NYMPHICIDAL EFFECT OF VEGETAL EXTRACTS OF *Annona mucosa* AND *Anonna crassiflora* (MAGNOLIALES, ANNONACEAE) AGAINST RICE STALK STINK BUG, *Tibraca limbativentris* (HEMIPTERA, PENTATOMIDAE)[1]

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ABSTRACT- This study aimed to verify the chloroform-methanol nymphicidal action of extracts of *Annona mucosa* leaves and seeds and of *A. crassiflora* seeds on second instar nymphs of rice stalk stink bug, *Tibraca limbativentris*. For each extract the concentrations of 0.5%, 1.0%, 2.0%, 4.0%, 8.0%, and two control treatments (water and Tween80®) were used. The results show that the seed extracts of *A. mucosa* and *A. crassiflora* have insecticidal activity against the *T. limbativentris* nymphs with statistical significance for all concentrations when compared with controls. The seed extract of *A. mucosa* showed the higher toxicity with greater than 75% mortality at a concentration of 1.0% in the first 24 h after application. The leaf extract of *A. mucosa* presented the lowest toxicity with no more than 40% mortality. The seed extract of *A. crassiflora* showed intermediate toxicity among all the tested extracts, and the nymph's mortality exceeded 80% for the highest concentration after 120 h of application. Considering these results, we were able to observe that the seeds extract of *A. mucosa* may be an alternative for the control of bed bug nymphs *T. limbatriventris*, especially for small producers.
Index terms: inseticidal plants, biological insecticide, alternative control, phyto-insecticide.

EFEITO NINFICIDA DE EXTRATOS VEGETAIS DE *Annona mucosa* E *Annona crassiflora* (MAGNOLIALES, ANNONACEAE) SOBRE O PERCEVEJO-DO-COLMO DO ARROZ, *Tibraca limbativentris* (HEMIPTERA, PENTATOMIDAE).

RESUMO - Este trabalho teve como objetivo verificar a ação ninficida dos extratos clorofórmio-metanólico de folhas e sementes de *Annona mucosa* e de sementes de *A. crassiflora* sobre ninfas de segundo instar do percevejo-do-colmo do arroz, *Tibraca limbativentris*. Para cada extrato, foram utilizadas concentrações de 0,5%; 1,0%; 2,0%; 4,0%; 8,0%; e dois tratamentos-controle (Tween 80® e água). Os resultados mostram que os extratos de sementes de *A. mucosa* e de *A. crassiflora* apresentaram atividade inseticida sobre ninfas de segundo instar de *T. limbativentris* em todas as concentrações, quando comparadas com os controles. O extrato de sementes de *A. mucosa* foi o que apresentou maior toxicidade com mortalidade maior que 75% na concentração de 1,0 % do extrato, nas primeiras 24 h após a aplicação. O extrato de folhas de *A. mucosa* apresentou a menor toxicidade, não ultrapassando 40% de mortalidade. O extrato de sementes de *A. crassiflora* apresentou toxicidade intermediária entre todos os extratos testados, e a mortalidade das ninfas passou dos 80% na maior concentração, após 120 h da aplicação. Diante destes resultados, nota-se que o extrato de *A. mucosa* pode ser uma alternativa para o controle de ninfas do percevejo *T. limbatriventris*, principalmente para pequenos produtores.
Termos para indexação: plantas inseticidas, inseticida biológico, controle alternativo.

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INTRODUCTION

Rice is the basic food for more than half of the world's population and presents one of the best nutritional balancing, responsible for providing 20% and 15% of the energy and protein needed for human nourishment (AZAMBUJA et al., 2004). During its cultivation a rice crop can be affected by invasive plants, phytophagous insects and diseases that can reduce significantly the productivity and grain quality up to 90% (FERREIRA et al., 1997; MARTINS et al., 2009). Among the main pests that attack rice crop the stink bug *Tibraca limbativentris* Stal, 1860 (Hemiptera: Pentatomidade), also known as brown stink bug, rice stem bug, rice stalk stink bug and stink bug large of rice, is one of the most important pests for rice crops in Brazil (FERREIRA et al., 1986). This species has a wide geographical distribution in the Neotropics (FERNANDES e GRAZIA, 1998) occurring in all regions of rice cultivation in Latin America (MARTINS et al., 2004). It is considered a pest in countries such as Argentina, Brazil, Colombia, Ecuador, Peru, Dominican Republic and Venezuela (PANTOJA et al., 2007).

The rice stalk stink bug is a hard insect pest to control in all rice production areas in Brazil, regardless of the production system used (MARTINS et al., 2009). According to Souza et al. (2009), the most used way to control *T. limbativentris* is through chemical products, and active registered ingredients are the phytosanitary pesticide system (AGROFIT) composed of tiometoxam (neonicotinoid), cyfluthrin (pyrethroid), lambda-cyhalothrin (pyrethroid) and malotina (organophosphate) (MAPA, 2010).

Considering the current effects caused by the use of chemicals in agriculture, a viable alternative for the control of this insect would be to use insecticidal plants extracts, which have low toxicity and little persistence in the environment (COSTA et al., 2004). In this context, plants of Annonaceae family come up as a promising alternative for general pest control, since acetogenins can be found within their composition, which are compounds that have a high insecticidal potential (ALALI et al., 1999). Some studies reporting the insecticidal properties of Annonaceae were primarily tested to control insect disease vectors such as mosquitoes (Diptera) and several species of triatomine bugs (Hemiptera, Reduviidae) (CARNEIRO et al., 2011; 2013; COSTA et al., 2012; DILL et al., 2012; MORAES et al., 2011). Researches that reported the Annonaceou's bioactivity on mortality of stink bugs (mainly on pentatomids) are nonexistent or incipient, and thus must be further studied. Therefore, due to the necessity of seeking alternative methods that stipulate a less impactful agriculture on the environment, this study aims to verify the nymphicidal action of chloroform-methanol extracts of *Annona mucosa* leaves and seeds and of *Annona crassiflora* seeds on second instar nymphs of rice stalk stink bug, *T . limbativentris*.

MATERIAL AND METHODS

Plant material and preparation of extracts - The plant species used on bioassays were *Annona mucosa* and *A. crassiflora* (Annonaceae). Fruits and leaves of *A. mucosa* were collected in the urban area of Tangará da Serra-MT and fruits of *A. crassiflora* in the Cerrado area, both in July 2012. Exsicates of these species were deposited at TANG Herbarium of the Universidade do Estado de Mato Grosso, campus of Tangará da Serra (UNEMAT/CUTS). To obtain the extracts, plant parts (seeds and leaves) were collected, dried in forced circulation stove air at 40 ° C for 72 h. The dried material was triturated in a grinder type knife and sent to the Laboratory of Carbohydrate Chemistry, at the Universidade Federal do Paraná (UFPR), where the de-lipidification during 3 days with organic solvents such as chloroform-methanol (2:1) using a soxhlet extractor apparatus warmed at 60 °C until exhaustion (when there had no more material for extraction). The extracted material was rota-evaporated at 40° C to remove the solvent and to obtain the crude extract. From these extracts, dilutions were made at the concentrations used in the bioassays.

Collection of nymphs - The nymphs were obtained from eggs laid by females of *T. limbativentris* established at greenhouse located at The Brazilian Agricultural Research, National Research Center of Rice and Beans (EMBRAPA/CNPAF), Santo Antônio de Goiás/GO, Brazil (6°28´S; 49°17´W; 823m). The egg-nymph development has been monitored daily, from hatching to the 2nd instar. In the bioassays there were used 2nd instar nymphs up to 24 h. This nymphal stage was used considering that within this period of development that the nymphs begin to attack the stalks of rice. And from this instar the nymphs begin to migrate for off the colonies (which are usually in the leaves) into the plant stems. This movement blocks further strategies to control this insect pest in subsequent instars (FERREIRA et al., 1997).

Bioassay - The bioassay was performed at the Laboratory of Entomology in EMBRAPA/CNPAF. We used a completely randomized design with five treatments and two controls with ten repetitions each containing ten 2nd instar nymphs of the stink bug *T. limbativentris*. For each extract that was used the concentrations varied from 0.5%, 1.0%, 2.0%, 4.0% and 8.0%, one including a negative control with the solubilizer Tween80® (10%) and another negative control containing only water. For each concentration topical applications of 2 µL were made with the assistance of a micropipette in the dorsum of each insect. After application of the extract, the nymphs were placed in plastic pots (245 ml) with a stem of rice for nourishment. The stems were changed every two days. The nymphs remained in climatized room with temperatures of approximately 25 ± 2 °C, relative humidity of 75 ± 0.60% and photoperiod of 14 h. The mortalty evaluation was performed daily and the live nymphs were evaluated until they reached the 3rd instar. Data from the experiment were subjected to analysis of variance (ANOVA) and the means were compared by the Scott Knott test at 5% probability using the SASM software (CANTERI et al., 2001). The lethal concentration to kill 50% and 90% of insects (LC50 and LC90) and the confidence intervals (CI) were evaluated by Probit analysis using the Statistica 7 software.

RESULTS AND DISCUSSION

The results of this study show that seed extracts of *A. mucosa*, and *A. crassiflora* exhibited insecticidal activity against 2nd instar nymphs of *T. limbativentris* and were statistically different in all concentrations when compared to controls ($p < 0.01$). However, the leaves extract of *A. mucosa* did not show insecticidal activity against insects in the first days after application, although mortality of nymphs has increased over time (Table 1).

Many studies have demonstrated that several species of plants of the Annonaceae family have insecticidal activity against several groups of arthropods (Acari, Blattodea, Coleoptera, Diptera, Hymenoptera, Hemiptera/Homopetera, Hemiptera/Heteroptera, Lepidoptera) (CASTILLO-SÁNCHES et al., 2010; BROGLIO-MICHELETTI et al., 2009; MAGADUM et al., 2009). Researches using Annonaceae in control of triatomine bugs were developed by Carneiro et al. (2011, 2013), using extracts of *Annona coriacea* against *Rhodnius neglectus* (Hemiptera: Reduviidae) and Parra-Henao et al. (2007) using the extract of *A. muricata* on *Rhodnius prolixus* and *Rhodnius pallescens*.

However, studies on the bioactivity of Annonaceae extracts about stink bugs pentatomids are still scarce, and the first studies performed in Brazil were made by Souza et al. (2007) using *A. coriacea* and Cordeiro (2007) with extracts of *A. crassiflora*, both on stink bug *Dichelops melacanthus* (Hemiptera: Pentatomidae). The most recent works are of Magalhães et al. (2011) and Bandeira et al. (2011) that evaluated the insecticide effect of *A. mucosa* extract against the brown stink bug, *Euschistus heros* (Hemiptera: Pentatomidae). This demonstrates the importance of increasing the studies using these plants against other insect pests, as conducted in this work with rice stalk stink bug nymphs, *T. limbativentris*.

In this study, the seed extracts of *A. mucosa* showed higher mortality at all concentrations when compared with the seed extracts of *A. crassiflora*, controling more than 75% of *T. limbativentris* nymphs of at the concentration of 1.0% after the first 24 hours, reaching 88% for this same concentration after 5 days from the application of the extract. Additionally, concentrations of 2.0, 4.0 and 8.0% killed within 24 h respectively 90, 92 and 100% of nymphs. This pattern was similar in the evaluations of 72 and 120 hours after application of the extract (Table 1).

Similar results were observed by Cordeiro (2007), using hexane extract of *A. crassiflora* on *D. melacanthus* nymphs and *E. heros* at concentrations of 8.0, 4.0 and 2.0%, achieving mortality of 98, 90 and 98% respectively for *D. melacanthus* and 64, 56 and 56% for *E. heros* respectively. In another study, Magalhães et al. (2011) used hexanic extract of *A. mucosa* seeds and verified 100% mortality of *E. heros* nymphs at concentrations of 2.0, 4.0 and 8.0% and 67.5% in the concentration of 1.0%, corroborating with the data shown in this research. Studying other species of Annonaceae, Souza et al. (2007) found the insecticidal activity of ethanolic and hexanic extracts of *A. coriacea* seeds on *D. melacanthus*, with 100% mortality with 4.0 and 8.0% for ethanol extract and 78, 86 and 68% with 8.0, 4.0 and 2.0%, respectively with the hexanic extract. This demonstrates that different species of Annonaceae can be used to control stink bugs from differents crops.

The leaves extracts of *A. mucosa* were less efficient when compared to extracts of seeds of both species used on the bioassays, although it showed differences among different concentrations and the controls (Table 1). Most research with Annonaceae use mainly extracts from seeds because the knowledge of some bioactive compounds that are present in higher quantities in these plant parts, especially from the acetogenins class, are promising for obtaining substances with insecticidal properties (CASTILLO-SÁNCHEZ et al., 2010; NASCIMENTO et al., 2003; ALALI et al., 1999). However, studies such as those of Cruz-Estrada et al. (2013), using extracts of *Annona squamosa* leaves showed high mortality rate of eggs and nymphs of whitefly, *Bemisia tabaci* (Hemiptera: Aleyrodidae), indicating that other plant parts such as Annonaceae leaves, are also sources of bioactive substances potential insecticide.

Therefore, is likely that the leaves extracts of *A. mucosa* did not present high mortality rate of *T. limbativentris* nymphs due to possible variation in the amount of acetogenins amount in the different plant parts (intra and inter-specific), showing that the leaves have less bioactive compounds that the seeds. Such is supported when the lethal concentrations (LC50 and LC90) found when the leaves extracts of *A. mucosa*, were taken into consideration (Table 1). Different from what was observed for the seeds extracts of *A. mucosa* where the LC50 values were lower when compared to the leaf extracts and also with the extract of *A. crassiflora* seeds. These findings highlights the high toxicity of seeds extract of *A. mucosa* on *T. limbativentris* nymphs, thereby causing the mortality of the insects with a small quantity of the extract (Table 1).

The high percentage of *T. limbativentris* nymphs' mortality within the first 24 h after application of the seeds extract of *A. mucosa* may be due to the excess of acetylcholine in the body of the insect caused by acetylcholinesterase inhibition, since it was observed that some nymphs "trembled" soon after the application of the extract, rapidly dying. Considering that the excess of acetylcholine causes something like a "short circuit" or excessive electrical discharge in the nervous system of the insect, this interferes in the balance of the body, causing the death of the insect by hyperexcitation (NELSON and COX, 2011).

Thus, the increase in *T. limbativentris* nymph's mortality during all the evaluation days with seed extracts of *A. crassiflora* might be a consequence of inanition since the nymphs do not feed or to the decrease in their feeding needs (feeding deterrence). Although this characteristic was not evaluated during the bioassays, authors such as Álvares-Colom et al. (2007) have reported that the effect of inanition is due to the use of Annonaceae extracts, especially when considering that the acetogenins present in these plants, which are potent inhibitors of mitochondrial complex I in NADH systems:oxidase of the plasma membrane, inducing apoptosis (programmed cell death) being thus a potential substance for use as pesticide (GONZÁLEZ-COLOMA et al., 2002; JOHNSON et al., 2000). These results corroborate whit those reported by Oliveira e Pereira (2009) that evaluated the antifeedant activity of *A. crassiflora* on adult brown stink bug, *E. heros*.

Also it was observed that some *T. limbativentris* nymphs lost their mobility after the use of extracts and this might also be a response of the acetogenins present in extracts of these species. It is known that the action of acetogenins, by contact or ingestion, interfere in sodium channels which is regulated by voltage, and this changes the balance of sodium and potassium, which impedes the normal nerve transmission, causing the insect paralysis followed by death ("knockdown") (HOLAN, 1969; COATS, 1990).

Considering these results, we have observed that the seed extract of *A. mucosa* might seen as an alternative for the control of *T. limbatriventris* nymphs, especially for small producers. However, further studies are needed in other developmental stages of the stink bug (eggs and adults, for example), as well as field research to check whether the same patterns found in laboratory bioassays are maintained under field contitions. Also it is to perform necessary additional researches with the aim of fractionate, isolate and identify the bioactive substances to be employed in the future for the development of new and promising biological insecticides for use in the integrated management of rice stalk stink bug and other insect pests.

TABLE 1- Mean mortality (%) of second instar nymphs of stink bugs *Tibraca limbativentris* in 24, 72 and 120 hours for each concentration of different plant extracts of Annonaceae (mean±SE)

[ ] [1]	***Annona mucosa* (seeds)***			***Annona mucosa* (leaves)***			***Annona crassiflora* (seeds)***		
	time after application			time after application			time after application		
	24 h	72 h	120 h	24 h	72 h	120 h	24 h	72 h	120 h
8,0%	100,0±0,0a[2]	100,0±0,0a	100,0±0,0a	26,0±1,6a	32,0±1,3a	38,0±12,2a	70,0±4,7a	78,0±4,9a	81,0±4,1a
4,0%	92,0±1,3b	96,0±1,6a	98,0±1,3a	14,0±2,7b	24,0±2,7b	26,0±3,1a	64,0±5,0a	72,0±4,9a	76,0±4,0a
2,0%	90,0±3,7b	96,0±2,7a	96,0±2,7a	10,0±4,2c	18,0±5,3b	22,0±4,5a	44,0±5,0b	54,0±4,5b	58,0±4,9b
1,0%	76,0±1,6c	86,0±3,4b	88,0±3,9a	6,0±1,6c	10,0±3,7c	26,0±7,5a	20,0±2,1c	30,0±3,0c	34,0±2,7c
0,5%	28,0±4,9d	42,0±7,1c	56,0±7,2b	4,0±1,6d	18,0±2,5b	28,0±3,2a	6,0±1,6d	10,0±3,0d	10,0±3,0d
H_2O + Tween80®	2,0±2,0e	4,0±2,2d	9,0±3,5c	2,0±2,0d	4,0±2,2d	9,0±3,7b	2,0±2,0d	4,0±2,2d	9,0±3,5d
H_2O	0,0±0,0e	2,0±1,3d	6,0±2,2c	0,0±0,0d	2,0±1,3d	6,0±2,2b	0,0±0,0d	2,0±1,3d	6,0±12,2d
LC_{50}	1,59% (0,49 - 2,69)[3]	1,18% (-0,24 - 2,61)	0,91% (-0,76 - 2,58)	15,10% (13,47 - 16,72)	12,99% (12,26 - 13,72)	12,61% (12,24 - 12,97)	4,46% (4,34 - 4,58)	3,64% (3,55 - 3,74)	3,30% (3,16-3,43)
LC_{90}	4,5% (3,25 - 5,92)	4,0% (2,61 - 5,51)	3,7% (2,28 - 5,27)	27,6% (24,42 - 30-80)	25,73% (24,14 - 27,31)	28,22% (27,31 - 29,14)	8,96% (8,72 - 9,21)	8,01% (7,82 - 8,20)	7,65% (7,38 - 7,92)
CV	14,59%	17,42%	17,77%	81,96%	61,76%	64,60%	37,03%	32,25%	28,90%
F	296,18	173,32	132,37	15,14	12,88	6,10	74,53	78,82	82,71

**(p<0,001); [1][ ]=Concentracion; [2]Means followed by same letter in the column do not differ by Scott Knott test at 5%; [3]Confidence Interval.*

CONCLUSIONS

1 - The seed extracts of *Annona mucosa* show high toxicity on the second instar of *Tibraca limbativentris* nymphs with mortality greater than 75% at an extract concentration of 1.0% already in the first 24 h after application.

2 - The leave's extracts of *A. mucosa* show low toxicity for 2nd instar of *T. limbativentris* nymphs and mortality did not exceed 40% even after 120 h of application.

3 - The seed extracts of *Annona crassiflora* present intermediate toxicity among all the extracts tested and mortality of nymphs exceeded 80% at the highest concentration after 120 h of applying the extract.

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