Synergism of anticholinesterase insecticides by non-insecticidal phosphorus esters in the boll weevil **Anthonomus grandis** Boheman*

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COMPENDIO

La actividad sinergística de tiol esteres de los ácidos fenilfosfórico y fosfórico aplicados con paration metílico, azinfos metílico, oxi-azinfos metílico, paraoxón metílico y carbofurano, fueron estudiados en el picudo del algodonero, Anthonomus grandis *Boheman.*

Los efectos debidos a la alteración de la cadena alifática de los sinergistas en el grado de sinergismo fue variable. La aplicación simultánea del producto S, S, S-*tributil-fosforotritioato (DEF) y de los homólogos* n-*propilo y* n-*butilo de la serie ácida* S, S-*dialquilfenilfosfónica resultó en un incremento de la toxicidad de azinfos metílico en el orden de 2,28 2,17 y 2,26 veces mayor que la del insecticida aplicado solo. Un incremento similar (2,14) fue observado con la combinación del carbofurano con el producto* S, S-*dietilfenilfosfonoditioato. La mayoría de los otros compuestos ensayados en combinación con azinfos metílico dieron incrementos sinergísticos entre 1,19 y 1,78 veces la toxicidad del insecticida solo.*

La toxicidad del paration metílico, bajo la forma de filme residual, se incrementó hasta cerca de 12 veces cuando fue aplicado después de una dosificación topical de varios sinergistas. La falta de interferencia en la penetración del insecticida a través de la cutícula de los picudos, por los sinergistas ensayados, es postulada como la causa de este alto efecto sinergístico.

Introduction

WITHIN a few years after the introduction of chlorinated hydrocarbon insecticides for the control of the boll weevil, *Anthonomus grandis* Boheman, high levels of resistance to these insecticides were observed in field populations. Organophosphorus insecticides (OP), in contrast, have not induced development of resistance in boll weevils even though these compounds have been used in their control since 1956 (10). Despite this somewhat intriguing susceptibility, resistance to OP insecticides in the boll weevil remains a real threat, for it was found that this insect, put under selective pressure with a mixture of DDT-Toxaphene, developed cross-resistance to azodrin (12 fold) and methyl parathion (5 fold) in 22 generations (9).

Combined applications of chemicals for the simultaneous control of unrelated harmful species is common practice under field conditions. For example, within recent years S,S, S,-tributylphosphorotrithioate (DEF) and S,S,S,-tributylphosphorotrithioite (Folex) have been introduced into practical use for the removal of leaves from cotton plants prior to the picking of cotton. These defoliants are normally applied in conjunction with the late season application of OP insecticides to reduce overwintering populations of weevils, prior to their going into diapause.

The apparent increase in effectiveness of OP insecticides applied along with DEF led Norment and Chambers (18) to investigate the effects of these combined applications under laboratory conditions. They found that EPN, azinphosmethyl, malathion, and methyl parathion were potentiated 3.5-, 3.4-, and 1.7 fold, respectively. Ganyard and Brazzel (8) however, were able to find only additive effects for the combinations of DEF and Folex with methyl parathion, azinphosme-

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thyl, and malathion in the same species. Further work done by Chambers (unpublished results) confirmed that DEF could synergize several other OP insecticides in the boll weevil. In general, those compounds having methyl groups attached to the side chain of the molecule were more prone to synergism, with the exception of EPN, for which the highest synergistic ratio was found.

The objective of this study was to further investigate synergism of insecticides in the boll weevil by considering the effects of altering the alkyl substituents of the synergists and their route of application.

Materials and methods

Test materials

All compounds tested for synergism in this study, except DEF and piperonyl butoxide (PB) were synthesized in the Toxicology Laboratory at Mississippi State University and purity was established by thin-layer chromatography.

Recrystalized samples (purity>98%) of methyl parathion (MPS), methyl paraoxon (MPO), azinphosmethyl (Apm), oxy azinphosmethyl (ApmO), and carbofuran (Cbf) were evaluated for toxicity.

Insects

The boll weevils employed in the tests were from a laboratory strain maintained at the Gast Rearing Laboratory, Mississippi State, Mississippi. Unsexed four day old weevils were utilized in all bioassays.

Bioassay

All insecticides and synergists were dissolved in acetone at the desired concentrations. Following preliminary assays to establish effective ranges, a minimum of five replicates of 20 insects/dosage/replicate and 4-5 dosages/treatment were used to define the dosage mortality response.

Treatments used were as follows: (1) Topical application of insecticide alone; (2) "simultaneous" applications of insecticide and synergists; and (3) exposure of untreated and treated (via topical application) weevils to insecticide impregnated filter paper.

In test 1, 1 μl of insecticide (in acetone) was applied to the dorsum of the weevils and allowed to dry. Controls received 1 μl of acetone. Mortality determinations (described below) were made 48 h post-treatment.

In test 2, 1 μl of synergist solution was applied as above. Insecticide solutions were applied immediately thereafter. Controls received synergist but no insecticide. Mortality was determined 48 h post treatment.

In test 3, 1 ml of insecticide solution was evenly distributed over 11 cm Whatman Nº 1 filter paper by using a calibrated pipette and allowed to dry. Nine centimeters petri dish bottoms containing 20 weevils each were covered with the treated filter papers, the lids were pressed on and the dishes were inverted to bring the weevils over the filter papers. Dishes were held in the dark for 24 h, then weevils were removed from exposure to the insecticide for a second 24-h period prior to determining the rate of mortality. Residual film assays were conducted with untreated weevils and with weevils immediately following topical application of a synergist. Controls were handled identically, except that untreated filter paper were used.

In all studies involving synergists, the chemicals were applied at a constant dosage with varying insecticide dosages. For DEF, the dosage was 100 μg/g of body weight. All other synergists were applied as molar dosages to adjust for differences in molecular weight. The dosage was 0.5 μmole/g, representing a range of 123-165 μg/g.

Except during exposure to insecticide treated filter paper, weevils were held in small glass jars and provided with fresh artificial diet. Temperature was laboratory ambient (approx. 23°C) and light regimen was not controlled (approx. 16 h light: 8 h dark). During the residual film treatment, no food was provided. After the initial 24 h, weevils were maintained as previously described.

For mortality determinations, death was defined as failure of the weevil to show coordinated response to a pinch on the proboscis. Experience has shown that live weevils in this condition following OP or carbamate poisoning die within the next 24 h. Observed mortality was corrected for mortality in control groups (1). LD_{50} values and 99 confidence parameters were calculated by linear regression analysis of the data following probit mortality/log dosage transformations. LD_{50}'s are given as μg/g of actual insecticide (alone or in combinations) for topical applications and as μg/cm^2 for residual film studies.

Results and discussion

The synergistic activities of thiol esters of phosphoric and phenylphosphonic acids, and piperonyl butoxide applied with the insecticides methyl parathion, methyl paraoxon, azinphosmethyl, oxy-azinphosmethyl, and carbofuran, are presented in Tables 1 and 2. The first section of Table 1 also shows the individual toxicities of the insecticides. This determination was deemed necessary for purposes of comparison with synergized toxicity data.

DEF was found to synergize all insecticides in the order of 1.58 fold to MPS and MPO, to 2.28 to Apm. Similarly, PB increased the toxicities of Apm, ApmO and Cbf in 1.66, 1.15, and 1.54 fold, respectively (Table 1).

A t-test by which the unsynergized versus DEF or PB synergized LD_{50} values were compared, showed that the increase in toxicity of all insecticides, except for the combination of ApmO plus PB, is highly significant. This indicates that the interactions of DEF or PB with the insecticides results in true synergism rather than merely additive effects. Similar phenomenon is observed for the combination of Apm with all other synergists

Table 1 —Toxicity data[1/] for 5 insecticides alone and in combination with DEF and piperonyl butoxide against the boll weevil.

Insecticide	Alone		+ DEF, 100 μg/g			+ piperonyl butoxide, 0.5 μmole/g		
	LD_{50}	Slope	LD_{50}	Slope	I/SI	LD_{50}	Slope	I/SI
Methyl parathion	4.99	8.95	3.16**	6.61	1.58			
Methyl paraoxon	3.17	3.49	2.00**	7.77	1.58			
Azinphosmethyl	5.29	4.90	2.43**	4.25	2.28	3.18**	3.75	1.66
Oxy-azinphosmethyl	1.82	3.25	1.00**	4.18	1.82	1.58	3.86	1.15
Carbofuran	7.31	3.40	4.11**	2.62	1.78	4.75**	3.21	1.54

1/ LD_{50} values are expressed as μg of insecticide/g body weight. LD_{50} value followed by** differs significantly ($P < 0.01$) from that of insecticide alone according to t-test. I/SI = LD_{50} of insecticide alone/LD_{50} of insecticidal component in binary treatments.

tested, and for the combination of Cbf with the ethyl and *n*-propyl homologs of the *S,S*-dialkyl phenylphosphonodithioate series (cmpds. I and II, Table 2). Synergistic ratios were rather small with either Apm or Cbf.

The interactions of DEF with MPO and ApmO result in homogenization of the response of the weevils to these compounds. The slope values increased from 3.49 to 7.77 for MPO and from 3,25 to 4.18 for ApmO. The opposite situation is observed with MPS, Apm, and Cbf. In this case the weevils responded more heterogeneously in the presence of DEF which is shown by the decrease in the slope values.

The significant increase in the toxicity of Apm by DEF merits a special comment. It has been postulated that DEF may act as non-specific esterase inhibitor in the boll weevil (Chambers, unpublished results), it has been demonstrated to be an esterase inhibitor in rats (16), and that it potentiates the toxicity of malathion, via carboxylesterase inhibition, in house flies and mosquitoes (3, 4, 5, 20), and in mice (6, 7).

On the other hand, Apm was found to be detoxified mainly by a glutathione dependent enzyme (GSH-transferase) in a predaceous mite and resistant house fly strains (14, 15). Moreover, DEF was found to inhibit the glutathione dependent desethylation of diazinon and diazoxon, and to a lesser extent, that of parathion in the resistant SKA strain of house flies (12).

Assuming that a similar mechanism of detoxication of Apm occurs in the boll weevil, then DEF might be inhibiting to a greater degree a GSH-alkyl or aryl transferase. If this is true, the mixed function oxidase enzymes will act more freely to desulfurate Apm, thus activating this compound by transforming it to its oxygen analog, ApmO.

There is also evidence that DEF inhibits the oxidative metabolism of hexobarbital in mice more than sesamex and about equal to sulfoxide, which are known mixed function oxidase inhibitors (21). Therefore, in the boll weevil, there are potentially two enzymes systems responsible for Apm detoxication which are inhibited by DEF. The actual degree of synergism could then be explained by the occurrence of a greater inhibition of the dealkylation or dearylation of the Apm molecule. The fact that ApmO is also synergized by DEF, and that the slope of the regression line is increased more than that of MPO, supports the above hypothesis. Of course, biochemical evidence should be sought to prove the validity of this speculation.

The same argument can be applied to the observed MPS and MPO synergism by DEF. In this case however, the ability of DEF to enhance cholinesterase inhibition by MPS *in vivo* has been shown to correlate with its ability to inhibit a soluble carboxylesterase (Chambers, unpublished results). Thus, esterase inhibition certainly plays a substantial, if only partial, role in MPS and MPO synergism by DEF. Similar correlations have been observed with the *n*-amyl and *i*-amyl homologs of the *S,S*-dialkyl phenylphosphonodithioate series (cmpds. VII and VIII, Table 2).

No antagonistic effects were registered in any combination of Apm and MPS with any synergist tested. MPS, however, was not tested with piperonyl butoxide, a combination which may yield antagonism. Antagonistic effects were found between Cbf and compounds IV, VII and VIII (Table 2). It is interesting to note that as the size of the alkyl substituents on the phosphonic acid increases there is a switch from synergism of Cbf (cmpds. I and II) to antagonism (cmpds. IV, VII, and VIII).

Table 2.—Toxicity data[1/] for azinphosmethyl and carbofuran in combination with potential synergists (at 0.5 μmoles/g)

Nº	Alkyl substituent	Azinphosmethyl[2/]			Carbofuran		
		LD_{50}	Slope	I/SI	LD_{50}	Slope	I/SI
	S,S-dialkyl phenylphosphonodithioates						
I	ethyl	3.49**	4.58	1.51	3.42**	3.28	2.14
II	*n*-propyl	3.38**	4.58	1.56	5.82**	3.28	1.25
III	*i*-propyl	4.09**	3.42	1.29			
IV	*n*-butyl	4.46**	5.95	1.19	9.85	2.78	0.74
V	*i*-butyl	4.08**	5.41	1.30			
VI	*t*-butyl	3.71**	4.68	1.43			
VII	*n*-amyl	3.90**	4.30	1.36	9.26	3.21	0.79
VIII	*i*-amyl	2.98**	4.70	1.78	8.91	2.11	0.82
	S,S,-dialkyl O-phenyl phosphorodithioates						
IX	*n*-propyl	2.44**	4.83	2.17			
X	*n*-butyl	2.35**	4.79	2.26			
			Other				
XI	propyl-DEF[3/]	3.25**	4.51	1.63			

1/ See Table 1 for toxicity data for insecticide alone and for explanations of abbreviations

2/ The combination of oxy-azinphosmethyl with compounds I, IV and X resulted in antagonistic effects ca I/SI = 0.80

3/ *S,S,S*-tri-n-propyl phosphorotrithioate

One posible explanation of the mechanism by which carbofuran can be antagonized by compounds IV, VII, and VIII is by the inhibition of an activation step carried out by mixed function oxidases (MFO's). The involvement of MFO's in detoxications in the boll weevil is demonstrated by the fact that Cbf is synergized by piperonyl butoxide. The latter compound is a known mixed function oxidase inhibitor.

Carbamates are generally more toxic when hydroxylated somewhere in the aromatic ring (aromatic carbamates), or when *N*-demethylated (13, 19). Further, aromatic carbamate insecticides are metabolized mainly by microsomal enzymes (MFO's), with aromatic hydroxylation and *N*-demethylation being the more pronoced mechanisms of degradation (17). This evidence fairly supports the above explanation

Influence of the route of application on the degree of synergism.

The n-propyl and n-butyl homologs of each series of synergists were tested with a residual film of methyl parathion. A rather high increase in the toxicity of MPS was registered after each combination, with synergistic ratios ranging from 10.89 for DEF to 12.13 for compound IV (Table 3)

Apparently, the cause for this increase in toxicity is the presence of a greater amount of methyl paraoxon at

Table 3.—Residual film toxicity[1/] of methyl parathion to the boll weevil applied along with a topical dose of synergist.

	LD_{50}	Slope	I/SI
Methyl parathion alone	2.20		
Methyl parathion + propyl DEF	0.20**	5.10	11.25
Methyl parathion + II	0.19**	3.40	11.44
Methyl parathion + IX	0.18**	4.05	12.12
Methyl parathion + DEF	0.20**	4.70	10.89
Methyl parathion + IV	0.18**	3.68	12.13
Methyl parathion + X	0.19**	4.24	11.62

1/ LD_{50} values are expressed as μg of toxicant/cm^2 of filter paper. Other abbreviations are as described in Table 1.

the target site, the enzyme acetylcholinesterase It appears that the penetration of MPS through the boll weevil cuticle is facilitated when the synergist does not interfere with it Interference in the absorption of insecticides by a synergist when both were applied to the same loci has been reported in house flies (2) and in rats (11).

By referring back to the procedure followed in tests 2 and 3 described previously, it can be seen that the synergists were always applied before the insecticide, as the dose (except DEF) and the route of application were also the same. Under these conditions, the degree of inhibition of the detoxifying enzymes should be expected to be very similar, if not the same, in both bioassay series. Hence, in the present case, the actual amount of MPO (MPS must be activated to exert its toxic effect) reaching the target site has to be higher, which in turn should be directly correlated to the greater uptake of the toxicant by the tarsi of the weevils.

Further support of this assumption is the fact that all synergists tested by this method behave in a very similar fashion regardless of the arrangement of the substituents around the phosphorus atom Other possible explanations are not excluded, however.

Since under field conditions the weevils must pick up contact insecticides mostly through their tarsi, the results of this investigation bear significance as they show that the dependence of the toxicity of an insecticide on its route of entry, may well be exploited favorably by the appropiate use of synergists

Conclusion

From the results obtained in this investigation it can be concluded that the combination of residual dry film, as a method of insecticide application, with topical treatment with the synergist, is the more sensitive procedure to search for effective synergists in the boll weevil.

Simultaneous application of insecticide and synergist to the same loci onto the dorsal surface of the weevils results in either poor synergism or antagonism.

S,S,S-tributylphosphorotrithioate (DEF) and propyl-DEF, increased the toxicity of azinphosmethyl 2.28 and 1.63 fold respectively, after simultaneous application The same homologs (butyl and propyl) of the *S,S,*-dialkyl O-phenyl phosphorotrithioate series, applied as before, increased azinphosmethyl toxicity 2 26 and 2.17 fold, respectively. On this basis, to carry out further screening tests of synergism of OP insecticides in the boll weevil, the butyl and higher homologs of the latter series should preferentially be assayed.

To investigate the relative importance of mixed function oxidases and glutathione alkyl or aryl transferases in detoxications in the boll weevil, the insecticide azinphosmethyl offers relevant features In addition, carbamate insecticides, which require activation to exert their toxic effects, should also prove valuable to clarify the involvement of mixed function oxidases in this insect.

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Reseña de Libros

LIKENS, G.E., BORMAN, F.H., PIERCE, R.S., EATON, J.S., and JOHNSON, N.M. Biogeochemistry of a forested ecosystem. New York, Springer, 1977. 146 p.

El libro de Likens y colaboradores presenta los resultados obtenidos a largo plazo de un estudio integral de un ecosistema forestal en Hubbard Brook, New England, USA. Se trata de una área boscosa de 3076 hectáreas en alturas entre 229 y 105 m s.n. en un clima húmedo continental sobre suelos podzolisados (haplorthods) limoarenosos desarrollados a partir de rocas graníticas y metamórficas. En esta área se han investigado entre 1956 y 1974 los ciclos bioegeoquímicos. Los resultados pertinentes se presentan en 5 capítulos del libro: 2) hidrología, 3) química, 4) balances de input-output, 5) meteorización y 6) ciclos de elementos nutritivos. En los últimos dos capítulos se comparan los resultados con datos de otros ecosistemas y se presentan las conclusiones.

Los datos hidrológicos corresponden a mediciones entre 1956 y 1974, arrojan en promedio una precipitación anual de 1295 mm, correspondiendo en 38,1 por ciento a evapotranspiración y el resto a la escorrentía. En último proceso fue medido como una cuenca hidrológica cerrada.

Para los ciclos biogeoquímicos se colectaron y analizaron muestras semanales entre 1963 y 1974. Las lluvias son bastante ácidas (promedio 4,14(y fueron analizadas para Na, K, Ca, Mg, Al, NH_4, SO_4, NO_3, Cl, PO_4, HCO_3, Si y C orgánico. Los elementos predominantes son H y SO_4; en el transcurso de las mediciones se encontró un aumento lineal del H (de 0,8 kequiv/ha en 1964 a 1,1 kequiv/ha en 1974), asociado con la polución reinante en la región. Basándose en los resultados se han calculado las ganancias y pérdidas de elementos nutritivos al comparar la deposición con la escorrentía. A largo plazo se encontraron balances positivos para N, Cl, S y P y negativos para Si, Ca, K, Mg, Na, y Al. En función de ellos consideran los autores los procesos de meteorización de minerales primarios del suelo. El proceso se explica teóricamente en el intercambio de H proveniente de las lluvias ácidas en los complejos metálicos y así la liberación de los elementos mencionados (Si, K, Ca, Mg, Al).

Basándose en una determinación de la biomasa de un rodal de 55 años y determinaciones en el suelo se calculan las cantidades de elementos acumulados en el ecosistema. Esos datos son punto de partida para el establecimiento de modelos matemáticos de los diferentes elementos nutritivos; de ellos se presentan en detalle el de calcio y de azufre. Si bien los modelos matemáticos son bastante interesantes llevan una serie de premisas que escatiman su valor; así por ejemplo no se considera al rodal mantillo humico del suelo y el "turnover" en el pasaje de las lluvias; los datos de mineralización y meteorización son también muy estimativos. A pesar de ello se trata de un estudio a largo plazo, bien conducido, el cual es pionero a nivel mundial. Para los especialistas es un ejemplo sin comparación que puede servir de guía en otros estudios.

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