baixo nas populações suscetíveis a Cry 1Ab e Cry 1F.

9 REFERÊNCIAS

ALVES, S. B. 1998. Patologia e controle microbiano: vantagens e desvantagens. In: ALVES, S. B. (Ed.). Controle microbiano de insetos. Piracicaba: FEALQ, 1, p:21-38.

ALVES, S. B.; LOPES, R. B. Controle Microbiano de Pragas na América Latina: avanços e desafios. Piracicaba: FEALQ, 2008, 414p.

ARANDA, E.; PEFEROEN, M.; GUURECA, L.; BRAVO, A. 1996. Interactions of *Bacillus thuringiensis* crystal proteins whith the midgut epithelial cell of *Spodoptera frugiperda* (Lepidoptera: Noctuidae). Journal of Invertebrate Pathology 68: 203-212.

BOBROWSKI, V. L.; FIUZA, L. M.; PASQUALI, G.; BODANESE-ZANETTINI, M. H. 2003. Genes de Bacillus thuringiensis: uma estratégia para conferir resistência a insetos em plantas. Ciência Rural, 33, n. 5, set-out, 2003.

BRAVO, A.; GILL, S.S.; SOBERON, M. 2007. Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. *Toxicon*. 49: 423-435.

BRAVO, A.; SOBERON, M. 2008. How to cope with resistance to *Bt* toxins? *Trends Biotechnol*. 26: 573-579.

CASTILHOS-FORTES R.; MATSUMURA A.T.S.; DIEHL E.; FIUZA L.M. 2002. Susceptibility of *Nasutitermes ehrhardti* (Isoptera: Termitidae) to *Bacillus thuringiensis* subspecies. Brazilian Journal of Microbiology. 33(3): 219-222.

CAVADOS, C. F. G.; FONSECA, R. N.; CHAVES, J. Q.; RABINOVITCH, L.; ARAÚJO-COUTINHO, C. J. P. C. 2001. Identification of Entomopathogenic *Bacillus* Isolated from *Simulium* (Diptera, Simuliidae) Larvae and Adults. Memórias do Instituto Oswaldo Cruz 96(7): 1017-1021.

CRICKMORE, N. 2011. Disponível em: http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/

DE MAAGD, R. A. ; BRAVO, A. ; BERRY, C. ; CRICKMORE, N. ; SCHNEPF, H. E. 2003. Structure, diversity and evolution of protein toxins from spore-forming entomopathogenic bacteria. Annual Review of Genetics 37: 409-433.

EMBRAPA. 2011. Cultivo do arroz irrigado no Brasil. Sistemas de Produção. Disponivel http://sistemasdeproducao.cnptia.embrpa.br, acessado em 01 de jul de 2012.

FAO 2011. World Production. Disponível em: <www.faostat.org.br>. Acesso em: 12 set. 2011.

FEITELSON, J. S.; PAYNE, J.; KIM, L. 1992. *Bacillus thuringiensis* insects and beyond. Biotecnology 10: 271-275.

FIUZA, L.M. 2009. Mecanismo de ação de *Bacillus thuringiensis*. Revista Biotecnologia Ciência e Desenvolvimento, 38: 32-35.

IBGE, 2011. Censo Demográfico de 2011. Fundação Instituto Brasileiro de Geografia e Estatística, fornecidos em meio eletrônico.

JOUZANI, G. S.; SEIFINEJAD, A.; SAEEDIZADEH, A.; NAZARIAN, A.; YOUSEFLOO, M.; SOHEILIVAND, S.; MOUSIVAND, M.; JAHANGIRI, R.; YAZDANI, M.; AMIRI, R. M.; AKBARI, S. 2008. Molecular detection of nematicidal crystalliferous *Bacillus thuringiensis* strains of Iran and evaluation of their toxicity on free-living and plant-parasitic nematodes. Canadian Journal of Microbiology, 54:812-822.

LIANG, G.M.; WU, K.M.; YU, H.K.; LI, K.K.; FENG, X. 2008. Changes of inheritance mode and fitness in *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) along with its resistance evolution to Cry1Ac toxin. *J Invertbr Pathol.* 97:142–149.

LOGAN, N. A.; BERGE, O.; BISHOP, A.H.; BUSSE, H.J.; DE VOS, P.; FRITZE, D.; HEYNGRICKX, M.; KÄMPFER, P.; RABINOVITCH, L.; SALKINOJA-SALONEM, M.S.; SELDIN, L.; VENTOSA, A. 2009. Proposed minimal standards for describing new taxa of aerobic, endorpore-forming bactéria. International Journal of Systematic and Evolutionary Microbiology. 59: 2114-2121.

MARROQUIN, L.D.; ELYASNIA, D.; GRIFFITTS, J.S.; FEITELSON, J.S.; AROIAN, R.V. 2000. *Bacillus thuringiensis*: toxin susceptibility and isolation of resistance mutants in the nematode *Caenorhabditis elegans*. Genetics 155: 1693-1699.

OLIVEIRA, J.V.; FREITAS, T. F. S.; FIUZA, L. M.; MENEZES, V. G.; DOTTO, G. 2010. Manual de Insetos Associados a cultura do Arroz Irrigado. IRGA, Boletim Técnico nº 8. 54p.

PARDO-LOPEZ, L.; GOMEZ, I.; RAUSELL, C.; SANCHEZ, J.; SOBERON, M. 2006. Structural changes of the Cry1Ac oligomeric pre-pore from *Bacillus thuringiensis* induced by N-acetylgalactosamine facilitates toxin membrane insertion. Biochemistry 45: 10329–10336.

PIGOTT, C.R.; ELLAR, D.J. 2007. Role of receptors in *Bacillus thuringiensis* crystal toxin activity*. Microbiol. Mol. Biol. Rev*. 71:255–281.

PINTO, L. M. N.; AZAMBUJA, A. O.; DIEHL, E.; FIUZA, L.M. 2003. Pathogenicity of *Bacillus thuringiensis* isolated from two species of *Acromyrmex* (Hymenoptera, Formicidae). Brazilian Journal of Biology. 63(2): 301-306.

POLANCZYK, R.A.; ALVES, S.B. 2005. Interação entre *Bacillus thuringiensis* e outros entomopatógenos no controle de *Spodoptera frugiperda*. Manejo Integrado de Plagas y Agroecología 74: 24-33.

SILVA, S.F.; DIAS, J.M.C.; MONNERAT, R.G. 2002. Isolamento, identificação e caracterização entomopatogênica de bacilos de diferentes regiões do Brasil. Brasília: Embrapa. 4 p. (EMBRAPA- Cenargen, Comunicado Técnico, 70).

SOSBAI. Sociedade sul-brasileira de arroz irrigado. 2007. Arroz irrigado: recomendações técnicas da pesquisa para o Sul do Brasil. Pelotas.154p.

VIEIRA, N.R.A.; SANTOS, A.B.; SANT'ANA, E.P. 1999. A cultura do arroz no Brasil. Embrapa Arroz e Feijão, Santo Antônio de Goiás. 633p.

ANEXO A – CARTA COORIENTADOR

>>> Blair Siegfried bsiegfried1@unl.edu> 10/08/12 6:49 pm >>

Dear Dr. Fiuza, I apologize for the slow reply to your email. I have been traveling for the last week and am just now getting a chance to review your email regarding Gabriela's thesis. I have only had a chance to briefly read the chapters that you forwarded to me and have not had time to review in detail.

The chapter describing biological characterization of Bt strains against *O. nubilalis* appears to be complete and provides important information regarding the activity of these new Bt isolates. I think that there are probably more appropriate ways to present the results of the bioassays against the various strains of corn borers, but I would need additional time to provide a more detailed review and suggestions for improvement. I do believe that there is significant new information in this chapter that could be published in a refereed journal article.

The chapter that describes activity of enzymes potentially associated with resistance to Bt toxins is very preliminary and unlikely to be accepted for publication. Although, I have not had a chance to review in detail, there is additional work that would be required to establish that the activity of either of these enzymes is significantly different among the strains examined. I also think that the results of experiments that are completedrequire additional analysis. I would be happy to provide review and suggestions for improvement in how these results are presented and interpreted but will not be able to do so in the time frame requested.

I hope that these comments provide enough detail for Gabriela to move forward with her defense. Please let me know if you need additional information.

Regards,
Blair

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ANEXO B - PUBLICAÇÕES DA AUTORA

- Artigos completes publicados em periódicos

ALLES, G. C.; HUBNER, M.; FIUZA, L.M. Toxiclogia de *Bacillus thuringiensis* às pragas urbanas e vetores. Biotecnologia Ciência & Desenvolvimento (Online), v. 38, p. 44-46, 2009.

AZAMBUJA, A.O.; **ALLES, G.C.**; FRITZ, L.L.; Reche, M.H.R.; FIUZA, L.M. Ecologia de *Bacillus* entomopatogênicos. Biotecnologia Ciência & Desenvolvimento (Online), v. 38, p. 14-23, 2009.

ALLES, G.C.; MACHADO, V.; FIUZA, L.M. Phenotypic characterization and the application of the rep-PCR technique in a study of new strains of *Bacillus thuringiensis* in the South of Brazil. In: Antonio Mendez-Vilas. (Org.). Microorganisms in Industry and Environment. From scientific and industrial research to consumer products. Microorganisms in Industry and Environment. From scientific and industrial research to consumer products. 2010, p. 96-100.

- Apresentação de trabalho em congressos:

CASSAL, M.C.; **ALLES, G. C**.; BERLITZ, D.L.; FIUZA, L.M. Seleção de novos isolados de *Bacillus thuringiensis* e *Lysinibacillus sphaericus* patogênicos a insetos-praga. In: CICPG Congresso de Iniciação Científica e Pós-Graduação, 2012, São Leopoldo. CICPG Congresso de Iniciação Científica e Pós-Graduação, 2012.

ALLES, G. C.; MACHADO, V. ; FIUZA, L. M. . Identificação molecular de novas cepas do entomopatógeno Bacillus thuringiensis, oriundas de amostras de solo do RS. 2009. XI Simpósio de Controle Biológico.

ALLES, G.C.; OLIVEIRA, J.V.; FIUZA, L.M. Efeito de Produtos Fitossanitários e Antimicrobianos às Bactérias Entomopatogênicas Presentes em Solos Orizícolas. VI Congresso naciona de Arroz Irrigado, 2009, Porto Alegre.

- Resumos publicados em anais de congressos

ALLES, G.C.; **CASSAL, M.C.**; MACHADO, V.; FIUZA, L. M. Caracterização de Novas Cepas de Bactérias do Gênero *Bacillus* frente a *Spodoptera frugiperda* pertencentes ao Banco de Bactérias Entomopatogênicas da Unisinos.. In: Simpósio Latino Americano de Coleções Biológicas e Biodiversidade- Conhecimento e Gestao, 2012, Teresópolis. Simpósio Latino Americano de Coleções Biológicas e Biodiversidade- Conhecimento e Gestao, 2012.

CASSAL, M.C.; **ALLES, G.C**.; BERLITZ, D.L.; FIUZA, L.M. Insecticidal potential of new *Bacillus thuringiensis* and *Lysinibacillus sphaericus* strains against *Spodoptera frugiperda* (Lep. Noctuidae). In: 45th Annual Meeting of the Society for Invertebrate Pathology, 2012, Buenos Aires. 45th Annual Meeting of the Society for Invertebrate Pathology, 2012.

ALLES, G.C.; FIUZA, L.M. Patogenecidade de *Bacillus thuringiensis* contra spodoptera frugiperda (Lepidoptera, noctuidae) em laboratório. In: XXIII Congresso Brasileiro de Entomologia, 2010, Natal/RN. Anais do XXIII Congresso Brasileiro de Entomologia, 2010.

ALLES, G. C. ; MACHADO, V.; FIUZA, L. M. . Análise molecular e atividade tóxica de cepas de Bacillus thuringiensis. In: Congresso Brasileiro de Entomologia, 2010, Natal/RN. Anais do XXIII Congresso Brasileiro de Entomologia, 2010.

ALLES, G. C.; MACHADO, V.; FIUZA, L.M. Caracterização Fenotípica de Cepas do Entomopatógeno *Bacillus thuringiensis* Oriundas das Regiões Orizícolas do Rio Grande do Sul. In: VI Congresso Nacional de Arroz Irrigado, 2009, Porto Alegre. Estresses e sustentabilidade: desafios para a lavoura arrozeira. Santa Maria: Pallotti, 2009. p. 52-52.

ALLES, G.C.; MACHADO, V.; FIUZA, L.M. 2009. RAPD-PCR caracterização fenotípica de novas cepas de *Bacillus thuringiensis.* XXV CBM, Porto de Galinhas, PE.

ALLES, G.C. & FIUZA, L.M. 2009. Novas cepas de *Bacillus thuringiensis* com atividade inseticida À *Spodoptera frugiperda* (LEPIDOPTERA, NOCTUIDAE). XXV CBM, Porto de Galinhas, PE.

ALLES, G.C.; MACHADO, V.; FIUZA, L. M. Caracterização de Estirpes de Bacillus Sphaericus Isoladas de Agroecossistemas Orizícolas. In: XI Simpósio de Controle Biológico, 2009, Bento Gonçalves. Anais do XI Siconbiol - CD do evento, 2009.

ALLES, G. C.; ALVES, C.M.; PANIZZON, J.; FIUZA, L.M. Interações de diferentes métodos de controle de Spodoptera frugiperda (Lepidoptera:Noctuidae) em laboratório. In: XI Simpósio de Controle Biológico, 2009, Bento Gonçalves. Anais Do XI Siconbiol - CD do evento, 2009.