Endophytic bacteria in the biological control of *Spodoptera frugiperda*

https://doi.org/10.56238/sevened2024.004-008

Irisléia Pereira Soares de Sousa[1], Martielle Batista Fernandes[2], Michelle de Oliveira Santos[3], Amanda Dayanne Malta Matos[4], Sabrina Gonçalves Vieira de Castro[5], Luciele Barboza de Almeida[6], Marielly Maria Almeida Moura[7], Helena Souza Nascimento Santos[8], Jaqueline Pereira Medeiros da Silva[9] and Edson Hiydu Mizobutsi[10]

ABSTRACT

The objective of this study was to evaluate the pathogenicity of bacteria isolated from neem, Azadirachta indica, on adults of Spodoptera frugiperda. All bacterial suspensions evaluated were calibrated to a concentration of 5.0 x 108 cells/mL. The adults evaluated were the survivors of caterpillars that ingested corn leaves treated with bacterial suspensions. With these surviving adults, couples were formed, which were kept in cages. The longevity of males and females, the pre-oviposition and fertile period, the total number of eggs, fecundity and fertility of females were evaluated. Of the total isolates evaluated, 64.0% of them caused some adverse effect to adults, to the point of affecting one or more of the variables evaluated. The ingestion of the bacteria by the caterpillars reduced the longevity of adults, both male and female. Females had a reduction in the fertile period, in the number of layings, in fecundity and in fertility. Only the pre-oviposition period was not affected. The isolates Bacillus sp. Epi 9, Bacillus subtilis and Neem 10 stand out because they affect the largest number of variables evaluated. The results obtained in this study are promising and important, as this is the first report of bacteria isolated from neem with pathogenic action to S. frugiperda.

Keywords: Azadirachta indica, Biological control, Fall armyworm.

[1] Doctoral student in Plant Production;
State University of Montes Claros, UNIMONTES
E-mail: pereira.irisleia@yahoo.com.br
[2] Dr. in Plant Production;
[3] Undergraduate student in Agronomic Engineering;
[4] Doctoral student in Plant Production;
[5] Master's student in Plant Production;
[6] Master in Plant Production;
[7] Doctoral student in Plant Production;
[8] Doctoral student in Plant Production;
[9] Master's student in Plant Production;
[10] Doctor in Phytopathology

INTRODUCTION

Plant species are symbiotically associated with an extensive and diverse community of microorganisms, including bacteria and fungi (HARDOIM et al., 2015; VERMA et al., 2009). These endophytic microorganisms do not cause disease symptoms in their hosts, but live symbiotically with the plant (WILSON, 1995).

One of the most studied plant species in terms of its microbiota is *Azadirachta indica*, neem, due to its importance as a medicinal plant, since it is used by about 80.0% of developing nations (EID et al., 2017; CHUTULO & CHALANNAVAR, 2018), in addition to its importance as an insecticidal plant (VERMA et al., 2011). All parts of this plant have already been evaluated for use in insect control and all of them have been shown to be harmful to pests. It has already been verified that neem has an anti-feeding effect on insects, repelling oviposition and inhibiting other biological and physiological activities of these organisms. In addition to these interferences, neem can reduce the growth of insects, inhibit ecdysis and reproduction, and cause their death (ROEL, 2010; COSTA et al., 2004). Azadirachtin, the main compound present in neem, affects exoskeleton shedding and insect growth, as it resembles the ecdysis hormone for this reason (ANURADHA et al., 2007; MARTINEZ, 2011).

In *A. indica*, in addition to the secondary metabolites, fungi and bacteria symbiotically associated with this plant have been isolated, characterized and identified (VERMA et al., 2007; VERMA et al., 2009; VERMA et al., 2011; CARDOSO, 2012; KUSARI et al., 2012; SINGH et al., 2017; D´LUIS et al., 2017). These endophytic microorganisms are responsible for the partial or complete biosynthesis of the secondary metabolites of their host plants (RAJAGOPAL et al., 2012; LUDWIG-MÜLLER, 2015). An example of this fact was found with the endophytic fungus, *Eupenicillium parvum*, which was isolated from the neem plant. According to the authors, the substances azadirachtin A and B were identified in the filtrate of its artificial growth medium (KUSARI et al., 2012). The fungus *Nigrospora* sp., also endophytic of neem, produced in its growth medium solanapyrones N, O and C, substances analogous to those produced in the plant (WU et al., 2009).

Among the groups of microorganisms most cited in the literature and that have already been isolated from the neem plant are fungi (VERMA et al., 2007; VERMA et al., 2011), actinomycetes (VERMA et al., 2009; VERMA et al., 2011) and bacteria (D´LUIS et al., 2017).

Cardoso (2012) isolated 33 bacteria associated with the neem plant, among them 16 have already been identified, *Bacillus pumilus* (7), *Bacillus methylotrophicus* (1), *Bacillus licheniformis* (2), *Bacillus subtilis* (2) and *Bacillus amyloliquefaciens* (1), in addition to two others belonging to the genus *Bacillus* sp. and one to the genus *Methylobacterium* sp. This author found that some of these isolates have potential for the production of indole-3-acetic acid (IAA). Soares (2013)

evaluated the 33 isolates of Cardoso (2012) for pathogenicity and virulence of *S. frugiperda larvae.*The author found that when 10-day-old caterpillars ingested corn leaves treated with bacterial suspensions, it caused an increase in the duration and mortality of the larval and pupal stages, a reduction in the weight of male and female pupae, and an increase in adult deformation, which reduced the number of viable adults in the population.

The results obtained by Soares (2013) demonstrate the great potential of these bacteria isolated from *A. indica* for the control of *S. frugiperda.* This author proved the pathogenic action of these microorganisms on the survival and development of fall armyworm, which enabled the selection of those bacteria that are more virulent to *S. frugiperda*. These more virulent bacteria reduced the number of viable *S. frugiperda* adults that would be incorporated into the population. Despite the recognized importance of the results obtained by this author, he did not evaluate the performance of the surviving adults. In the present study, these adults were evaluated, since it is postulated that the ingestion of bacterial isolates by caterpillars negatively affects the fecundity and fertility of adults. Thus, based on the results obtained by Soares (2013), this study aimed to evaluate the fecundity and fertility of adults of *S. frugiperda* after the caterpillars ingested corn leaves treated with bacteria isolated from *A. indica*.

MATERIAL AND METHODS

The experiment was carried out in laboratories, under controlled conditions (temp.: 25±2ºC, U.R.: 60±10 % and photophase: 12 h), using newly hatched caterpillars of *Spodoptera frugiperda* taken from the stock rear, where they were fed an artificial diet (GREENE *et al.*, 1976). Adults were fed 10.0% honey solution.

The bacterial isolates evaluated were: Epi 1, Epi 7 (*Bacillus pumilus* - E value of 0.0, identity of 99.0% and number of access to Genbank - KR010188.1), Epi 9 (*Bacillus* sp. - E value of 3.e-50, identity of 95.0%, Genbank access number - KM678261.1), Epi 12, Epi 13 (*Bacillus* sp. - E value of 3.e-55, identity of 97.0%, Genbank access number - JN700129.1), Nim 5 (*Bacillus methylotrophicus* - E value of 0.0, identity of 99.0%, Genbank access number - KM659219.1), Neem 8 (*Bacillus subtilis* - E value of 0.0, identity of 98.0%, Genbank access number - KF818630.1), Neem 10, Neem 12, Neem 14 and Neem 15 (*Bacillus pumilus* - And value of 0.0, identity of 99.0%, Genbank access number - KR010188.1). These isolates were obtained from the Bacterioteca of the Phytopathology Laboratory of UNIMONTES, Janaúba Campus, MG, where they are stored in sterile mineral water and kept under controlled conditions (temp.: 25±2ºC, U.R.: 60±10 % and photophase: 14 h). These bacteria were isolated from the surface (epiphytic - Epi) and from the fermented extract (Neem) of *Azadirachta indica leaves* (CARDOSO, 2012).

CULTIVATION OF MAIZE PLANTS

Creole maize seeds, susceptible to *S. frugiperda,* were sown in plastic pots (3.0 dm3). The plants were kept under screened conditions. During the cultivation period, the plants did not receive any phytosanitary treatment for pest control. Leaves from the plant cartridge region, 15 days after germination, were used to feed newly hatched caterpillars of *S. frugiperda*, in order to carry out the assay with the adult insects of the pest.

MULTIPLICATION OF BACTERIAL ISOLATES

For the multiplication of bacterial isolates, TSA (Tryptic Soy Agar) solid culture medium (40 g in 1,000 mL distilled water) was used. The medium was sterilized in an autoclave, set to 120oC/1.0 atm, for 20 minutes. The bacterial isolates were multiplied in Petri dishes (90 mm x 15 mm) from the transfer of an aliquot (1.0 mL) obtained from the storage suspension. The isolates were incubated at room temperature for 24 hours.

To obtain the bacterial suspension, 5.0 mL of sterile saline solution (0.85% NaCl) was added to the incubated bacterial colonies. The bacterial cells were disaggregated using a sterile microscope glass slide and then transferred to test tubes (2.5 cm x 15 cm) for homogenization in a vortex apparatus. In a spectrophotometer set to 540 nm optical density, the absorbance of the bacterial suspensions of each of the isolates was adjusted so that the concentration reached 5.0 x 108 cells/mL. The absorbance adjustments were based on the growth curves of these bacterial isolates established by Silva (2014). Sterile NaCl (0.85%) was added to adjust the concentration of bacterial suspensions.

OBTAINING PUPAE OF *SPODOPTERA FRUGIPERDA*

Leaves removed from the central region of the corn plant cartridge were cut into fragments (5.0 cm x 5.0 cm) that were immersed for 20 seconds in the bacterial suspensions. This procedure was performed in a laminar flow chamber. As a control of the experiment, the fragments were immersed in sterile distilled water. The treated and untreated fragments were placed on filter paper to cause excess moisture loss.

In transparent plastic containers (250 mL), containing a thin layer of sterilized agar-agar medium, five fragments of treated or untreated corn leaves were distributed, depending on the treatment or control to be evaluated. Agar-agar was used to prevent the corn leaves from curling and also to maintain their turgidity. For each treatment (isolates and control), three of these containers were prepared. In each of the containers, approximately 200 newly hatched caterpillars of *S. frugiperda* were transferred over the corn leaves, which were closed with the lids. The caterpillars were left feeding on these leaves for three days, when the food was replaced by untreated ones. The

larvae remained feeding on the untreated leaves for another five days, when they were individualized in glass tubes with flat bottoms (2.5 cm x), also containing a thin 8,5 cmagar-water layer at the bottom and a fragment of inserted corn leaf, as previously described. In the tubes, the caterpillars were fed until pupation. Food exchange at this stage of the experiment was performed whenever necessary. The control caterpillars were reared on untreated corn during the entire larval period.

The pupae obtained were removed from the tubes, cleaned and sexed, as described by Butt and Cantu (1962). After sexing the pupae, the males and females were individualized in new glass tubes, where they remained until emergence.

EVALUATION OF *SURVIVING SPODOPTERA FRUGIPERDA* ADULTS

The emerged adults, males and females, up to two days of age and without deformations were used in the formation of S . *frugiperda pairs*. The couples were transferred, individualized, to a cage consisting of a PVC tube (7.0 cm in diameter x 10 cm in height), with the inner wall covered with sulfite paper, which served as a substrate for laying. The lower end of the cage was closed with a Petri dish (80 mm x 80 mm) and the upper end with a thin Voil-type fabric . The adults were fed in the cages with a 10.0% honey solution, which was changed every two days. The insects were kept in these conditions until death.

On a daily basis, during the oviposition period of the females, the eggs of *S. frugiperda* were removed from the cages, accounted for and placed on transparent acrylic plates (50 mm x 50 mm) lined with filter paper moistened with distilled water, aiming at hatching. The plates were kept in the laboratory under controlled conditions (temp.: 25±2ºC, RH: 60±10% and photophase: 12 h).

The longevity of males and females, the pre-oviposition and fertile period of females, the total number of lays, the total number of eggs per female (fecundity) and the viability of the eggs (fertility) were evaluated. The longevity of males and females corresponded to the time elapsed between emergence and death of the insect. The fertile period of the females corresponded to the time elapsed between the first and the last day of oviposition. The pre-oviposition period corresponded to the time elapsed between the emergence of the female until the day she started oviposition.

The experimental design was completely randomized and consisted of eleven treatments (corn leaves treated with bacterial isolates) and one control (untreated corn leaves). Each treatment consisted of 15 replicates (cages), each containing a pair of *S. frugiperda*.

Tests of homogeneity of variances and normality of errors were performed and, as the variables evaluated did not fit these requirements, the results were submitted to the Kruskal-Wallis analysis and the means were compared by the Bonferroni test, at 5% probability. The statistical program used in all analyses was Statisticx, version 9.0.

RESULTS

The ingestion of *corn leaves immersed in the suspensions of bacteria isolated from the neem plant,* A. indica, by the caterpillars of *S. frugiperda,* affected all the variables evaluated, except for the pre-oviposition period of the females of this insect (Table 1).

For longevity, a reduction in the life span of females was observed after the caterpillars of *S. frugiperda* ingested leaves containing the isolates Epi 9 and Neem 8, which caused them to live 5.0 days less than those of the control ($X2 = 88.1989$; $P < 0.00001$) (Table 1). The caterpillars that ingested the other isolates resulted in females that were as long-lived as those of the control. Males also had their longevity reduced ($X2 = 68.2614$; $P < 0.00001$). This reduction occurred after the caterpillars ingested the isolates Epi 7, Epi 9, Epi 13, Neem 8 and Neem 12, which made them live around 3.0 days less than those of the control. For the other treatments, the males were as long-lived as the control ones.

The pre-oviposition period of *S. frugiperda females* was not altered by the ingestion of bacterial isolates by the caterpillars, which were similar to the control ($X2 = 13.9380$; $P = 0.2364$) (Table 1). However, the fertile period of females was reduced by the ingestion of bacteria by the caterpillars ($X2 = 38.3200$; $P < 0.0001$). This occurred when the caterpillars ingested the isolates Epi 9, Epi 12, Neem 8 and Neem 10. There was a reduction of up to 4.0 days when they ingested the Epi 9 treatment. In the other treatments, the fertile period of the females was similar to that observed for the control.

The number of eggs laid by females was reduced after ingestion by the caterpillars of isolates Epi 9 and Neem 10 ($X2 = 27.0767$; $P < 0.0045$) (Table 1). The females of these treatments performed a maximum of up to 3.1 postures. In the other treatments, the number of eggs per female was similar to the control group.

The fecundity of females was reduced after the larvae ingested the isolates Epi 9, Neem 8, Neem 10 and Neem 12 ($X2 = 40.6837$; $P < 0.00001$) (Table 1). The number of eggs laid by these females ranged from 230 to 370. In the other treatments, the females laid an average number of eggs similar to the control. Fertility was also reduced by the ingestion of Epi 9 and Neem 12 isolates ($X^{2=}$ 87.8114; $P < 0.00001$). The viability of the eggs of these females ranged from 32.6% to 46.6%. For the other females evaluated, egg viability was similar to the control control.

Table 1. Longevity of male and female (days), pre-oviposition and fertile periods of females (days), number of eggs, fecundity (number) and fertility (%) of *Spodoptera frugiperda*, from caterpillars fed corn leaves treated with suspension of bacteria isolated from *Azadirachta indica*.

Isolated	Longevity*		Period*				
	Female	Male	Pre-oviposition	Fertile	No. of postures*	Fertility*	Fertility*
Control	13,8 ± 0,5** bc	12,3 ± 0,4 bc	4,0 ± 0,3 a	6,3 ± 0,3 c	7,1 ± 0,4 b	884,8 ± 76,3 c	96,9 ± 2,2 bc
Epi 1	16,3 ± 0,6 c	12,3 ± 0,8 bc	3,5 ± 0,3 a	5,5 ± 0,7 bc	6,3 ± 1,0 ab	750,6 ± 118,3 bc	100,0 ± 0,0 c
Epi 7	11,9 ± 1,3 ab	9,3 ± 0,7 a	3,8 ± 0,4 a	4,5 ± 0,8 abc	4,7 ± 0,5 ab	434,0 ± 52,6 abc	62,4 ± 9,4 ab
Epi 9	8,5 ± 0,5 a	9,5 ± 0,7 a	4,7 ± 0,9 a	1,8 ± 0,5 a	2,7 ± 0,8 a	230,7 ± 78,2 a	46,6 ± 15,7 a
Epi 12	15,3 ± 0,8 bc	12,9 ± 0,7 c	5,4 ± 0,6 a	3,1 ± 0,6 ab	4,0 ± 0,8 ab	413,9 ± 77,7 abc	84,6 ± 10,4 bc
Epi 13	11,7 ± 0,5 ab	9,3 ± 0,5 a	3.6 ± 0,2 a	4,0 ± 0,6 abc	4,3 ± 0,7 ab	536,1 ± 98,3 abc	100,0 ± 0,0 c
Nim 5	13,6 ± 0,5 bc	10,3 ± 0,4 abc	3,5 ± 0,2 a	4,8 ± 0,6 abc	5,3 ± 0,7 ab	659,9 ± 114,6 abc	96,6 ± 1,7 bc
Nim 8	8,5 ± 0,5 a	9,3 ± 0,5 a	3,6 ± 0,2 a	3,2 ± 0,5 ab	5,1 ± 0,8 ab	370,7 ± 60,3 ab	60,7 ± 11,2 ab
Nim 10	12,5 ± 0,8 abc	12,5 ± 0,5 c	4,4 ± 0,6 a	2,9 ± 0,8 ab	3,1 ± 0,9 a	307,6 ± 91,4 ab	100,0 ± 0,0 c
Nim12	11,3 ± 0,8 ab	9,5 ± 0,6 a	5,9 ± 0,9 a	3,9 ± 0,9 abc	4,3 ± 0,8 ab	308,2 ± 58,2 ab	32,6 ± 11,3 a
Nim 14	13,7 ± 0,8 bc	10,5 ± 0,5 abc	4,0 ± 0,2 a	4,8 ± 0,7 abc	5,3 ± 0,9 ab	746,9 ± 133,6 abc	97,7 ± 1,2 bc
Nim 15	16,9 ± 0,7 c	13,1 ± 0,5 c	4,1 ± 0,3 a	3,7 ± 0,8 abc	4,0 ± 0,8 ab	472,6 ± 113,0 abc	100,0 ± 0,0 c
X^2	88,20	68,26	13,94	38,32	27,08	40,68	87,81

* Means followed by the same letter in the columns do not differ significantly from each other, according to the Kruskal-Wallis test, at 5% probability.

DISCUSSION

In this study, 64.0% of the bacteria isolated from the neem plant caused some harmful effect to the adults of *Spodoptera frugiperda*, to the point of affecting one or more of the variables evaluated (Table 1). The bacteria Epi 7 (*Bacillus pumilus*), Epi 9 (*Bacillus* sp.), Epi 12 (unidentified), Epi 13 (*Bacillus* sp.), Neem 8 (*Bacillus subtilis*), Neem 10 (unidentified) and Neem 12 (unidentified) were entomopathogenic to adults. These isolates differed in terms of their virulence to adults of *S. frugiperda*. The isolates Epi 1, Neem 5, Neem 14 and Neem 15 were not pathogenic to adults, since they did not cause any change in the variables evaluated.

Gram-positive bacteria of the genus *Bacillus* have several mechanisms to infect and kill insects. Several species of this genus are entomopathogenic to several orders of insects and, for this reason, used for pest control (RAJASHEKHAR et al., 2017).

The longevity of males and females of *S. frugiperda* was reduced by the ingestion of entomopathogenic bacteria to adults (Table 1). Two of the isolates evaluated, Epi 9 and Neem 8, were effective in causing this reduction in both females and males. On the other hand, the isolates Epi 7, Epi 13 and Neem 12 only reduced the longevity of the males. Of these three isolates, Epi 7 and Epi

13, affected only males. The same was not found for the Neem 12 isolate, which also affected females, as it reduced their fecundity and fertility. None of the isolates affected the pre-oviposition period of the females.

Among the 11 isolates evaluated on adults of *S. frugiperda*, Epi 9 stood out from the others, because in addition to reducing longevity (females and males), it also affected the fertile period, the number of eggs, fertility and fecundity of females. This bacterium reduced the life span of females by 38.0%, the fertile period by 71.0%, the number of eggs by 62.5%, and its females were 74.0% less fertile and 51.0% less fertile.

Several hypotheses may explain this type of action observed for the Epi 9 isolate. The pathogenicity of this bacterium may be linked to the intracellular presence of a protein crystal such as that found in the bacterium *Bacillus thuringiensis*, but the genus *Bacillus* can produce a wide range of active substances (BRAVO et al., 2007; CRICKMORE et al., 2008; GUTIÉRREZ-MANERO et al., 2001).

At first, it can be suggested that the Epi 9 bacterium was able to produce active crystals containing both the Cry and Cyt proteins. It has been proven in several studies that the Cry and Cyt proteins synergistically occur, which causes greater toxicity of the bacterium to the hosts (BRAVO et al., 2007; CRIALESILEGOR et al., 2014; ÖSKAN et al., 2003; VILAS-BÔAS et al., 2012; RIBEIRO et al., 2017).

The proteins Cry and Cyt are synthesized in the form of protoxins. Thus, its action depends on activation processes, which occur inside the digestive tract of the insect (ANGELO et al., 2010). It has been shown that the Cyt protein can increase the toxicity of the Cry protein that functions as a receptor molecule (CRICKMORE et al., 1995; PÉREZ et al., 2005; PONCET et al., 1995). Thus, the higher efficiency of the Epi 9 isolate is probably linked to the production of these two proteins acting synergistically, i.e., Cry as a toxin and Cyt as its receptor. Thus, the synergism between Cry and Cyt may have caused damage to the digestive system of *S. frugiperda caterpillars* and, concomitantly, a reduction in the variables evaluated, due to an anti-feeding effect, for example, as a consequence, malnutrition in the insects. The lack of nutrients or even the low amount assimilated by the caterpillars may have affected the maturation of the ovaries of the females and the number of ovarioles present, which caused a reduction in the fecundity and fertility of the females (CHAPMAN, 2013).

Bacillus sp. (Epi 9) showed promise for use in integrated management programs of *S. frugiperda.* This is an important fact, because in Brazil most of the products sold are based on the bacterium *B. thuringiensis* (Bt) and for the domestic market this technology is imported, which results in an increase in the final price of this product to the consumer and, consequently, a decrease in the competitiveness of these biological products in relation to synthetic insecticides (ANGELO et

al., 2010). Another important fact to be highlighted is that, in the world, *B. thuringienis* is the only microbial insecticide with widespread use, and there are already several cases of insect species that have developed resistance to the toxin produced by this bacterium. This is one of the reasons why research has been carried out to evaluate other species of bacteria (BERGAMASCO et al., 2013; RAJASHEKHAR et al., 2017). The information obtained for the Epi 9 isolate sets precedents for the realization of new experiments, now carried out in the field, aiming to study and prove the efficiency of this entomopathogen under Brazilian crop conditions.

ACKNOWLEDGMENT

The Foundation for Research Support of the State of Minas Gerais (FAPEMIG) for financial support to the project and for the granting of Research Incentive Scholarships, the Coordination for the Improvement of Higher Education Personnel (CAPES) (for the granting of a Master's scholarship), and the National Council for Scientific and Technological Development (CNPq) (for the granting of a Research scholarship).

REFERENCES

1. Angelo, E. A., Vilas-Bôas, G. T., Castro-Gómez, R. J. H. (2010). Bacillus thuringiensis: características gerais e fermentação. Semina: Ciências Agrárias, 31(4), 945-958.

2. Anuradha, A., Annadurai, R. S., Shashidhara, L. S. (2007). Actincytoskeleton as a putative target of the neem limonoid azadirachtin A. Insect biochemistry and molecular biology, 37(6), 627-634.

3. Bergamasco, V. B., Mendes, D. R. P., Fernandes, O. A., Desidério, J. A., Lemos, M. V. F. (2013). Bacillus thuringiensis Cry1Ia10 and Vip3Aa protein interactions and their toxicity in Spodoptera spp. (Lepidoptera). Journal of invertebrate pathology, 112(2), 152-158.

4. Bravo, A., Gill, S. S., Soberon, M. (2007). Mode of action of Bacillus thuringiensis Cry and Cyt toxins and their potential for insect control. Toxicon, 49(4), 423-435.

5. Butt, B. (1962). Sex determination of lepidopterous pupae. USDA, Agricultural Research Service Report, 33(75), 1-7.

6. Cardoso, A. M. S. (2012). Caracterização fisiológica de isolados bacterianos obtidos de Azadirachta indica e de bananeira 'Prata-anã'. Monografia (Graduação em Agronomia) – Universidade Estadual de Montes Claros, Janaúba.

7. Chutulo, E.C., Chalannavar, R.K. (2018). Endophytic mycoflora and their bioactive compounds from Azadirachta Indica: A comprehensive review. Journal of Fungi, 4(42), 1-12.

8. Costa, E. L. N., Silva, R. F. P. da, Fiuza, L. M. (2004). Efeitos, aplicações e limitações de extratos de plantas inseticidas. Acta Biologica Leopoldensia, 26(2), 173-185.

9. Crickmore, N., Bone, E. J., Williams, J. A., Ellar, D. J. (1995). Contribution of the individual components of the δ-endotoxin crystal to the mosquitocidal activity of Bacillus thuringiensis subsp. israelensis. FEMS Microbiology Letters, 131(3), 249-254.

10. D´Luis, R. L., Chamorro, A. L., Pérez, C. A. (2017). Diversidad de bacterias endófitas aisladas de árbol de neem y su actividad inhibitoria contra el Colletotrichum gloesporioides causante de la antracnosis del ñame en el departamento de Sucre. Revista Colombiana de Ciência Animal-RECIA, 9, 48-54.

11. Eid, A., Jaradat, N., Elmarzugi, N. (2017). A Review of chemical constituents and traditional usage of Neem plant (Azadirachta Indica). Palestinian Medical and Pharmaceutical Journal (PMPJ), 2(2), 75-81.

12. Greene, G.L., Lepla, N.C., Dickerson, W.A. (1976). Velvetbean caterpillar: a rearing procedure and artificial medium. Journal of Economic Entomology, 69(4), 488-497.

13. Hardoim, P. R., Overbeek, L. S. V., Berg, G., Pirttilä, A. M., Compant, S., Campisano, A., Döring, M., Sessitsch, A. (2015). The hidden world within plants: Ecological and evolutionary considerations for defining functioning of microbial endophytes. Microbiology and Molecular Biology Reviews, 79, 293-320.

14. Kusari, S., Hertweck, C., Spiteller, M. (2012). An endophytic fungus from Azadirachta indica A. Juss. that produces azadirachtin. World journal of microbiology and biotechnology, 28(3), 1287-1294.

15. Ludwig-Müller, J. (2015). Plants and endophytes: Equal partners in secondary metabolite production?. Biotechnology letters, 37, 1325-1334.

16. Martinez, S. S. (2011). O nim - Azadirachta indica - um inseticida natural. Londrina: IAPAR. 2° Ed., 205 p.

17. Öskan, M., et al. (2003). Nutritional and cultural parameters influencing antidpteran delta-endotoxin production. Research in Microbiology, 154(1), 49-53.

18. Pérez, C., Fernandez, L. E., Sun, J., Folch, J. L., Gill, S. S., Soberón, M., & Bravo, A. (2005). Bacillus thuringiensis subsp. israelensis Cyt1Aa synergizes Cry11Aa toxin by functioning as a membrane-bound receptor. Proceedings of the National Academy of Sciences, 102(51), 18303-18308.

19. Poncet, S., Delécluse, A., Klier, A., Rapoport, G. (1995). Evaluation of synergistic interactions among the CryIVA, CryIVB, and CryIVD toxic components of B. thuringiensis subsp. israelensis crystals. Journal of Invertebrate Pathology, 66(2), 131-135.

20. Rajagopal, K., Maheswari, S., Kathiravan, G. (2012). Diversity of endophytic fungi in some tropical medicinal plants - A report. African Journal of Microbiology Research, 6, 2822-2827.

21. Rajashekhar, M., Shahanaz, E., Vinay, K. (2017). Biochemical and molecular characterization of Bacillus spp. isolated from insects. Journal of Entomology and Zoology Studies, 5(5), 581-588.

22. Ribeiro, B. M., Martins, É. S., De Souza Aguiar, R. W., Corrêa, R. F. T. (2017). Expression of Bacillus thuringiensis toxins in insect cells. In: Bacillus thuringiensis and Lysinibacillus sphaericus. Springer, Cham, 99-110.

23. Roel, A. R., Dourado, D. M., Matias, R., Porto, K. R., Bednaski, A. V., Costa, R. B. D. (2010). The effect of sub-lethal doses of Azadirachta indica (Meliaceae) oil on the midgut of Spodoptera frugiperda (Lepidoptera, Noctuidae). Revista Brasileira de Entomologia, 54(3), 505-510.

24. Silva, H. D. (2014). Virulência de bactérias associadas a Azadirachta indica sobre Spodoptera frugiperda. Dissertação (Mestrado em Produção Vegetal no Semiárido) - Universidade Estadual de Montes Claros, Janaúba, MG, 73 p.

25. Singh, A. K., Sharma, R. K., Sharma, V., Singh, T., Kumar, R., Kumari, D. (2017). Isolation, morphological identification and in vitro antibacterial activity of endophytic bacteria isolated from Azadirachta indica (neem) leaves. Veterinary world, 10(5), 510-516.

26. Soares, E. P. S. (2013). Patogenicidade de bactérias isoladas de nim à Spodoptera frugiperda (J. E. Smith). Dissertação (Mestrado em Produção Vegetal no Semiárido) - Universidade Estadual de Montes Claros, Janaúba, MG, 82 p.

27. Verma, V. C., Gond, S. K., Kumar, A., Kharwar, R. N., Strobel, G. (2007). The endophytic mycoflora of bark, leaf, and stem tissues of Azadirachta indica A. Juss. (Neem) from Varanasi (India). Microbial Ecology, 54(1), 119-125.

28. Verma, V. C., Gond, S. K., Kumar, A., Mishra, A., Kharwar, R. N., Gange, A. C. (2009). Endophytic actinomycetes from Azadirachta indica A. Juss.: isolation, diversity, and anti-microbial activity. Microbial Ecology, 57(4), 749-756.

29. Verma, V. C., Gond, S. K., Kumar, A., Kharwar, R. N., Boulanger, L. A., Strobel, G. A. (2011). Endophytic fungal flora from roots and fruits of an Indian neem plant Azadirachta indica A. Juss., and impact of culture media on their isolation. Indian Journal of Microbiology, 51(4), 469-476.

30. Vilas-Bôas, G. T., Alvarez, R. C., dos Santos, C. A., Vilas-Boas, L. A., et al. (2012). Fatores de virulência de Bacillus thuringiensis: o que existe além das proteínas Cry. EntomoBrasilis, 5(1), 1-10.

31. Wilson, D. (1995). Endophyte: The evolution of a term, and clarification of its use and definition. Oikos, 73, 274-276.

32. Wu, S. H., Chen Y. W., Shao, S. C., Wang, L. D., Yu, Y., Li, Z. Y., Yang, L. Y., Li, S. L., Huang, R. (2009). Two new solanapyrone analogues from the endophytic fungus Nigrospora sp. YB-141 of Azadirachta indica. Chemistry & Biodiversity, 6(1), 79-85.