



UNIVERSITY OF BRASÍLIA

DEPARTMENT OF CELL BIOLOGY

GRADUATE PROGRAM IN MOLECULAR BIOLOGY

**STUDY OF THE EFFECT OF CRY34AB1 AND CRY35AB1 PROTEIN EXPRESSION IN
TOMATO PLANTS ON THE LIFE CYCLE OF *Tuta absoluta* AND *Spodoptera frugiperda*
HERBIVORY**

**ESTUDO DO EFEITO DA EXPRESSÃO DAS PROTEÍNAS CRY34AB1 E CRY35AB1 EM
PLANTAS DE TOMATE NO CICLO DE VIDA DE *Tuta absoluta* E NA HERBIVORIA DE
*Spodoptera frugiperda***

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Brasília, DF.

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A Dissertation submitted to the Department of Cell
Biology of the Institute of Biological Sciences at the
University of Brasília, in partial fulfillment of the
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“With me, the present is forever, and forever is always shifting, flowing, melting. This second is life. And when it is gone it is dead. But you can't start over with each new second. You have to judge by what is dead”

Sylvia Plath

ABSTRACT

Solanum lycopersicum, the tomato, is one of the most widely cultivated and commercially produced crops worldwide. Among the many crop varieties, Micro-Tom's small size and rapid fruiting make it a model for biotechnology projects, given its short life cycle and fruiting time, both desirable traits. Many pests affect tomato production, diminishing productivity and preventing marketability of produce. Among these, *Tuta absoluta* and *Spodoptera frugiperda* can cause total yield loss if not managed in the field in time. Dependence on agrochemical insecticides imposes selection pressure on pests, resulting in the emergence of resistant populations, therefore genetic engineering provides alternatives by inducing endogenous resistance through plant breeding consequently diminishing reliance on chemical control methods. This work generated transgenic Micro-Tom lines expressing the Cry34/35Ab1 insecticidal binary proteins as a biotechnological alternative for agrochemicals. Micro-Tom explants were transformed using *Agrobacterium tumefaciens*, and the generated events were tested using NPTII enzyme-detecting immunochromatographic strips and PCR. Bioassays conducted with MicroTom-Cry34/35Ab1 expressing proteins showed reduced herbivory by *Spodoptera frugiperda* and diminished *Tuta absoluta* emergence compared with wild type controls. These results regarding *Spodoptera frugiperda* are promising, given the need for alternative effective control methods. As for the Tomato Leafminer, further research is needed.

RESUMO

Solanum lycopersicum, o tomateiro, é uma das culturas mais amplamente cultivadas no mundo e de grande valor comercial. MicroTom é uma variedade de porte pequeno utilizada como uma das plantas modelo para projetos de biotecnologia vegetal, considerando o curto ciclo de vida e frutificação rápida, que são características desejadas. Diversas pragas afetam a produção de tomate, reduzindo a produtividade e impedindo a comercialização dos frutos. Dentre essas, *Tuta absoluta* e *Spodoptera frugiperda* podem causar a perda total da produção se não forem controladas a tempo em campo. A dependência de inseticidas agroquímicos exerce pressão seletiva, o que favorece o surgimento de populações de insetos resistentes. A engenharia genética vem como alternativa para induzir resistência endógena por meio do melhoramento genético de plantas, auxiliando, assim, o cultivo em campo. O presente trabalho gerou linhagens transgênicas de Micro-Tom expressando as proteínas inseticidas Cry34/35Ab1. A transformação de MicroTom foi realizada com *Agrobacterium tumefaciens* EHA105, e os eventos gerados foram testados por meio de imunocromatografia de fluxo lateral para detecção da enzima NPTII, presente no vetor binário, e por PCR com *primers* específicos para os genes *Cry34* e *Cry35*. Bioensaios conduzidos com MicroTom-Cry34/35Ab1 demonstraram redução da herbivoria em *Spodoptera frugiperda* e diminuição da emergência de adultos de *Tuta absoluta*, quando comparados às plantas controle. Esses resultados referentes a *Spodoptera frugiperda* são promissores, considerando a necessidade de métodos de controle no campo. Quanto à traça-do-tomateiro, são necessárias pesquisas adicionais e testagem de outras proteínas inseticidas.

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INTRODUCTION

Tomato (*Solanum lycopersicum*) is a crucial agronomic crop for food production, but its cultivation is affected by many organisms that prey on tomato plants, leading to significant productivity losses and fruit unmarketability due to pest damage. *Spodoptera frugiperda* and *Tuta absoluta* are insects belonging to the order Lepidoptera, which cause damage in the tomato by laying eggs in various organs that hatch and become larvae, which leave holes that facilitate the entry of pathogens and also interfere with the homeostasis of the tomato plant, decreasing productivity and market values (Biondi *et al.*, 2018; Han *et al.*, 2018). Insect control in tomato crops is done using agrochemicals, creating an issue which is the continuous use of a limited number of active insecticide ingredients that select for resistant individuals and pose environmental risks, as they affect several non-target insects (Roditakis *et al.*, 2018; Guedes *et al.*, 2019; Pereira *et al.*, 2023). The bacterium *Bacillus thuringiensis* produces several insecticidal toxins that target a wide range of individuals in the orders Diptera, Coleoptera, and Lepidoptera, making it an important alternative for the biological management of insects in agronomic crops (Wang *et al.*, 2019). After ingestion by feeding, the protoxins are cleaved by digestive enzymes, usually proteases, inside the gut and become toxins that bind to glycoprotein receptors on the membranes of microvilli in the intestine of insects. After binding to the gut epithelial membranes, the protoxins form pores that allow the indiscriminate passage of molecules, which interfere with cell homeostasis and lead to cell lysis and, eventually, the insect's death due to nutrient deprivation (Heckel, 2020). Among the toxins found in *Bacillus thuringiensis* (Bt), the Cry group includes several toxins described in the literature for their cytotoxic effects against insects. Agronomical management methods that use Cry toxins are limited by environmental factors such as temperature, photoinactivation, rain, and humidity, which can reduce the toxins' efficacy when applied directly to plants and may not reach the insects thoroughly (Liu *et al.*, 2021). In contrast, the endogenous expression of several Cry proteins in genetically modified (GM) plants of great economic importance has proven to be an alternative and efficient method for controlling entomological pests that do not pose great risks to non-target organisms compared to agrochemicals (Fernandes *et al.*, 2023; Liu *et al.*, 2022; Sohrabi *et al.*, 2016; Liu *et al.*, 2016). The present project aims to obtain genetically modified tomato plants expressing the protoxins Cry34Ab1 and Cry35Ab1 to control *Tuta absoluta* and *Spodoptera frugiperda* in tomato crops.

LITERATURE REVIEW

Solanum lycopersicum

The tomato, *Solanum lycopersicum* L., is a herbaceous plant in the family *Solanaceae*. Its genome is diploid ($2n=24$) and compact in size (900 Mb), and it was fully sequenced in 2012, comprising 34,727 coding sequences (Tomato Genome Consortium, 2012). The tomato is a perennial plant, but is more often cultivated as an annual due to agronomic challenges in the field. It is covered in glandular trichomes that secrete exudates responsible for the plant's aroma and for defense against herbivores. The leaves have alternate and imparipinnate architecture, and the flowers are pentamerous and hermaphroditic, which favors autogamy. The fruit is a fleshy berry containing seeds embedded in a gelatinous matrix derived from the placenta (Padmanabhan *et al.*, 2016; Simmons; Gurr, 2005). The Micro-Tom tomato cultivar (fig. 1) is characterized by a determinate growth habit, conferred by the *self-pruning* (*sp*) gene, which causes the shoot apical meristem to terminate in a flower cluster, which ensures a short life cycle. A recessive mutation in the *dwarf* (*d*) gene is the main reason for the small stature, allowing for a higher number of plants in limited spaces during commercial cultivation (Meissner *et al.*, 1997; Martí *et al.*, 2006; Gonzalez *et al.*, 2015). The Micro-Tom completes its life cycle in up to 90 days, much faster than commercial cultivars (Meissner *et al.*, 1997). Regarding its role as a model crop, Micro-Tom exhibits high transformation efficiency with *Agrobacterium tumefaciens*, making it a good model for functional genomics studies (Sun *et al.*, 2006). In 2024 alone, *Solanum lycopersicum* production from 20 countries totaled over 158 million tonnes, led by China and India, while Brazil stands as the largest producer in South America (FAOSTAT, 2026). Beyond its economic value, tomatoes are a good source of dietary antioxidants such as lycopene and β -carotene (Ali *et al.*, 2020). Tomato commercial production relies mainly on monocultures, which, along with genetic uniformity in its cultivars, have created high susceptibility to biotic and abiotic stress (Keneni *et al.*, 2012). Intensive cultivation, particularly in tropical regions like Brazil, increases pest pressure by providing year-round pest infestation, most notably by *Bemisia tabaci*, *Spodoptera frugiperda*, thrips (*Frankliniella* spp.), and *Tuta absoluta*, which alone can cause 100% yield loss (Mahmoud *et al.*, 2020; WU *et al.*, 2021; Wang *et al.*, 2026). Therefore, integrated pest management and the incorporation of molecular resistance approaches into cultivars are crucial for food security, given the rise of pest resistance to synthetic insecticides (Green *et al.*, 2020; Al Naggar *et al.*, 2025).



Fig. 1 Micro-Tom (Gasparini *et al.*, 2025). Micro-Tom short stature.

Tuta absoluta

Tuta absoluta (Meyrick), also known as the Leafminer, is a holometabolous and oligophagous insect (fig. 2). Host range includes plants in the Solanaceae family, such as potato and eggplant, although it shows an inclination toward *Solanum lycopersicum*. Literature also demonstrates that it feeds on alternative plant species, hence its polyphagous behavior. (Bawin *et al.*, 2016). Morphologically, it belongs to the order Lepidoptera and the Gelechiidae family. Attributable to its rapid life cycle, which completes in an average of 31 days, ten to twelve generations are produced per year (Vidyapeeth *et al.*, 2021). In tomato plants, it can cause up to 100% yield loss if not controlled. The females show high fecundity, depositing up to 260 eggs over their lifespans on the abaxial side of leaves. The leafminer life cycle has four developmental stages: egg, four larval instars, pupa, and adults (Rostami *et al.*, 2017; Dalbianco *et al.*, 2024). The larvae feed on the leaves' mesophyll, forming internal mines that result in transparent lesions on the epidermis. *T. absoluta* larvae can exit a mine to invade new tissue or pupate on the soil (Biondi *et al.*, 2018). Apart from tissue depletion from larval activity on tomato, secondary infections render production unmarketable, contributing to investment loss for producers (Biondi *et al.*, 2018; Pandey *et al.*, 2023).



Fig. 2 *Tuta absoluta*. (A) Adult Leafminer (CABI, 2025). (B) Larvae feeding on tissue; (C) Galleries on leaves; (D) Larvae feeding on tomato (Blancard/INRAe, 2022).

Spodoptera frugiperda

The fall armyworm (FAW), *Spodoptera frugiperda* (fig. 4), is native to South America and belongs to the order Lepidoptera and the family Noctuidae. It is a highly prolific pest of major crops, particularly corn, and significant efforts have been made to control its impact. Due to its polyphagous behavior, *S. frugiperda* also infests other crops, including tomato (Herrero *et al.*, 2016a). During reproduction, the larvae penetrate and destroys plant tissue, which poses a challenge for commercial production because damaged fruits are unmarketable. The life cycle of *S. frugiperda* (fig. 3) is completed in approximately 35 days and consists of four stages: eggs, six larval instars, pupa, and adults (Li, *et al.*, 2023; Bakry, 2024). After hatching, the larvae migrate to the ground, where pupation is completed in about 8 days on average (Shi *et al.*, 2021; Masaya *et al.*, 2022). Given the destructive impact of *S. frugiperda* on crops such as tomato, multiple control methods are necessary to ensure yield production and reduce plant damage (WU *et al.*, 2021b).

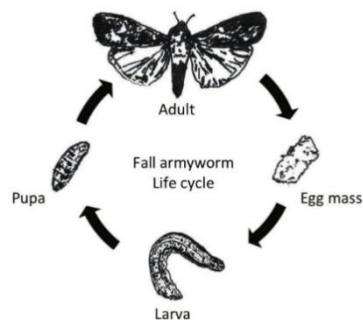


Fig. 3 *Spodoptera frugiperda* life cycle (Joseph, 2025).



Fig. 4. *Spodoptera frugiperda* (Owens; Purser, 2021).

Bacillus thuringiensis

Bacillus thuringiensis (Bt) is a bacterium found in the soil that produces endotoxins targeting the midgut epithelium of insects and causing death by disrupting the epithelial midgut. These proteins trigger pore formation and death in insects, thereby serving as biopesticides in genetically modified crops (GMOs) and in biological formulations. Discovered in 1901, *B. thuringiensis*' toxin diversity was still manageable up until 1989, when Höfte and Whiteley proposed a nomenclature based on toxicity towards the host: CryI for Lepidoptera, CryII for both Lepidoptera and Diptera, CryIII is lethal to Coleoptera, and CryIV to Diptera. Alternatively, Cyt proteins exhibit cytolytic activity against diverse cell types (Höfte; Whiteley, 1989). As research progressed, Bt proteins exhibited cross-order toxicity, others showed sequence homology to known toxins but lacked the desired insecticidal result, which led to Crickmore *et al.*, in 1998 and 2021 to propose better nomenclature alternatives which affect most proteins and are based primarily on structure, rather than sole activity (Crickmore *et al.*, 2020; Schnepf *et al.*, 2021). Cry34Ab1 and Cry35Ab1, for instance, are not 3D Cry toxins and were reclassified as Gpp34Ab1 and Tpp35Ab1, respectively, based on structural criteria (Crickmore *et al.*, 2020; Schnepf *et al.*, 2021). Gpp (Gram-positive pesticidal proteins) are characterized by their structural resemblance to Aegerolysin proteins, whereas Tpp (Toxin pesticidal proteins) are pore-forming β -domain proteins in the Toxin_10 family domain (Crickmore *et al.*, 2021). Regarding the mode of action of three-domain (3D) Cry toxins (δ -endotoxin), it demands the completion of five steps, including crystal solubilization, proteolytic activation, receptor binding (Fig. 6), membrane insertion, and pore formation (Fig. 5). Missing a step deems the process inefficient, hence accounting for the high specificity of Bt toxins and their safety for non-target organisms (Resende *et al.*, 2016).

Fig. 5. Cry proteins mechanism of action (Fernández-Chapa *et al.*, 2019).

Fig. 6. Bt toxins and their respective binding sites (Herrero *et al.*, 2016) .

As for structure, Cry δ -endotoxins are composed of a three-piece domain (Fig. 7). Domain I is composed of α -helices, acting as a driver for pore formation and membrane penetration. Domain II is formed by three β -sheets intercalated with loops and is responsible for receptor binding and specificity in insect midgut membranes. Domain III, a β -sandwich, is associated with toxin stability and receptor binding as well (Deist *et al.*, 2014). The N- and C-terminal regions are responsible for Cry protoxin stability and consequent activation. The C-terminal domain is stabilized by disulfide bonds, forming the insoluble crystalline structure that protects the toxin until it is exposed to specific insect pH conditions. Once solubilized, the protoxins undergo proteolytic processing, where enzymes, which digest the C-terminal fragment and cleave Helix α -1 from the N-terminus, which destabilizes Domain I and then exposes the hydrophobic surfaces required for membrane insertion and pore formation. (Gill; Soberón, 2007; de Maagd, 2001; Pardo-López; Soberón; Bravo, 2013).

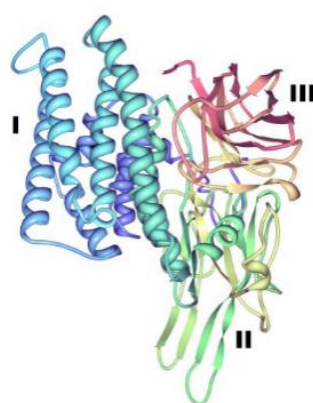


Fig. 7. 3D Cry protoxins Structure
(Deist *et al.*, 2014).

Cry34Ab1 (Gpp34Ab1) and Cry35Ab1 (Tpp35Ab1) binary cooperation

Cry34Ab1 is a small protein (14 kDa) composed of two antiparallel β sheets that fold into a β -sandwich, which forms a hydrophobic core. Unlike 3D classical Cry proteins, the C-terminal strand is contained within the structure rather than exposed. Interestingly, the protein is fully active without requiring proteolytic processing. Cry35Ab1 (44 kDa) folds into two domains, N-terminal β trefoil and a C-terminal region consisting of antiparallel β sheets and a three-helix motif (fig. 8). Activation involves proteolytic activity at the C-terminus, distinct from classical 3D Cry toxins, which require removal of larger crystallization domains. This small C-terminal region is responsible for maintaining the protein's insolubility in inclusion bodies, and after removal, it produces a truncated 40 kDa version, trCry35Ab1, that is highly soluble but still retains full toxicity (de Maagd, 2001; Masson *et al.*, 2004; Bravo; Gill; Soberón, 2007; Soberón; Bravo, 2013; Li *et al.*, 2013; Kelker *et al.*, 2014; Narva *et al.*, 2017). Histopathology midgut assays performed by Bowling *et al* (2017) on *Diabrotica virgifera virgifera* artificially fed with Cry34Ab1 and Cry35Ab1 individually showed mild epithelial disturbance and produced no significant epithelial perturbation, respectively. Interestingly, simultaneous ingestion of Cry34Ab1/Cry35Ab1 resulted in damage to the anterior midgut, enterocyte hypertrophy, cytolysis, microvilli desquamation as well as the accumulation of necrotic debris within the cell lumen (fig. 9). The study also reported an increase in stem cell in the area, which could be a regenerative response to epithelial injury, which suggests a potential route for toxin resistance (Bowling *et al.*, 2017). Contrasting 3D Cry toxins that can function independently, Cry34Ab1 and Cry35Ab1 are obligatory partners, as neither protein exhibits significant insecticidal activity alone, and toxicity occurs only as a binary cooperation. Still, the precise molecular mechanism of the Cry34/35Ab1 activity remains not fully understood (Li, *et al.*, 2013; Bowling *et al.*, 2017).

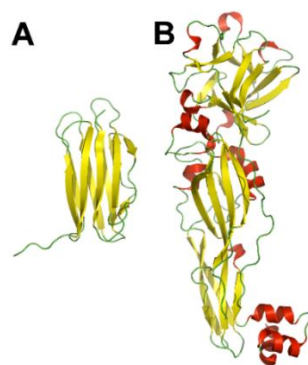


Fig. 8. Cry34Ab1 and Cry35Ab1 structure (Kelker *et al.*, 2014).

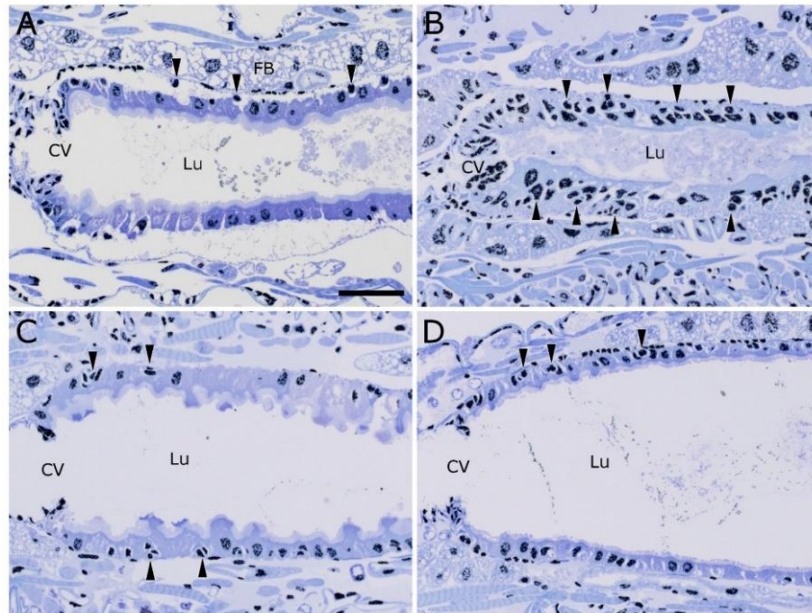


Fig. 9. Cry34/35Ab1 activity in *Diabrotica virgifera virgifera* (WCR) midgut. (A) Ordinary *Diabrotica virgifera virgifera* midgut (B) Complete midgut disruption after 48h of Cry34/35Ab1 ingestion. (C) Cry34Ab1 and (D) Cry35Ab1 isolate effect on *D. virgifera virgifera* midgut (Bowling *et al.*, 2017). LU: lumen; CV: cardiac valve; Arrowheads: stem cells.

A definitive receptor for Cry34/35Ab has yet to be determined. Data show Cry34Ab1 binds nonspecifically to WCR Brush Border Membrane Vesicles (BBMVs), which are laboratory preparations of microvillar intestinal membranes isolated from insect larvae midgut. Upon binding, Cry35Ab1 is recruited, and ion-conducting channels are formed in lipid membranes, leading to indiscriminate permeability and consequent cell death (Masson *et al.*, 2004). Interestingly, Tan *et al.* (2016) examined Cry34/35Ab1 binding to WCR membranes in the absence of cadherin receptors, and this did not affect overall toxicity. Structural description defines Cry34Ab1 within the aegerolysins (Fig. 10), a protein group exhibiting specificity for sphingolipids such as ceramide phosphoethanolamine (CPE). Cry34Ab1 shows a generic association on BBMVs, thus, a definitive receptor remains unidentified (Panevska *et al.*, 2021). Cry35Ab1 is classified as an aerolysin β toxin that forms membrane pores, and the n-terminus forms a β -trefoil domain that contains repeated QxW motifs, associated with lectin receptor binding. The current hypothesis supports the binary Cry34/35Ab1 system attaches to glycosylated targets on the insect midgut, although, as Cry34Ab1 the specific targets are not elucidated yet (Schnepf *et al.*, 1998; Kelker *et al.*, 2014).

PlyA	MAYAQWVIIIIHNVGSKDVKIKNLKPSWGKLHADGDKDTEVSASKYE	47
Cry34Ab1	-MSAREVHIDVNNKTGHTLQLED-----KTKLDGGRWR	35
	* * * *	*
PlyA	GTVIK-PDEKLQINACGRSDAAEGTTGTFDLVDPADGDKQVRHFYWD	93
Cry34Ab1	TSPTNVANDQIKTFVAESNGFMTGTEGT--IYYSINGEAEI-SLYFD	80
	** ** *	** *
PlyA	CPWGSKTNTWTVSGSNTKW-MIEYSGQNLDGALGTITVDTLKKGN--	138
Cry34Ab1	NPFAG-SNKYDGHSNKSQYEIITQGGSGNQSHVTYTIQTSSRYGHKS	126
	* * *	** *

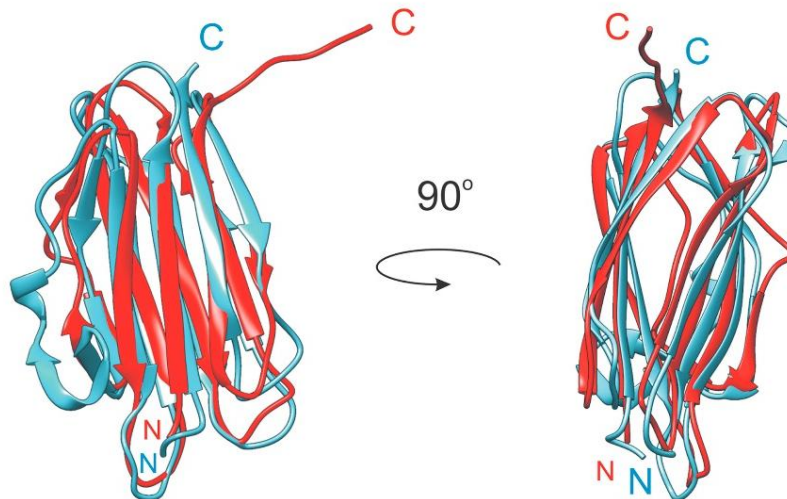


Fig. 10. Structure of pleurotolysin A, PlyA (*Pleurotus ostreatus*) and Cry34Ab1 (*Bacillus thuringiensis*). Overlay of the crystal structures of aegerolysin family proteins PlyA and Cry34Ab1 (Butala *et al.*, 2017).

Agrobacterium tumefaciens

Agrobacterium tumefaciens, a gram-negative bacterium, is used for genetic engineering of crops and for general gene testing in plants. Initially described as a phytopathogen for causing tumors in plants (crown gall disease), the mechanism behind the disease would become a landmark for plant breeding and the answer for agricultural challenges. Back in 1983, the first genetically modified plants (GM) were created by three distinct research groups (Fraley, 1983). The disarming of the Ti plasmid present in the bacterium's machinery prevents tumorigenesis, thus exchanging the nucleic sequence present in the T-DNA region allows the transfer of genes from the bacterium into the plant's genome (Grace Sunday *et al.*, 2024; Goralogia *et al.*, 2025). Present in the bacterium membrane, the kinase sensor VirA binds to phenolic compounds exuded by wounded plants, thereby phosphorylating VirG, the transcriptional regulator of the Vir operon. Now transcribed, this cluster of genes are the reason for the mechanism behind bacterium-plant gene transfer. The VirD1 and VirD2 endonuclease complex cleaves on 25bp repeats present at the left and right borders, which in return generates a DNA molecule named T-DNA. This single stranded molecule is 5' capped by VirD2 and translocated towards the plant

cytoplasm via a pore formed by VirB/VirD4. In the cytoplasm, VirE2 forms the T-complex. This structure protects the DNA from degradation, compacts it for transport, and facilitates nuclear entry through the host's importin- α pathway. In the nucleus VIP1 factors guide the complex to euchromatin regions, which allows the T-DNA to integrate into the genome by Non-Homologous End Joining (NHEJ) on a double-strand break (Park *et al.*, 2015; Ohmine *et al.*, 2018; Miroshnichenko *et al.*, 2020; Jin *et al.*, 2022; Yu *et al.*, 2025). Transformation of Micro-Tom tomatoes utilizing *Agrobacterium* strain EHA105 renders high success rate and efficient selection of positive integrated cells utilizing neomycin phosphotransferase II (NPTII) has been shown. EHA105 was engineered to be sensitive to kanamycin, ensuring that when you use the kanamycin resistant pCAMBIA2300 plasmid, any surviving bacteria are transformants. EHA105 is known to be super virulent by possessing the hyperactive gene system VirA/VirG, facilitators of the infection mechanisms employed by *A. tumefaciens* (Hood *et al.*, 1993; Sun *et al.*, 2006).

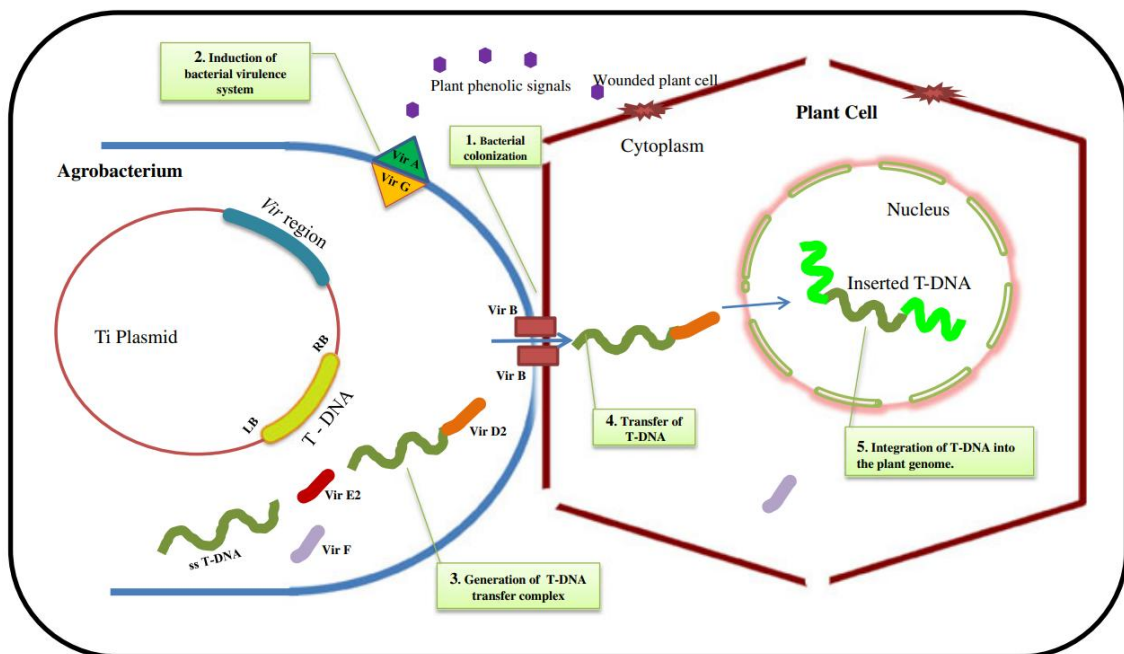


Fig. 11. *A. tumefaciens* mechanism of action (Mehrotra; Goyal, 2012).

MOTIVATION

Tomato is one of the most consumed crops and is rich in vitamins and antioxidants of great relevance to human nutrition. *Solanum lycopersicum* is preyed upon by *Tuta absoluta* and *Spodoptera frugiperda*, causing loss of productivity due to the destruction of leaves, stem and fruits. The use of pesticides to control these pests generates environmental issues by affecting non-target species and selecting for resistance, resulting in an increase in the number of agrochemicals applied. Lepidopteran insects such as *Tuta absoluta* and *Spodoptera frugiperda* have shown to be negatively affected at different stages of their life cycles when feeding on protoxins of the Cry subgroup. This project aims to generate tomato plants (variety Micro-Tom) resistant to *Tuta absoluta* and *Spodoptera frugiperda* by expressing the protoxins Cry34Ab1 and Cry35Ab1.

HYPOTHESIS

The genes encoding the protoxins Cry34Ab1 and Cry35Ab1, when present in the tomato genome, influence the success of *Tuta absoluta* and *Spodoptera frugiperda* herbivory.

Main goal

The development of *Solanum lycopersicum* cv. Micro-Tom plants resistant to *Tuta absoluta* and *Spodoptera frugiperda* by the expression of the protoxins *Cry34Ab1* and *Cry35Ab1*.

Specific objectives

- Production of the GS74103-2 pCry34_35Ab1_NPT vector.
- Transformation of *Agrobacterium tumefaciens*, via electroporation, using the GS74103-2 pCry34_35Ab1_NPT vector.
- Production of genetically modified Micro-Tom events expressing Cry34ab1 and Cry35ab1 proteins.
- Evaluation of tomato GMO events by ImmunoStrip® NPTII/Bt-Cry34Ab1 and PCR.
- *Tuta absoluta* bioassay to evaluate life cycle disruption.
- *Spodoptera frugiperda* herbivory success bioassay.

MATERIALS AND METHODS

Synthesis of the GS74103-2 pCry34_35Ab1_NPT vector

The vector containing the cassettes for expression of the Cry34Ab1 and Cry35Ab1 protoxins and the Neomycin phosphotransferase type II (NPTII) enzyme was synthesized by Epoch Life Science (USA, www.epochlifescience.com). The coding regions of the genes *Cry34* and *Cry35* were cloned under the control of the tomato vacuolar promoter. The genes were controlled by the H⁺-pyrophosphatase (*SlAVP1*) and *Arabidopsis thaliana* *ACTIN2* gene promoters, respectively. In contrast, the *nptII* gene was cloned under the control of the 35S RNA promoter of the cauliflower mosaic virus (CaMV 35S) to ensure constitutive expression in all plant tissues, preventing herbivory bias, thus ensuring proper bioassay results.

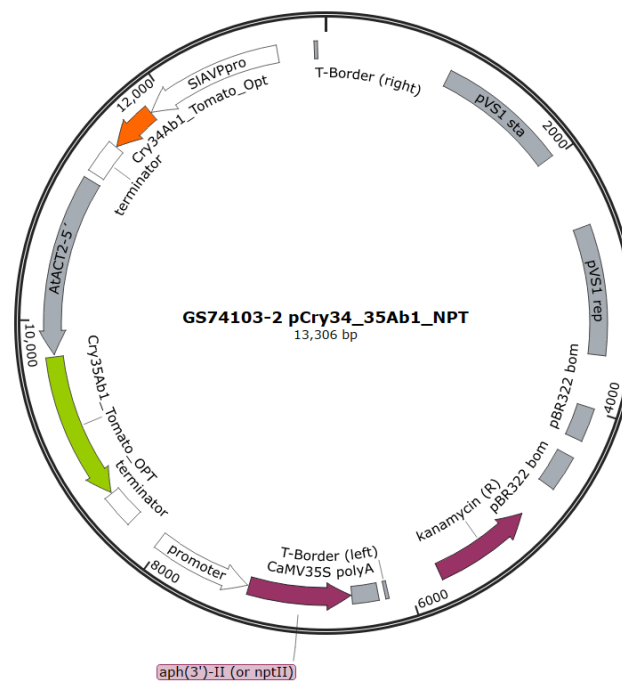


Figure 12. Vector GS74103-2 pCry34_35Ab1_NPT. Vector containing the coding sequence for the genes *Cry34*, *Cry35*, and *nptII*.

Transformation of *Agrobacterium* using the GS74103-2 pCry34_35Ab1_NPT vector

The transformation of *Agrobacterium tumefaciens* strain EHA105 was performed using electroporation, following the protocol described by Brasileiro and Carneiro (2015). The protocol consists of adding 2.0 µL of the vector to 40 µL of competent *Agrobacterium tumefaciens* cells. This mixture is transferred to an electroporation cuvette containing electrode poles and placed in an electroporator, where it is subjected to an electric potential difference to

permit DNA uptake by the bacterial cells. Following transformation, the cells were suspended in liquid LB medium and incubated for 2 hours at 28°C. The resulting solution was plated onto solid LB medium supplemented with rifampicin (100 mg/mL) and kanamycin (100 mg/mL), and incubated for 72 hours at 28 °C. Three isolated colonies of *A. tumefaciens* were selected and cultured in liquid LB medium with shaking at 160 rpm for 72 hours at 28°C. Vector integration in the selected colonies was confirmed by PCR and analyzed via gel electrophoresis. Positive inocula were mixed with 50% glycerol, aliquoted into 200 µL volumes, and stored at -80 °C for cryopreservation.

***Agrobacterium*-Mediated Transformation of Micro-Tom Tomato**

The protocol for the transformation of tomato cotyledons, variety Micro-Tom, followed the method described by Sun *et al.* (2006). *Micro-Tom* seeds were sterilized using a solution made of 70% alcohol and 2% hypochlorite. Germination was carried out in flasks containing MS medium (Murashige and Skoog, 1962) in the dark for 7 days. Forty-eight hours prior to the start of the transformation protocol, an aliquot of *EHA-105-GS74103-2 pCry34_35Ab1_NPT* cells, previously stored at -80°C, was cultured on solid AB supplemented with rifampicin (100 mg/mL) and kanamycin (100 mg/mL). The resulting bacterial culture was resuspended in liquid co-culture medium (Brasileiro and Carneiro, 2015), which contains MES buffer (pH 5.2) to maintain the acidic environment required for expression of *vir* genes, and Acetosyringone (200 µM) to simulate natural plant wound signals and enhance phosphorylation of the *VirA/VirG* system, thereby activating T-DNA transfer. Cysteine (400 mg/L) was included as an antioxidant to deal with oxidative stress caused by tissue wounding, and PVP (200 mg/L) was added to absorb polyphenols, thereby preventing tissue necrosis during transformation. After the co-culture medium was prepared, cotyledonary sections of Micro-Tom tomatoes were submerged in this solution for 20 minutes, and the explants were then maintained for 48 hours in an incubator at 20°C on a solid agar version of this co-culture medium (Brasileiro and Carneiro, 2015).

After this period, the explants were transferred to induction and selection medium. This medium was supplemented with Timentin and Cefotaxime to eliminate *Agrobacterium* without harming plant cells, and kanamycin (100 mg/mL) as the selective agent. Kanamycin inhibits protein synthesis in non-transformed tissues, but since the T-DNA contains the *nptII* gene, only transgenic events survive. To induce organogenesis, the medium contained Zeatin and IAA, therefore preventing apical dominance, as well as Silver Nitrate (AgNO₃ 2 mg/L), which acts

as a competitive inhibitor of ethylene, a critical step for reducing senescence and improving shoot regeneration. The explants were maintained in this medium in a tissue culture growing room for 15 days, either for eventual fresh medium transference or for continued maintenance if cotyledons formed regenerative shoots. Subsequently, the now differentiated explants were transferred to rooting medium (Brasileiro and Carneiro, 2015), remaining there until root formation and subsequent greenhouse acclimatization (Sun *et al.*, 2006).

Molecular characterization of transgenic events by polymerase chain reaction (PCR) and gel electrophoresis

DNA was extracted from plant tissue and subjected to polymerase chain reaction, also known as PCR, and gel electrophoresis analysis. The CTAB DNA extraction method described by Rogers and Bendich (1985) was employed. For gene amplification, the enzyme Recombinant Taq DNA Polymerase from Invitrogen® and the forward and reverse primers of each gene were used. Primer synthesis was ordered from Exxtend® (São Paulo, Brazil) (<https://www.exxtend.com.br/>) with the appropriate instructions and the necessary information regarding the DNA sequence.

Analysis of NPTII enzyme and Cry34Ab1 presence by ImmunoStrip® Test

To detect the NPTII enzyme and Cry34Ab1 protein, immunodetection strips were used according to the manufacturer's instructions. The methodology consisted of macerating plant tissue in 1X phosphate-buffered saline (PBS), centrifuging for 3 minutes at 400 RPM, and placing one strip per tube.

Progeny analysis

Seeds from the T₀ generation were harvested and germinated in a greenhouse using a substrate composed of an equal mixture of vermiculite, latosol, and Bioplant®. Sixteen germinated plants were randomly selected for gene segregation analysis. For statistical analysis, a Mendelian ratio and the chi-square test were used to determine the Mendelian pattern of transgene segregation.

GMO Tomatoes x *Tuta absoluta*

Tuta absoluta adult insects were obtained from Embrapa Hortaliças (CNPq, Brazil) and subjected to feeding on GMO tomatoes expressing Cry34AB1 and Cry35AB1 proteins as well as the wild-type (WT) Micro-Tom cultivar. The experiment evaluated four transgenic lines:

22, 47, 84, and 86. Nine genetically modified plants per line and nine wild type (WT) plants were randomly placed in four distinct insect cages (Rescholar Equipment, India) measuring 45 x 45 x 55 cm, for a total of 45 plants. Four adult Leafminers per plant were introduced into the cages, totaling 180 insects. One cotton ball embedded in 10% aqueous honey solution was available in each cage to nourish adult insects during oviposition. Insect herbivory was observed throughout the experiment. Plants from each line and WT were distributed equally between three breathable plastic containers, totaling three plants of each line (and WT) in three distinct containers. Insect emergence and quantification were recorded for 35 days until no adults were present. Data was analyzed using GraphPad Prism 10 software. This bioassay followed the methodology of Fernandes *et al.*, 2023.

Herbivory Bioassay with *Spodoptera frugiperda*

Spodoptera frugiperda first-instar larvae were exposed to detached leaves from each transgenic line (22, 47, 84, and 86) and compared with tissue consumption from wild-type controls. The experimental design consisted of placing one leaf per Petri dish, with its petioles wrapped in water-moistened cotton balls to maintain tissue turgidity. The larvae were obtained from a laboratory colony at Embrapa Cenargen (Brazil) and maintained under environmental conditions of 25 °C, 70% relative humidity, and 16/8 h photoperiod (Ge *et al.*, 2024). Photos were taken from each leaf before larval consumption. One first instar larva was introduced into each dish, with three biological replicates (leaves) for each line and WT. The experiment lasted 15 days, and herbivory damage was quantified by comparing the leaf area after larval feeding with the initial, undamaged area to calculate the percentage of tissue consumed using ImageJ® software. Herbivory percentages were obtained and analyzed using GraphPad Prism 10 software.

RESULTS

Plant transformation, tissue culture, and acclimatization of transgenic events

The tomato transformation protocol developed by Sun *et al.* (2006) was repeated twenty-two times using transformed EHA-105-GS74103-2 pCry34_35Ab1_NPT *A. tumefaciens* cells. Independent shoots were generated and transferred to elongation and rooting media (Brasileiro & Carneiro, 2015) until acclimatization (fig. 13). Generated events were confirmed transgenic for *cry34*, *cry35*, and *nptII* genes via PCR and lateral flow immunochromatographic strips. Four independent events were selected for progeny as well as *T. absoluta* and *S. frugiperda* bioassays. Considering *A. tumefaciens* randomness of transgene insertion into genomic loci, it

is of paramount importance to obtain multiple transgenic events to ensure high expression and non-interference with other crucial genes for plant survival (Shahnam; Mahin, 2023).



Fig. 13. In-vitro explants and acclimatized GMO plant containing *cry34*, *cry35*, and *nptII* genes.

Analysis of NPTII enzyme and Cry34Ab1 proteins via ImmunoStrip® Tests

Lateral flow immunochromatographic assays were conducted using the NPTII ImmunoStrip® test (ImmunoStrip® - Agdia, USA) in GM events and one WT plant. Leaf tissue was macerated in 1X phosphate-buffered saline (PBS) and centrifuged, the resulting supernatant was used for the insertion of one ImmunoStrip® per event sample, which detected the NPTII enzyme in all four events but not in the WT samples, confirming the integration of the GS74103-2 pCry34_35Ab1_NPT vector in the genome (fig. 14). While PCR analysis confirms the genomic integration of transgenes, easy and direct protein detection by ImmunoStrips® simplifies characterization of GM events, indicating proper post-transcriptional gene regulation and expression (Katta *et al.*, 2021).



Figure 14. Immunostrips® for NPTII protein detection in transgenic lines 22, 47, 84, and 86 and wild-type control.

As shown in the table below, the amino acid sequence for the Cry34Ab1 protein expressed on the GM lines utilized in this project differs from the sequence present in commercial transgenic crops expressing Cry34/35Ab10 (ISAAA, 2026), which explains the inability of protein detection using ImmunoStrip® Bt-Cry34Ab1 (Fig. 15). Interestingly, protein folding remains unaltered (Fig. 16).

Table 1. Amino Acid sequence from GS74103-2 pCry34_35Ab1NPT (Cry34Ab1) and commercial crops utilizing Cry34/35Ab1 proteins (ISAAA, 2026).

Cry34Ab1	MSAREVHIDVNNKTGHTLQLEDKTKLDGGRWRTSPTNVANDQIKTFVAESHGFM TGTEGT
Cry34AB1 (commercial)	MSAREVHIDVNNKTGHTLQLEDKTKLDGGRWRTSPTNVANDQIKTFVAESNGFM TGTEGT
Cry34Ab1	IYYSSINGEAEISLYFDNPYSGSNKYDGHSNKNQYEVIITQGGSGNQSHVTTYTIQT VSSRYG
Cry34Ab1 (commercial)	IYYSSINGEAEISLYFDNPFAGSNKYDGHSNKSQYEIITQGGSGNQSHVTTYTIQT TSSRYG



Fig. 15. ImmunoStrip® Bt-Cry34Ab1. Unsuccessful Bt-Cry34Ab1 protein detection in GS74103-2 pCry34_35Ab1NPT events. (A) Tested in WT MicroTom plant and (B) tested in MicroTom-Cry34/35Ab1.



Fig. 16. Protein structure comparison between GS74103-2 pCry34_35Ab1NPT (left) and Cry34Ab1 (commercial version, right). Highlighted colors indicate loops (ISAAA, 2026; NCBI iCn3D, 2026).

Molecular characterization of GMO events by polymerase chain reaction (PCR) and gel electrophoresis analysis.

DNA was extracted from foliar tissue using the CTAB method (Rogers & Bendich, 1985) and subjected to polymerase chain reaction for gel electrophoresis analysis. For *cry34* and *cry35* genes, the primers Cry34F/Cry34R and Cry35F/Cry35R were used, respectively. The banding pattern obtained by the polymerase chain reaction demonstrates the expected presence of amplicons containing 312 bp for Cry34 and 328 bp for Cry35 (Fig. 17). The transmission of transgenes to subsequent lines is essential, therefore, it is crucial to possess multiple transgenic plants, as not every event can produce lines and pass the transgenes to the next generation (Chaudhury, 2006). The CaMV35S promoter was used to enable constitutive expression of the NPTII enzyme, ensuring the ingestion of both protoxins regardless of the tissue fed upon by *Tuta absoluta* and *S. frugiperda* (AMACK; ANTUNES, 2020).

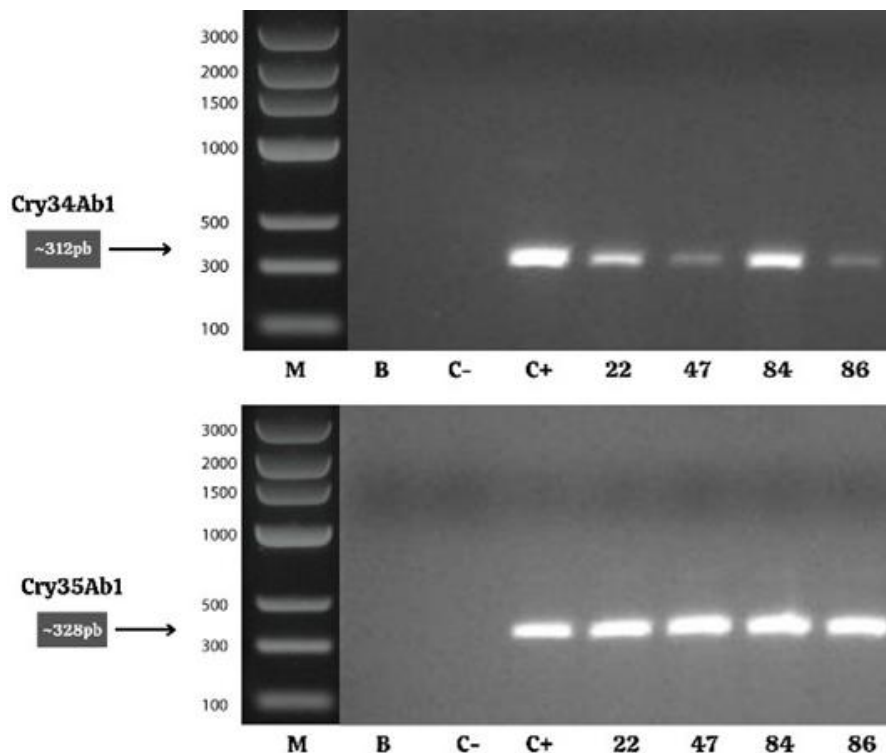


Fig. 17. Polymerase chain reaction for *cry34* and *cry35* genes. The 1kb Plus molecular weight marker (Invitrogen, USA) was used to assist in band differentiation. The no template control (NTC) had no nucleic acids in the reaction. The positive control (C+) was obtained by amplifying the transgenes

contained in the GS74103-2 pCry34_35Ab1_NPT vector. The negative control (C-) was obtained by amplifying DNA from a wild type plant.

Progeny analysis

Molecular progeny analyses demonstrated genomic integration and segregation of *Cry34* and *Cry35* transgenes in F1 plants following a Mendelian pattern, as shown in the table below:

Table 2. Segregation of transgenic lines in the T₁ generation compared with expected Mendelian ratios using a chi-square (χ^2) test with Yates' correction. In contrast to lines 22, 47, and 86, line 84 presents a distinct segregation pattern.

Lines	Positive	Negative	X ²	P-Value (Yates)	Mendelian Ratio
L22	14	2	0.26	0.60	15:1
L47	14	2	0.26	0.60	15:1
L84	13	3	0.08	0.77	3:1
L86	14	2	0.26	0.60	15:1

The observed segregation patterns of *cry34* and *cry35* genes were evaluated in the T₁ progeny using a chi-square (χ^2) test with Yates' correction. The analysis showed two distinct different patterns among the four transgenic events. Lines 22, 47, and 86 exhibited a 14:2 segregation ratio, which is more consistent with the 15:1 model ($P = 0.60$), suggesting the presence of two autonomously segregating loci. In contrast, line 84 exhibited a 3:1 segregation ratio, which is consistent with a high probability of fit ($P = 0.77$) to the expected Mendelian 3:1 ratio, consistent with a single-locus insertion. Regarding transgene stability, single-locus insertion are preferred to guarantee desirable inheritance and stable gene expression, thus, obtaining single-locus events is better for breeding programs, as simple Mendelian inheritance facilitates trait stabilization. Multi-copies could be undesirable due to the risk of forming inverted repeats within the genome, which folds on itself, forming hairpin-like double-stranded RNA (dsRNA) structures (Fig. 17). These molecules are characteristic of pathogen infections, triggering gene silencing by the RNA interference (RNAi) pathway both at the transcriptional (DNA

methylation) and post-transcriptional (mRNA cleavage) levels (Basso *et al.*, 2020; Willoughby, 2025; Zhan; Meyers, 2023).

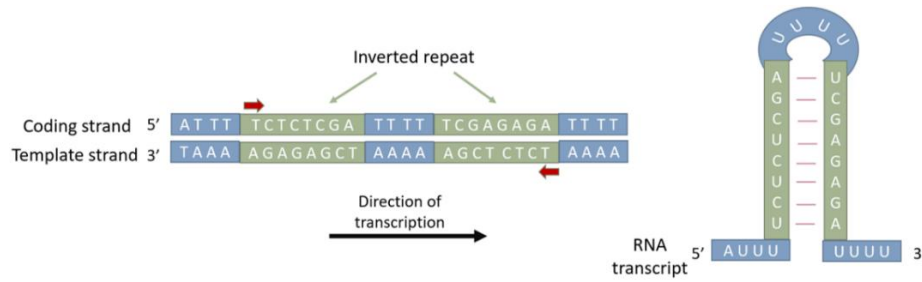


Fig. 18. RNA hairpin-like structure via inverted repeats. Transcription of RNA from inverted repeats. Adapted: (Shen, 2019).

***Tuta absoluta* life cycle completion bioassay**

Herbivory bioassays were conducted for 30 days and demonstrated that lines 22, 47, 84, and 86 (T₄ generation) expressing Cry34/35Ab1 protoxins failed to prevent tissue consumption. All plants exposed to the Leafminer insects were decimated and died in this period, suggesting the inefficiency of the Cry34/35Ab1 protoxins in disrupting larvae's ability to feast on MicroTom-Cry34/35Ab1 tissue (fig. 19).



Fig. 19. *T. absoluta* life cycle completion bioassay on MicroTom-Cry34/35Ab1 lines.

Analysis utilizing one-way ANOVA (Fig. 20) and Dunnett's tests concluded that *T. absoluta* life cycle completion in Cry34/35Ab1- MicroTom plants was disturbed by reducing the number of emerged adults when compared to WT plants, which is in accordance with the results obtained by Fernandes *et al* (2023) in their work on MicroTom expressing Cry10a plants when fed by the leafminer. Interestingly, full adult emergence in the transgenic lines analyzed was delayed relative to WT plants. González-Cabrera *et al* (2011) observed Leafminer mortality, as well as reduced adult emergence in tomato by applying Biolep® (Biorun Company, Iran), a commercial biological pesticide formulated with *B. thuringiensis subsp. Kurstaki*. These results

demonstrate the efficacy of Bt proteins in managing *T. absoluta* and the potential of endogenous expression of Bt protoxins in crops.

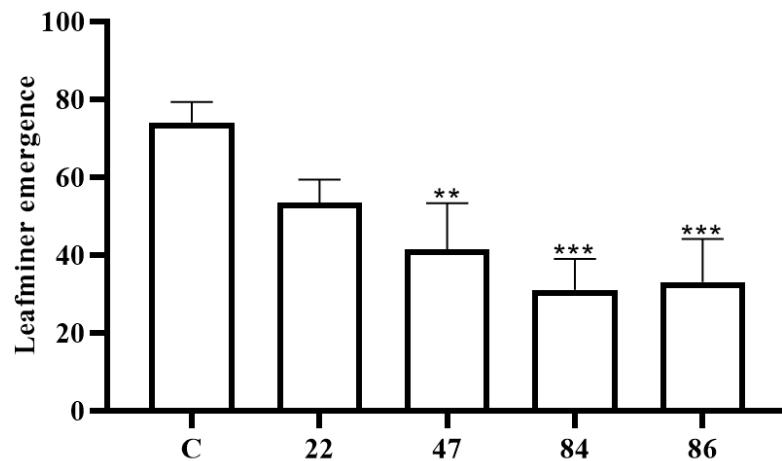


Fig. 20. *T. absoluta* life cycle completion bioassay. Adult insect emergence on WT MicroTom when compared to Cry34/35Ab1 transgenic lines by ANOVA and Dunnett's tests. Two asterisks indicate values less than $P < 0.01$, whereas three asterisks indicate values less than $P < 0.001$, which are considered highly and very highly significant, respectively. P-values: 22 (0.0532), 47 (0.0038), 84 (0.0005), and 86 (0.0007).

Evaluation of Cry34Ab1 and Cry35Ab1 efficacy against *Spodoptera frugiperda*

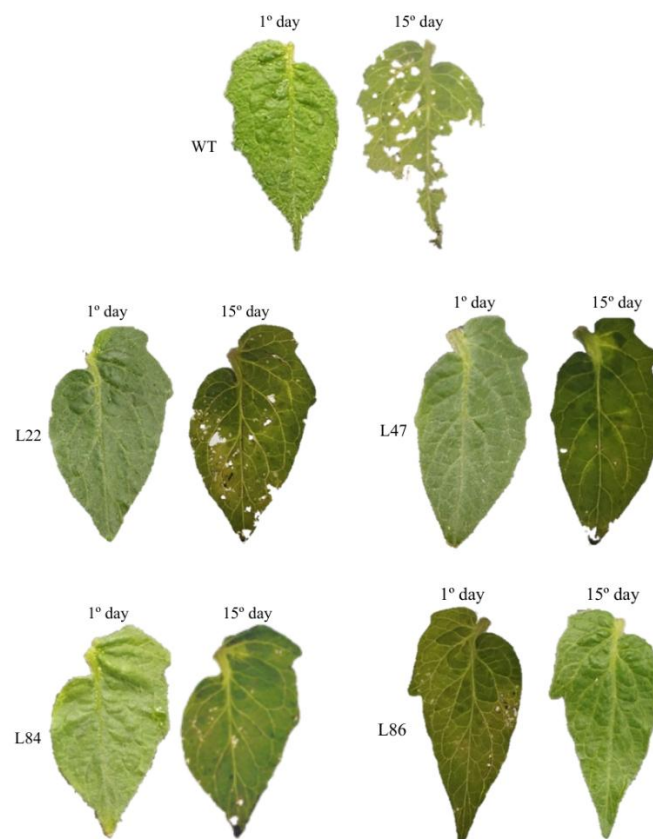


Fig. 21. GM leaves showing *S. frugiperda* feeding damage.

Leaves from Cry34/35Ab1 transgenic lines and wild-type (WT) control after 15 days of herbivory.

Three bioassays were conducted over 15 days to evaluate the efficacy of the Cry34/35Ab1 GM lines against Fall Armyworm. Results showed a significant reduction in leaf consumption compared to the WT control group (Fig. 20). First-instar larvae, when allowed to eat detached leaves, showed delayed development by the end of the experiment, with a clear size difference compared to larvae feeding on WT leaves. Quantification of leaf damage and ANOVA tests revealed that the percentage of tissue consumed was significantly lower in the GM lines than in the WT control. L47 and L84 demonstrated the lowest consumption compared to L22 and L86. Regardless, treatment groups differed extensively compared with the control group, with all treatment groups demonstrating p-values lower than $p < 0.0001$ (Fig. 21). This difference in consumption could be associated with protein expression. This result aligns with studies indicating that distinct classes of Bt proteins associated with toxicity against Coleoptera can also exhibit cross-order toxicity towards Lepidoptera (van Frankenhuyzen, 2013; Fernandes *et al.*, 2023; Silva *et al.*, 2024;). The level of control observed may vary depending on the expression levels of Cry34/35Ab1 proteins and the susceptibility of certain insect populations. This study used laboratory *S. frugiperda* colonies, although field populations could employ different molecular strategies to evade the toxin mechanism of Bt protoxins (Legan *et al.*, 2024; Silva *et al.*, 2024).

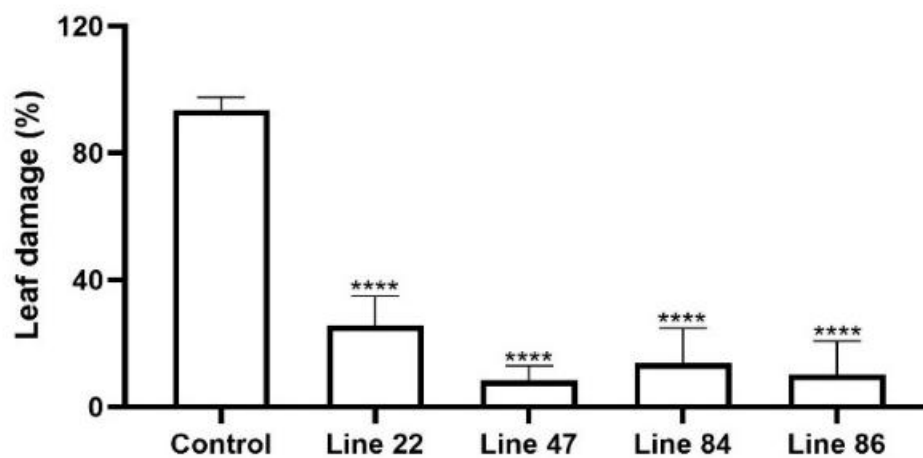


Fig. 22. *S. frugiperda* herbivory bioassay. Percentage of leaf area consumed by *S. frugiperda* on Cry34/35Ab1 transgenic lines when compared to non-GMO plants. Four asterisks indicate values are very highly significant ($P < 0.0001$). All P-values were lower than 0.0001.

CONCLUSION

The present work demonstrates that transgenic MicroTom-Cry34/35Ab1 lines prevent *Spodoptera frugiperda* (FAW) herbivory. This finding aligns with the field efficacy of commercial GM corn Herculex Xtra® against *S. frugiperda*, although in this cultivar, this trait is solely recognized as the action of Cry1F toxicity, the expression of Cry34/35Ab1 may also contribute. In contrast, toxicity against *Tuta absoluta* was modest and not sufficiently relevant for plant breeding. Larvae herbivory was demonstrated to be equal to that of wild-type MicroTom plants, resulting in a mild reduction and delay in adult emergence. This partial tolerance in *T. absoluta* might be explained by a lack of binding sites in the epithelial midgut, or possibly by an increase in stem cells in the adjacent tissue, as observed in the CRW midgut in response to Cry34/35Ab1 feeding (Bowling *et al.*, 2017). To confirm this hypothesis, histopathology assays should be conducted to visualize the cellular integrity of the midgut in Leafminer larvae fed by Bt proteins before plant expression, saving time and resources that plant genetic engineering demands. Increased insect resistance could also be achieved by high Cry34/35Ab1 tissue accumulation. Unfortunately, expression quantification via commercial immunoassay detection methods was not possible in this study because of a six-amino acid difference between the Cry34Ab1 variant expressed here and the antigen used for commercial antibody production. Given that these transgenic MicroTom-Cry34/35Ab1 lines are now available, bioassays targeting other insect tomato pests would be a valuable next step to characterize the agronomic potential of this genotype. The release of these Bt proteins into a tomato cultivar presents a facilitated path to commercialization in Brazil, as Cry34/35Ab1 has a long history of safe use in GM maize. This should aid regulatory approval by CTNBio. Genetically engineering plants for endogenous resistance prevents the overuse of chemical insecticides, thereby mitigating pest selection pressure, one of the main issues in agriculture today. To make sure this technology for Fall Armyworm is effective in the long term for control, it must be integrated into an Insect Resistance Management (IRM) strategy. This includes crop rotation, refuge areas for pests, different and alternating chemical control strategies, and the stacking of multiple Bt proteins in a single cultivar (Al Naggar *et al.*, 2025b).

PERSPECTIVES

- RT-PCR for transgene expression quantification.
- Development of immunoassays for Cry34/35Ab1 detection.
- Bioassays against other tomato insect pests.
- Field bioassays.
- Comparative study of Cry34Ab1 commercial protein variants.
- Stacking of multiple Bt protoxins.
- Midgut histopathology of *Tuta absoluta*.

REFERENCES

AL NAGGAR, Y. *et al.* Mechanisms and genetic drivers of resistance of insect pests to insecticides and approaches to its control. **Toxics**, v. 13, n. 8, p. 681, 2025. Available at: <https://doi.org/10.3390/toxics13080681>.

ALI, M. Y. *et al.* Nutritional composition and bioactive compounds in tomatoes and their impact on human health and disease: a review. **Foods**, v. 10, n. 1, p. 45, 2020. Available at: <https://doi.org/10.3390/foods10010045>.

AMACK, S. C.; ANTUNES, M. S. CaMV35S promoter – A plant biology and biotechnology workhorse in the era of synthetic biology. **Current Plant Biology**, v. 24, p. 100179, 2020. Available at: <https://doi.org/10.1016/j.cpb.2020.100179>.

BAKRY, M. M. S.; ABDEL-BAKY, N. F. Impact of the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae) infestation on maize growth characteristics and yield loss. **Brazilian Journal of Biology**, v. 84, 2024. Available at: <https://doi.org/10.1590/1519-6984.274602>.

BALDONI, A. B. *et al.* Immunolocalization of Ricin accumulation during castor bean (*Ricinus communis* L.) seed development. **International Journal of Plant Biology**, v. 1, n. 2, p. e12, 2010. Available at: <https://doi.org/10.4081/pb.2010.e12>.

BASSO, M. F. *et al.* Insights into genetic and molecular elements for transgenic crop development. **Frontiers in Plant Science**, v. 11, 2020. Available at: <https://doi.org/10.3389/fpls.2020.00509>.

BAWIN, T. *et al.* Ability of *Tuta absoluta* (Lepidoptera: Gelechiidae) to develop on alternative host plant species. **The Canadian Entomologist**, v. 148, n. 4, p. 434–442, 2016. Available at: <https://doi.org/10.4039/tce.2015.59>.

BEVAN, M. W.; CHILTON, M. D. Multiple transcripts of T-DNA detected in nopaline crown gall tumors. **Journal of Molecular and Applied Genetics**, v. 1, n. 6, p. 539–546, 1982.

BIONDI, A. *et al.* Ecology, worldwide spread, and management of the invasive South American tomato pinworm, *Tuta absoluta*: past, present, and future. **Annual Review of**

Entomology, v. 63, n. 1, p. 239–258, 2018. Available at: <https://doi.org/10.1146/annurev-ento-031616-034933>.

BLANCARD, D. Tomate: Tuta absoluta. **Ephytia (INRAe)**, 2022. Available at: <https://ephytia.inra.fr/pt/C/5150/Tomate-Tuta-absoluta>.

BOWLING, A. *et al.* Histopathological effects of Bt and TcdA insecticidal proteins on the midgut epithelium of western corn rootworm larvae (*Diabrotica virgifera virgifera*). **Toxins**, v. 9, n. 5, p. 156, 2017. Available at: <https://doi.org/10.3390/toxins9050156>.

BRASILEIRO, A. C. M.; CARNEIRO, V. T. C. (org.). **Manual de Transformação Genética de Plantas. 2. ed.** Brasília: Embrapa, 2015. v. 1.

BRAVO, A.; GILL, S. S.; SOBERÓN, M. Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. **Toxicon**, v. 49, n. 4, p. 423–435, 2007. Available at: <https://doi.org/10.1016/j.toxicon.2006.11.022>.

BUTALA, M. *et al.* Aegerolysins: lipid-binding proteins with versatile functions. **Seminars in Cell & Developmental Biology**, v. 72, p. 142–151, 2017. Available at: <https://doi.org/10.1016/j.semcdb.2017.05.002>.

CABI. **CABI BioProtection Portal**. 2025. Available at: <https://bioprotectionportal.com/resources/tuta-absoluta-control-what-is-it-and-how-do-you-get-rid-of-it/>.

CHAUDHURY, D. *et al.* *Agrobacterium tumefaciens*-mediated high frequency genetic transformation of an Indian cowpea (*Vigna unguiculata* L. Walp.) cultivar and transmission of transgenes into progeny. **Plant Science**, v. 172, n. 4, p. 692–700, 2006. Available at: <https://doi.org/10.1016/j.plantsci.2006.11.009>.

CHILTON, M. D. *et al.* *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. **Proceedings of the National Academy of Sciences**, v. 71, n. 9, p. 3672–3676, 1974. Available at: <https://doi.org/10.1073/pnas.71.9.3672>.

CRICKMORE, N. *et al.* A structure-based nomenclature for *Bacillus thuringiensis* and other bacteria-derived pesticidal proteins. **Journal of Invertebrate Pathology**, v. 186, p. 107438, 2021. Available at: <https://doi.org/10.1016/j.jip.2020.107438>.

DALBIANCO, A. B. *et al.* Development of *Tuta absoluta* (Meyrick, 1917) (Lepidoptera: Gelechiidae) in tomato genotypes. **Comunicata Scientiae**, v. 16, e4224, 2024. Available at: <https://doi.org/10.14295/cs.v16.4224>.

DE MAAGD, R. How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. **Trends in Genetics**, v. 17, n. 4, p. 193–199, 2001. Available at: [https://doi.org/10.1016/S0168-9525\(01\)02237-5](https://doi.org/10.1016/S0168-9525(01)02237-5).

DEIST, B. *et al.* Bt toxin modification for enhanced efficacy. **Toxins**, v. 6, n. 10, p. 3005–3027, 2014. Available at: <https://doi.org/10.3390/toxins6103005>.

EXPRESSION of the Cry10Aa toxin in transgenic tomato confers tolerance to the tomato leafminer (*Tuta absoluta*). **Scientia Horticulturae**, v. 319, p. 112161, 2023. Available at: <https://doi.org/10.1016/j.scienta.2023.112161>.

FAO. **Food and Agriculture Organization of the United Nations**. 2025. Available at: <http://www.fao.org/home/en/>.

FERNÁNDEZ-CHAPA, D.; RAMÍREZ-VILLALOBOS, J.; GALÁN-WONG, L. Toxic potential of *Bacillus thuringiensis*: an overview. In: Protecting Rice Grains in the Post-Genomic Era. **IntechOpen**, 2019. Available at: <https://doi.org/10.5772/intechopen.85756>.

FRALEY, R. T. Liposome-mediated delivery of tobacco mosaic virus RNA into petunia protoplast. **Plant Molecular Biology**, v. 2, n. 1, 1983. Available at: <https://doi.org/10.1007/BF00187570>

GASPARINI, K. *et al.* Micro-Tom tomato: from ornamental horticulture to fundamental research. **Horticulture Advances**, v. 3, n. 1, p. 8, 2025. Available at: <https://doi.org/10.1007/s44281-025-00062-x>.

GE, S. *et al.* An internal marking method for adult *Spodoptera frugiperda* Smith using an artificial diet containing Calco Oil Red N-1700. **Insects**, v. 15, n. 8, p. 561, 2024. Available at: <https://doi.org/10.3390/insects15080561>.

GONZALEZ, C. *et al.* Tomato cv. ‘Micro-Tom’ as a model system to study postharvest chilling tolerance. **Scientia Horticulturae**, v. 184, p. 63–69, 2015. Available at: <https://doi.org/10.1016/j.scienta.2014.12.020>.

GONZÁLEZ-CABRERA, J. *et al.* Efficacy of *Bacillus thuringiensis* (Berliner) in controlling the tomato borer, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). **BioControl**, v. 56, n. 1, p. 71–80, 2011. Available at: <https://doi.org/10.1007/s10526-010-9310-1>.

GORALOGIA, G. S.; WILLIG, C.; STRAUSS, S. H. Engineering *Agrobacterium* for improved plant transformation. **The Plant Journal**, v. 121, n. 5, 2025. Available at: <https://doi.org/10.1111/tpj.70015>.

GRACE SUNDAY, G. N. *et al.* *Agrobacterium tumefaciens*: biology and application in genetic engineering. **GSC Advanced Research and Reviews**, v. 20, n. 1, p. 389–398, 2024. Available at: <https://doi.org/10.30574/gscarr.2024.20.1.0272>.

GUEDES, R. N. C. *et al.* Insecticide resistance in the tomato pinworm *Tuta absoluta*: patterns, spread, mechanisms, management and outlook. **Journal of Pest Science**, v. 92, n. 4, p. 1329–1342, 2019. Available at: <https://doi.org/10.1007/s10340-019-01086-9>.

HAN, P. *et al.* *Tuta absoluta* continues to disperse in Asia: damage, ongoing management and future challenges. **Journal of Pest Science**, v. 92, n. 4, p. 1317–1327, 2018. Available at: <https://doi.org/10.1007/s10340-018-1062-1>.

HECKEL, D. G. How do toxins from *Bacillus thuringiensis* kill insects? An evolutionary perspective. **Archives of Insect Biochemistry and Physiology**, v. 104, n. 2, 2020. Available at: <https://doi.org/10.1002/arch.21673>.

HERRERA-ESTRELLA, L. *et al.* Chimeric genes as dominant selectable markers in plant cells. **The EMBO Journal**, v. 2, n. 6, p. 987–995, 1983. Available at: <https://doi.org/10.1002/j.1460-2075.1983.tb01532.x>.

HERRERO, S. *et al.* Susceptibility, mechanisms of response and resistance to *Bacillus thuringiensis* toxins in *Spodoptera* spp. **Current Opinion in Insect Science**, v. 15, p. 89–96, 2016. Available at: <https://doi.org/10.1016/j.cois.2016.04.006>.

HÖFTE, H.; WHITELEY, H. R. Insecticidal crystal proteins of *Bacillus thuringiensis*. **Microbiological Reviews**, v. 53, n. 2, p. 242–255, 1989. Available at: <https://doi.org/10.1128/mr.53.2.242-255.1989>.

HOOD, E. E. *et al.* New *Agrobacterium* helper plasmids for gene transfer to plants. **Transgenic Research**, v. 2, n. 4, p. 208–218, 1993. Available at: <https://doi.org/10.1007/BF01977351>.

HUDSON, W.; WALTZ, F.; JOSEPH, S. Armyworms in Sod. **University of Georgia Cooperative Extension**, 2025. Available at: <https://fieldreport.caes.uga.edu/publications/C1130/armyworms-in-sod/>.

ISAAA. cry34Ab1: Genes List. **GM Approval Database**, 2026. Available at: <https://www.isaaa.org/gmapprovaldatabase/gene/default.asp?GeneID=39&Gene=cry34Ab1>.

JIN, K. *et al.* Comparative transcriptome analysis of *Agrobacterium tumefaciens* reveals the molecular basis for the recalcitrant genetic transformation of *Camellia sinensis* L. **Biomolecules**, v. 12, n. 5, p. 688, 2022. Available at: <https://doi.org/10.3390/biom12050688>.

KARLSSON GREEN, K.; STENBERG, J. A.; LANKINEN, Å. Making sense of Integrated Pest Management (IPM) in the light of evolution. **Evolutionary Applications**, v. 13, n. 8, p. 1791–1805, 2020. Available at: <https://doi.org/10.1111/eva.13067>.

KATTA, S.; ADDEPALLY, U.; GARLADINNE, M. An efficient and rapid kanamycin screening assay for identifying true transgenics in cotton. **Journal of Pharmacognosy and Phytochemistry**, v. 10, n. 3, p. 357–359, 2021. Available at: <https://doi.org/10.22271/phyto.2021.v10.i3e.14096>.

KELKER, M. S. *et al.* Structural and biophysical characterization of *Bacillus thuringiensis* insecticidal proteins Cry34Ab1 and Cry35Ab1. **PLoS ONE**, v. 9, n. 11, e112555, 2014. Available at: <https://doi.org/10.1371/journal.pone.0112555>.

KENENI, G. *et al.* Genetic vulnerability of modern crop cultivars: causes, mechanism and remedies. **International Journal of Plant Research**, v. 2, n. 3, p. 69–79, 2012. Available at: <https://doi.org/10.5923/j.plant.20120203.05>.

LACORTE, C. *et al.* Molecular characterization of genetically manipulated plants. In: DAVEY, M. R.; ANTHONY, P. Plant cell culture: essential methods. **Chichester**: John Wiley & Sons, 2010. p. 261–280. Available at: <https://onlinelibrary.wiley.com/doi/book/10.1002/9780470686522>.

LEGAN, A. W. *et al.* Mismatch between lab-generated and field-evolved resistance to transgenic Bt crops in *Helicoverpa zea*. **Proceedings of the National Academy of Sciences**, v. 121, n. 47, 2024. Available at: <https://doi.org/10.1073/pnas.2416091121>.

LI, H. *et al.* Bacillus thuringiensis Cry34Ab1/Cry35Ab1 interactions with western corn rootworm midgut membrane binding sites. **PLoS ONE**, v. 8, n. 1, e53079, 2013. Available at: <https://doi.org/10.1371/journal.pone.0053079>.

LI, T. H. *et al.* Variation in egg mass scale thickness of three Spodoptera species and its effects on egg parasitoid performance. **Journal of Pest Science**, v. 96, n. 4, p. 1393–1402, 2023. Available at: <https://doi.org/10.1007/s10340-023-01608-6>.

LIU, J. *et al.* Environmental fate of Bt proteins in soil: transport, adsorption/desorption and degradation. **Ecotoxicology and Environmental Safety**, v. 226, p. 112805, 2021. Available at: <https://doi.org/10.1016/j.ecoenv.2021.112805>.

LIU, Y. *et al.* Engineered chimeric insecticidal crystalline protein improves resistance to lepidopteran insects in rice (*Oryza sativa* L.) and maize (*Zea mays* L.). **Scientific Reports**, v. 12, n. 1, 2022. Available at: <https://doi.org/10.1038/s41598-022-16426-6>.

LIU, Y. *et al.* Ingestion of Bt corn pollen containing Cry1Ab/2Aj or Cry1Ac does not harm *Propylea japonica* larvae. **Scientific Reports**, v. 6, n. 1, 2016. Available at: <https://doi.org/10.1038/srep23507>.

MAHMOUD, Y. A. *et al.* Susceptibility of different tomato, *Solanum lycopersicum* L., varieties to infestation with some insect pests in Egypt. **Bulletin of the National Research Centre**, v. 44, n. 1, p. 46, 2020. Available at: <https://doi.org/10.1186/s42269-020-00300-4>.

MASAYA PANDURO-TENAZOA, N. *et al.* The life cycle of Fall Armyworm (FAW), **Spodoptera frugiperda** and toxicity of botanicals against 2nd instar larvae on maize. 2022. Available at: www.isisn.org.

MASSON, L. *et al.* A novel Bacillus thuringiensis (PS149B1) containing a Cry34Ab1/Cry35Ab1 binary toxin specific for the western corn rootworm *Diabrotica virgifera virgifera* LeConte forms ion channels in lipid membranes. **Biochemistry**, v. 43, n. 38, p. 12349–12357, 2004. Available at: <https://doi.org/10.1021/bi048946z>.

MEHROTRA, S.; GOYAL, V. Agrobacterium-mediated gene transfer in plants and biosafety considerations. **Applied Biochemistry and Biotechnology**, v. 168, n. 7, p. 1953–1975, 2012. Available at: <https://doi.org/10.1007/s12010-012-9910-6>.

MEISSNER, R. *et al.* A new model system for tomato genetics. **The Plant Journal**, v. 12, n. 6, p. 1465–1472, 1997. Available at: <https://doi.org/10.1046/j.1365-3113x.1997.12061465.x>.

MURASHIGE, T.; SKOOG, F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. **Physiologia Plantarum**, v. 15, n. 3, p. 473–497, 1962. Available at: <https://doi.org/10.1111/j.1399-3054.1962.tb08052.x>.

NARVA, K. E.; WANG, N. X.; HERMAN, R. Safety considerations derived from Cry34Ab1/Cry35Ab1 structure and function. **Journal of Invertebrate Pathology**, v. 142, p. 27–33, 2017. Available at: <https://doi.org/10.1016/j.jip.2016.07.019>.

OHMINE, Y. *et al.* Successful transfer of a model T-DNA plasmid to *E. coli* revealed its dependence on recipient RecA and the preference of VirD2 relaxase for eukaryotes rather than bacteria as recipients. **Frontiers in Microbiology**, v. 9, 2018. Available at: <https://doi.org/10.3389/fmicb.2018.00895>

ORTIZ, A.; SANSINENEA, E. Genetically modified plants based on *Bacillus* genes and commercial *Bacillus*-based biopesticides for sustainable agriculture. **Horticulturae**, v. 9, n. 9, p. 963, 2023. Available at: <https://doi.org/10.3390/horticulturae9090963>.

PADMANABHAN, P.; CHEEMA, A.; PALIYATH, G. Solanaceous fruits including tomato, eggplant, and peppers. In: **Encyclopedia of Food and Health**. Elsevier, 2016. p. 24–32. Available at: <https://doi.org/10.1016/B978-0-12-384947-2.00696-6>.

PANDEY, M. *et al.* A review on biology and possible management strategies of tomato leaf miner, *Tuta absoluta* (Meyrick), Lepidoptera: Gelechiidae in Nepal. **Heliyon**, v. 9, n. 6, e16474, 2023. Available at: <https://doi.org/10.1016/j.heliyon.2023.e16474>.

PANEVSKA, A. *et al.* Aegerolysins from the fungal genus *Pleurotus* – Bioinsecticidal proteins with multiple potential applications. **Journal of Invertebrate Pathology**, v. 186, p. 107474, 2021. Available at: <https://doi.org/10.1016/j.jip.2020.107474>.

PARDO-LÓPEZ, L.; SOBERÓN, M.; BRAVO, A. *Bacillus thuringiensis* insecticidal three-domain Cry toxins: mode of action, insect resistance and consequences for crop protection. **FEMS Microbiology Reviews**, v. 37, n. 1, p. 3–22, 2013. Available at: <https://doi.org/10.1111/j.1574-6976.2012.00341.x>.

PARK, S. *et al.* Agrobacterium T-DNA integration into the plant genome can occur without the activity of key non-homologous end-joining proteins. **The Plant Journal**, v. 81, n. 6, p. 934–946, 2015. Available at: <https://doi.org/10.1111/tpj.12779>.

PEREIRA, D. L. *et al.* Recent assessment and characterization of *Tuta absoluta* resistance to cartap hydrochloride. **Pesticide Biochemistry and Physiology**, v. 193, p. 105420, 2023. Available at: <https://doi.org/10.1016/j.pestbp.2023.105420>.

PHULE KRISHI VIDYAPEETH, M. *et al.* Seasonal abundance of tomato pinworm, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) on tomato in Western Maharashtra, India. **Journal of Entomology and Zoology Studies**, v. 9, n. 2, p. 220–223, 2021. Available at: <http://www.entomoljournal.com>.

RESENDE, D. C. *et al.* Does Bt maize cultivation affect the non-target insect community in the agro ecosystem? **Revista Brasileira de Entomologia**, v. 60, n. 1, p. 82–93, 2016. Available at: <https://doi.org/10.1016/j.rbe.2015.12.001>.

RODITAKIS, E. *et al.* A four-year survey on insecticide resistance and likelihood of chemical control failure for tomato leaf miner *Tuta absoluta* in the European/Asian region. **Journal of Pest Science**, v. 91, n. 1, p. 421–435, 2018. Available at: <https://doi.org/10.1007/s10340-017-0900-x>.

ROGERS, S. O.; BENDICH, A. J. Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. **Plant Molecular Biology**, v. 5, n. 2, p. 69–76, 1985.

ROSTAMI, E. *et al.* Life table parameters of the tomato leaf miner *Tuta absoluta* (Lepidoptera: Gelechiidae) on different tomato cultivars. **Journal of Applied Entomology**, v. 141, n. 1–2, p. 88–96, 2017. Available at: <https://doi.org/10.1111/jen.12319>.

SCHNEPF, E. *et al.* *Bacillus thuringiensis* and its pesticidal crystal proteins. **Microbiology and Molecular Biology Reviews**, v. 62, n. 3, p. 775–806, 1998. Available at: <https://doi.org/10.1128/MMBR.62.3.775-806.1998>.

SHAHNAM, A.; MAHIN, P. *Agrobacterium tumefaciens* - mediated plant transformation: a review. **Molecular Biotechnology**, v. 66, n. 1, p. 1563–1580, 2023. Available at: <https://doi.org/10.1007/s12033-023-00788-x>.

SHEN, C. H. Gene expression: transcription of the genetic code. In: **Diagnostic Molecular Biology**. Elsevier, 2019. p. 59–86. Available at: <https://doi.org/10.1016/B978-0-12-802823-0.00003-1>.

SHI, Y. *et al.* Effect of soil moisture on pupation behavior and inhabitation of *Spodoptera frugiperda* (Lepidoptera: Noctuidae). **Applied Entomology and Zoology**, v. 56, n. 1, p. 69–74, 2021. Available at: <https://doi.org/10.1007/s13355-020-00709-3>.

SILVA, A. F. T. *et al.* Susceptibility of Fall Armyworm field populations to Vip3Aa/Cry Bt maize in a tropical agricultural region. **Agronomy**, v. 14, n. 3, p. 451, 2024. Available at: <https://doi.org/10.3390/agronomy14030451>.

SIMMONS, A. T.; GURR, G. M. Trichomes of *Lycopersicon* species and their hybrids: effects on pests and natural enemies. **Agricultural and Forest Entomology**, v. 7, n. 4, p. 265–276, 2005. Available at: <https://doi.org/10.1111/j.1461-9555.2005.00271.x>.

SOHRABI, F. *et al.* Evaluation of ten tomato cultivars for resistance against tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) under field infestation conditions. **Entomologia Generalis**, v. 36, n. 2, p. 163–175, 2016. Available at: <https://doi.org/10.1127/entomologia/2016/0350>

SOUZA, B. H. S. de. *et al.* Feeding non-preference by *Spodoptera frugiperda* and *Spodoptera eridania* on tomato genotypes. **Revista Ceres**, v. 60, n. 1, p. 21–29, 2013. Available at: <https://doi.org/10.1590/S0034-737X2013000100004>.

SUN, H. J. *et al.* A highly efficient transformation protocol for Micro-Tom, a model cultivar for tomato functional genomics. **Plant and Cell Physiology**, v. 47, n. 3, p. 426–431, 2006. Available at: <https://doi.org/10.1093/pcp/pci251>.

TAN, S. Y. *et al.* RNAi induced knockdown of a cadherin-like protein (EF531715) does not affect toxicity of Cry34/35Ab1 or Cry3Aa to *Diabrotica virgifera virgifera* larvae (Coleoptera: Chrysomelidae). **Insect Biochemistry and Molecular Biology**, v. 75, p. 117–124, 2016. Available at: <https://doi.org/10.1016/j.ibmb.2016.06.006>.

TOMATO GENOME CONSORTIUM, THE. The tomato genome sequence provides insights into fleshy fruit evolution. **Nature**, v. 485, p. 635–641, 2012. Available at: <https://doi.org/10.1038/nature11119>.

TUNCEL, A. *et al.* Genome-edited foods. **Nature Reviews Bioengineering**, 2023. Available at: <https://doi.org/10.1038/s44222-023-00115-8>.

VAN FRANKENHUYZEN, K. Cross-order and cross-phylum activity of *Bacillus thuringiensis* pesticidal proteins. **Journal of Invertebrate Pathology**, v. 114, n. 1, p. 76–85, 2013. Available at: <https://doi.org/10.1016/j.jip.2013.05.010>.

WAGNER, E. M. Monitoring gene expression: quantitative real-time RT-PCR. In: FREEMAN, L. **Lipoproteins and Cardiovascular Disease**. Totowa, New Jersey: Humana Press, 2013. p. 19-45. (Methods in Molecular Biology). Available at: https://doi.org/10.1007/978-1-60327-369-5_2.

WANG, F. *et al.* Associations between western flower thrips and tomato spotted wilt virus. **Insect Science**, 2026. Available at: <https://doi.org/10.1111/1744-7917.70243>.

WANG, K. *et al.* Effective bacterial insecticidal proteins against coleopteran pests: a review. **Archives of Insect Biochemistry and Physiology**, v. 102, n. 3, 2019. Available at: <https://onlinelibrary.wiley.com/doi/10.1002/arch.21558>.

WILLOUGHBY, A. C. Aberrant RNA identification reveals triggers of transgene silencing. **The Plant Cell**, v. 37, n. 11, 2025. Available at: <https://doi.org/10.1093/plcell/koaf274>.

WU, L. *et al.* Fitness of fall armyworm, *Spodoptera frugiperda* to three solanaceous vegetables. **Journal of Integrative Agriculture**, v. 20, n. 3, p. 755–763, 2021. Available at: [https://doi.org/10.1016/S2095-3119\(20\)63476-1](https://doi.org/10.1016/S2095-3119(20)63476-1).

YU, W. *et al.* Stable transformation mediated by *Agrobacterium tumefaciens* in Jonquil is better than *Agrobacterium rhizogenes*. **Frontiers in Plant Science**, v. 16, 2025. Available at: <https://doi.org/10.3389/fpls.2025.1594197>.

ZHAN, J.; MEYERS, B. C. Plant Small RNAs: their biogenesis, regulatory roles, and functions. **Annual Review of Plant Biology**, v. 74, n. 1, p. 21–51, 2023. Available at: <https://doi.org/10.1146/annurev-arplant-070122-035226>.