The Mindanao Forum, Vol XIII, No. 2 (December 1998)

Effects of Oil Extracts of Neem *Azadirachta indica* A. Juss, Sweetflag *Acorus calamus* L. on the Rice Weevil *Sitophilus Oryzae*

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Abstract

The biochemical effects of neem oil, sweetflag oil and "Margosan-O" on the rice weevil *Sitophilus oryzae* were investigated by looking at percent mortality and enzyme activity on treated insects. Toxicity results show "Margosan-O" as the most effective when compared to sweetflag oil and neem oil.

Amylase, a- and β -esterase activities exhibited a general trend of decreasing enzyme activity in response to increased oil concentration.

Key Words: *Azadirachta indica, Acorus calamus, Sitophilus oryzae*

Introduction

To keep up with the ever increasing human population, the Philippine government has centered much of its resources to boost food production. The intensification of crop production has given risce to many postharvest problems like pest infestation during storage. In grain storage, it has been approximated that around 34% of the weight of maize is lost when stored for around 8 months in the absence of protection from insects (Caliboso 1977). Based on the 1993 procurement of the NFA, the Philippines can be expected to lose US$8.8 million worth of 44.8 million kg of maize if not pest control measures are undertaken. For milled rice, unchecked insect infestation can lead to a loss of about 18.5 million kg valued at US$6.2 million. The government stockpiles 260 million kg to constitute 45 days consumption requirement (Caliboso et al 1985).

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S. oryzae is one significant rice insect pest during storage. It belongs to Family Curculionidae and is listed as one of the major pests of stored grain (Cotton and Wilbur 1974). Irabagon (1959) reported that the rice weevil is capable of consuming 74.7% of a single kernel and could cause 12.9% to 25.9% loss in weight in bulk grains. They are particularly well adapted to life in the stringent environment imposed by a bin of grain (Cotton and Good 1937).

The use of insecticide, fumigation or other protectant is therefore, absolute. In recent years, the use of insecticides on stored grain products has progressed because of their effectiveness. But some of these effective compounds, such as DDT and dieldrin, have been discarded because of their potential hazards to human health, wildlife and the environment (The Council of Europe 1973). Without the proper management and supervision on these chemicals, they can be detrimental to life. Aside from toxic residues, the selection of insecticide resistant pest strains can be considered as an additional problem. Likewise, the increase in prices of commercial petroleum-based pesticides has made them more difficult to acquire by small farmers especially those in developing countries (Morallo-Rejesus 1987).

Given this situation, interest is now turned to possible agents which are safe, biodegradable, less prone to insect resistance, pest-specific and cheap yet effective (Saxena 1988). A possibility would be in botanicals or derivatives from plants such as neem (*Azadirachta indica* A. Juss) and sweetflag (*Acorus calamus*).

A. indica is an evergreen tree in India which is found in almost all of its dry forests. Almost every part of the tree is bitter but it is its seed kernel which is most bitter. Nearly every part of this plant has been used in the country's folk medicine (Mahato et al 1987). Neem has an insect antifeedant and repellent action. This is attributed to triterpenoid azadirachtin and other related compounds (Butterworth and Morgan 1968, Nakanishi 1975, Zanno et al 1975). Azadirachtin posses growth regulating activities towards the insect (Rembold et al 1981). Like neem, sweetflag also has an active component, asarone (Baxter al 1960). Their being safe to target organisms including humans make them ideal insecticides.

Food crops in the Philippines should be given enough protection from stored pests. One of these pests is *Sitophilus oryzae* L. Neem oil, sweetflag oil, "Margosan-O" (a commercial neem-based insecticide) were found to be antifeedant and toxic to stored pests, including *S. oryzae*.

It is therefore, the objective of the study to evaluate the effects of different plant derivatives, such as neem oil and Margosan-O to the physiology of *S. oryzae* to be able to understand the biochemical basis of antifeeding and toxicity.

In evaluating the effects of different plant derivatives on the physiology of an insect at the biochemical level, it is essential to consider variations in enzyme activity. Enzymes a such esterases, amylases and acetylcholinesterases are those that show evident changes in their activity in response to different concentrations of the botanical extracts.

Materials and Methods

Insect samples. *S. oryzae* were obtained from inbred cultures at the Department of Entomology, IRRI.

Treatments. Eight dosages of neem crude oil and its processed oil "Margosan-O" and sweetflag oil (200, 400, 800, 1600, 3200, 5000, 10,000, 25,000 ppm) were used in treating *S. oryzae*. These oils were also provided by the Entomology Department of the International Rice Research Institute (IRRI).

For each treatment, three replicates were prepared. In each replicate, a clean container had 50 grams of flour. The cans were properly labeled with their corresponding oil, dosage and replicate number.

For each 50 gram flour, the weighed amount of the oil was dissolved in 20 ml acetone until it became a homogeneous solution. Then the mixture was poured into the media while constantly stirring to ensure even distribution of the oil. The treated media was allowed to stand for 24 hours to facilitate maximum evaporation of acetone.

After 24 hours, 40 adult individuals were introduced per replicate and allowed to feed on the treated flour for 7 days.

Collection and counting of live insects after a week was done by straining the media. They were placed in properly labeled test tubes and kept in a freezer at a constant temperature of -10° C. The mortality of *S. oryzae* was determined.

The frozen insects were washed thoroughly with cold distilled water, homogenized in 0.04 M phosphate buffer, pH 8 (a ratio of 4 insects per ml of 0.04 M, pH 8, phosphate buffer was used), thoroughly crushed and ground. Then the supernatant was poured into labeled test tubes, The tubes were placed in the freezer after the debris had settled and removed from the solution. This was done in all replicates.

Esterase Assay. Four ml of 0.04 M phosphate buffer, pH 8, was pipetted into each test tube (including a blank) while the samples were thawed in ice cold water. Twenty-five microliters of B-napthyl acetate was added. Enzyme extracts of 50 microliters were added to the blank. After 30 minutes, one ml of diazoblue-lauryl sulfate (DBLS) solution was added as color reagent. Transmittance readings at 555 nm (for B-naphtol) and 600 nm (for x naphtol) were recorded using a Bausch and Lomb spectronic 20 spectrophotometer. The values were converted to absorbance using the formula T = 2-logT.

Amylase Assay, Five hundred microliters (0.5 ml) of 1% starch solution was pipetted into each test tube and a blank. Five hundred microliters of each sample was added to each test tube while an equivalent amount of dH_20 was added to the blank. The tubes were incubated for three minutes after which 1 ml of dinitrosalicylic acid (DNSA) color reagent was added to each test tube. For 5 minutes, the test tubes ere then placed in a hot water bath and then allowed to cool at room temperature. To each test tube, 3 ml dH_20 was added. Transmittance values ere obtained at 640 nm using a spectrophotometer. The readings were converted to absorbance values. The amount of amylase (micromoles) was computed by dividing the absorbance values by 0.02784. Then the gram amylase values were obtained by multiplying the micromoles amylase by molecular wight of maltose (360.3), then divided by 1000 micromoles.

Toxicity. The ANOVA performed on the results of toxicity tests of neem oil and sweetflag oil concentrations on *S. oryzae* was significant ($P \geq 0.05$). However, the ANOVA conducted on mortality of *S. oryzae* due to Margosan-O showed no significance among treatments. In the test for neem oil, there was significant increase in percent mortality at 10000 ppm to 25000 ppm compared with the control. Difference in mortality between treatment concentration 1000 ppm and control was significant (Table 1). The percent mortality of *S. oryzae* at concentrations 200 to 5000 ppm did not vary significantly from that of the control.

Margosan-o toxicity revealed higher mortality values in all treatments compared with the control. However, only the concentrations of 10000 ppm showed a significant difference against the control. Against all treatments, however, there was no significant difference for 10000 ppm. Increasing the concentrations from 200 to 5000 ppm likewise did not show significant differences towards the control.

In sweetflag oil, increasing the concentrations caused a corresponding increase in percentage mortality. At 200 ppm, mortality was similar to that of the control. There was steady increase in the percent mortality starting with 6.67 at 200 ppm to 81.67 at 5000 ppm. Concentrations of 200, 400, and 800 showed no significant difference towards the control.

Table 1. Mortality effects of neem oil, Margosan-O and sweetflag oil in *S. oryzae*.

CONCENTRATION (PPM)	NEEM	MARGOSAN-O	SWEETFLAG
Control	6.67 c	6.67 a	6.67 c
200	3.33 c	45.00 ab	6.67 c
400	10.00 c	40.83 ab	7.50 c
800	1.67 c	34.17 ab	15.83 c
1600	0.83 c	49.17 ab	36.67 b
3200	5.00 c	52.50 ab	69.17 a
5000	14.17 c	37.50 ab	81.67 a
10000	44.17 b	55.83 b	
25000	73.33 a		

But increasing the concentrations to 1600, 3200, and 5000 showed significant difference towards the control and treatments below 1600 ppm.

Table 1 shows that in a certain high concentration, there is increased percent mean mortality towards the insect population. This can be attributed to the toxic effects of the oil because if we would look at the alpha and beta-esterase activities at 25000 ppm concentration of neem oil (Table 2, 3), there is a significant reduction. This would mean that the neem oil could have an effect on the detoxification system of the insect. Esterases are considered part of the system. They cleave the ester bonds of certain compounds like insecticides.

The antifeedant properties of the oil are probably shown at the lower concentrations. As the oil concentrations are increased, there is a decrease in amylase activity which thus,

Results and Discussion

Table 2. Effect of neem oil, Margosan-O and sweetflag oil on α - esterase activity in *S. oryzae*.

CONCENTRATION (ppm)	NEEM	MARGOSAN-O	SWEETFLAG
Control	0.396 ab	0.396 a	0.396 a
200	0.480 b	0.124 b	0.153 b
400	0.395 ab	0.069 b	0.141 b
800	0.391 ab	0.087 b	0.193 b
1600	0.357 a	0.107 b	0.144 b
3200	0.404 ab	0.072 b	0.159 b
5000	0.358 a	0.135 b	0.128 b
10000	0.403 ab	0.09 b	
25000	0.299 a		

Table 3. Effect of neem oil, Margosan-O and sweetflag oil on ß esterase activity in *S. oryzae*

CONCENTRATION (ppm)	NEEM	MARGOSAN-O	SWEETFLAG
Control	0.563 b-d	0.563 a	0.563 a
200	0.749 a	0.192 b	0.440 b
400	0.612 bc	0.105 b	0.403 bc
800	0.628 b	0.161 b	0.381 bc
1600	0.512 b-e	0.150 b	0.360 bc
3200	0.570 b-d	0.157 b	0.314 c
5000	0.482 de	0.248 b	0.166 d
10000	0.492 c-e	0.219 b	
25000	0.394 e		

imply unpalatability of the flour (Table 4). The unpalatability of the oil-starch mixture shows the antifeedant and repellent properties of the extracts. At increasing oil concentrations, there is an observed repellency. This conforms with the study of Jilani et al (1988) on *T. castaneum*. The toxic and antifeedant effects are also shown in the alpha esterase, beta-esterase and amylase activity of "margosan-O" and sweetflag oil-treated insects (Table 4). The results indicate varying dosages to effect at least 50% mortality. The highest mortality with only very minimal amount of the formulation was the sweetflag oil indicating that

sweetflag oil has an advantage over neem oil and "Margosan-O". However, if we look at Table 6 showing the comparison of the effects of the different botanical extracts, "Margosan-O" showed the highest mean mortality for all the treatments (from 200 to 5000 ppm) with 43.19% compared with neem (6.94% mean mortality) and sweetflag (36.25% mean mortality). This result is similar to the study of Jilani et al (1988) which reveals that at 10000 ppm, Margosan-O showed the most toxic results, it can be said that since "Margosan-O" showed the highest mean mortality, then probably the oil has the greatest repellency. This is followed by sweetflag then neem oil.

alpha-esterase. The ANOVA performed on the effect of Margosan-O and sweetflag oil on alpha-esterase activity was significant ($P \geq 0.05$). However, for neem oil, the ANOVA was not significant.

The effect of neem on the activity of alpha-esterase revealed fluctuating values starting at 1600 ppm. However, no significant difference in the results was observed even as the concentration of the oil was increased to 25000 ppm. Results showed fluctuating values but if we would correlate the alpha-esterase activity to the toxicity values shown in Table 1, it would be observed that generally, the higher the percent mortality, the lower the enzyme

Table 4. Effect of neem oil, Margosan-O and sweetflag oil on amylase activity in *S. oryzae* (gm/insect).

CONCENTRATION (ppm)	NEEM	MARGOSAN-O	SWEETFLAG
Control	0.968 b	0.968 a	0.968 a
200	0.854 ab	0.814 a	0.743 b
400	0.893 ab	0.591 a	0.826 ab
800	0.911 ab	0.589 a	0.724 b
1600	0.892 ab	0.707 a	0.671 b
3200	0.974 ab	0.518 a	0.689 b
5000	0.761 a	0.536 a	0.743 b
10000	0.781 a	0.658 a	
25000	0.779 a		

activity and vice versa. This result show the oil's toxicity and antifeedant properties. It can be said that at higher concentrations where the insect takes in lesser starch-toxin mixture, the insects died of starvation. The principle is the same with the results in esterase activity as affected by sweetflag oil and Margosan-O.

In "Margosan-O", all treatments showed decreased esterase values compared to the control. Fluctuating values were also observed in the treatments. While there were significant differences between treatments and the control, no difference was observed among the treatments.

In sweetflag, all treatments revealed lower esterase activity as compared to the control. The concentrations from 200 to 5000 ppm showed results which are significantly differ-

ent towards the control. However, there was no significant difference within the treatment results.

Results show that there is a trend of decreasing esterase activity with increasing oil concentration. However, the ANOVA performed on the effect of neem on the enzyme activity showed an insignificant test. Every organism has a natural tendency of defense against certain changes in the environment. In the experiment, the detoxification mechanism is observed against the different oils. Esterases serve to cleave bonds of specific insecticides. The enzyme is thus produced as part of the insect's detoxification mechanism. The results however, are contradicting to that of Ziegler et al (1986) which shows that an increase in toxin concentration would result in corresponding increase in enzyme activity. A probable explanation of the results is traced to the unpalatability of the oil-starch mixture. Because of the insects' capability to take in the mixture at lower concentrations, there is a probability of higher amounts of toxin. Thus there is more enzyme activity observed in this lower concentration level. In comparison, at higher oil concentration, the more the insect is incapable of feeding, the lower will be the amount of toxin, thus a lower esterase activity needed.

Table 6 shows that neem oil had the highest esterase activity on all treatments compared to sweetflag oil and "Margosan-O". "Margosan-O" had the lowest enzyme activity mean. Since it had been said that neem oil had the least repellency effect in this study, then the insect was able to have greater starch intake compared with sweetflag and "Margosan-O". This increase in feeding probably caused the higher toxin content, thus causing in turn, an increase in detoxifying activity of alpha-esterase.

Beta-esterase. The ANOVA performed on the effect of "Margosan-O", sweetflag oil and neem oil on the β -esterase activity was significant ($P \geq 0.05$).

The effect of "Margosan-O" on β -esterase activity showed fluctuating results. All treatment values were lower than the control. Despite the fluctuations, all treatment values showed a significant difference towards the control but had no significant difference towards each other.

When the values of toxicity is correlated with that of the β -esterase activity, it is observed that at concentrations with a higher percent mortality, there is a corresponding lower enzyme activity and vice versa. This result is similar to that of the α -esterase activity. The principle is consistent with the effects of neem oil, "Margosan-O" and sweetflag oil on α -esterase activity.

The enzyme activity as affected by sweetflag oil showed a steady decrease. All treatment values showed significant differences towards the control. The same results were observed with neem oil-treated insects.

In similarity to the effect of neem, sweetflag and "Margosan-O" on -esterase activity, increased in oil concentration caused a corresponding decrease in -esterase activity. This trend is evident especially in neem and sweetflag oil. Margosan-O revealed deviations from the trend at concentrations 5000 and 10000 ppm. However, this deviation did not show any significant difference towards all the other treatments. Not much difference is expected in the results for α - esterase and β -esterase activities.

Table 3 indicated that neem had the highest α -esterase activity when compared to Margosan-O and sweetflag oil. As earlier reported on this study because of neem's lower repellency and antifeedant effect, the insect was able to take in more starch (which means a corresponding increase in toxin intake) thus requiring a higher detoxifying effect of -

esterase activity. The results in Table 3 also show that -esterase as affected by neem oil, Margosan-O and sweetflag oil has a consistently higher activity relative to -esterase. This implies that α -esterase is produced in a larger amounts as compared to α -esterase for detoxification.

Amylase. The ANOVA performed on the effect of the neem oil and Margosan-O on the amylase activity showed an insignificant test at 0.05% level of significance. However, it was a significant test for sweetflag oil.

In neem oil, the trend was generally decreasing while the concentration of the oil was increasing. Significant decrease however,.was observed in 5000 and above. Since amylase functions in starch hydrolysis, the rate of amylase activity can be correlated with the amount of starch present which was taken in by the insect. This is seen in the results for neem. At higher concentrations, there is lower amylase activity because of low amount of starch taken in due to the unpalatability of the starch. This is in contrast to the higher enzyme activity in lower concentrations where the antifeedant effects of the oil is relatively at the minimum.

In "Margosan-O", although no increase in concentrations up to 10000 ppm showed significant differences towards the control, amylase activity was lower when compared to the control.

Decreasing trend in activity was also observed with sweetflag oil-treated insects. Significant differences in activity was observed between treated insects than the control.

The decreasing trend of amylase activity with increasing concentration of the oils is due to the unpalatability of the starch. Amylase functions in the hydrolysis of starch and so the amount of starch present affects the rate of enzyme activity. High amounts of starch taken in by the insect would trigger the increased activity of amylase while low consumption of starch would significantly show low enzyme activity.

The oils, with their antifeedant and repellent properties, showed a trend of amylase activity inhibition which is expected of them. This conforms with a study on *S. litura* where extracts of neem seed kernel inhibited amylase activity. Margosan-O the lowest amylase activity because it has already been shown that it has the greater repellency effect.

Summary and Conclusion

The storage pest *S. oryzae* was treated with different concentrations of neem oil, sweetflag oil and Margosan-O. Their toxicity on the insect was noted. Results show that among the three plant derivatives, sweetflag oil affected significant insect mortality at a lower concentration, followed by neem oil and Margosan-O. However, comparison of the mortality effects of the three oils (from 200 ppm to 5000 ppm), Margosan-O had the highest mean mortality followed by sweetflag oil and neem oil. The principle that at higher concentrations, where the insect takes in lesser starch-toxin mixture and its consequential dying to starvation is shown.

The live insects were homogenized and the enzyme extracts were subjected to amylase, α -esterase and β -esterase , assay using spectrophotometry.

In the test for β -esterase activity, result showed that Margosan-O and sweetflag oil had significant difference towards the control Comparison of the three oils' effect on the enzyme activity showed that neem oil-treated insects had the highest enzyme activity while Margosan-O exhibited the lowest enzyme activity.

For β -esterase activity, significant results were obtained at just 200 ppm for Margosan-O. Comparison of three oils' effect on the enzyme activity showed neem oil affecting the highest α -esterase activity while Margosan-O had the lowest.

In both α - and β -esterase activity, it was observed that generally, increased in concentration would have a corresponding decrease in enzyme activity. The detoxification mechanism of the enzyme becomes inactive at high concentrations because of lesser starch-toxin mixture intake due to its unpalatability.

For amylase activity, sweetflag oil showed significant results at 800 ppm. Generally, there was a decreasing trend of enzyme activity as oil concentration is increased. Comparison of the three botanical extracts' effect on amylase activity (from 200-5000 ppm) showed that neem oil-treated insects had the highest enzyme activity while Margosan-O registered the lowest activity.

Results, thus, showed that the antifeedant nature of the oils directly affect enzyme activities. At high concentrations, it decreases amylase, and α -β esterase activity while increasing acetylcholinesterase activity.

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