UFSM

Ciência Florestal

ISSN 1980-5098
OPEN ACCESS

Ci. Fl., Santa Maria, v. 34, n. 1, e71211, p. 1-23, Jan./Mar. 2024 • https://doi.org/10.5902/1980509871211
Submitted: 5th/08/2022 • Approved: 21st/09/2023 • Published: 20th/02/2024

Articles

Activity of an azadirachtin-based product against *Gyropsylla spegazziniana* (Lizer and Trelles, 1919) and its interaction with the entomopathogenic fungus *Beauveria bassiana*

Atividade de um produto à base de azadiractina contra *Gyropsylla spegazziniana* (Lizer e Trelles, 1919) e sua interação com o fungo entomopatogênico *Beauveria bassiana*

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ABSTRACT

Yerba mate ampoule, *Gyropsylla spegazziniana* (Lizer and Trelles, 1919) is one of the main pests of yerba mate crops. This insect attacks sprouts leading to gall formation and defoliation. The nymph's habit of living inside galls makes them difficult to access with chemical insecticides, hampering the effectiveness of chemical control. This study aimed to evaluate the systemic and contact activity of the azadirachtin-based product (via irrigation and spraying) on nymph and adult stages of the ampoule, both in laboratory and field conditions. In vitro interaction of the product with the fungus *Beauveria bassiana* (Balls.-Criv.) Vuill. (Hypocreales: Cordycipitaceae) was also evaluated. After the exposure of nymphs to plants treated with azadirachtin, a reduction in adult emergence was recorded (65–99% depending on the concentration applied). It was observed 80% of adult mortality in plants previously treated with the product (residual effect) and 90% of mortality after direct pulverization of the product on the insects. The product did not affect *B. bassiana* fungal growth, conidial viability, and conidial production on culture media. A synergistic interaction was observed between the azadirachtin insecticide and the fungus in laboratory. In the field trial, after three applications by irrigation of azadirachtin, 67.9% reduction in the number of galls was achieved five weeks after the first application. No phytotoxic effect was observed on yerba mate plants treated with the product. The results of our study demonstrate that the azadirachtin-based product and its association with the fungus *B. bassiana* have potential in controlling *G. spegazziniana*.

Keywords: Yerba mate ampoule; Alternative control; Botanical insecticide; Compatibility

RESUMO

A ampola-da-erva-mate, *Gyropsylla spegazziniana* (Lizer e Trelles, 1919) é uma das pragas mais importantes da erva-mate. O inseto ataca as brotações, levando à formação de galhas e desfolhação. As ninfas vivem no interior das galhas, o que dificulta o contato com inseticidas químicos, reduzindo a eficiência dessa tática de controle. Este estudo visou avaliar a atividade sistêmica e de contato de um produto à base de azadiractina (via irrigação e pulverização) sobre ninfas e adultos da ampola, em condições de laboratório e campo. A interação *in vitro* do produto com o fungo *Beauveria bassiana* (Balls.-Criv.) Vuill. (Hypocreales: Cordycipitaceae) também foi avaliada. Após a exposição de ninfas a plantas tratadas com azadiractina, houve redução na emergência de adultos (65–99% variando conforme a concentração utilizada). A mortalidade de adultos foi de 80% quando mantidos em plantas previamente pulverizadas com o produto (efeito residual) e de 90% quando receberam a pulverização diretamente de azadiractina. O produto não afetou o crescimento, viabilidade e a produção de conídios em meio de cultura. Foi observado sinergismo entre o produto e o fungo em condições de laboratório. Em condições de campo, após três aplicações de azadiractina via irrigação, houve redução de 67,9% na formação de galhas, após cinco semanas do início do experimento. Nenhum sintoma de fitotoxicidade foi observado nas plantas de erva-mate tratadas com o produto. Conclui-se que a o produto à base de azadiractina associado ao fungo *B. bassiana* tem potencial para controle de *G. spegazziniana*.

Palavras-chave: Ampola-da-erva-mate; Controle alternativo; Inseticida botânico; Compatibilidade

1 INTRODUCTION

Yerba mate, *Ilex paraguariensis* (St.-Hil.) (Aquifoliaceae), is a native species native from Argentina, Brazil, and Paraguay. Its leaves are used as a raw material to produce drugs, cosmetics, beverages, and pigments (CROGE; CUQUEL; PINTRO, 2021). Extensive monoculture crops of yerba mate in these countries have been diminishing the biodiversity of the agroecosystem, which favor an increase in phytophagous species. Yerba mate ampoule, *Gyropsylla spegazziniana* (Lizer and Trelles, 1919) (Hemiptera: Aphalaridae), is considered one of the most important pests of this crop (BORGES; LÁZZARI; LÁZZARI, 2003).

Before ovipositing, the female injects saliva into the shoots that causes hypertrophy and the formation of globous galls, called ampules. Nymphs develop and feed continuously inside the galls (LEITE; ZANOL, 2001). The attack of this pest on seedlings causes a reduction in plant growth and an increase in the number of branches and sprouting. When fully developed plants are attacked, the leaves fall off,

and the plants compensate by producing new shoots (PENTEADO, 1995). Chemical insecticides are not allowed to control of this insect (AGROFIT, 2023), therefore, ecologically appropriate methods for pest control are needed.

Azadirachtin is the main compound of the seed neem oil, extracted from the Indian neem tree *Azadirachta indica* A. Juss. (Meliaceae) and can be an important biorational tool for pest control (WEATHERSBEE; MCKENZIE, 2005). Previous studies had proved the effectiveness of the neem oil against *G. spegazziniana* (FORMENTINI; ALVES; SCHAPOVALOFF, 2016). Unlike neem oil commercial products, the formulations of azadirachtin have greater quality control, with a standardized concentration of the active ingredient. Although, azadirachtin-based product has not yet been evaluated against *G. spegazziniana*.

Furthermore, entomopathogenic fungi have been found associated with the *G. spegazziniana* in field conditions (SOSA-GÓMEZ; KITAJIMA; ROLON, 1994; ALVES; LEITE; OLIVEIRA, 2009). Also, *Beauveria bassiana* (Balls.-Criv.) Vuill. (Hypocreales: Cordycipitaceae) was proved the potential as biocontrol agent to the yerba mate ampoule (ALVES; FORMENTINI; FANTI; SCHAPOVALOFF; BARZOTTO, 2013; FORMENTINI; ALVES; SCHAPOVALOFF; MAMPRIM; BONINI; PINTO, 2015; LOEBLEIN; ALVES; NASCIMENTO; RODE; ALMEIDA, 2022). Thus, this study aimed to evaluate the systemic (via irrigation) and contact activity (spraying) of the commercial azadirachtin-based product on nymph and adult stages of the ampoule, both under laboratory and field conditions. In vitro interaction of the product and the fungus *B. bassiana* was also evaluated.

2 MATERIALS AND METHODS

2.1 Insects and plants

Twigs infested with galls were collected in a commercial yerba mate crop without phytosanitary management. In the laboratory, the galls were opened. Those in which

ampoule nymphs were at the 4th and 5th instar were incubated in plastic containers with a screened lid and a bottom covered with filter paper in a controlled room (26 ± 1 °C, RH 60 ± 10%, 12-h daylight cycle) (LOEBLEIN; ALVES; CORACINI; SCHAPOVALOFF, 2019). Emerged adults (24 to 48 h after emergence) were used in bioassays.

Yerba mate seedlings (approximately 20 cm high) were grown in plastic containers, containing organic substrate (earth, earthworm humus, charcoal, and ground pine bark), kept under a 50% shade screen, and irrigated every two days.

2.2 Bioassays

2.2.1 Azadirachtin activity against nymphs

Yerba mate seedlings were pruned and fertilized with of urea. Five groups of ten yerba mate seedlings each were placed into wood cages covered with an anti-aphid screen (60 × 40 × 40 cm) with thirty adults of *G. spegazziniana*. After 15 days, the formation of the galls in the plants was verified. The plants with galls were then selected for the bioassays, with the number of galls per seedling standardized to three by removing excessive galls. The removed galls were opened and observed under a stereomicroscope (40×) to check for the presence of nymphs and honeydew, indicating the presence of the insects infesting the plants. The commercial product Azamax® (12 g of azadirachtin/L, in concentrated vegetal oil emulsion) was evaluated in distilled water solutions (30, 48 or 66 mg a.i./L), as recommended by the product manufacturer (UPL Brasil - https://www.upl-ltd.com/br).

2.2.1.1 Irrigation

To evaluate the activity of Azamax®, 70 mL of product solutions (30, 48 or 66 mg a.i./L) were weekly applied to the base of the plants using a pipette. Applications were performed three times for each concentration. In the control group, the plants were treated with 70 mL of distilled water. After the first application, the plants were transferred to individual cylindrical cages made of colourless polyvinyl chloride (PVC)

(13 cm in diameter × 40 cm in height), with the top and side openings covered by the sheer fabric. The PVC cages were kept under controlled conditions (26 ± 1°C, RH 60 ± 10%, 12-h daylight cycle) during all experimental period. The plants were irrigated with 20 mL of water daily for five days. Water irrigation was then suspended for 24 hours, the number of emerged adults of *G. spegazziniana* in each cage was counted, and the second application was performed. The same procedures described before were used for the third application. After that, the plants were irrigated daily (20 mL of water) and the number of adults in the cages was counted weekly for a month. The experiment had a completely randomized design with four replicates (cage with a single seedling) for each treatment. The experiment was repeated twice, after 30 days, with a different group of plants.

2.2.1.2 Spraying

A new group of galled seedlings was treated with a single application of 1 mL of the Azamax® solutions (30, 48 or 66 mg a.i./L) using a micro-spray connected to an air compressor (0.5 kgf/cm²). The solutions were sprayed over the whole plant (total spray) or only on the leaves (leaf spray), with galls covered by a PVC plastic film layer to avoid contact with the solution. For the control treatment, the plants were sprayed with 1 mL of distilled water. After drying, the plants were transferred to individual cylindrical PVC cages and were incubated as described before. Every day, the plants received 20 mL of water. The adult emergency was assessed weekly for a month. The experiment had a completely randomized design with four replicates (cages with a single seedling) for each treatment. The experiment was repeated twice, after 30 days, with a different group of plants.

2.2.2 Azadirachtin activity against adults

2.2.2.1 Residual contact - leaf spray

Yerba mate seedlings were sprayed with 1 mL of Azamax® solutions (30, 48 or

66 mg a.i./L) with a micro-sprayer coupled to an air compressor (0.5 kgf/cm²). After drying, the plants were placed individually in PVC cages and then infested with 20 adults each. Plants sprayed with distilled water were used as control treatment. Five replicates were performed for each treatment (n = 100 insects). Plants were incubated as described before and irrigated with 20 mL of distilled water daily. The mortality of adults was assessed daily for 10 days. Those insects who did not react to the touch of a brush were considered dead. The experiment was developed in a completely randomized design. The experiment was repeated twice, after 30 days, with a different group of plants.

2.2.2.2 Direct contact - Spraying on insects

Five groups of 20 adults were transferred to plastic containers with screened lid and bottom. The insects were sprayed with 0.2 mL of the product solutions (30, 48 or 66 mg a.i./l), using a micro-spray connected to an air compressor (0.5 kgf/cm²), inserted in an opening on the container lid. In the control treatment, the insects were sprayed only with distilled water. After spraying, the insects were transferred to a PVC cage with a yerba mate seedling. Plants were incubated and evaluated for 10 days, as previously described. The experiment was performed in a completely randomized design, with each treatment consisting of five replicates (cages with 20 adults). The experiment was repeated twice, after 30 days, with other groups of plants.

2.2.3 Fungal and azadirachtin interaction

2.2.3.1 In vitro evaluation

The fungus *Beauveria bassiana* Unioeste 44 (GenBank sequence OK004060) was previously selected as virulent to the yerba mate ampoule (FORMENTINI; ALVES; SCHAPOVALOFF; MAMPRIM; BONINI; PINTO, 2015). The fungus was multiplied in Petri dish with potato-dextrose-agar (PDA) culture medium. After 10 days (26 ± 1 °C, RH

60 ± 10%, 12-h daylight cycle), conidia were collected by scraping the surface of the culture medium and immediately used to prepare the suspensions. The interaction of the fungus and the product was evaluated based on the conidial viability, vegetative growth, and conidial production (SILVA; NEVES, 2005), as follows.

Conidial viability: 250 µL-droplet of a conidial suspension was applied to the surface of the PDA culture medium, in the center of the Petri dish (9 cm diameter). After drying, 250 µL of the Azamax® solutions (30, 48 or 66 mg a.i./L) was sprayed with a micro-spray connected to an air compressor (0.5 kgf/cm^2). After 18 hours (26 ± 1°C, 12-h daylight cycle), germinated and non-germinated conidia were evaluated with a 400× optical microscope. Conidia that displayed a germ tube with a length equal to or greater than the diameter were considered viable.

Vegetative growth: the fungus was inoculated at three points on the surface of the PDA medium in Petri dishes. After 48 hours (26 ± 1°C, 12-h daylight cycle), the plates were sprayed with the Azamax® solutions (30, 48 or 66 mg a.i./L) as described before and were again incubated at the same conditions for another seven days. In the control treatment, the Petri dishes were sprayed with distilled water. The average diameter of colonies with uniform development was obtained with two perpendicular measurements at the edges of these colonies.

Conidia production: the previously evaluated colonies were removed from the plates and transferred individually to sterile glass tubes containing 10 mL of solution with 0.01% of the surfactant Tween 80. Tubes were vortexed by 1 minute to remove the conidia from the colony and conidia were counted with the aid of a Neubauer chamber. The experimental design was completely randomized, with five plates (replicates) for each treatment. All experiments were repeated twice.

2.2.3.2 In vivo evaluation

Yerba mate seedlings (residual contact) and adult insect groups (direct contact) were sprayed, as previously described (respectively 2.2.2.1 and 2.2.2.2 items) with a mixture of conidia and Azamax® solution in distilled water (30, 48 or 66 mg a.i./L).

Another group of plants was sprayed only with conidia suspended in the distilled water. In all treatments with fungus the final concentration was 1×10^9 conidia/mL. In control treatment, plants and/or insects sprayed only with distilled water. Seedlings were kept at 26±1 °C, RH 60±10%, 12-h daylight cycle, and evaluated for 10 days, as previously described. Insects were evaluated daily and were considered dead when they did not react to the touch of a brush. In the treatments with the fungus, the dead insects were removed and placed in a moistened chamber to confirm the mortality by the pathogen. The experiment was performed in a completely randomized design, with each treatment consisting of five replicates (cages with 20 adults). The experiment was repeated twice, after 30 days, with other groups of plants.

2.2.4 Field trial

The experiment was conducted between November 12th and December 10th, 2020, in a commercial yerba mate crop (2 m between plants × 3 m between lines; plants with approximately 2 m high) naturally infested (with developed galls). The plants were randomly chosen (with at least 5 m between them), the infested twigs were identified, and the galls on the twigs were counted to assess the initial infestation. After that, 500 mL of Azamax® solution (24 mg a.i./L) was sprayed using a 15 L electric knapsack sprayer with Jacto 11003 fan jet tip, directly to infested twigs. In the irrigation treatment, 500 mL of the product solution was manually distributed on the soil surface, near to the trunk. In the control, the plants did not receive treatment. Treatments were performed 3× (0, 7, and 14 days). Nine replicates (one plant for each replicate) were used in each treatment. The evaluations were performed weekly before each application of the treatments for 5 weeks. The efficacy percentage (EP) of the insecticides was determined by Abbott's formula.

2.3 Statistical analysis

Data normality (Shapiro-Wilk test) and homogeneity (Levene test) were checked. In the assessment of azadirachtin activity against nymphs and adults after three

applications of the product by irrigation in different concentrations, the data were submitted to ANOVA - Repeated Measures followed by Tukey-HSD test. When assessing the azadirachtin activity against adult after one application on plants (residual) or spraying on insects (direct) and, adult mortality data, considering a single application of spraying on plants (residual) or spraying on insects (direct), the data were submitted to One-way ANOVA, following by post-hoc Tukey-HSD ($p<0.05$).

The interaction of the product and the fungus was analysed, according to the Equation (1):

$$BI = [47\ (VG) + 43\ (SPO) + 10\ (GERM)] \times 100 \quad (1)$$

where BI = biological index; VG = percentage of vegetative growth compared to the control; SPO = percentage of conidia production in the colonies compared to the control; GERM = germination percentage compared to the control.

The classification of the effect of the product on the fungus follows: BI> 66 compatible; moderately toxic 42 <BI <66; and toxic BI <42 (0–41) (ROSSI-ZALAF; ALVES; LOPES; SILVEIRA NETO; TANZINI, 2008). Also, the effect was classified as either nonadditive, synergistic (S), or antagonistic (A) (KOPPENHÖFER; BROWN; GAUGLER; GREWAL; KAYA; KLEIN, 2000). The interaction analysis of the product and the fungus was determined by the Pearson chi-squared test.

Data from the field trial (the galls in the yerba mate tree), were submitted to the Kruskal-Wallis test ($p <0.05$), followed by the post-hoc Dunn test. All the statistical analyses were performed in Statistica software, version 7.

3 RESULTS

3.1 Azadirachtin activity against nymphs: irrigation and spraying

The product Azamax® affected the development of nymphs, reducing the number of adults emerged when compared to the control treatment (irrigation: $F_{3,36}$

= 6.85; p < 0.001; total spray: $F_{3,36}$ = 6.53; p < 0.001; foliar spray: $F_{3,36}$ = 6.33; p < 0.001). There was no significant interaction between the number of emerged adults and the applications or concentration of the product in the irrigation treatment, ranging from 44 to 63 insects ($F_{2,72}$ = 0.34; p = 0.713). In the spray treatments, the increase of product concentration caused a significant reduction in adult emergence, mainly in the total spray. Significantly fewer adults emerged in spray treatments with azadirachtin at 48 and 66 mg a.i./L (total spray) and 66 mg a.i./L (foliar spray) (Table 1).

The reduction in adult emergence due to the presence of the product in the irrigation treatment was close to 80%. For the total spray treatment, the reduction varied from 69 to 99%, according to the tested concentration. In foliar spray, the reduction in adult emergence was lower (65.9 to 78.9%). Regardless the form of use, the percentage of adult emergence reduction in treatments was higher as the concentration of the product increased (Table 1).

Table 1 – Number of adults of *Gyropsylla spegazziniana* emerged in yerba mate seedlings irrigated or sprayed with Azamax® in different concentrations

		Azadirachtin concentration (mg a.i./L)			
Treatment	**applications**	**control**	**30**	**48**	**66**
	1	10.3±5,4a	0.0±0.0b	0.0±0.0b	1.8±0.9 b
Irrigation	2	7.7±7.4a	6.0±2.8b	3.7±1.5 b	2.6±2.3 b
	3	17.9±7.5a	0.3±0.3b	0.9±0.5 b	0.0±0.0 b
Total spray	1	374 ± 108a	116 ± 89b (69.0)	16 ± 7b (95.7)	3 ± 2 c (99.2)
Foliar spray	1	462 ± 101a	162 ± 63b (64.9)	144 ± 31b (68.8)	98 ± 35c (78.9)

Source: author (2020)

In where: Mean (± SE) followed by the same letter in the row did not significantly different (Tukey HSD test, p> 0.05); values in parentheses indicate the percentage of reduction in the emergence compared to the respective control.

3.2 Azadirachtin activity against adults

Different levels of activity were observed on adult after product spray on plants or on insects with significant effect for all tested concentrations ($F_{3,26}$ = 123.3, p <0.001;

$F_{3,26}$ = 200.11, p <0.001, respectively). For the residual effect (spraying on plants), the mortality was greater as the concentration of the product increased, reaching 80% at a concentration of 66 mg a.i./l. For the direct spray on insects, the highest mortality was observed at 48 and 66 mg a.i./L (77 and 90%, respectively) (Table 2).

Table 2 – Mortality (%) of adults of *Gyropsylla spegazziniana* in yerba mate seedlings treated with Azamax® in different concentrations by spraying on plants or on insects

Treatment	Applications	Azadirachtin concentration (mg a.i./L)			
		Control	30	48	66
Spraying on plants (residual)	1	5.0±1.1d	31.0±2.9c	48.0±3.7b	80.0±6.9a
Spraying on insects (direct)	1	4.0±1.0c	43.0±4.1b	77.0±6.6a*	90.0±3.5a

Source: author (2020)

In where: Means (± SE) followed by the same letter in the row, did not differ significantly (Tukey-HSD test, p<0.05); *indicates a significant difference in insect mortality between the spraying on plants and on insects.

3.3 Fungal and Azadirachtin Interaction

No negative effect on fungal growth, conidial viability, and conidial production was observed by contact with Azamax® *in vitro*. Regardless of the treatment or the tested concentration, conidia viability ranged from 95 – 98% ($F_{3,26}$ = 0.3, p = 0.792); the colony growth was between 1.8 – 2 cm2 ($F_{3,26}$ = 6.02, p = 0.003); and conidia/colony production was between 1.2 - 1.3 × 10^8 ($F_{3,26}$ = 1.16, p = 0.343). Furthermore, the BI values obtained in all product concentrations were above 90, indicating the compatibility of the botanical insecticide and the fungus.

In vivo, different levels of total mortality were observed after spraying on yerba mate seedlings or on insects ($F_{1, 80}$ = 250.10, p <0.001). Differences were also observed for the product concentrations ($F_{4,80}$=169.55; p<0.001) and the interaction of spray method and concentrations ($F_{4,80}$=27,39; p<0,0001). Total mortality was significantly higher with azadirachtin + fungus than the fungus alone, in both spraying on seedlings

and on insects. Direct insect spray (all treatments) led to higher total mortality, even in the treatment only with the fungus (Table 3). Confirmed mortality in residual treatment occurred in azadirachtin + fungus (9, 22, 30%, respectively to Azamax® at 30, 48 and 66 mg a.i./L concentration). The product at 48 and 66 mg a.i./L affected the mycosis (confirmed mortality) in *G. spegazziniana* for direct spray treatment ($F_{4, 80}$=28.54; p<0.001) (Table 3). The product at 48 and 66 mg a.i./L had a significant residual effect.

The synergistic effect of azadirachtin + fungus (in all concentrations) was observed in the residual treatment ($\chi 2$ = 39.6, 44.3, 24.4; df = 1; p <0.05, respectively, for concentrations of 30, 48 or 66 mg a.i./L). For spraying on insects (direct), there was an additive effect ($\chi 2$ = 1.9, 0.03, 0.015; df = 1, p <0.05, df = 1, p <0.05, respectively for the concentrations of 30, 48 or 66 mg a.i./L) (Table 3).

Table 3 – Mortality (%) of adult *Gyropsylla spegazziniana* on yerba mate seedlings sprayed with Azamax® in different concentrations, associated with the fungus *Beauveria bassiana*

Treatment	Mortality	
	Total	Confirmed fungal
Spraying on plants (residual)		
Control	7.3 ± 0.95b	0.0 ± 0.00c
Fungus	8.0 ± 3.00 b	0.0 ± 0.00 c
30	38.0 ± 2.55a (S)	9.0 ± 2.92b
48	48.0 ± 4.64a (S)	22.0 ± 4.06a**
66	45.0 ± 3.16a (S)	30.0 ± 5.48a**
Spraying on insects (direct)		
Control	7.7 ± 0.96c	0.0 ± 0.00c
Fungus	47.0 ± 2.62b*	24.0 ± 2.85b**
30	79.0 ± 4.85a* (A)	39.0 ± 6.78a**
48	87.0 ± 5.83a* (A)	0.0 ± 0.00c
66	93.0 ± 4.90a* (A)	0.0 ± 0.00c

Source: author (2020)

In where: Means (± SE) followed by the same letter in the row did not differ significantly (Tukey-HSD test, p <0.05); *indicates a significant difference in the comparison of total mortality between spray types; **indicates a significant difference in the comparison of confirmed mortality between spray types, using the t-test (p <0.05); A = additive; S = synergistic

3.4 Field trial

Different numbers of *G. spegazziniana* galls were observed depending on the application method. After three applications of 500 mL-solution of 66 mg a.i./L, a significant reduction in the mean number of galls was observed. Before treatment, insect infestation was similar between control and treatments (H = 4.5196, df = 2, p = 0,1044, Kruskal-Wallis test). On the fourth week of sampling (after three applications), the mean number of galls per tree in treatment by irrigation was 3.1 ± 1.14 (n = 22) and lower than the other treatments (H = 6.9138, df = 2, p = 0.0315, Kruskal-Wallis test). At the last sampling, the mean number of galls per plant in Azamax® irrigation also differed from the other treatments (3.3 ± 1.36 n = 21) (H = 4.7906, df = 2, p = 0.0326, Kruskal-Wallis test). The mean number of galls per plant was 67.9% less than the initial mean value. However, the spray of a 500-mL solution of 66 mg a.i./L had no significant effect on the *G. spegazziniana* infestation. At the last sampling, the mean number of galls per plant was 6% higher than the initial mean value (Table 4).

Table 4 – Mean number of *Gyropsylla spegazziniana* galls on yerba mate plants treated with Azamax® via irrigation or pulverization (November 12 to December 10, 2020)

	Weekly sampling				
Treatments	**1***	**2***	**3***	**4**	**5**
Control	11.0 ± 0.55 Aab	14.5 ± 1.02 Aa	12.9 ± 1,66 ABab	11.4 ± 5.88 Ab	9.8 ± 4,9 Ab
Irrigation	10.3 ± 0.33 Aa	12.6 ± 1.10 Aa	9.4 ± 1.12 Ba	3.1 ± 1.14 Bb	3.3± 1.36 Bb
Pulverization	10.3 ± 0.33 Aa	11.7± 1.28 Aa	10.7 ± 2.01 Aa	9.9 ± 2.40 Aa	11.0 ± 2.43 Aa

Source: author (2020)

In where: Means (± SE) followed by the same lowercase letter in the row and uppercase in the column did not differ significantly (Kruskal-Wallis test; p <0.05); *Azamax® application; **based on the value initially observed in the respective treatment

Furthermore, as observed in the laboratory, the product applied at 66 mg a.i./L did not cause apparent phytotoxicity on the plants treated in the field.

4 DISCUSSIONS

The insecticidal activity of azadirachtin on nymphs and adults of *G. spegazziniana* was confirmed in the present study. A significant effect of Azamax® (at 48.6 mg a.i./L) against nymphs was also observed for *Diaphorina citri* Kuwayama, 1908 (Hemiptera: Liviidae) maintained on citrus plants treated with azadirachtin solution (SANTOS; ZANARDI; PAULI; FORIM; YAMAMOTO; VENDRAMIM, 2015). Both *D. citri* and *G. spegazziniana* are sucking insects that feed on the sap of the plant. In turn, the systemic action of the azadirachtin was previously proved by analyzing samples from different parts of white spruce seedlings [*Picea glauca* (Moench) Voss] at different times after immersing their roots in a nutrient solution containing azadirachtin-A. There was observed the uptake, translocation, persistence, and dissipation of azadirachtin (SUNDARAM, 1996; THOEMING; DRAEGER; POEHLING, 2006). Also, azadirachtin was found in litchi (*Litchi chinensis* Sonn.) ripe fruits after azadirachtin solution injection in litchi tree trunks (SCHULTE; MARTIN; SAUERBORN, 2006).

The reduction of emergence of adults in azadirachtin-treated plants is due the interference of the azadirachtin on the production of juvenile and ecdysone hormones by the insects, which affects their molting process and development (MORDUE; NISBET, 2000). Azadirachtin also has sublethal effects related to the feeding behavior of the insects, which result in a sum of actions that leads to the reduction of the pest population (HASAN; ANSARI, 2011; TOMÉ; MARTINS; CORRÊA; GALDINO; PICANÇO; GUEDES, 2013). Thus, we proved for the first time the effect of irrigation of yerba mate plants with azadirachtin solution on nymph mortality.

The galls are closed structures, and they open only when the nymphs reach the fifth instar and leave the galls to finish their development (LEITE; ZANOL, 2001). Even though in this case the effect of direct contact of the product on insects during the product spray is practically null, the systemic action of azadirachtin was detected for *G. spegazziniana*, as previously observed for other insect species (CARVALHO; VENDRAMIN; SÁ; SILVA; RIBEIRO; ROSSI, 2015; SANTOS; ZANARDI; PAULI; FORIM;

YAMAMOTO; VENDRAMIM, 2015). This is confirmed by the lower number of adults emerged from plants that received the azadirachtin in total spray treatment, which led to a greater amount of active ingredient absorbed and translocated in plant.

The high mortality of adults in the plant spray treatment observed here contrasts with what was observed by Santos; Zanardi; Pauli; Forim; Yamamoto; Vendramim (2015) for *D. citri*, in which Azamax® had low activity (10% mortality) after 5 days of contact with citrus plants. A longer time of exposure of yerba mate plants to the product (10 days) may have induced greater adult mortality. The feeding behavior of sucking insects may be affected by phagodeterrence and repellency caused by the systemic azadirachtin, leading them to starvation and death (WEATHERSBEE; MACKENZIE, 2005). Reduced feeding activity and longevity have been reported in *Nezara viridula* (Linnaeus, 1758) (Hemiptera: Pentatomidae) (ABUDULAI; SHEPARD; MITCHEL, 2003), and adult mortality was observed in *Halyomorpha halys* (Stål, 1855) (Hemiptera: Pentatomidae) (MOREHEAD; KUFAR, 2007; LEE; SHORT; NIELSEN; LESKEY, 2014).

Although treatment with azadirachtin is focused on nymph mortality, the percentage of adult mortality by direct spraying was considerable and relevant in the context of pest control. The direct action on adults could be caused by the oily nature of the formulation (azadirachtin in emulsifiable vegetable oil). After application, the adhesion of insects on the treated surface leading to death was visible. Furthermore, oily substances are liable to cause insect mortality by suffocation or anoxia, due to the clogging of the spiracles. It cans also act as fumigants and cause nervous disruption and narcosis, corrosion, cell rupture, and desiccation (TAVERNER, 2002; LEONG; ABANG; BEATTIE; KUEH; WONG, 2012).

The systemic action on yerba mate represents a possibility for the effective control of *G. spegazziniana*, as nymphs develop inside the galls, making them unattainable by the spraying of a contact-acting insecticide. Furthermore, the systemic action reduces possible impacts on pollinators and natural enemies that can be reached with the spraying, facilitating the recolonization by these organisms and the restoration of the ecological balance (YAMAMOTO; BASSANEZI, 2003).

The BI value above 90 demonstrates the high compatibility of the product and the fungus (BI> 66 is the minimum value for compatibility). Previous studies have demonstrated the compatibility of azadirachtin formulations (NeemAzal and Azamax®) with the fungi *Metarhizium anisopliae* (Metsch.) Sorok. and *M. rileyi* (Farl.) Kepler, Rehner, and Humber (Shumacher; Poehlling, 2012). The non-fungitoxic effect in all concentrations tested here (0.25–0.55%) corroborates with a previous study that showed the compatibility between *B. bassiana* and a different commercial product based on azadirachtin 0.3%. It is possible that the fungus metabolizes the product, and the components may have been used as nutrients (ROCHA; MELO; SANTOS; BITTENCOURT, 2012) or the adjuvants disperse conidial agglomerations, increasing the number of free propagules in the suspension, explaining the better vegetative growth on the culture medium surface when both *B. bassiana* and azadirachtin are present (ISLAM; OLLEKA; REN, 2010).

The increase on the activity of the association of with azadirachtin and *B. bassiana* spraying on plants, compared to the fungus alone, was also observed against whitefly (ISLAM; OLLEKA; REN, 2010), and *Spodoptera littura* (Fabricius, 1775) (Lepidoptera: Noctuidae) (MOHAN; REDDY; DEVI; KONGARA; SHARMA, 2007; ISLAM; OMAR, 2012). The oily formulation increased the adhesion of the conidia on the adults or improved its dispersion, increasing the amount of inoculum on host cuticle (PRIOR; JOLLANDS; PATOUREL, 1988; IBRAHIM; BUTT; BECKET; CLARK, 1999). Furthermore, oily formulations protect conidia against environmental factors (OLIVEIRA; LOPES; REZENDE; DELALIBERA JR, 2018), increasing the survival of the fungus and lengthening the contamination of adults on conidia-impregnated surfaces (LOEBLEIN; ALVES; NASCIMENTO; RODE; ALMEIDA, 2022).

Even though it was compatible, the association with the fungus interfered with the infection when applied to insects. There was a lower percentage of confirmation, and the interaction was additive. The death of insects was probably due more to the presence of azadirachtin or the oily formulation than the fungus itself.

In the field experiment, the irrigation application was more effective than the azadirachtin foliar application. Azadirachtin exhibited a significant effect on the population reduction only after the third application. This observation is consistent and occurred in pistachio trees (*Pistacia vera* L.) treated with a commercial neem product, aiming to control *Agonoscena targionii* (Lichtenstein, 1874) (Hemiptera: Aphalaridae) (LABABIDI, 2002). Control of phytophagous mites in papaya (*Carica papaya* L.) with an azadirachtin-based product was also achieved only after the third azadirachtin application (ABATO-ZÁRATE; VILLANUEVA-JIMÉNEZ; OTERO-COLINA; ÁVILA-RESÉNDIZ; HERNÁNDEZ-CASTRO; REYES-PÉREZ, 2012). In contrast to the greater efficiency of spraying yerba mate seedlings, irrigation was more efficient in the treatment of trees in the field. Due to the greater number of leaves in the canopy, the volume of the applied product was probably insufficient for the azadirachtin be absorbed in quantities to affect the nymphs in developing galls.

Corroborating previous studies (RIBEIRO; BLUME; BOGORNI; DEQUECH; BRAND; JUNGES, 2012; HERNÁNDEZ; MARTÍNEZ-VILLAR; PEACE; PÉREZ-MORENO; MARCO, 2012), azadirachtin proved to be a sustainable alternative for the *G. spegazziniana* management in nurseries, due to its innocuousness, either in the combined applications with *B. bassiana* or allowing the natural occurrence of other entomopathogenic fungi. Thus, in addition to reducing the negative effect of synthetic chemical insecticides, the use of this botanical insecticide in the production system of yerba mate is an eco-friendly alternative, adding quality and value to the final product. Despite the absence of phytotoxicity of the product at the concentrations evaluated on yerba mate plants, it is important to establish safer limits for the concentrations, since phytotoxicity of neem-based products has been reported for more concentrated solutions (VENZON; TOGNI; PEREZ; OLIVEIRA, 2020). Furthermore, the application time considering the presence of the insect, both in nurseries and the field, and the persistence of the product or possible organoleptic changes in the final product must be better determined.

5 CONCLUSIONS

Azamax® affected the development of nymphs and caused the death of adults. In the seedlings, its action on the nymphs was more expressive in irrigation treatment. Spraying on plants or insects was more effective against adults. The product was compatible with the fungus *B. bassiana* (which is a natural enemy of the ampoule and other pests of yerba mate). In the field, the infestation of the *G. spegazziniana* was reduced with the irrigation treatment. Thus, the product showed great potential for use in the biorational control of yerba mate ampoule in nurseries and yerba mate crops.

ACKNOWLEDGMENT

Parry America Inc - Murugappa Group, for support part of the research and Erva-mate Laranjeiras Ltda. for access to the cultivation areas for insect collection, to CNPq and CAPES for granting scholarships to authors.

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How to quote this article

LOEBLEIN-VERDÉRIO, J. S.; ALVES, L. F. A.; RODE, P. A.; BORDIN, C.; FETTER, I.; GUIMARÃES, A. T. B. Activity of an azadirachtin-based product against *Gyropsylla spegazziniana* (Lizer and Trelles, 1919) and its interaction with the entomopathogenic fungus *Beauveria bassiana*. **Ciência Florestal**, Santa Maria, v. 34, n. 1, e71211, p. 1-23, 2024. DOI 10.5902/1980509871211. Available from: https://doi.org/10.5902/1980509871211. Accessed in: day month abbr. year.