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Laboratory Evaluation of Boric Acid and Azadirachtin in Combating *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae: Dryophthorinae)

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Abstract: Background: the red palm weevil, *Rhynchophorus ferrugineus* (F.) (Col.: Curculionidae: Dryophthorinae) is a pest species that infests many palm and coconut tree species across 50 countries. Larval feeding damages the palm heart and/or the palm stipe meristem, often causing the tree's death. The damage caused by the larvae also allows phytopathogens and other insect pests to enter the tree, which can prove lethal. Methods: two active ingredients, azadirachtin and boric acid, were incorporated into an artificial diet fed to the weevils. The efficacy of these compounds was evaluated through bioassays conducted under laboratory conditions. Results: the most effective concentration for azadirachtin (95.0±5.0 %) was observed at 50.0 ppm on day 20; for boric acid (100 %), it was most efficacious at 2,500.0 ppm on day 12. Conclusions: boric acid shows significant potential in controlling the larvae of this weevil pest species. It does so in two ways - firstly, through its direct action against the red palm weevil larvae, and secondly, by its interaction with the micro-organisms associated with the larvae, which may result in more rapid mortality amongst the pest larvae themselves.

Keywords: red palm weevil; palm pest; larval stage; chemical control; effectiveness; microbiota

1. Introduction

The red palm weevil *Rhynchophorus ferrugineus* (Fabricius) (Col.: Curculionidae: Dryophthorinae) is a pest species that affects numerous palm and coconut tree species in at least 50 countries. It's distribution has been recorded over four continents: Africa (6 countries) - Djibouti, Egypt, Libya, Mauritania, Morocco, and Tunisia; The Americas: (3 countries) - Aruba, Guadeloupe, and the Netherlands Antilles; Asia (25 countries) - Bahrain, Bangladesh, Cambodia, China, India, Iran, Iraq, Israel, Japan, Kuwait, Lebanon, Malaysia, Myanmar, Oman, Pakistan, The Philippines, Qatar, Saudi Arabia, Sri Lanka, Syria, Taiwan, Thailand, United Arab Emirates, Vietnam, and Yemen; and Europe (15 countries) - Albania, Bosnia and Herzegovina, Bulgaria, Croatia, Cyprus, France, Georgia, Greece, Italy, Malta, Montenegro, Portugal, Russia, Spain, and Turkey [1]. It has also recently been found in Uruguay [2]. The biology and ecology of the red palm weevil have been reviewed in several publications (e.g., [3–6]).

The first symptoms of *R. ferrugineus* damage are exceedingly difficult to detect during the initial stages of infestation. Therefore, emphasis should be placed on preventive methods although this is not always possible. The most common approach is the chemical control of this pest species using insecticides [7]; however, again, this has proven difficult to implement due to the location of the larvae deep inside the stipe. Successful chemical control depends on: (i) the efficacy of the active ingredient (e.g., [8,9]); (ii) the timing of the application (e.g., [10]); (iii) the location of the palm plants

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– in an urban environment or in the field – due to the hazardous nature of some of the active ingredients (e.g., [11]); and (iv) the application method (e.g., [12–14]).

The aim of this work was to determine the effectiveness of one of the active ingredients, boric acid, which is minimally toxic to humans and the environment under normal handling and usage conditions (e.g., [15,16]).

2. Materials and Methods

The insects used in these trials came from a colony kept in the Agricultural Entomology Laboratory at the University of Almeria.

The *R. ferrugineus* larvae were reared on an artificial diet that was optimal from a biological [3] and physiological standpoint [17]. The management of the species was conducted according to Martin and Cabello's method [18].

A completely randomized experimental design was employed for the two trials. The method used in the two bioassays was described by Cabello et al. [8] and Barranco et al. [9]. The active ingredients evaluated (at the doses mentioned below) were incorporated into the artificial diet at the time of its preparation. The two active ingredients tested were boric acid (boric acid 99.5% wp, Panreac Quimica S.A., Castellar del Valles, Barcelona, Spain) and azadirachtin (Align 3.2% ls, Sipcam Inagra S.A., Valencia, Spain), with the following active ingredient doses per liter: 6.25, 12.5, 25 and 50 ppm of azadirachtin and 312.5, 625, 1250, 2500 and 5000 ppm of boric acid, respectively. Four replicates per treatment were performed for each bioassay. For each replicate, five second-instar larvae were taken from the colony and placed in Coulter vials (20 ml) containing a portion of the artificial diet (10-11 g) with the corresponding dose of active ingredient. The bioassays were conducted in a controlled environment cabinet at 25±2 ºC, 65±10% R.H. and a photoperiod of total darkness (0:24h; Light: Dark). Neither of the active ingredients added to the diet was used in the controls. Larval mortality was evaluated at 3, 6, 9, 12, 15 and 20 days after the start of the trials.

The number of live larvae and mortality percentage values were analyzed using generalized linear models (GZLM), having Poisson as the probability distribution and logarithm as the link function, in which dose was considered as a variable and days as a covariate. IBM SPSS version 28 software was used to carry out this analysis.

3. Results

When azadirachtin was used as the active ingredient, the number of live larvae did fall dramatically from day 15, but only at the 50 ppm dose. In contrast, when boric acid was the active ingredient, all the larvae died from day 12 at the two highest doses - 2,500 and 5,000 ppm (**Table 1**).

When analyzing the number of live larvae, model significance was found for the active ingredient azadirachtin (Omnibus test: Chi-square = 35.90, df = 5; $P < 0.01$) with significant effects for the dose variable (Chi-square = 17.54; df = 4; $P < 0.01$) and the day covariate (Chi-square = 18.36, df = 1; $P < 0.01$). For boric acid as the active ingredient, model significance was high (Omnibus test: Chi-square = 143.36, df = 6; $P < 0.01$), with effects for the dose variable (Chi-square = 112.39; df = 5; $P < 0.01$) and the day covariate (Chi-square = 30.97, df = 1; $P < 0.01$).

In the control treatments, none of the larvae died during the two bioassays (**Table 1**). Therefore, the mortalities found for each active ingredient and dose (**Figs. 1a**, **b**) corresponded to the efficacy percentage, which was determined using the Abbott formula [19].

When analyzing the mortality rate for the active ingredient azadirachtin, significant model effects were found (Omnibus test: Chi-square = 3,858. 13, df = 5; $P < 0.01$) with significant effects for the dose variable (Chi-square = 1,944.32; df = 3; $P < 0.01$) and the day covariate (Chi-square = 3,244.65, df = 1; $P < 0.01$). For boric acid as the active ingredient, model significance was high (Omnibus test: Chi-square = 7,628.36, df = 6; $P < 0.01$), with effects for the dose variable (Chi-square = 6,166.67; df = 2; $P < 0.01$), and the day covariate (Chi-square = 6,563.25, df = 1; $P < 0.01$).

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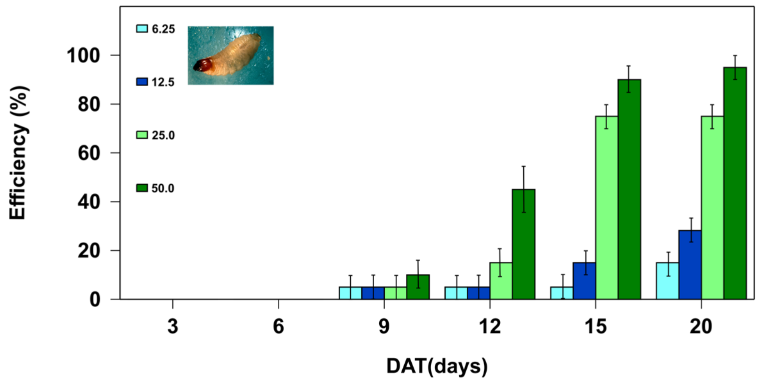
Table 1. Average number (±SE) of live *Rhynchophorus ferrugineus* larvae (II-instar) as a function of time, when fed an artificial diet with two active ingredients at different doses (ppm) under laboratory conditions (25 ºC and 0:24 hours of Light/Dark).

Active Ingredient	Dose (ppm)	Day after treatments (DAT)					
		3	6	9	12	15	20
Azadirachtin	0 (control)	5.0 (0.0)	5.0 (0.0)	5.0 (0.0)	5.0 (0.0)	5.0 (0.0)	5.0 (0.0)
	6.3	5.0 (0.0)	5.0 (0.0)	4.8 (0.3)	4.8 (0.3)	4.8 (0.3)	4.3 (0.3)
	12.5	5.0 (0.0)	5.0 (0.0)	4.8 (0.3)	4.8 (0.3)	4.8 (0.3)	4.3 (0.3)
	25.0	5.0 (0.0)	5.0 (0.0)	4.8 (0.3)	4.8 (0.3)	4.3 (0.3)	4.0 (0.0)
	50.0	5.0 (0.0)	5.0 (0.0)	4.5 (0.3)	2.8 (0.5)	0.5 (0.3)	0.3 (0.3)
Boric Acid	0 (control)	5.0 (0.0)	5.0 (0.0)	5.0 (0.0)	5.0 (0.0)	5.0 (0.0)	5.0 (0.0)
	312.5	5.0 (0.0)	5.0 (0.0)	5.0 (0.0)	5.0 (0.0)	5.0 (0.0)	5.0 (0.0)
	625.0	5.0 (0.0)	5.0 (0.0)	5.0 (0.0)	5.0 (0.0)	5.0 (0.0)	5.0 (0.0)
	1250.0	5.0 (0.0)	5.0 (0.0)	4.8 (0.3)	3.8 (0.3)	0.5 (0.3)	0.3 (0.3)
	2500.0	5.0 (0.0)	4.3 (0.3)	2.3 (0.5)	0	0	0
	5000.0	5.0 (0.0)	1.8 (0.5)	0.3 (0.3)	0	0	0

For azadirachtin, the highest larval mortality (95%) was only found at the highest dose (50.0 ppm) from day 20 of the trial. Lower azadirachtin doses were less effective on RPW (**Figure 1a**). In contrast, the efficacy achieved with the boric acid was more rapid and more efficient. The mortalities achieved with the two highest doses evaluated (2,500 and 5,000 ppm) were 100% from day 12 of the start of the trial (**Figure 1b**).



(**a**)

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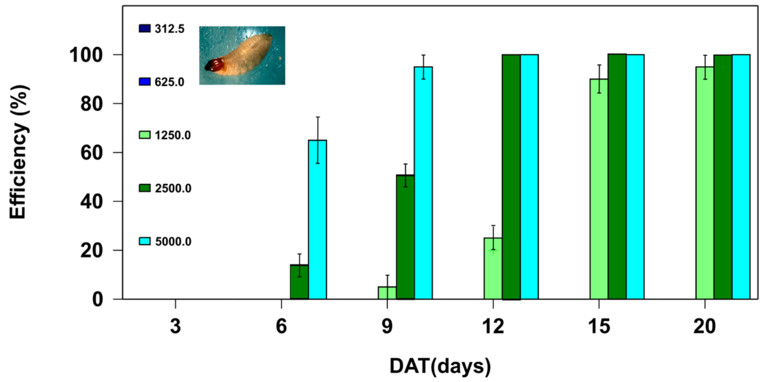

(**b**)

Figure 1. Efficacy percentage of the two active ingredients: (a) azadirachtin and (b) boric acid, at different doses (ppm), as a function of the number of days post application (DAT), when incorporated in an artificial diet and offered to II-instar *Rhynchophorus ferrugineus* larvae under laboratory conditions (25 ºC and 0:24 hours of light/dark).

3. Discussion

The mortality results obtained for azadirachtin (Figure 1a) were better than others reported in earlier works [9,20]. The reason for this difference might be attributable to the use of different commercial products in the two assays. At the larval level, azadirachtin is a chemical that exhibits antifeeding effects that impact the insects through chemoreception (the primary effect) and by reducing their food consumption (the secondary effect). Furthermore, it affects the synthesis of vital proteins in the insect larvae's digestive system – the more concentrated the azadirachtin dose, the greater the malfunction and mortality of the larvae [21,22]. Commercial compounds containing this active ingredient are used to control various crop pest species, including Coleoptera, Hemiptera, Diptera, Orthoptera, and Isoptera [23]. A variety of insecticides have been the subject of experimental laboratory and field trials worldwide seeking to control *R. ferrugineus*; of these, azadirachtin appears to show real potential for controlling this pest species [24] and other species of the genus *Rhynchophorus* [25] when used as the active ingredient.

In contrast, the active ingredient boric acid proved most efficacious at the highest doses used, after 12 days (**Figure 1b**). This is the first time, according to the existing literature, that this compound's toxicity has been evaluated for *R. ferrugineus* larvae.

Boric acid has been considered an insecticide since 1948, when it began to be used to control ants, cockroaches, silverfish, wood destroying insects (including termites), and fleas [15,26–29]. As an insecticide, it is employed in dry-powder form, to which a food attractant is added. It is then sprinkled into cracks and crevices in a thin powder layer [26]. On contact, the boric acid adheres to the insects' legs as they move through the powder. Later, the insects ingest the poison when cleaning themselves; this leads to death by starvation and dehydration after 3-10 days. However, boric acid's mode of action on insects has not been satisfactorily explored [15].

Initial studies on the cockroach species *Blattella germanica* (Blattodea: Ectobiidae) reported that the insects eventually died of starvation. Through macroscopic examination of the insects' viscera on days 1 and 2 following treatment, it was revealed that their intestines were empty and slightly enlarged. By days 3 and 4, the intestines of all the treated insects examined were empty, enlarged and filled with gas bubbles. The average food consumption during this period was less than 1 mg/insect and a 50% mortality rate was achieved after 3.8 days [30].

Habes et al. [31] investigated the same species. They concluded that boric acid exhibited insecticidal activity. According to their histological studies, they postulated that death may be due to alteration of the midgut structure. They also proved that this compound exerted a neurotoxic action on this species.

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Likewise, in the ant species *Linepithema humile* (Hym.: Formicidae), electron-microscopic studies revealed ants that were fed on low concentrations of boric acid (0.5%) presented gross abnormalities in the microvilli and cells lining the midgut [32].

More recently, in the larvae, pupae and adults of *Aedes aegypti* (Dip.: Culicidae), boric acid has been reported to produce malformations as well as mortality [33].

From another perspective, some organisms and/or microbial structures, known as bacteriocytes, exist in specialized cells within insects; these are endosymbionts. Others are ectosymbionts - they are found on the body surface or on the surface of internal organs. Symbionts, which comprise mainly actinomycete fungi and bacteria, play a fundamental role in insect nutrition, as they allow many species to develop normally even though their food has limited nutritional value. Many symbiotic relationships are casual, especially for ectosymbionts, which often make up rich gut microbiota [34].

All insects (including insect pests) have symbiotic bacteria inside their body, particularly those insects that feed on restricted diets such as plant sap, vertebrate blood, or woody material. The symbionts play a prominent role in insect ecology; they aid in food digestion or provide nutrients, influence insect–plant interactions, host population, heat tolerance, and pesticide detoxification, as well as protect against natural enemies [35].

The syntrophic relationship within ectosymbiosis provides an important ecological evolutionary advantage (e.g., [35–37]). However, knowledge regarding several aspects of these associations is yet to be fully unraveled [35].

It has already been documented that palm weevils (including *R. ferrugineus*) host a diverse array of protozoan, fungal, viral, and bacterial species [38,39]. This is due to them having to feed on material, such as palm stipe, that has limited nutritional value (as mentioned before), thus the ectosymbionts provide them with a much-needed microbiota.

The intestinal microbiota of *R. ferrugineus* is composed of facultative and obligate anaerobic bacteria that metabolize through fermentation. It is assumed that these bacteria manage the fermentation of the palm tissue in the tunnels where the larvae develop and might play a key role in providing nutrition to the insect [40,41]. The contribution of microbial ectosymbionts to the physiology, reproduction, and detoxification of secondary plant metabolites in palm weevils, and in *R. ferrugineus*, is not yet fully understood [39]. Nevertheless, we do know how important this gut microbiota is in *R. ferrugineus* development [42,43].

Boric acid possesses insecticidal properties (as shown above). It is also an effective biocide, exhibiting bactericidal, fungicidal, and virucidal properties [44,45]. Furthermore, boric acid is approved for use in several countries to protect wooden structures, timber, and related products (e.g., [26,46,47]) whereas in other areas, such as in the E.U., an exclusion criterion applies that is subject to derogation [48]. Indeed, efforts are underway for boric acid to be re-evaluated and reapproved.

The results presented into the mortality-effectiveness of boric acid (**Figure 1b**) could be due to the compound's direct toxic effect on the larvae; this cannot be ruled out. On the other hand, it may also be because the larvae starve as a result of an alteration in the midgut, as has been demonstrated in other insect species (see above) along with the elimination of their intestinal microbiota. In this regard, boric acid has been shown to induce dysbiosis in termite and cockroach species. Apart from a general decrease in microbial diversity, the relative abundance of some symbionts essential for nutrition in these insects decreased in response to higher boric acid concentrations [49,50].

Moreover, boric acid has been shown to have a synergistic effect when entomopathogens are applied, as occurs in other insect species [51–55].

The findings of this study are very promising and important for increasing our knowledge into the effects of boric acid (and its derivatives) as an insecticide and/or synergist in the control of this destructive species. However, we must still fully investigate any possible undesirable effects that this compound might have on the palm trees themselves. In general, the importance of boron is undeniable. It is an essential micronutrient for cultivated plants since both its deficiency and overabundance (toxicity) in the soil can negatively affect plant growth and development [56], especially for the date palm [57].

5. Conclusions

-The active ingredient azadirachtin demonstrated good potential in controlling the pest species, with an efficacy of 95.0 ± 5.0 % at a dose of 50.0 ppm on day 20.

-In contrast, the active ingredient boric acid showed even greater potential, showing 100 % efficacies at doses of 2,500 and 5,000 ppm at day 12.

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