Revista Colombiana de Entomología 2022, 48 (2): e11537 • https://doi.org/10.25100/socolen.v48i2.11537

Sección Control / Control
Artículos de investigación / Research paper

Toxicity of *Agave sisalana* extracts on *Cordyceps* and their effect and the association with fungi on *Nasutitermes corniger* (Isoptera: Termitidae)

Toxicidad de los extractos de *Agave sisalana* en *Cordyceps* y su efecto y asociación con hongos en *Nasutitermes corniger* (Isoptera: Termitidae)

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Suggested Citation
LOPES, R. S.; MARTINS, M. C. B.; LIMA, G.; OLIVEIRA, L. COSTA, A. F.; SANTOS, V. F.; CORREIA, M. T. S.; DA SILVA, N. H.; ALBUQUERQUE, A.C; LUNA-ALVES LIMA, E. A.; LIMA, V. L. M. 2022. Toxicity of *Agave sisalana* extracts on *Cordyceps* and their effect and the association with fungi on *Nasutitermes corniger* (Isoptera: Termitidae). Revista Colombiana de Entomología 48 (2): e11537. https://doi.org/10.25100/socolen.v48i2.11537

Received: 20-Ago-2021
Accepted: 20-May-2022
Published: 1-Nov-2022

Revista Colombiana de Entomología
ISSN (Print): 0120-0488
ISSN (On Line): 2665-4385
https://revistacolombianaentomologia.univalle.edu.co

Publishers: Sociedad Colombiana de Entomología SOCOLEN (Bogotá, D. C., Colombia)
https://www.socolen.org.co
Universidad del Valle (Cali, Colombia)
https://www.univalle.edu.co

Abstract: *Nasutitermes corniger* is an arboreal termite that causes economic damage in urban areas, and its control is for chemical insecticides. An alternative for insect control is the use of entomopathogenic fungi and plant extracts, or the synergistic effect of the association of these. The toxicity of aqueous and hydroethanolic extracts of *Agave sisalana* on the biological aspects of the fungal *Cordyceps farinosa*, *C. fumosorosea* and *C. javanica* and the action of the extracts and their synergistic effect on *N. corniger* was examined. The effect of the extracts on fungi was evaluated by germination, sporulation and mycelial growth of fungi in Sabouraud medium through the study of biological aspects under laboratory conditions; and control of *N. corniger* were examined, by ingestion of extracts and of the association extract plus fungus on filter paper, being the percentage of mortality of soldiers and workers of the termite daily. In general, the extracts were inoffensive to the fungi at concentrations 10, 25, 50, 100 and 200 mg.mL^{-1}. The extracts demonstrated termiticidal actions at concentrations tested, causing the mortalities about 100% of the workers after the third and sixth days (LC_{50}: 0.778 to 0.803 mg.mL^{-1}) and 100% of the soldiers between the fourth and seventh days (LC_{50}:0.146 to 0.956 mg.mL^{-1}). The association of the extracts with *C. farinosa* ESALQ1355 was more efficient in inducing the mortality in *N. corniger.* These results demonstrated the efficiency *in vitro* of the sisal extracts in controlling *N. corniger* termites, alone or in combination with fungi, suggesting their potential joint usefulness in the biological control of insect pests.

Keywords: Biological control, entomopathogenic fungi, plant extracts, sisal, termite.

Resumen: *Nasutitermes corniger* es una termita arbórea que causa daños económicos en las zonas urbanas, y su control es mediante insecticidas químicos. Una alternativa para el control de insectos es el uso de hongos entomopatógenos y extractos de plantas, o el efecto sinérgico de la asociación de estos. Se examinó la toxicidad de los extractos acuosos e hidroetanólicos de *Agave sisalana* sobre los aspectos biológicos de los hongos *Cordyceps farinosa*, *C. fumosorosea* y *C. javanica* y la acción de los extractos y su efecto sinérgico sobre *N. corniger*. Se evaluó el efecto de los extractos sobre hongos por germinación, esporulación y crecimiento micelial de los hongos en medio Sabouraud mediante el estudio de aspectos biológicos bajo condiciones de laboratorio y se examinó el control de *N. corniger*, por ingestión de extractos y de la asociación extracto y hongo sobre papel de filtro, siendo el porcentaje de mortalidad de soldados y obreras de la termita diaria. En general, los extractos resultaron inofensivos para los hongos a concentraciones de 10, 25, 50, 100 y 200 mg.mL^{-1}. Los extractos demostraron acciones termiticidas a las concentraciones ensayadas, provocando la mortalidad de alrededor del 100% de los trabajadores después del tercer y sexto día (LC_{50}: 0.778 a 0.803 mg.mL^{-1}) y del 100% de los soldados entre el cuarto y séptimo días (LC_{50}: 0.146 a 0.956 mg.mL^{-1}). La asociación de los extractos con *C. farinosa* ESALQ1355 fue más eficiente

para inducir la mortalidad en *N. corniger*. Estos resultados demostraron la eficiencia *in vitro* de los extractos de sisal en el control de las termitas de *N. corniger*, solas o en combinación con hongos, lo que sugiere su potencial utilidad conjunta en el control biológico de plagas de insectos.

Palabras clave: Control biológico, hongos entomopatógenos, extractos de plantas,termitas.

Introduction

Insect pests are controlled using chemical insecticides, which can contaminate man and the environment – and their continued use usually results in the appearance of resistant insects (Chen *et al*. 2004; Pourseyed *et al.* 2010). Plant extracts and entomopathogenic fungi have been found to be efficient alternatives for controlling insect pests by maintaining insect populations at low-level equilibriums and limiting their spread (Marques *et al.* 2004; Sabbour & Abdel-Rahman 2013).

Plant extracts have been tested for insect pest control, due to the insecticidal action of their secondary compounds, besides being easily degradable and not contaminating the environment (Oliveira *et al.* 2007; Souza *et al.* 2011). These substances are more beneficial than chemicals, since they are renewable, easily degradable and do not pollute the environment (Oliveira *et al.* 2007; Souza *et al.* 2011).

Agave sisalana Perrine ex Engelm (Asparagales: Agavaceae) or sisal is an herbaceous plant whose fiber of its leaves is used as raw material in the manufacture of ropes, threads, and rugs in Brazilian industry (Costa *et al.* 2014). Residues and extracts of sisal are constituted by alkaloids, tannins, triterpenoids, saponins and flavonoids, which have insecticidal properties (Costa *et al.* 2014) being efficient controlling arthropods such as the mosquito *Aedes aegypti* (L., 1762) (Diptera: Culicidae), the red mite *Tetranychus urticae* (Koch,1836) (Acari: Tetranychidae), and the carmine cochineal *Dactylopius opuntiae* Cockerell, 1896 (Hemiptera: Dactylopiidae) (Barreto *et al*. 2010; Nunes *et al.* 2015; Lopes *et al.* 2018).

The fungus *Cordyceps fumosorosea* (= *Isaria fumosorosea*) (Wize) Kepler, B. Shrestha & Spatafora, *C. farinosa* (= *Isaria farinosa*) (Holmsk.) Kepler, B. Shrestha & Spatafora and *C. javanica* (= *Isaria javanica*) (Bally) Kepler, B. Shrestha & Spatafora are efficient in controlling *in vitro* the termites *Coptotermes formosanus* Shiraki 1909, *Heterotermes tenuis* (Hagen, 1858) (Isoptera: Rhinotermitidae), *Coptotermes gestroi* (Wasmann, 1896) (Isoptera: Rhinotermitidae), and *Nasutitermes corniger* (Motschulsky, 1855) (Isoptera: Termitidae) (Lopes *et al.* 2011; Wright & Lax 2013; Lopes *et al.* 2017).

Nasutitermes corniger is the most common and most important termite pest of the genus, being widely distributed in the Americas, from southern Mexico to northern Argentina causing considerable damage to historical buildings, collections, and documents, and likewise attacks urban constructions and ornamental tree. In Brazil, it is found in all its territory, especially in northern and northeastern states (Piauí, Bahia, Paraíba, and Pernambuco) (Milano & Fontes 2002; Albuquerque *et al.* 2012; Mello *et al.* 2014). *N. corniger* is highly adaptable to colonization of contrasting habitats in urban, agricultural, and natural environments (Breznak *et al.* 1982), being found on *Moringa oleifera* Lam. plantations in Nicaragua (Quiroz-Medina *et al.* 2021) and it is also encountered in the Bahamas and New Guinea and is responsible for enormous economic damage to wood products (Scheffrahn *et al.* 2006).

The use of entomopathogenic fungi in combination with plant oils and extracts may enhance their efficiency in insect pest control, as well as reducing the damage to the environment caused by commercial insecticides (Marques *et al.* 2004; Santos *et al.* 2015; Silva *et al.* 2015). In this sense, the objective of this study was to analyze the insecticidal potential of the extracts of *A. sisalana* and the combination of these with *Cordyceps* spp. in the control of *N. corniger.*

Materials and methods

Preparation of the plant extracts. The leaves of *A. sisalana* were collected from plants growing at the Agronomic Institute of Pernambuco (Brazil) and identified by the Botany Department there. The reference samples were deposited at "Dárdano de Andrade Lima Herbarium" at Agronomic Institute of Pernambuco. After collection, the leaves were washed in distilled water, dried at room temperature, and subsequently triturated. The extracts were obtained according to Lopes *et al.* (2018). Aqueous extracts of *A. sisalana* (AELAs) were prepared from 20 g the plant material mixed with 80 mL of a 0.15 M NaCl solution, for a final concentration of 250 $mg.mL^{-1}$ (w/v). The suspension was agitated for 16 hours at 4 °C, followed by filtration and centrifuging at 10.000 rpm for 15 min (4 °C). Hydroethanolic extracts (HELAs) were prepared from 20 g of the plant material infused in 70 % ethyl alcohol (80 mL) for two hours, followed by filtering. The alcohol was then evaporated for 16 hours at 45 °C. The extracts (250 $mg.mL^{-1}$) were then diluted (in a 0.1 % solution of Tween 80) to+ final concentrations of 100, 50, 25, and 10 $mg.mL^{-1}$.

Species of *Cordyceps.* For the pathogenicity bioassay, three species of *Cordyceps* were selected due to the potential in termite *in vitro* control, using the strains *C. farinosa* ESALQ1355, *C. fumosorosea* ESALQ1297 and *C. javanica* URM4993 in the lethal concentration pre-established, having presented excellent percentages of germination and being effective in the control of *N. corniger* workers, according to Lopes *et al.* (2017) (Table 1). *C. javanica* URM4993 from the URM collection at the Federal University of Pernambuco/UFPE; *C. farinosa* ESALQ1355 and *C. fumosorosea* ESALQ1297 from the ESALQ Collection of Microorganisms at Escola Superior de Agricultura Luiz de Queiroz – ESALQ of the University of São Paulo/USP (Table 1). The fungi were cultivated in Sabouraud-Dextrose-Agar (SDA) for 12 days, after that time, the conidia of each species were transferred to 10 mL of a Tween 80 (0.1 %) solution and quantified using a Neübauer chamber, being then diluted to a final concentration of 1 x 10^7 $conidia.mL^{-1}$. This conidia solution was used for experiments of compatibility with sisal extracts and to obtain LC_{50} values using *N. corniger* workers.

Nasutitermes corniger. Parts of termite mounds located at Federal University of Pernambuco (UFPE), State of Pernambuco (Brazil). They were collected with the aid of a hatchet and conditioned in plastic pots. The samples were identified by Dr. Auristela Correia de Albuquerque, from the Federal Rural University of Pernambuco (UFRPE) / Brazil. The identification of termites was carried out using the key for neotropical termite genera (Constantino 2002) and by comparing the morphological characteristics with the specimens of the species deposited in the Collection of the Order Isoptera of the Department of Biology at UFRPE.

Table 1. The strains of *Cordyceps* used in the experiments.

Strains	Origin	Germination (%)	LC_{50}(CI)* (conidia.mL^{-1})
Cordyceps farinosa ESALQ1355	ESALQ Culture Collection	91	6.66x10^4 (12.47-3.10)
Cordyceps javanica URM4993	URM Culture Collection	98	7.22x10^5 (20.25-2.43)
Cordyceps fumosorosea ESALQ1297	ESALQ Culture Collection	91	4.60x10^5 (13.10-1.38)

Source: Lopes *et al*. (2017). *95% confidence interval.

Effects of the plant extracts on the fungi strains tested. The effects of the extracts on the *Cordyceps* spp. were evaluated by measuring germination, vegetative growth, and the sporulation of the fungal. These experiments were performed on the three strains of *Cordyceps* used in the toxicological studies, using the two sisal extracts at five different concentrations, as well as a control (without the sisal extract), totaling 33 treatments, with three repetitions. To evaluate conidia germination, a 1 mL suspension (1 x 10^8 conidia.mL^{-1}) of each of the fungal specie was inoculated into 9 mL of each extract concentration with Tween 80 (0.1 %) (Tween solution without any extract served as the control) to obtain suspensions with 1 x 10^7 conidia.mL^{-1}. After one hour, 0.1 mL of those suspensions were inoculated into Petri dishes containing SDA, which were subsequently incubated in a BOD chamber (26 ± 1 °C, 80 ± 10 % RH). Germination was determined after 16 hours by observing 500 conidia (both germinated and non-germinated); the germination percentages were calculated using the formula (G = n x 100/500), following Alves & Pereira (1998) To evaluate vegetative growth and sporulation, the extracts were added to the SAD media in autoclaved Petri dishes while it was still liquid (45 °C) to final concentrations of 10, 25, 50, 100 and 200 mg.mL^{-1}. Filter paper disks (0.3 mm diameter) with 0.01 mL of a conidia suspension (1 x 10^7 conidia.mL^{-1}) of each strain of *Cordyceps* tested were placed in the Petri dishes with SDA containing the sisal extracts at test concentrations and subsequently incubated in a BOD chamber (26±1 °C, 80 ± 10 % RH) for 12 days. Mycelial growth was determined by measuring the diameters of the colonies. To evaluate sporulation, fragments of the vegetative colonies (cultured as above) were transferred to test tubes containing 10 mL of Tween 80 (0.1 %) solution, and the suspensions agitated for approximately 2 min in a vortex mixer; the conidia from each were then quantified by counting using a Neübauer chamber.

Toxicity efficiency of the sisal extracts on *Nasutitermes corniger*. The toxicity efficiency analyses of sisal extracts on *N. corniger* were according to Kang *et al.* (1990). Filter paper disks (4 cm diameter) were impregnated with 0.2 mL of the extract solutions (10, 25, 50, 100, and 200 mg.mL^{-1}) as well as with a 0.1% Tween 80 solution (control) and were subsequently dried at room temperature (24 ± 1 °C). The treated disks were then transferred to Petri dishes (90 x 15 mm) containing a small amount of humid cotton to maintain humidity levels. A total of 20 termites (4 soldiers and 16 workers) were carefully transferred to the Petri dishes, in the pre-established proportion of 1:4 for *N. corniger* to maintain the natural interdependence of the castes and guarantee the maximum survival of those insects outside of their nest, following Vasconcellos & Bandeira (2006). The insects were then carefully transferred to the Petri dishes and maintained at 26 ± 1 °C, relative humidity ± 80 %, in the dark. The experiment was therefore composed of 11 treatments (two types of extracts, five different concentrations of each, and one control) with five repetitions, totaling 100 insects per treatment (20 soldiers and 80 workers). Mortality was evaluated daily until the death of the last insect; the survival percentages and lethal concentrations (LC_{50}) were subsequently calculated. The LC_{50}values obtained were compared by the Chi-square Test.

Evaluations of associations plant extracts with *Cordyceps* spp. on *Nasutitermes corniger*. This experiment sought to determine the LC_{50} of each plant extract with the LC_{50} of the fungi (Tables 1 and 2). Paper filter disks (4 cm in diameter) were impregnated with 0.2 mL of suspensions containing the plant extracts and the fungi, as well as with extracts and fungi separately (which would serve as control treatments). The disks were dried at room temperature and transferred to plastic Petri dishes (90 x 15 mm) containing 16 worker termites, which were then maintained at 26 ± 1 °C, relative humidity ± 80 %, in the dark. The experiment was composed of 17 treatments (with five repetitions), totaling 80 insects per treatment; the percent mortality was evaluated after four days. In order to confirm fungal infection and consequent insect death, disinfection of insects with 70% alcohol, 4% sodium hypochlorite and sterile distilled water was carried out to eliminate microorganisms on the surface of the insect and subsequently transferred for a wet BOD chamber (26 ± 1 °C and 80% ± 10% RH) to the exteriorization of the fungal mycelium, according to Alves (1998). Afterwards, the material was inoculated in test tubes containing SAD + Chloramphenicol, to obtain the fungal growth in the period of 12 days, without the presence of bacteria. Afterwards, the aspects of the colonies and the characteristics of the microstructures (hyphae, mycelium, conidiophores and conidia) of the re-isolated species were verified by means of mycelial growth techniques in Petri dishes and cover crops, respectively, according to Domsch *et al.* (2007). The results obtained were compared with the literature (Samson *et al.* 1988; Lopes *et al.* 2016) and for the confirmation of re-isolated species and the percentage of mortality of the workers of *N. corniger.*

Statistical analyses. Data concerning the effects of extracts on biological aspects (germination, mycelial growth, and sporulation) of *Cordyceps* spp. were submitted to analysis of variance (ANOVA) using the SAS ANOVA Proc (SAS - Institute, 1999-2001) and the means were compared by the Tukey test at a 5 % level of probability. The extracts toxicity on *Cordyceps* spp. was determined by the Biological Index (IB), obtained by means of the formula IB = 47 [CV] +43 [ESP] +10 [GERM] / 100 (CV = the percentage of vegetative growth; ESP = % of sporulation; GERM = % conidia germination), all in relation to the control. The IB index may vary from: 0 - 41 (toxic), 42 - 66 (moderately toxic) and more than 66 (compatible) (Rossi-Zalaf *et al.* 2008). Using the termite

mortality data from the extracts, the survival rates (%) were determined for each treatment and the data were submitted to the Long-Rank test, using the Kaplan-Meyer method by pairs of isolates, through the SAS Proc Lifetest (SAS - Institute 1999-2001). The mean Lethal Concentrations (LC_{50}) was determined after the fourth day of treatment, using Proc Probit Software (SAS - Institute 1999-2001). Data on mortality of termites caused by the combination of fungi + extracts were submitted to analysis of variance (ANOVA) using the SAS ANOVA Proc (SAS - Institute, 1999-2001) and the means were compared by the Tukey test at a 5 % level of probability. The control data of the termites were presented graphically using Software GraphPad Prism (Software Prism 2016).

Results

The *A. sisalana* extracts reduced the survival indices of both the workers and soldiers of *N. corniger* at all of the concentrations tested, differing from the control treatment (in which the insects survived until the 11th day) **($p < 0.05$).** Exposure to the aqueous (AELAs) and hydroethanolic (HELAs) extracts at concentrations of 200 mg.mL and 100 mg.mL resulted in the deaths of 100 % of the workers after the third and fourth days of exposure; the other concentrations resulted in the deaths of 100 % of the workers between the fifth and sixth day (Figs. 1A and 1C). The soldiers treated with the EAAs solutions survived until the fourth day when exposed to extract concentrations of 50 $mg.mL^{-1}$, 100 $mg.mL^{-1}$, and 200 $mg.mL^{-1}$ (Fig. 1B), whereas the HELAs solutions resulted in deaths of 100 % of the soldiers after the fifth day at the same concentrations (Fig. 1D). At lower concentrations the soldiers survived until between the sixth and seventh day. These data differed significantly from the control treatment, in which all insects remained alive until the 11th day **($p < 0.05$)**.

There were no significant differences between the LC_{50} indices of the workers and soldiers of *N. corniger* exposed to the AELAs and HELAs, with LC_{50} of 8.0 $mg.mL^{-1}$and 7.0 $mg.mL^{-1}$, and 9.7 $mg.mL^{-1}$and 9.5 $mg.mL^{-1}$, respectively (Table 2).

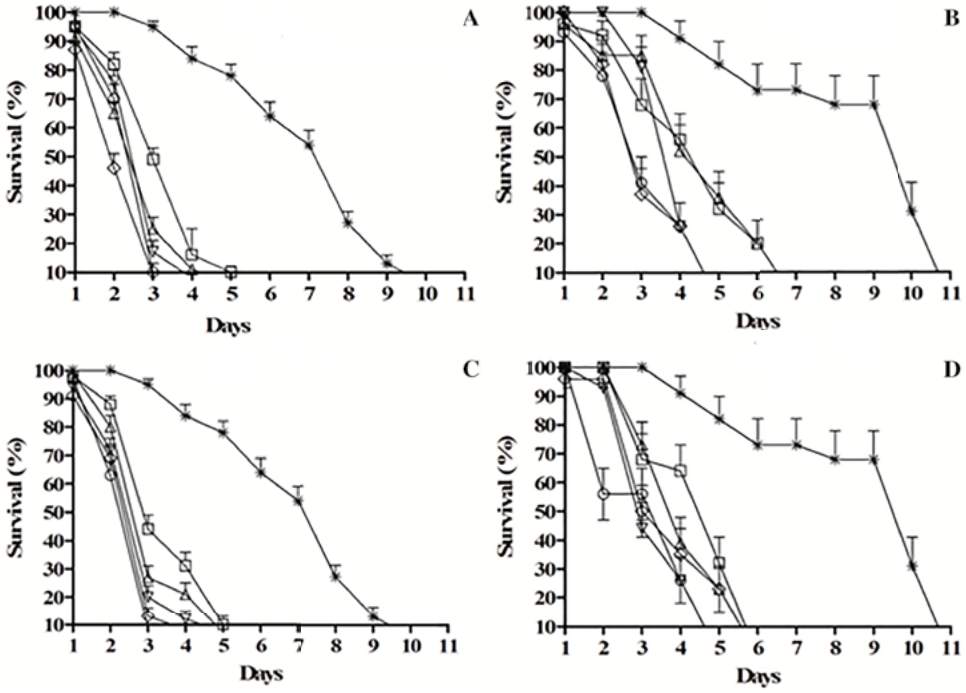

Figure 1. Daily survival (%) of workers and soldiers of *Nasutitermes corniger* treated with aqueous and hydroethanolic extracts of *Agave sisalana* evaluated until death of the last individual by the Long-Rank test: AELAs applied to workers (A) and soldiers (B). HELAs applied to workers (C) and soldiers (D), at concentrations of 10 (⊟), 25 (△), 50 (▽), 100 (◇), 200 mg/L (○), and the control (✳). Each point represents the mean ± SE of five repetitions.

Table 2. Lethal concentrations (LC_{50}) of *Agave sisalana* extracts on *Nasutitermes corniger.*

	Extracts Workers			Soldiers		
	LC_{50} (CI) * ($mg.mL^{-1}$)	**Regression equation**	**(χ^2)****	**LC_{50} (CI) ($mg.mL^{-1}$)**	**Regression equation**	**(χ^2)**
Aqueous	0.803 (1.108-0.489)	Y=5.15707+1.65228*logX	86.894	0.146 (0.657-0.000)	Y=5.9102+1.09267*logX	17.836
Hydroethanolic	0.778 (1.100-0.451)	Y=5.1666+1.52468*logX	113.918	0.956 (1.604-0.288)	Y=5.0325+1.566254*logX	76.449

* 95% confidence interval. ** Significant analyses by the Chi-square Test.

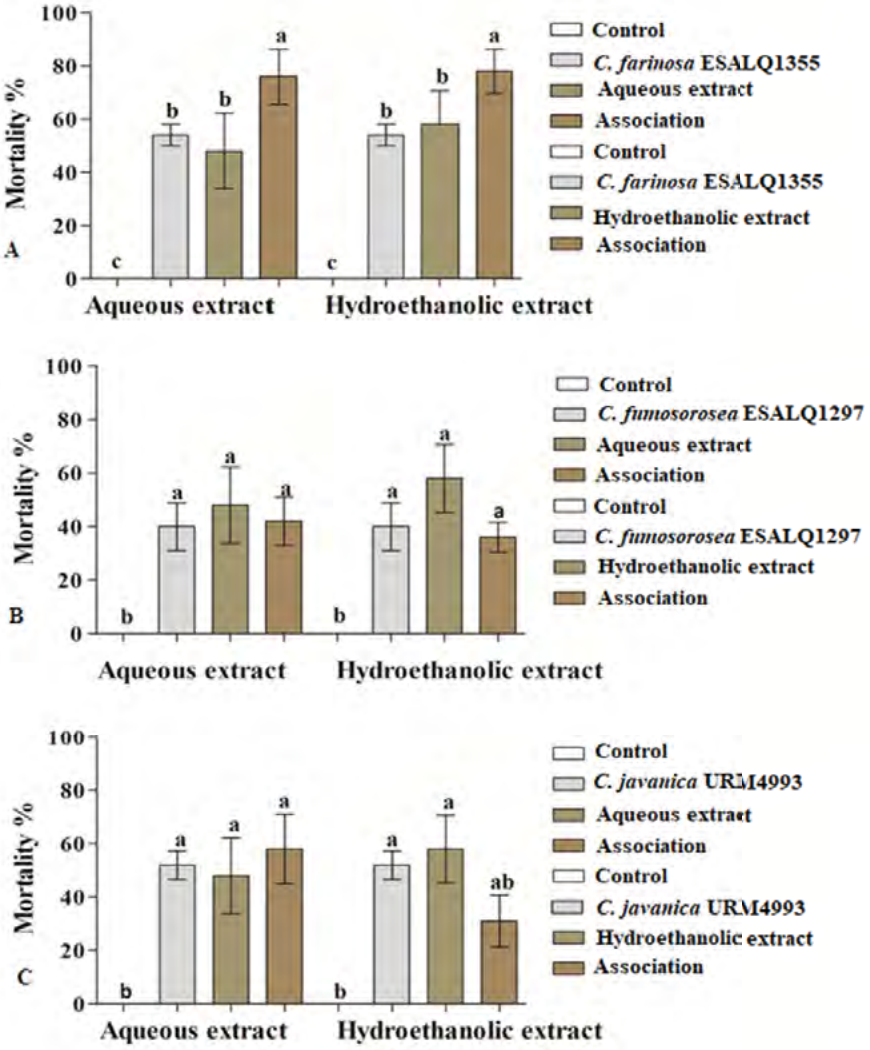

Figure 2. Effects of the association of fungal strains and aqueous and hydroethanolic extracts of *Agave sisalana* on the workers of *Nasutitermes corniger*: *Cordyceps farinosa* ESALQ1355 (A) (df: 5, F= 8.7812, $p < 0.01$), *Cordyceps fumosorosea* ESALQ1297 (B) (df = 5, F = 5.8253 $p < 0.01$), and *Cordyceps javanica* URM4993 (C) (df = 5, F = 5.7618, $p < 0.01$). Different letters on the bars indicate statistical differences between them by the Tukey test ($p = 0.05$).

The effects of the *A. sisalana* extracts on the biological aspects of the fungal strains can be observed in Table 3. No decreases in the germination percentages of the conidia of any of the strains were noted at the different extract concentrations, except with *C. fumosorosea* ESALQ1297 (which demonstrated a significant decrease in germination (33%) when exposed to the highest concentration of the aqueous sisal extract ($p < 0.05$). The different AELAS did not negatively affect the fungal strains at lower concentrations (10 mg.mL^{-1} and 25 mg.mL^{-1}) in terms of micellar growth and sporulation, which did not differ from the control treatment ($p < 0.05$). The HELAs, however, did promote diminished micellar growth of *C. farinosa* ESALQ1355 and *C. fumosorosea* ESALQ1297, although these extracts were well-tolerated by *C. javanica* URM4993 at low concentration (10 mg.mL^{-1} and 25 mg.mL^{-1}). These hydroethanolic extracts also negatively impacted sporulation, except in *C. farinosa* ESALQ1355, which demonstrated compatibility with extract treatments at concentrations of 10, 25, and 50 mg.mL^{-1}, which did not differ from the control ($p < 0.05$).

According to the value of the Biological Index (BI), the extracts were considered compatible with the fungi, at the concentrations tested, except aqueous, classified as toxic for *C. fumosorosea* ESALQ1297, at 200 mg.mL^{-1} concentration , presenting a biological index of 36.34 (Table 4).

The termiticidal activities associated with the sisal extracts combined with the *Cordyceps* strains against *N. corniger* workers can be seen in Figure 2. The associations of the AELAs and HELAs extracts with *C. farinosa* ESALQ1355 were efficient in causing the death of approximately 78% of the workers, with significant increases in the mean mortality as compared to applications of only the aqueous (47%) hydroethanolic (61%) extracts, or just the *C. farinosa* ESALQ1355 strain (53%) ($p < 0.05$) (Fig. 2A). The associations of the extracts with the fungal strains *C. javanica* URM4993 and *C. fumosorosea* ESALQ1297 did not result in synergetic actions in controlling *N. corniger*, as their combined the uses did not result in increases in the mean mortality rates caused by the fungi acting alone ($p < 0.05$) (Fig. 2B; C).

Table 3. Effects of aqueous and hydroethanolic extracts of *Agave sisalana* on the germination (%), growth (cm), and sporulation (1 x 10^7 conidia.mL^{-1}) of *Cordyceps*.

	Biological Aspects of Strains								
Extracts (mg.mL^{-1})	***Cordyceps farinosa* ESALQ1355**			***Cordyceps javanica* UM4993**			***Cordyceps fumosorosea* ESALQ1297**		
AELAs	**G***	**C****	**E*****	**G**	**C**	**E**	**G**	**C**	**E**
Control	100.00±0.00a	3.85±0.33a	4.17±0.13a	98.66±0.55a	3.20±0.00a	2.40±0.00a	98.40±0.00a	3.54±0.10a	2.06±0.34a
10	98.30±0.26a	3.53±0.33a	3.92±0.50a	98.00±0.00a	3.01±0.28a	1.90±0.15a	96.00±0.00a	3.47±0.01a	1.96±0.03a
25	97.60±0.46a	3.45±0.02a	2.80±0.25b	97.00±0.33a	3.00±0.00a	1.71±0.49a	95.70±0.26a	3.05±0.10b	1.59±0.21b
50	98.40±0.00a	2.91±0.28b	2.79±0.11b	96.00±0.33a	2.71±0.07b	0.88±0.14b	81.06±1.06a	2.81±0.04c	1.53±0.45b
100	95.66±0.70b	2.93±0.08b	2.20±0.05bc	96.00±0.33a	2.51±0.01b	0.86±0.06b	74.93±7.06b	2.41±0.06d	1.04 ±0.08c
200	95.67±0.33b	2.16±0.00c	1.46±0.24d	96.00±0.00a	2.00±0.00c	0.53±0.06b	33.60±6.80c	2.33±0.01d	0.57±0.00d
HELAs	**G***	**C****	**E*****	**G**	**C**	**E**	**G**	**C**	**E**
Control	100.00±0.00a	3.85±0.12a	4.17±0.08a	98.66±0.53a	3.20±0.04a	2.40±0.18a	98.40±0.96a	3.54±0.57a	2.06±0.34a
10	98.93±0.26a	2.55±0.07b	3.37±0.43a	96.55±1.06a	3.91±0.0a	2.05±0.09a	96.55±0.96a	3.16±0.04a	1.74±0.08a
25	98.66±0.55a	2.53±0.08b	3.17±0.09a	98.13±0.55a	3.74a±0.08a	1.75±0.16ab	98.13±0.46a	2.83±0.06ab	1.51±0.30ab
50	98.13±0.26a	2.55±0.10b	3.16±0.32a	97.87±0.26a	3.40±0.15b	1.48±0.19ab	97.87±0.26a	2.81±0.04ab	1.24±0.37abc
100	97.86±0.26a	1.10±0.15c	2.17±0.08b	96.53±0.96a	3.05±0.05c	1.26±0.20ab	96,53±3.00a	2.50±0.05b	1.77±0.04bc
200	97.67±0.26a	0.00±0.00d	0.00±0.00d	96.80±2.01a	2.59±0.19c	1.15±0.13ab	96.80±3.66a	1.91±0.01b	1.50c±0.10c

Means followed by the same letter in the same column do not differ significantly at a 5% level of probability by the Tukey test. *Germination, **Growth, ***Sporulation

Table 4. Compatibility of *Cordyceps* with extracts of leaves of *Agave sisalana.*

Extract	**Concentration (mg.mL^{-1})**	**Strains/ IB Values and classification**		
		***Cordyceps farinosa* ESALQ1355**	***Cordyceps javanica* URM4993**	***Cordyceps fumosorosea* ESALQ1297**
Aqueous	10	94.72 C	94.83 C	91.98 C
	25	94.06 C	88.48 C	90.62 C
	50	91.31 C	88.56 C	78.63 C
	100	89.30 C	87.78 C	69.67 C
	200	89.30 C	84.25 C	36.34 T
Hydroethanolic	10	96.01 C	92.37 C	95.90 C
	25	95.95 C	94.81 C	96.07 C
	50	94.56 C	94.81 C	94.84 C
	100	89.56 C	91.35 C	82.24 C
	200	85.75 C	89.46 C	73.89 C

*Biological Index C compatible (above 66), MT moderately toxic (42-66), T toxic (0-41).

Discussion

Our data showed the efficiency of *A. sisalana* extracts in diminishing the survival rates of workers and soldiers of the termite pest *N. corniger*, indicating that the extracts contain primary and/or secondary metabolites with insecticidal properties. Plants are known to be rich sources of bioactive compounds that demonstrate toxicity to termites, repel them, or inhibit their consumption of potential food sources (Scheffrahn 1991; Chen *et al.* 2004) but that are, at the same time, inoffensive to humans and the environment (Omena *et al.* 2007).

The AELAs and HELAs extracts showed insecticidal activity in relation to *N. corniger*, causing the deaths of all the workers and soldiers at a concentration of 10 mg.mL^{-1} (after five and seven days, respectively); at higher concentrations, insect survival varied between three and five days, with LC_{50} values varying between 7.0 mg.mL^{-1} and 9.7 mg.mL^{-1}. These data corroborate those of Santana *et al.* (2010), who reported the susceptibility of *N. corniger* to extracts of *Bowdichia virgilioides* Kunth (Fabaceae), with all the termites dying after the fourth day when treated with extract concentrations of 100 mg.mL^{-1} (LC_{50} 7.2 mg.mL^{-1}); likewise ethyl acetate extracts of *Anadenanthera colubrina* (Vell.) Brenan (Fabaceae), provoked the death of 100 % of the insect individuals after seven days when tested at concentrations of 25 mg.mL^{-1}, 50 mg.mL^{-1}, and 100 mg.mL^{-1} (LC_{50} 17.3 mg.mL^{-1}). Other studies proved that a lectin (CrataBL) extracted from the bark of *Crataeva tapia* L. (Capparaceae) was an efficient insecticide against *N. corniger* workers, causing the deaths of all of the individuals after the sixth day of treatment (LC_{50} 0.475 mg.mL^{-1}) (Araújo *et al.* 2012).

The termiticidal actions of sisal extracts are apparently related to the toxicity of primary and secondary metabolites produced by that plant, indicating that these compounds contain toxic properties against *N. corniger* workers and soldiers (or render their natural foods impalpable). Studies of the residual liquids and extracts of *A. sisalana* have been shown to be efficient against *A. aegypti* mosquito and the red mite *T. urticae* (Barrêto *et al.* 2010; Nunes *et al.* 2015). Bioactive compounds produced by plants, such as alkaloids, tannins, terpenoids, glycosides, phenolic compounds, flavonoids, and phenylpropanoids are known to have attractant, deterrent, and insecticidal properties (Chen *et al.* 2007; Melo-Santos *et al.* 2009); flavonoids are widely distributed among plant

species and show insecticidal actions against termites (Ohmura *et al.* 2000). Primary metabolic compounds such as lectins have been shown to control *N. corniger* under laboratory conditions, and the lectins extracted from the bark (MuBL) and heartwood (MuHL) of *Myracrodruon urundeuva* Fr. All. (Anacardiaceae) were also found to be efficient in controlling that termite, causing the deaths of 100 % of the workers (LC_{50} 0.374 mg.mL^{-1}and 0.974 mg.mL^{-1}) and soldiers (LC_{50} 0.432 mg.mL^{-1}and 0.78 mg.mL^{-1}) (Napoleão *et al.* 2011). Similarly, a lectin (BmoRoL) extracted from the roots of *Bauhinia monandra* Kurz (Fabaceae) demonstrated termiticidal activities against workers and soldiers of an arboreal termite after 12 days of exposure (LC_{50} 0.09 mg.mL^{-1}and 0.395 mg.mL^{-1}), demonstrating its potential bio-technological use against those pests (Souza *et al.* 2011).

The utilization of fungi as bio-insecticides requires the previous selection of fungal species and strains to determine their virulence, their reproductive aspects, and their mass production under artificial culture conditions (Ambethgar 2009; Lopes *et al.* 2011). These pathogens can be used in association with plant extracts to increase their effectiveness, although it will be necessary to examine the extract concentrations most compatible with them.

In general, the *A. sisalana* extracts were compatible with the fungal strains tested here at the lowest concentrations, not causing any detectable inhibitory effects to their sporulation, germination, or growth. Studies show that the compatibility of entomopathogenic fungi with insecticides or chemical substances derived from plants is important when considering their combination for the control of insect pests (Amjad *et al.* 2012, Silva *et al.* 2015; Santos *et al.* 2015). Studies examining the compatibility of chemical products natural our metabolic compounds with entomopathogenic species of *Cordyceps* have revealed diverse effects on their growth, sporulation, and germination (Marques *et al.* 2004; Demirci *et al.* 2011; Amjad *et al.* 2012). The effects of the oil derived from *Azadirachta indica* A. Juss. (Meliaceae) on the vegetative growth, sporulation, and germination of the fungus *Metarhiziuim anisopliae* (Metchnikoff) Sorokin*, Beauveria bassiana* (Balsamo) Vuiillemin*,* and *C. farinosa* were examined by Marques *et al.* (2004), who found that the plant oil reduced fungal growth and sporulation but did not affect conidia viability. Xu *et al.* (2011) likewise examined the larvicide synergism of *C. fumosorosea* in combination with the secondary metabolic compound 20-hydroxyecdysone and concluded that the latter did not negatively influence germination, vegetative growth, or fungal conidia production. Matsuura & Matsunaga (2015), however, reported that the pheromones ethyl n-butyl-n-butyrate and 2-methyl-1-butanol extracted from the termite queen of *Reticulitermes speratus* (Kolbe, 1885) (Isoptera: Rhinotermitidae) had antifungal properties that significantly reduced growth, sporulation and conidia germination in *C. farinosa* and *M. anisopliae.* The effects of certain chemical products on micellar growth and conidia germination in *C. farinosa* were reported by Demirci *et al.* (2011), although tebuconazole, penconazol, and nuarimol were not found to affect conidia germination or micellar growth. Likewise, Gurulingappa *et al.* (2011) evaluated the actions of nine insecticides on the fungus *Lecanicillium lecanii* (Zimmermann) Gams & Zare and reported that thiamethoxam and acetamiprid did not affect conidia germination or micellar growth. Micellar growth and conidia germination in *C. fumosorosea* and *Lecanicillium muscarium* (Zimmerman) Viegas varied according to the concentrations and types of pesticides they were exposed to, with azocyclotin being the most toxic to conidia germination; acetamiprid was found to be the insecticide most compatible with *C. fumosorosea* (Amjad *et al.* 2012).

The extracts of *A. sisalana* did not cause severe damage to the conidia germination, the mycelial growth of *Cordyceps* strains, this fact was confirmed by the values of the biological indexes, which, in general, did not cause toxicity to the tested strains. The selection of entomopathogenic fungi compatible with plant extracts is essential to determine the most efficient strains to be tested in association with these products in the control of insect pests.

Previous studies have reported the effectiveness of entomopathogenic fungi in the laboratory to control *Nasutitermes* species, with mortality percentages greater than 40%, highlighting the *Isaria* (*Cordyceps*) and *Metarhizium* species (Lopes *et al.* 2017, Quiroz-Medina *et al.* 2021). However, the effectiveness of entomopathogenic fungi is not always reproduced in field experiments, as reported by Quiroz-Medina *et al.* (2021), who found that *M. anisopliae* and *B. bassiana* caused the death of 24% and 7%, respectively, on *N. corniger* castes. The failure of fungal infections can be attributed to the actions of insects against the pathogens, as they isolate and remove infected individuals from termite mounds, preventing the spread of fungal spores; in addition, they produce inhibitory and defensive metabolites, such as pheromones and terpenoids with antimicrobial action (Rath, 2000; Rosengaus, 2000; Yanagawa *et al.* 2012; Matsuura and Matsunaga, 2015). The use of plant extracts in association with fungal species may have stressed those termites and facilitated the fungal infection. As such, associations of entomopathogenic fungi with chemical insecticides or plant extracts can amplify their effects against insect pests while diminishing environmental damage (Amjad *et al.* 2012).

The associations of the EAAs and EHEAs extracts with *C. farinosa* ESALQ1355 increased its pathogenicity to *N. corniger* workers, with percentage mortalities much greater than either the fungus or the extracts alone – demonstrating that the extracts increase the potency of *C. farinosa* ESALQ1355 in infecting the termites, making them most potent at concentrations of 6.66 x 10^4 conidia.mL^{-1} in conjunction with LC_{50} doses of the extracts. Similar results were reported by Xu *et al.* (2011) in their analyses of the action of *C. fumosorosea* against *Plutella xylostella* L., 1758 (Lepidoptera: Plutellidae) when associated with different concentrations of 20-hydroxyecdysone; the percentage mortalities of the insects were related to the concentrations of each component in the solution, with their cumulative effects being more evident with greater exposure times. Similarly, Santos *et al.* (2015) examined the insecticidal actions of extracts (water and hydro ether) of *Ricinus communis* L. (Euphorbiaceae) and *Poincianella pyramidalis* (Tul.) L.P. Queiroz (Fabaceae) and their effectiveness in association with the *F. incarnatum-equiseti*species complex (FIESC) against the cochineal insect *D. opuntiae*, finding a synergistic effect of the fungus with an aqueous extract of *R. communis* that caused 100% mortality of the insects. The combined utilization of plant extracts with entomopathogenic fungi can increase the efficiency of the biological control of insect pests and reduce the economic costs and environmental impacts of chemical insecticides (Marques *et al.* 2004; Ambethgar 2009). The association of extracts of *A. sisalana* with *C. farinosa* ESALQ1355 may facilitate the penetration of the teguments of the worker termites by their conidia due

to stress provoked by the extracts. It has been shown that after the deaths of termite workers exposed to entomopathogenic fungi, the conidia are easily dispersed to other members of the colony during their social interactions, increasing the killing potential of the fungal inoculum in the nest environment (Chouvenc *et al.* 2011; Lopes *et al.* 2011). Termites' frequent diverse environments, which can make them susceptible to infections and promote the rapid transmission of pathogens (Hamilton *et al.* 2011).

Conclusions

The termite *N. corniger* causes severe economic damage in urban areas in northeastern Brazil as well as other countries throughout the world, creating the necessity for modern techniques that can control those pests without impacting the environment with chemical pollutants. Our results demonstrated the extracts of *A. sisalana* is compatibility with all the *Cordyceps* fungal strains tested, end the insecticidal activities of *A. sisalana* extracts on the soldiers and workers of *N. corniger*, as well as the efficiency of the association of those extracts with *C. farinosa* ESALQ1355 on workers, indicating their potential joint usefulness in the biological control of *N. corniger*.

Acknowledgements

Appreciate the Brazilian agencies: "Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco" (FACEPE) grant (APQ-1133-2.07/12, and IBPG-0040-2.08/09), "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (CNPq; grant no. 314082/2009-3), "Coordenação de Aperfeiçoamento de Pessoal de Nível Superior" (CAPES; grant PROCAD/NF 519/2010), and "Banco do Nordeste do Brasil" (BNB) for their financial support.

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Origin and funding

The present work is a result from a essay on biological and alternative control of insect pests carryed out at the Federal University of Pernambuco - UFPE and the Agronomic Institute of Pernambuco - IPA, Brazil. The financial for the research activities, was provided by the Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco - FACEPE, grants APQ-1133-2.07/12, and IBPG-0040-2.08/09; the National Scientific Development Council – CNPq, grant no. 314082/2009-3; the High Education Human Development Coordination – CAPES, grant PROCAD/NF 519/2010, and the Brazilian Northeastern Bank - BNB.

Author contribution

Rosineide da Silva Lopes and Vera Lúcia de Menezes Lima conceived and designed research. Elza Áurea de Luna Alves Lima, Luciana Gonçalves de Oliveira and Rosineide da Silva Lopes analyzed data. Rosineide da Silva Lopes, Mônica Cristina Barroso Martins and Geiziquele de Lima conducted experiments. Auristela Correia de Albuquerque identified the termite species. All authors wrote, critically revised and approved the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.