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CROP PROTECTION

Caenorhabditis elegans as an indicator of toxicity of *Bacillus thuringiensis* strains to *Meloidogyne incognita* race 3

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ABSTRACT: *The cotton plant (Gossypium hirsutum) is affected by several diseases of economic importance, among them root-knot nematode (Meloidogyne incognita races 3 and 4). Methods to control this disease include the application of nematicides, solarization, deep plowing, crop rotation and use of antagonistic microorganisms. Among species of Bacillus, there are strains that act as bioregulators and antagonists of several pathogens. Tests to identify these strains are hampered by the difficulty of obtaining large populations of the pathogen and by the time of execution of the in vivo tests that should be conducted for about 90 days. The objective of this research was to compare the toxicity of B. thuringiensis strains to two nematodes, M. incognita and Caenorhabditis elegans, evaluating the possibility of using C. elegans as an indicator for the selection of strains with biocontrol potential against M. incognita. Therefore, the toxicity of nine B. thuringiensis strains on C. elegans and M. incognita was evaluated under laboratory and greenhouse conditions. Most strains toxic to C. elegans in vitro were also toxic to M. incognita, and three of them (S906, S1192, S2036) significantly reduced the populations of the two nematodes. The toxic effect of B. thuringiensis strains on C. elegans was like that reported for the same bacterial isolates on M. incognita in vivo. These results suggested that it is plausible to use C. elegans as an indicator of toxicity for selection of B. thuringiensis strains toxic to M. incognita.*
Key words: *Biocontrol, phytonematode, root-knot nematode, rhizobacteria.*

Caenorhabditis elegans como indicador de toxicidade de estirpes de *Bacillus thuringiensis* a *Meloidogyne incognita* raça 3

RESUMO: *O algodoeiro (Gossypium hirsutum) é acometido por várias doenças de importância econômica, dentre as quais a meloidoginose (Meloidogyne incognita raças 3 e 4). Entre os métodos de controle dessa doença, destacam-se as aplicações de nematicidas, a solarização, a aração profunda, a rotação de culturas e o uso de microrganismos antagonistas. Dentre as espécies do gênero Bacillus, existem estirpes que atuam como biorreguladores e antagonistas de vários patógenos. Os testes para identificação dessas estirpes são prejudicados pela dificuldade de se obter grandes populações do patógeno e pelo tempo de execução dos testes in vivo que devem ser conduzidos por cerca de 90 dias. Diante disso, o presente trabalho teve como objetivo comparar a toxicidade de estirpes de B. thuringiensis a dois nematoides, M. icognita e Caenorhabditis elegans, verificando a possibilidade de empregar C. elegans como indicador para a seleção de estirpes com potencial de biocontrole contra M. incognita. Para tanto, a toxicidade de nove estirpes de B. thuringiensis para C. elegans e M. incognita foi avaliada em laboratório e em casa de vegetação. A maioria das estirpes tóxicas ao C. elegans in vitro, também foi tóxica ao M. incognita, sendo que três delas (S906, S1192, S2036) reduziram significativamente as populações dos dois nematoides. O efeito tóxico apresentado pelas estirpes de B. thuringiensis contra C. elegans foram similares aos apresentados pelos mesmos isolados contra M. incognita in vivo. Esses resultados sugerem que é plausível o uso do C. elegans como indicador de toxicidade para seleção de estirpes de B. thuringiensis* tóxicas a M. *incognita.*
Palavras-chave: *Biocontrole, fitonematoide, nematoide das galhas, rizobactérias.*

INTRODUCTION

Cotton (*Gossypium hirsutum* L.) is grown in more than 60 countries, covering an area of more than 35 million hectares and producing around 25 million tons of plume per year, and is; therefore, one of the most important agricultural commodities in the world (ABRAPA, 2015).

Soil and climatic conditions of the producing regions, associated with their cultivation systems, sometimes in extensive areas and with few cultivated varieties, facilitate the appearance of diseases (SUASSUNA & COUTINHO, 2007; AMORIM et al., 2011). Among them, root-knot nematode caused by *Meloidogyne incognita* race 3 stands out. This nematode, after penetrating the host root, moves to the root cortex where it establishes the infection site, initiating the feeding process (MOENS et al., 2009). It remains at this site feeding and undergoing several ecdysis until

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emerging as a male or female adult (EISENBACK & TRIANTAPHYLLOU, 1991; MOENS et al., 2009). This characteristic offers several benefits, such as protection against inclement weather and natural enemies, and makes it difficult to develop effective control methods. The rich food available favors its reproduction and a single female can produce from 200 to 1,000 eggs (FREIRE, 2007). *M. incognita* stands out today as the main plant-parasitic nematode of the cotton crop in Brazil, causing losses of more than 40% in highly infested areas (FREIRE et al., 2015).

Among the control methods are the prevention of pathogen entry into areas free of infection, use of resistant varieties, seed nematicide applications, solarization, deep plowing, crop rotation and incorporation of organic residues and the use of antagonists (FERRAZ et al., 2012). There is a wide range of rhizosphere bacteria antagonistic to phytopathogens that colonize roots of plants, and act as bio-controllers producing substances toxic to pathogens(LUZ,1996;TURAetal.,2007;MACHADO et al., 2012). However, no method is fully effective.

Bacillus are recognized as bioregulators and natural antagonists of several phytopathogens, in addition to promoting growth and inducing resistance in plants (MAHDY et al., 2001; VONDERWELL et al., 2001; ONGENA & JACQUES, 2007; RUSSI, 2012). These bacteria are gram-positive, spore forming, and some have the ability to produce protein crystals during their sporulation phase, such as *B. thuringiensis*. Proteins contained in these crystals may be toxic to insects of different orders and to nematodes, as is the case of the previously described nematocidal proteins Cry5B, Cry6A, Cry14A, Cry21A and Cry55A (WEI et al., 2003; MONNERAT et al. 2001; GUO et al., 2008). There is great demand to identify and select bacterial strains with high control activity on *M. incognita* to enable their use as tools in biological control programs. However, *in vitro* and *in vivo* assays with this nematode are hampered by the difficulty in obtaining large populations in a short period of time.

In addition, *in vivo* tests should be performed to verify results obtained *in vitro* and are quite laborious and time consuming, as the cycle of *M. incognita* lasts about 28 days and to be safe in the evaluations of the experiments, they should be conducted for approximately 90 days. In addition, *M. incognita* is an obligate plant parasite, making it difficult to use in *in vitro* selection trials. Thus, it would make sense to substitute this nematode with an organism that can be more easily managed. *Caenorhabditis elegans*, a free-living nematode inhabitant of moist soils that uses atmospheric oxygen and feeds on bacteria, appears to be a plausible alternative (BRENNER, 1974). This nematode is now used as a model organism for the most varied types of research (SCHIERENBERG & WOOD, 1985; DONALD, 1997). The present research aimed to evaluate the potential of *C. elegans* as an indicator organism for selection of strains of *B. thuringiensis* with biocontrol potential against *M. incognita* Race 3.

MATERIALS AND METHODS

Bacillus thuringiensis strains

Bacterial strains used in these experiments were isolated from soil from different regions of Brazil using the methodology of World Health Organization, (1985), they were: S906, S1185, S1192, S2036, S2038, S2193, S2493 and S2496 (all strains are deposited in the Invertebrate Bacteria Collection of Embrapa Genetic Resources and Biotechnology). These bacteria were cultured in Erlenmeyer flasks (500mL) containing 150mL of Embrapa medium (MONNERAT et al., 2007), in a rotary incubator at 150RPM (28± 2ºC) for 72h. After this culture period the morphology of strains was analyzed with a phase contrast microscope to observe the presence of the protein crystal (MONNERAT et al., 2001) and its concentration was quantified according to the methodology of ROMEIRO (1989) and then adjusted with saline solution to $3X10^{7}mL^{-1}$.

Maintenance of the C. elegans population

Maintenance of the *C. elegans* colony was carried out according to the protocol of STIERNAGLE (2006) with modifications. This nematode was given to Embrapa by Dr. Colin Berry of Cardiff University. The nematode feeding was done using *E. coli* bacteria, OP50 strain, multiplied in a rotary shaker (150RPM, 36ºC, 12h) in Luria-Bertani medium (LB) (SAMBROOK & RUSSELL, 2001). Every two days 1mL of the bacterial suspension was given to the nematodes in the culture plates. The colonies of *C. elegans* were kept in an incubator (Marconi, mod. MA 403) in the absence of light (21ºC).

Maintenance of the population of Meloidogyne incognita

The initial inoculum of *M. incognita* Race 3, provided by the Phytonematode Laboratory at Embrapa Genetic Resources and Biotechnology, were

multiplied in tomato plants (*Solanum lycopersicum* cv. Santa Clara) for 3 months in greenhouse. After this period, eggs were extracted from the plants according to the method described by HUSSEY & BARKER (1973), modified by BONETI & FERRAZ, (1981). After extraction, eggs were surface disinfected by a method described by ZUCKERMAN & BRZESKI (1966). Eggs were suspended in 30mL of commercial PerioGard® product (chlorhexidine gluconate solution 0.12%) with antibiotics added (10µg/mL erythromycin, 2.5g/L streptomycin) for 30min. They were then centrifuged (3min, 360g), and after this, the supernatant was discarded and the eggs present in the pellet were suspended in sterile distilled water. The procedure was repeated twice. In a sterile environment eggs were placed in a modified Baermann funnel (FLEGG, 1967) for hatching. Second-stage juveniles (J2) of *M. incognita* were collected every 2 days in a sterile environment and placed in a capped vial under refrigeration (4±2°C).

Selection of strains toxic to C. elegans

The assay was performed in Petri dishes (90X15mm) to which 7.5mL of the *C. elegans* suspension (concentration of 300 nematodes/mL) and 2.5mL of the bacterial suspension (concentration of $3X10^7$CFU/mL) were added. As controls, pure Embrapa medium (EM), Embrapa medium (MONNERAT et al., 2007) plus erythromycin (10µg/mL), carbofuran (a liter of the comercial product/20L of water) and saline solution (8,5g of HCl/water liter) were used. Plates were identified, dated and their edges were sealed with plastic film, to avoid contamination. The assay was incubated in the dark at 21°C for 48h. After this period, number of nematodes was evaluated with an optical microscope. For this, 2mL of the contents of each plate was collected, diluted 30 times (in tap water) and with the help of a Peters chamber and an optical microscope, counts of live individuals (they are very agile and easily identified under an optical microscope) were performed in the suspension. For each plate three counts were performed, and the mean of these counts was used for statistical analysis.

The experiment was a completely randomized in design with three replicates per treatment. The data obtained from the number of live nematodes per plates were transformed into √x, to facilitate the statistical analyses, submitted to analysis of variance and the means compared by Duncan test (P≤0.05) [Assistat version 7.7 (SILVA & AZEVEDO, 2014)].

This bioassay was performed using nematodes obtained with the Baermann funnel, following the same methodology as described for *C. elegans*.

Selection of strains toxic to M. incognita Race 3 in vivo

This experiment was carried out under greenhouse conditions. Tomato seedlings (*Solanum lycopersicum* cv. Santa Clara) produced in Styrofoam trays were transplanted one week after emergence into 2-liter vessels with Bio-Plant® compost (sphagnum peat, coconut fiber, rice husk, pine-bark, vermiculite, and nutrients) and autoclaved soil (1:1). One week after transplantation, the seedlings were inoculated, with the aid of micropipette, with 5mL of the bacterial suspension ($3x10^7$CFU/mL), this suspension was inoculated in the soil next to the base of seedlings. For the controls, distilled water was inoculated instead of suspension of bacteria. Five days after the first inoculation, nematode inoculation was performed. For this, a suspension containing 10,000eggs was deposited in three equidistant furrows dug approximately 2cm deep. Immediately after inoculation of the nematodes, the bacteria were again inoculated as at the start of the assay. Plants were kept in a greenhouse for a period of three months (28°C±80% RH). Irrigation was performed daily at the end of the afternoon and monthly fertilizations used three grams of the Dimy® (granulated commercial fertilizer 04 - 14 - 08). Weekly thinning of senescent leaves was performed to avoid pest emergence; flowers were also removed to avoid fruit production.

The assay was evaluated after 3 months. The aerial part of the plants was discarded and the root system were taken to the Laboratory of Nematology where eggs were extracted according to the method of HUSSEY & BARKER (1973) modified by BONETI & FERRAZ, 1981). Egg suspensions were homogenized and with a Pasteur pipette, 2mL of the suspension were transferred to beakers (100mL) and the volume was adjusted to 60mL with water. Three samples of 1mL of each suspension were counted in a Peters chamber and the mean of these was used to calculate egg concentration and reproduction factor (OOSTENBRINK, 1966).

The experiment was a completely randomized in design with four replicates per treatment. The data obtained from the RF calculation were transformed into √x, to facilitate the statistical analyses, submitted to analysis of variance and the means compared by Duncan test (P≤0.05) [Assistat

version 7.7 (SILVA & AZEVEDO, 2014)]. All the assays were replicate two times.

Molecular characterization of toxic strains

The strains were characterized for the presence of *cry* genes of *B. thuringiensis*. The procedure of DNA extraction of the selected bacteria to carry out the Polymerase Chain Reaction was done as described by BRAVO et al. (1998), in which the bacteria were cultured in solid Embrapa medium (MONNERAT et al., 2007) for 16h at 30°C. After this period, for each sample, the bacterial growth was collected with a loop and transferred to a 1.5mL polyethylene tube properly identified and containing 200µL of sterile ultra-purified water (Mili Q). Samples were then homogenized in Vortex apparatus and frozen at -80°C for 20min and then boiled in a 100°C water bath for 10min and finally incubated on ice for 2min. The supernatant obtained was used for reactions. Different oligonucleotides were used in the PCR reaction, 5µL of the DNA from each strain was transferred to a 0.2mL polyethylene tube containing 0.5µM of each oligonucleotide, 0.2mM dNTP, 1X Taq buffer and 2.5U of Taq DNA polymerase (5.0U), totaling a final volume of 30µL. Oligonucleotides were used to identify the following genes: *cry1, cry2, cry3, cry4, cry5, cry8, cry9, cry10, cry11, cry12, cry13, cry14, cry17, cry19, cry21, cry24, cry25, cry32, cry39, cry40, cyt1* and *cyt2*. PCR conditions were performed for each oligonucleotide as described by CERON et al. (1994), CERON et al. (1995), BRAVO et al. (1998) and IBARRA et al. (2003). For analysis of the result, 25µL of the mixed PCR product was applied to 5µL of 10X run buffer in 1.5% agarose gel. Electrophoresis run was done in TBE 1X buffer (Tris base; boric acid; 0.5M EDTA - pH 8.0). Subsequently, the gel was stained with ethidium bromide at 1µg/mL for 20min and followed by distilled water for 15min. The gel was visualized by transilluminator under UV light and photo-documented (Eagle Eye, Stratagene).

RESULTS AND DISCUSSION

Assay with C. elegans in vitro and M. incognita in vitro and in vivo

Of the eight *B. thuringiensis* strains used in this assay, six were toxic to *C. elegans*. The other two matched the control. The highest response was presented by strain S906 with greater effectiveness than the control (saline solution), and reduction by more than 51% of the nematode population, followed by strain S2493 with 41.8% control. Other strains caused a reduction in the nematode population ranging from 14.7 to 25.6% of the control.

Molecular characterization of strains showed that 4 of them had known *cry* genes and 4 of them did not present expected PCR products for the detection of *cry* genes. As all showed protein crystals when observed in phase contrast microscopy, it is likely that other genes are involved in the formation of the protein evidenced by the presence of crystals. The most toxic strain S906 was one of the strains that despite presenting crystals, did not present expected PCR products with the primers tested. In addition, the S2493 strain, which resulted in a reduction of 41.8% in the nematode population, shows the *cry6* gene, which encodes the *Cry6* toxin described as a nematicide (BRAVO et al., 2012; PALMA et al., 2014), corroborating the results we obtained in this assay. SILVERA & LENGUA (2015) tested the activity of *Cry6* on gastrointestinal nematodes of sheep (*Nematodirus spathiger*), reporting control of 44.7% and 45.6% in eggs and larvae, respectively. The strain S2038 presented the *cry11* gene, which encodes proteins that are toxic to mosquito larvae (FERNÁNDEZ et al., 2005), and recently reported as toxic to *Haemonchus contortus* (LARAet.al., 2016). Strains S2193 and S2496 did not differ from the control, presenting *cry1*, *cry2* and *cry52* genes that encode proteins that were not described as toxic to nematodes. Other strains that had an antagonistic effect on the nematode (S1192, S1185 and S2036) did not show PCR products for the genes tested, indicating that these strains should have toxin genes different from those known. The nematicide carbofuran presented a statistically superior result to the other treatments.

The *in vitro* test results with *M. incognita* (Table 1) showed the strains caused mortality, between 4 and 41.1%, lower than that obtained in the *C. elegans* assays. The S2036, S1192 and S906 strains caused mortality higher than the control, respectively of 41.1, 19.7 and 24.9%. However, because the protein crystal is too large to be ingested by *M. incognita*, making it impossible for it to be toxic, it is possible that the toxicity reported is attributed to other substances produced, such as enzymes, which may be acting through contact with the nematode (URWIN et al., 1997). In the *in vivo* assay, four strains (S2036, S1185, S1192 and S906) reduced the nematode population from 17.81 to 46.79%. This fact was also evidenced by the reproduction factor (RF), which ranged from 9.74 to 10.15 (Table 1).

The three strains toxic to *M. incognita in vitro* were toxic to *M. incognita in vivo* and *C.*

Table 1 - Number [transformed (√x)] of Caenorhabditis elegans in vitro and Meloydogine incognita in vitro and in vivo (NN), percentage of control (%C) and genes present in Bacillus thuringiensis strains.

Treatments	*cry gene*	Presence protein crystal	C. elegans		M. incognita *in vitro*		M. incognita *in vivo*	
			NN	% C	NN	% C	RF	% C
Control EM + erythromycin	-		81.9 a	----	13.7 ab	---	12.33 a*	0.0*
Control EM	-		78.3 ab	----	14.3 a	---	----	---
Initial inoculum	-		71.4 cd	----		---	---	---
Control saline solution	-		76.1 bc*	0,0*	13.5 ab*	0.0*	----	---
S2496	*cry52*	+	72.4 cd	9.5	12.8 ab	10.6	10.84 ab	22.69
S2193	*cry1Aa, cry1Ab, cry1Ad, cry1C, cry1D, cry1F, cry2Ab*	+	72.1 cd	10.2	13.1 ab	6.7	10.32 ab	30.02
S907	-	+	70.6 cd	13.6	12.8 ab	9.7	8.99 d	46.79
S2036	-	+	70.2 de	14.7	10.4 e	41.1	10.15 bc	32.21
S1185	-	+	68.1 de	19.5	13.3 ab	4.0	9.32 cd	44.82
S1192	-	+	67.3 de	21.6	12.1 cd	19.7	9.89 bc	35.64
S2038	*cry11*	+	65,6 e	25.6	14.0 ab	0.0	11.14 ab	17.81
S2493	*cry6*	+	58.0 f	41.8	12.6 ab	13.8	10.88 ab	22.10
S906	-	+	53.2 f	51.1	11.7 de	24.9	9.74 bc	37.64
Carbofuran	-		42.7 g	68.7	0.0 f	100	Nr	Nr

Values followed by the same letter do not differ by Duncan's test (P≤0.05). The original data was transformed (√x). *Reference value.

elegans. Results obtained in the bioassay with *M. incognita in vivo* were more similar to those obtained with *C. elegans* than with *M. incognita in vitro*. This can be explained by the fact that *M. incognita in vitro* does not feed on the Cry toxins of *B. thuringiensis*. In contrast, studies showed that strains of *B. thuringiensis* can colonize plants in a systemic way and facilitate the acquisition of toxin by pests (MONNERAT et al., 2009). Complementarily, *C. elegans*, being free-living and feeding on bacteria, and having a greater movement on the plates, has more contact with the bacteria and proteins dispersed in the medium in which it is found. Devidas & Rehberger (1992) working with *M. incognita* and *C. elegans in vitro* also noticed a greater sensitivity of the free-living nematode to the compounds produced by *B. thuringiensis* and attributed this fact to *C. elegans'* dietary habits, which predispose it to another mode of action of the *Bt* toxins where they would act on its gut and not just on the epidermis.

In all three assays the *M. incognita* toxic strains were also toxic to *C. elegans* and that those that were not toxic to *M. incognita* were also not toxic to *C. elegans*. Some toxic to *C. elegans* were not toxic to *M. incognita*. Conversely, no strain that killed *M. incognita* both *in vivo* and *in vitro* killed *C. elegans*.

Results allow us to infer that *C. elegans* can be an indicator of toxicity of *B. thuringiensis* strains to nematodes and this methodology can be useful when it is desired to carry out a selection of strains from *B. thuringiensis* collections.

CONCLUSION

The *M. incognita* toxic strains (S906, S1192, S2036) of *B. thuringiensis* were toxic to *C. elegans* and those that were not toxic to *M. incognita* were not toxic to *C. elegans*. In addition, *C. elegans* can be an indicator of toxicity of *B. thuringiensis* strains to *M. incognita* and this approach can be useful when it is desired to carry out a selection of strains from *B. thuringiensis* collections.

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REFERENCES

ABRAPA – Associação Brasileira dos Produtores de Algodão. **Relatório de Gestão da Abrapa Bienio 2013/2014**. Available from: <htttp://www.abrapa.com.br/Documents/relatorio_gestao-13-14.pdf>. Accessed:July,13,2015.

AMORIM, L. et al. **Manual de Fitopatologia**. Piracicaba: Agronômica Ceres. v.1, 4. Ed, 2011. p.383-387.

BONETI, J. I. S.; FERRAZ, S. Modificação do método de Hussey e Barker para extração de ovos de *Meloidogyne incognita* de raízes de cafeeiros. **Fitopatologia Brasileira**, 1981. v.6, 553p.

BRAVO, A. et al. Characterization of *cry* genes in a mexican *Bacillus thuringiensis* strain collection. **Apllied and Enviromental Microbiology,** v.64, p.4965-4972, 1998.

BRAVO, A. et al. Evolution of *Bacillus thuringiensis* Cry toxins insecticidal activity. **Microbial Biotechnology**, v.6, p.17–26, 2012. Available from: <https://onlinelibrary.wiley.com/doi/epdf/10.1111/j.1751-7915.2012.00342.x>. Accessed: Apr. 15, 2016. doi: 10.1111/j.1751-7915.2012.00342.x.

BRENNER, S. The genetics of *Caenorhabditis elegans*. **Genetics**, v.77, p.71–94, 1974.

CERON, J. et al. PCR analysis of the cryI insecticidal crystal family genes from *Bacillus thuringiensis*. **Applied and Environmental Microbiology**, v.60, p.353-356, 1994.

CERON, J. et al. Specific PCR primers directed to identify cryI and cryIII genes within a *Bacillus thuringiensis* strain collection. **Applied and Environmental Microbiology**, v.61, p.3826-3831, 1995.

IBARRA, J.E. et al. Diversity of *Bacillus thuringiensis* strains from Latin America with insecticidal activity against different mosquito species. **Applied and Environmental Microbiology**, v.69, p.5269-5274, 2003. doi: 10.1128/AEM.69.9.5269-5274.2003.

DONALD, D.L. (Ed.). ***C. elegans* II**. Cold Spring Harbor Laboratory Press. New York. 1997.

EISENBACK, J.D.; TRIANTAPHYLLOU, H.H. Root-knot nematode: *Meloidogyne* spp. and races. *In*: Nickle, W.R (ed.) **Manual of Agricultural Nematology**. Marcel Dekker, Inc., New York, USA, v.74, p.191-192, 1991.

FERNÁNDEZ, L.E. et al. Cry11Aa toxin from *Bacillus thuringiensis* binds its receptor in *Aedes aegypti* mosquito larvae trough loop α-8 of domain II. **FEBS Letters,** v.79, p.3508–3514, 2005. Available from: <https://febs.onlinelibrary.wiley.com/doi/epdf/10.1016/j.febslet.2005.05.032>. Accessed: May. 15, 2015. doi: 10.1016/j.febslet.2005.05.032.

FERRAZ, S. et al. **Manejo Sustentável de Fitonematoides**. Viçosa, MG, Ed. UFV, 2012. 306 p.

FLEGG, J. J. M. Extraction of *Xithinema* and *Longidorus* species from soil by a modification of Cobb's decanting and sieving technique. **Annual Applied Biology**, v.60, p.429-437, 1967. doi: 10.1111/j.1744-7348.1967.tb04497.x.

FREIRE, E. F. Algodão no Cerrado do Brasil. Brasília: **Associação Brasileira dos Produtores de Algodão**. 2007. 918 p. I1.

FREIRE, E. F. Algodão no Cerrado do Brasil. Brasília: **Associação Brasileira dos Produtores de Algodão**. 2015. 956 p.

GUO, S.X. et al. New strategy for isolating novel nematicidal crystal protein genes from *Bacillus thuringiensis* strain YBT-518. **Applied and Environmental Microbiology**, v.74, p.6997-7001, 2008. doi: 10.1128/AEM.01346-08.

HUSSEY, R. S.; BARKER, K. R. A comparison of methods of collecting inocula of *Meloidogyne incognita* spp., including a new technique. **Plant Disease Report**, v.57, p.1025-1028, 1973.

LARA, A.P.S.S. et al. Larvicidal activity of *Bacillus thuringiensis* var. *israelensis* Cry11Aa toxin against *Haemonchus contortus*. **Parasitology**, v.143, p.1665-1671, 2016. doi: 10.1017/S0031182016001451.

LUZ, W. C. Rizobactérias promotoras de crescimento de plantas e bioproteção. **Revisão Anual de Patologia de Plantas**, Passo Fundo v. 4, p. 1-49. 1996.

MACHADO, V. et al. Bactérias como agentes de controle biológico de fitonematóides. **Oecologia Australis**, Rydalmere, v.16, p.165-182, 2012. doi: 10.4257/oeco.2012.1602.02.

MAHDY, M. et al. Influence of plant species on the biological control activity of the antagonistic rhizobacterium *Rhizobium etli* strain G12 toward the root-knot nematode, *Meloidogyne incognita*. **Mededelingen Faculteit Landbouwwetenschappen Rijksuniversiteit Gent**, v.66, p.655-662, 2001.

MOENS, M. et al. *Meloidogyne s*pecies – a diverse group of novel and important plant parasites. In R.N. PERRY, M. MOENS, & J.L. STARR (Eds.), *Root-knot nematodes* (pp.1-17). Wallingford: **CABI International**. 2009.

MONNERAT, R.G. et al. Catálogo do banco de germoplasma de bactérias do gênero *Bacillus*. Brasília: **Embrapa-Cenargen**, 2001. 65p.

MONNERAT, R.G. et al. Characterization of Brazilian *Bacillus thuringiensis* strains active against *Spodoptera frugiperda*, *Plutella xylostella* and *Anticarsia gemmatalis*. **Biological Control**, v.41, p.291-295, 2007.

MONNERAT, R.G. et al. Translocation and insecticidal activity of *Bacillus thuringiensis* bacteria living inside of plants. **Microbial Biotechnology**, v.2, p.1560-1562, 2009. doi: 10.1111/j.1751-7915.2009.00116.x.

ONGENA, M.; JACQUES, P. *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. **Trends in Microbiology**, v.16, p.115-125, 2007. doi: 10.1016/j.tim.2007.12.009.

OOSTENBRINK, M. Major characteristics of the relation between nematodes and plants. **Mendelingen Landbouwhogeschool Wageningen**, v.66, p.1-46, 1966.

PALMA, L. et al. *Bacullus thuringiensis* Toxins: An overview of their Biocidal activity. **Toxins**, v.6, p.3296-3325, 2014. doi: 10.3390/toxins6123296.

ROMEIRO, R. S. **Técnica de microgota para contagem de células bacterianas viáveis em uma suspensão**. In: ROMEIRO, R. S. (ed.). Métodos em bacteriologia de plantas. Viçosa: Editora UFV, 1989. p.117-126, 1989.

SAMBROOK, J. & RUSSELL, D. W. **Molecular Cloning: A Laboratory Manual**, 3 ed. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press. pp. A2.2, 2001.

SCHIERENBERG, E.; WOOD, W. B. Control of cell-cycle timing in early embryos of *Caenorhabditis elegans*. **Developmental Biology**, v.107, p.337-354, 1985. doi: 10.1016/0012-1606(85)90316-1.

SILVA, F.A.; AZEVEDO, C.A.V. ASSISTAT. Software Assistência Estatística. 2014.

SILVERA, A. A. O.; LENGUA, F. R. M. Actividad Letal *in vitro* del Extracto Proteico Total de *Bacillus thuringiensis* sobre Huevos y Larvas (L3) Infectivas de *Nematodirus Spathiger*. **Revistas de Investigación UNMSM**, v.26, p.509-518, 2015. doi: 10.15381/rivep.v26i3.11187.

STIERNAGLE, T. Maintenance of *C. elegans*. WormBook. ed. The *C. elegans* Research Community, WormBook. 2006.

SUASSUNA, N. D.; COUTINHO, W. M. Manejo das principais doenças do algodoeiro no cerrado brasileiro. In FREIRE, E. C. (Ed.). Algodão no cerrado do Brasil, **Gráfica e Editora Positiva.** 2007. p.479-521.

URWIN, P. E. et al. Continual green-fluorescent protein monitoring of cauliflower mosaic virus 35 S promoter activity in nematode-induced feeding cells in Arabidopsis thaliana. **Molecular Plant-Microbe Interactions Journal**, v.10, p.394-400, 1997. doi: 10.1094/MPMI.1997.10.3.394.

WEI, J. Z. et al. *Bacillus thuringiensis* Crystal proteins that target nematodes. **Proceedings of the National Academy of Sciences of the United States of America**, v.100, p.2760-2765, 2003. doi: 10.1073/pnas.0538072100.

WORLD HEALTH ORGANIZATION. Informal consultation on the development of Bacillus sphaericus as a microbial larvicide. Geneva: UNDP: World Bank: WHO, 1985. 24p. Special Programme for Research and Training in Tropical Diseases (TDR).

ZUCKERMAN, B.M.; BRZESKI, M.W. Methods for the study of plant-parasitic nematodes in gnotobiotic root culture. **Nematologica**, v.11, p.453-466, 1966. doi: 10.1163/187529265X00636.