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Controlling Properties of the Hydro-Alcohol-Glycol Extract of Plant Species, Myrtaceae Family, on *Callosobruchus maculatus* in Grass Legume

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Some plants of the Myrtaceae family have active principles that inhibit the development and growth of many insects, according to experts, with antifeedant effect, repellent and insecticide. In the present study, we evaluated the bioactivity of three doses of compound extract from seeds of *Myrciaria dubia* (Kunth) McVaugh, Myrtaceae, bioprocessed in order to use them for the biocontrol of *Callosobruchus maculatus* in the grass legume *Vigna unguiculata* (L.) Walp (cowpea) without previous treatment. The sequential probability ratio test for the period and minimum dose of extract application was applied with and without the presence of *M. dubia*, inter-related to the health and physiological behavior of cowpea, evaluated initially at six and fifty days of infestation. The positive effect was obtained around 94% for the health and in the physiological evaluation, an average value of 73% of effectiveness on the doses tested, which showed no significant difference with each other. The extract caused the death of adult *C. maculatus* and the immobilization of hatchings in cowpea. In the *In vitro* conditions, it has insecticidal and controlling bioactive properties with potential for use in preservation of stored seeds and grain of grass legumes such as *Vigna unguiculata* (L.) Walp.

1. Introduction

Many plant families such as Myrtaceae, Asteraceae Piperaceae are well known to have active ingredients like terpenoids and amide groups, which have antifeedant, repellent and insecticide effect that inhibits the development and growth of many insects of the *Lepidoptera* order (Srivastava et al., 2000; Mendoza et al., 2008). Researchers have recently found that aqueous extracts of the leaves of the Myrtaceae species have bioactivity (Imatomi et al., 2015), i.e., ability to bind to tissues (Silva Junior and Orefice, 2001). The extracts tested showed potential for the isolation of active compounds that can be used for the production of natural herbicides in the future (Imatomi et al., 2015).

Myrciaria dubia (camu-camu) of the Myrtaceae family is rich of secondary metabolites already proven in studies published by researchers in the field (Fracassetti et al., 2013). It grows along the banks of rivers and lakes throughout the Amazon basin (Delgado and Yuyama, 2010; Yuyama, 2011). It is known for high content of vitamin C, 800-6100 mg/100 g, according to Yuyama (2011), present in the pulp of its fruit, antioxidant capacity (Correa et al., 2011) and has astringent properties found both in the pulp and in shell (Chirinos et al., 2010). It is also a high percentage of antioxidants in the oil extracted from the seeds (Diogenes Filho et al., 2018). The secondary metabolites produced by plants, highly regarded, were considered as special metabolites (Gottlieb, 1996). These are specific of the species and participate in intra- and intercellular interactions of the organism itself or with cells of other organisms (Montanari and Bolzani, 2001). Therefore, the exploitation of biological activity of secondary compounds present in extract or essential oil of plants can be an option to safely reduce the use of pesticides (Schwan-Estrada; Stangarlin; Cruz, 2003).

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Active compounds found in plants have been commercially valued, via biotechnological processes for various industries. They contribute to the resistance against pests and diseases, among others, while maintaining favorable environmental conditions (Braz-Filho, 2010). The greatest difficulty is the technical-scientific finding of bioactivity in extracts developed fast, and the determination of an appropriate concentration (Bogorni and Vendramim, 2003).

Quick verifications of bioactivity were identified in aqueous extract of medicinal plants in fava bean seeds via physiological and health behavior (Nobre et al., 2014). Germination test, first count of germination, germination speed index, root length in the first and last count, and sanity test were performed (Nobre et al., 2014). Thus, the present study aimed to evaluate the bioactivity of extract produced using seeds of *Myrciaria dubia* (Kunth) McVaugh, Myrtaceae, bioprocessed in *in vitro* bioassay, via physiological and health behavior of *Vigna unguiculata* (L.) Walp (cowpea) without prior treatment in order to use them for the biocontrol of *Callosobruchus maculatus*.

2. Materials and Method

2.1 Collection, preparation of sample and obtaining cowpea samples and preparation of compound extract of camu camu seeds

The experimental studies were conducted from May to December 2015, in the waste and seeds laboratories of the Brazilian Agricultural Research Corporation, located (02º45'28" N 60º43'54" W) in Boa Vista-Roraima, Brazil. The BR samples were obtained from 10 materials that were stored and preserved in a freezer since the collection period, 2012-2014.

The cowpea samples, BRS Aracê, not previously treated and maintained in uncontrolled environment, was obtained in June from a production performed in technological showcase the company headquarters. They were taken to the seed laboratory (21 ± 3°C and 65 ± 5% relative humidity) in November, already contaminated by weevils (*Callosobruchus maculatus*).

Based on expertise procedures (Mapric, 2001 and Ardisson et al., 2002), the extract composed of camu-camu seed was obtained in May, in the company's waste laboratory, from a mixture of distilled water-based solvents, ethyl alcohol (95%) and PA glycerin and powders of seeds, pre-dried and crushed, grown in the northern Amazon. Termed as concentrate hydro-alcohol glycol extract (Hag) - bioproduct (Bp) bioprocessed *M. dubia* seeds-based bioproduct.

To perform preliminary *in vitro* bioassay, the Hag extract, concentrated and maintained under semi-controlled room temperature, was diluted at 8% for performing the treatment on contaminated cowpea sample, applying three doses (1, 2 and 3 mL) of Hag.

From the sample of approximately 2 kg of cowpea seeds, contaminated by weevils, three sub-samples of 600 g were weighed and split into three aliquots of work, 200 g each. These were identified and prepared as Bioproduct (Bp), control (C) and Witness (W).

In the samples Bp and C containing cowpea seeds, three doses (1, 2, 3 mL) of Hag extract were applied, previously prepared, with and without plant product (seed powder of *M. dubia*), both unprecedented. In the T sample containing the same seeds, only water was applied. Later, these were manually shaken for 1 minute to homogenize the surface of the seeds and then kept at constant temperature of 24 ºC in the seed laboratory.

Work samples, properly treated were divided into four replicates (I, II, III and IV) of 50 g and placed in glasses with screened lids for the establishing itself of the bioassay.

The bioassay was established based on the sequential probability ratio test (Wald, 1947), statistical method characterized by the fact that the number of observations is not fixed before conducting the experiment. The decision to continue or complete the assessment depends on the results obtained at each stage (Wald, 1947).

Thus, taking into account the partial information obtained from sampling, health (Nobre et al., 2014) and germination tests (Brazil, 2009) of work samples were established to verify the bioactivity, pre-selection of a minimum dose and appropriate period of application of the product in contaminated cowpea samples with postures of pest insects, *C. maculatus* (weevil)

2.2 Biossay assessment - Health and physiological behavior of work samples

The bioassay was evaluated in a randomized experimental design, arranged in a factorial 2 x 4, according to results obtained in the sequential probability ratio test for the period and minimum dose of application interrelated to health and physiological performance of work samples. We used two extracts (C and Bp) and four doses (0, 1, 2 and 3 mL), with four replications, and the dose 0 related to the T sample and the others, C and Bp. Thus, after six days of bioassay installation, the hatched weevils were removed from the glass jars, counted and discarded, remaining only the seeds. Completing 10 days, new withdrawal and counting of insect pests were held successively at 21, 30, 40 and 50 days and the population level evaluated by measures of relative population (number of insects per survey) and population indices (average products and the effects caused by insects) according to Castellani and Brandao (2000).

The assessment of the damage to seeds and adult emergence of *C. maculatus* on cowpea was held at fifty days of the application of bioproducts. Based on Almeida et al. (2015), the number of hatched insects was counted, mass loss of seeds in study quantified, and by weighing the remaining mass by the difference of the initial weight, the loss percentage was calculated.

In order to quantify the mass and evaluate the number of grains with holes caused by emerged adults, and set the percentage and number of holes per grain, 100 grains were separated from the sub-sample. These were classified into grains with 1, 2, 3, 4, 5, and 6 holes in each repetition. Then, with these same grains, germination and vigor tests were carried out as Brazil (2009) to detect the bioactivity itself of Hag extracts.

2.3 Statistical analysis

The data obtained in the bioassay were subjected to analysis of variance and the means were compared by Tukey test ($p \leq 0.05$) with the help of the software SISVAR (Ferreira, 2011) and for bioproduct doses, regression in the analysis of variance was performed considering the equation of largest coefficient R^2.

3. Results and discussion

3.1 Minimum dose and duration of the studies on the bioactivity of Hag extracts on *C. maculatus*

In Figure 1-a, we can observe that over time, the tested Hag extracts began to show a potent insecticidal action on *C. maculatus* (weevil). It is efficient in reducing the hatched adults when applied directly to the stored cowpea grains (Figure 1b). The Cd1 and Bpd1 extracts significantly reduced the number of insect pests, from 1246 to 73 in average (Figure 1-a), with a positive effect around 94% by the end of the evaluation period of the bioassay at 50 days (Figure 1-b).

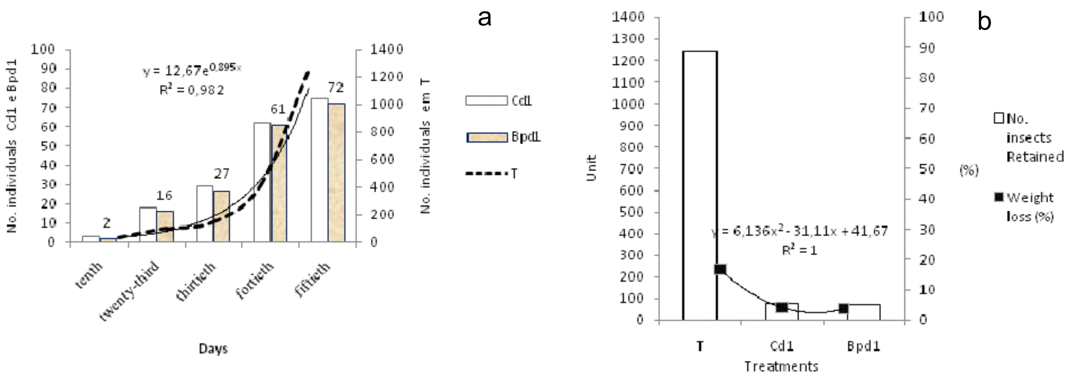

Figure 1: Effectiveness values obtained from the tenth to the fiftieth day between treatments (a) and number of hatched and reduced individuals at fifty days in Cd1 and Bpd1, compared to T and related weight loss (b).

Other researchers had observed that the *A. indica* extract, whose active ingredient is azadirachtin, caused 100% mortality in adults when using a dose of 9 ml of extract (Almeida et al., 1999; Almeida et al., 2005; Azevedo et al., 2007). In addition, when it was evaluated the effects of plant alcohol extracts on this insect pest, they observed the efficiency of *Callopogonium caeruleum,* a root containing rotenone (Almeida et al., 1999). The extract inhibited the insect postures performance, thus, inducing fewer eggs laid on the cowpea grains (Azevedo et al., 2007). This behavior was observed in the assessments (Figure 1-b).

The adults insect pest *C. maculatus* are fully mature 24 to 36 hours after emergence. Males seek females to inseminate and females store viable sperm in their spermatheca. Neither male nor female adults require food or water during their short adult lifetime (10-14 days) (Beck and Blumer, 2014).

Thus, from the analysis of results (Figure 1), in continuity with the proposed bioassay reviews, we obtained the average values of mass and perforated grains (Table 1) after 50 days of monitoring, related to damage to the seeds and emergency of *C. maculatus* adults in the witness and three doses (1, 2, 3 mL) of Hag extract with and without the presence of bioprocessed *M. dubia* seeds, respectively, control and byproduct (C and Bp).

Table 1: Average mass values of 100 seeds and percentage of perforated grains after applying doses of Hag extracts, Cd1, Cd2, Cd3, Bpd1, Bpd2 and Bpd3 compared to the Witness (W) at 50 days of monitoring of C. maculatus.

Variables in treatments	Witness and doses of Hag extracts (C,Bp)						
	T	Cd1	Cd2	Cd3	Bpd1	Bpd2	Bpd3
Mass 100 seeds[1] (g)	**13.92[a]**	**16.21[b]**	**16.64[b]**	**16.83[b]**	**16.27[b]**	**16.77[b]**	**16.89[b]**
Variance	1.794	0.118	0.039	0.426	0.164	0.187	0.279
Standard deviation	1.340	0.343	0.198	0.653	0.405	0.433	0.528
Coefficient of variation	9.62	2.12	1.19	3.88	2.49	2.58	3.13
% Perforated grains[1]	**88.25[a]**	**51.00[b]**	**55.00[b]**	**55.00[b]**	**49.00[b]**	**51.00[b]**	**51.00[b]**
Variance	30.917	1.333	38.667	18.667	22.667	12.00	38.667
Standard deviation	5.560	1.155	6.218	4.320	4.761	3.464	6.218
Coefficient of variation	6.30	2.26	11.31	8.00	9.72	6.79	12.19

[1]Means followed by the same letters on the line do not differ by Tukey test, at 5% probability.

The results (Table 1) showed that both doses had not significant differences; however, they interchangeably caused reduction of hatching around 33% of *C. maculatus* in the development period as shown in Figure 2, when compared to the efficiency of W (witness) to Hag extracts for the first dose (Cd1 and Bpd1).

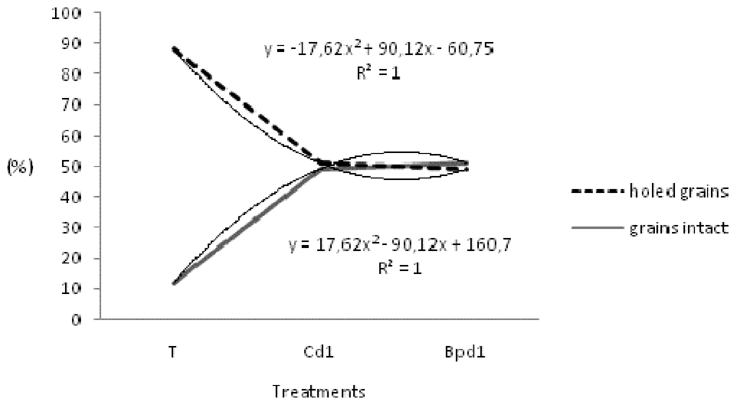

Figure 2: Comparison of efficiency percentage values obtained in the reduction of hatching (intact grains) and in the development of pest insects (holed grains) in the Witness treatment, Cd1 and Bpd1.

The results obtained (Figure 2) from the health test, it was found that in the damage assessment and emergency of adult pest insects, both tested extracts (bioproducts) were effective for the biocontrol of *C. maculatus*. They caused mortality of adult insect pests and the stopped the hatchings, indicating that they also have repellent bioactive properties against adult pest insects.

Thus, in general, the results obtained revealed the product potential with positive biocontrol effects shown in the health and physiological quality of cowpea seeds treated with any of the doses tested for fifty days.

3.2 Physiologic Evaluation of cowpea seeds - bioactivity of Hag extracts

The most surprising results for the evidence of bioactivity of Hag extracts prepared from bioprocessed *M. dubia* seeds were obtained in the physiological assessment through germination tests of cowpea seeds treated with Bp, but already contaminated with pest insect postures, weevil, at fifty days of monitoring (Figure 3). This can be understood as the ability of the material to interact with living tissues in such a way to stimulate physicochemical processes inherent to biological systems that allow integration of the biomaterial in the receiving environment (Silva Junior and Orefice, 2001).

Seed germination bioassays are usually conducted in germination camera under controlled conditions of temperature and light, lasting a variable time, usually between 7 and 10 days. In some cases, at the end of the incubation period, the germinated seeds are counted and the allelopathic effects are calculated comparatively to the W treatment (Souza Filho et al., 2010).

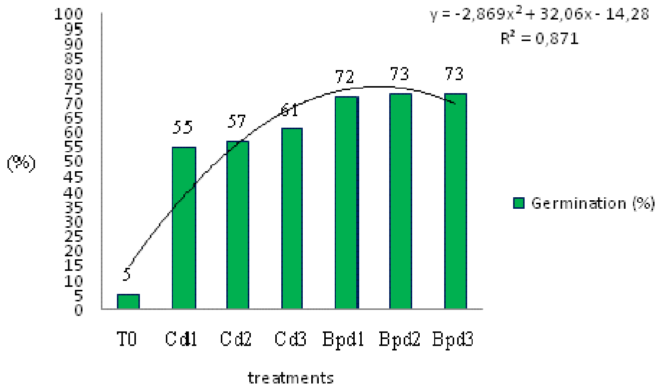

Figure 3: Percentage values obtained in the germination test with cowpea seeds in treatments established for verification and evidence of bioactivity of the prepared extracts.

4. Conclusions

In *in vitro* conditions, hydro-alcohol glycol extract based on *Myrciaria dubia*, Myrtaceae family, has bioactive and controlling properties with insecticide potential for use in the preservation of stored seeds and grains of grass legumes such as *Vigna unguiculata* (L.) Walp. It causes mortality of adult *C. maculatus* and the stops the hatchings, suggesting that it has bioactive properties equally repellent against adult insects.

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