THE EFFECT OF INSECT GROWTH REGULATORS AND THEIR BINARY MIXTURES ON LABORATORY STRAIN OF *SPODOPTERA LITTORALIS* (LEPIDOPTERA: NOCTUIDAE).

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INTRODUCTION

Recently, many of the conventional methods of insect control by broad spectrum synthetic chemicals have come under assault and scrutiny because of their undesirable effects on human health and the environment (Perry, *et al.*, 1998). Furthermore, resistance has been recorded for most conventional insecticides. As a consequence, it provides impetus to study new alternatives and more ecologically acceptable methods for insect control. One of these approaches which have captured worldwide attention is the development of novel compounds capable of interfering with the processes of growth, development and metamorphosis of the target insects such as insect growth regulators (Ishaaya & Horowitz, 1997).

Benzoylphenylurea (BPU's) are insecticides acting on various insect orders by reducing chitin deposition into the insect's cuticle, which in turn disrupts normal molting (Mülder & Gijswijt, 1973; Retnakaran *et al.*, 1985). Carrol Williams coined the term 'Third Generation Pesticides' to describe hormone-based on the role of juvenile hormone. The major drawback to the use of juvenile hormone analogs (JHA's) as insecticides is that they are only active during a short period at the end of the larval instars when endogenous titer of juvenile hormone is low (Jennings, 1984). Early developed analogs such as methoprene and hydroprene have a chemical structure which resemble that of JH, modified chiefly to improve resistance to degradation in the insects. More recently, nonterpenoidal analogs such as fenoxycarb and pyriproxyfen were developed (Cusson & Palli, 2000). While success in the much earlier, it is only that insecticide came much earlier, it is only recently that insecticides

which act as agonists of 20-hydroxyecdysone (20E) have been discovered (Dhadialla & Jasson 1999). These ecdysone agonists bind to the ecdysone receptor (EcR) and at molecular level through initiating and accelerating moulting process by gene regulation (Oberlander *et al.*, 1995 and Retnakaran *et al.*, 1995).

Three compounds (Chlorfluazuron, tebufenozide and pyriproxyfen) belong to the class of IGR's insecticides. Each one has a specific mode of action was selected in this study. The present work emphasizes the effectiveness of the three IGR's interaction and their binary mixture to the 4th instar larvae of the cotton leafworm, *Spodoptera littoralis*.

MATERIAL AND METHODS

1- Insect Rearing:

The susceptible laboratory strain of *Spodoptera littoralis* (Boisd.) was reared under laboratory conditions at 25±2 °C and 75±5 relative humidity for several generations as described by El-Defrawi *et al.* (1964).

2- Chemicals:

Tebufenozide 24% EC was purchased from DOW Agroscience Egypt, Pyriproxyfen 10% EC from Sumitomo Chemical CO., Egypt, and Chlorfluazuron 5% EC from Syngenta, Egypt.

3- Insecticidal activity:

In all tests, newly moulted (within 24h after ecdysis) fourth instar larvae of *S. littoralis* were used. Fourth instar larvae was identified by width of head capsule. The larvae were starved for 2-4h before transferring onto the treated leaves.

In preliminary assays, serial dilutions of each insecticide were prepared in water based on percentage of active ingredient (a.i.). Castor bean leaves were dipped in either water (control) or in one of the five previously prepared dilutions for 20 seconds and were air dried under room temperature. Larvae were placed in glass jars (12cm high and 7cm diameter) and offered treated food for 24h and then replaced by untreated leaves. Castor bean leaves were renewed daily.

On the basis of preliminary experiments, at least 5 concentrations of insecticides that caused mortality ranged from 15 to 90% were used to determine their LC_{50}. Fifty newly moulted fourth instar larvae used for each concentration were replicated at least 5 times (10 larvae/ replicate).

Larval mortality was used as the response criterion and observations were recorded until all test larvae had either died or pupated. These data were corrected for untreated mortalities using Abbott's formula (Abbott, 1925) and analyzed with probit analysis (Finney, 1971).

4- Joint action of IGR's:

Based on the toxicity lines of tebufenozide and chlorfluazuron, some concentrations were selected from the two compounds to be used in these experiments. The fourth instar larvae and leaf-dipping technique was used as described above.

Firstly, the recommended concentration of pyripoxyfen (75ppm) simultaneously was mixed with the sublethal concentrations (in the range between LC_{10} and LC_{70}) of either tebufenozide or chlorfluazuron based on their LCP-line. Secondly, the LC_{10} of tebufenozide was assayed simultaneously with sublethal concentrations of chlorfluazuron based on LCP-line, in the range between LC_{10} and LC_{50} and *vice versa.* Fourth instar larvae were fed on leaves previously dipped in the prepared mixtures for 24h and the accumulated mortality of the treated larvae was recorded up to pupation and evaluated as mentioned above.

RESULT AND DISCUSSION

1- Insecticidal activity:

In the present study, the efficacy of different insect growth regulators (IGR's), chlorfluazuron (BPU's), Pyriproxyfen (JH mimic) and tebufenozide (ecdysone agonist) were tested against 4th instar larvae of *S. littoralis*. Each of these has its own mode of action.

Numerous studies reported that chlorfluazuron had an insecticidal potency on lepidopteran insects. It seemed to be effective against *Platynota stultana* (Hejazi & Granett, 1986); *S. littoralis* (Ishaaya *et al.,* 1986 and Guyer & Neumann 1988); *S. exigua* (Van Laecke *et al.* 1989); *Earias insulana* (Horowitz *et al.,* 1992) and *Spodoptera litura* F. (Perveen, 2000) by inhibiting chitin biosynthesis, thereby abortive molting process.

Tebufenozide has a selective toxicity to lepidopteran pests (Dhadialla *et al.,* 1998). Previous studies reported its efficiency on *S. littoralis* (Ishaaya et *al.*, 1995; Smagghe *et al.* 1995 and Smagghe & Degheele, 1997). The potency of insecticidal activity of tebufenozide is correlated with binding affinity to ecdysteroid receptor complex (Smagghe *et al.*, 2000 and 2002).

The majority of research on juvenile hormone in Lepidoptera concentrated on the last instar larvae (*i.e.* Hatakoshi *et al.,* 1988). Pyriproxyfen was registered in Egypt against purple scale and whiteflies by the field rates 50 and 75 ml /100L in citrus and cucumber fields, respectively.

The newly molted 4th instar of *S. littoralis* larvae were treated within the first 24h after ecdysis. This early part of the stadium is the optimal phase for treatment with ecdysone agonists, before the appearance of the natural ecdysteroid peak (Palli *et al.,* 1995). In the later treatment, the most of larvae underwent a normal molt but followed with another incomplete lethal molt immediately (Dhdialla *et al.* 1998). This probably indicated that the molting cascade is a one-way process that cannot restart once initiated by the 20E (Blackford & Dinan, 1997).

In the current assays, the length of the bioassay was an important factor affecting larval response to each insecticide. In this connection, Ismail and Wright (1991) reported that acylurea compounds tested on second instar larvae of *Plutella xylestella* (L.) took a long time (9-17 days) to reach end point mortality when compared with the conventional neuroactive insecticides. A study on IGR's that used shorter assay periods may markedly underestimate mortality. In this shorter time interval, 50% larval mortality would not have occurred even at the highest doses of the IGR's (Mascarenhes *et al.,* 1998). It is clear that larval mortality was more dependent on the time after treatment than on concentration used (Malinowski & Maria, 1992). Furthermore, Biddinger *et al.,* (1996) and Knight *et al.,* (2001) published similar results with ecdysone agonists.

Ecdysone agonists and bezoylphenylureas are differing markedly in their mode of action, although both classes of compounds cause abortive ecdysis. The benzoylphenylureas disrupt chitin biosynthesis while it appears that ecdysone agonists exert their effect by binding with ecdysteroid receptors and induce premature lethal molts.

In preliminary experiments, all levels of that abnormal ecdysis described in obvious studies were noticed. The phenotypic effects seemed to be similar in both classes. The difference in toxicity symptoms between the two classes is the onset of these symptoms. These symptoms were manifested after 24h in ecdysone agonists case vs. in normal ecdysis time in BPU's.

In the current study, no significant effect of pyriproxyfen on 4th instar larvae was found to evaluate the LC_{50}. There are many authors reported that pyriproxfen had no direct effect on larval stage in *Bemesia tabasi* (Ishaaya &

Horowitz, 1992 and 1995) *Shistocerca gregaria* (Vennard *et al.*, 1998). In this frame, Perry *et al.*, (1998) indicated that young larvae were not killed by JHA's. Generally, the periods of great sensitivity of JHA's are very restricted to the last larval instar and the beginning of the pupal stage (Staal, 1975).

The insecticidal activity results of chlorfluazuron and tebufenozide are presented in Table (1). The LC_{10}, LC_{50} and LC_{90} of chlorfluazuron and tebufenozide were determined as 0.024, 0.13 and 0.77 ppm and 5.27, 9.76 and 18.07 ppm, respectively. The slope of tebufenozide (4.79±0.457) was higher than that of chlorfluazuron (1.71±0.127) *i.e.* the LCP-line of tebufenozide was steeper than LCP-line of chlorfluazuron. However, the data showed that chlorfluazuron was more efficient than tebufenozide.

TABLE (I)

Insecticidal activity of chlorfluazuron and tebufenozide in leaf-dipping bioassay fourth instar *Spodoptera littoralis* larvae up to pupation.

Insecticide	[1]LC_{10} (95% CL)[2]	LC_{50} (95% CL)	LC_{90} (95% CL)	Slope±SE
Chlorfluazuron	0.024 (0.01-0.033)	0.13 (0.089-0.218)	0.768 (0.57-1.99)	1.71±0.13
Tebufenozide	5.27 (2.51-6.81)	9.76 (7.87-12.84)	18.07 (13.47-44.41)	4.79±0.46

[1] LC_{10}, LC_{50}, LC_{90}; Concentration levels required for 10%, 50% or90% mortality expressed as ppm (a.i.).
[2] CL, Confidence Limits

2- Cumulative mortality of IGR's:

LC_{50} were selected to assess the cumulative toxicity on the fourth instar larvae (Fig. 1). The mortality percent in the tebufenozide treated 4th instar was 46.3% followed by 27.7% in the 5th instar while no mortality was observed in the 6th instar. In some cases, additional molt (7th instar) was recorded. This phenomenon had previously been reported in larvae of *Laconobia olercea* (Blackford & Dinan, 1997) and in *Ostrinia nubilalis* larvae (Trisyono & Chippendale, 1997) after treatment with tebufenozide. In addition, the same result was recorded in the 4th instar larvae of *S. littoralis* treated by methoxyfenozide (Adel & Sehnal 2000).The mortality was 2.7% during 7th instar (addional instar) and was 30.6% during pupal stage. Previously, many authors reported that mortality of insect growth regulators (IGR's) extended to subsequent instars and pupal stage after treatment (Herbert & Harper 1985; Guyer & Neumann 1988; Biddinger *et al.*, 1996 and Mascarenhes *et al.*, 1998).

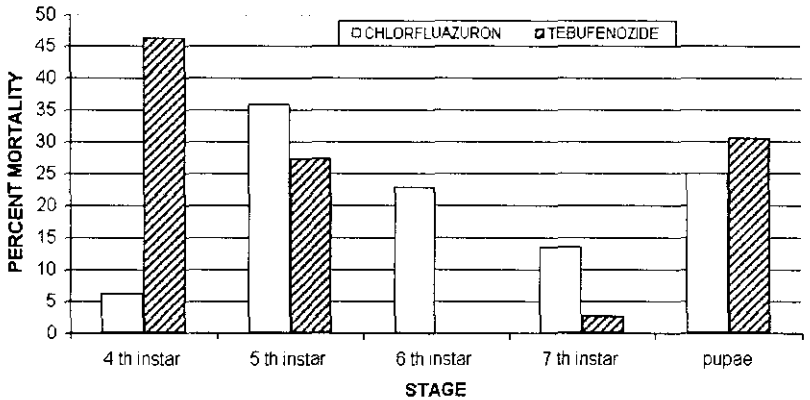

Fig. (1): Distribution of mortality (%) during development time till pupation of chlorfluazuron and tebufenozide treated fourth instar larvae of *S. littoralis*.

It is expected that tebufenozide causes most mortality during 4th instar (treated instar) via binding with ecdysteroid receptor (Wing, 1988) and induces premature lethal molt (Wing *et al.*, 1988; Smagghe & Degheele 1994a, 1995, 1997 and Palli *et al.*, 1995). The decrease in mortality percent during subsequent instars may be due to the metabolic process of the compound persistence in the larval body. Smagghe *et al.* (1999) indicated that, in last instar larvae of *S. littoralis*, [^{14}C]-tebufenozide amount was 33% at 6h after ingestion and 8% after a further 6h.

On the other hand, the mortality percent of chlorfluazuron was 6.25, 36 and 22.9 for 4th, 5th and 6th instars, respectively. In some cases some larvae accessed additional molt when benzoylphenylurea compounds was used. In this frame, Emam & Degheele (1993) treated 4th instar larvae of *S. littoralis* with sublethal doses of benzoylphenylureas (teflubenzuron, chlorfluazuron, triflumuron and diflubenzuron) and found that in some cases, an extra moult was induced. Also, chlorfluazuron induced additional molt in some larvae of *Rhyzopertha dominica* (Elek, 1994).The mortality in 7th instar and pupal stage was 13.5 and 25%, respectively.

In chlorfluazuron case, complete inhibtion of moulting process was low (6.25%) in treated 4th instar. The higher mortality in 5th instar can be attributed to two damage levels: Firstly; some larvae in 4th instar succeeded in partly splitting the exuvia and survive some days but finally died. Secondly; some larvae that succeeded to complete molting was unable to feed and their weight decreased. The latter damage may be attributing to chlorfluazuorn effect on production of the

peritrophic membrane (Clarke *et al.*; 1977; Becker, 1978 and Soltani, 1984). Moreover, the high mortality caused by the compound during the subsequental instar may be attributing to the high persistence of the compound inside larval bodies. Guyer & Neumann (1988) reported that using radiolabelled assay explained that the effectiveness of chlorfluazuron is due to its retention and low elimination. Half–life of chlorfluazuron was estimated to be about 50h.

3- Joint action of IGR's:

In this part, the research was directed to emphasize the joint action of the three tested IGR's, *i.e.* to clarify if either their combinations give a synergistic or antagonistic effect. The impact of simultaneous treatment effect of newly 4th instar *S. littoralis* larvae by tebufenozide or chlorfluazuron plus pyriproxyfen; and tebufenozide plus chlorfluazuron was investigated to elucidate more fully the nature of their joint action.

3.1- Tebufenozide plus Chlorfluazuron:

Benzoylphenylureas (BPU's) inhibit chitin synthesis during growth and development in insects and acts as moult disruptants (Retnakaran *et al.*, 1985). However, ecdysone agonists exhibit a precocious and incomplete molt (Wing *et al.*, 1988 and Retnakaran *et al.*, 1995). With the recent progress in insecticides development, the use of additional methods of population control was deemed considerably.

TABLE (II)

Toxicity values of accumulative mortality up to pupation of 4th instar *S. littoralis* larvae fed on treated leaves by tebufenozide alone or in combination with either pyriproxyfen or chlorfluazuron.

Insecticide	[1]LC_{50} (95% CL)[2]	LC_{90} (95% CL)	Slope±SE
Tebufenozide alone	9.76 (7.87-12.84)	18.07 (13.47-44.41)	4.79±0.46
+ Pyriproxyfen (75ppm)	4.764 (---------)	480.7 (----------)	0.64±0.44
+ Chlorfluazuron (LC_{10})	7.29 (7.092-7.513)	10.45 (9.79-11.47)	8.19±0.764

[1] LC_{50}, LC_{90}; Concentration levels required for 10%, 50% or90% mortality expressed as ppm (a.i.).
[2] CL, Confidence Limits

The newly molted 4th instar *S. littoralis* larvae were forced to feed on castor bean leaves dipped in various concentrations of tebufenozide and fixed concentration (LC_{10}=0.024 ppm) of chlorfluazuron. The results presented at Table

(2) show that this combination caused accumulative mortality percentages up to pupation much higher than tebufenozide alone. The simultaneous treatment caused reduction in the LC_{50} and LC_{90} to 7.29 and 10.45 ppm, respectively. The potential effects of chlorfluazuron were shown in the increasing of slope combination's LCP-line (8.19±0.764) compared with tebufenozide alone (4.79±0.457).

At the same manner, tebufenozide seemed to be potential to the effectiveness of chlorfluazuron. In this combination fashion, the reduction showed in the LC_{50} and LC_{90} (0.063 and 0.183 ppm respectively) compared to chlorfluazuron alone (0.13 and 0.77 ppm, respectively). Additionally, the slope of LCP-line for the mixture also increased to 2.776±0.325 compared to 1.711±0.127 of chlorfluazuron alone Table (3).

TABLE (III)

Toxicity values of accumulative mortality up to pupation of 4th instar *S. littoralis* larvae fed on treated leaves by chlorfluazuron alone or in combination with either pyriproxyfen or tebufenozide.

Insecticide	[1]LC_{50} (95% CL)[2]	LC_{90} (95% CL)	Slope±SE
Chlorfluazuron alone	0.137(0.089-0.218)	0.77 (0.567-1.997)	1.711±0.127
+ Pyriproxyfen (75ppm)	0.018 (0.0024-0.033)	1.46 (0.543-21.72)	0.673±0.164
+ Tebufenozide (LC_{10})	0.063 (0.3058-0.071)	0.183 (0.142-0.273)	2.776±0.325

[1] LC_{50}, LC_{90}; Concentration levels required for 10%, 50% or90% mortality expressed as ppm (a.i.).

[2] CL, Confidence Limits

To our knowledge, no information has been reported in the literature concerning the possible interaction between benzoylphenylurea and ecdysone agonists when simultaneously treated.

Marks *et al.* (1982) reported that insect tissues treated with 20E and Dimilin (diflubenzuron) produced an imperfect proteinoceous cuticle that was devoid of chitin. Moreover, CSI's inhibit chitin synthesis in cultured imaginal wing discs after only a very brief exposure whether it applied before, during or after treatment with 20E (Oberlander & Silhacek, 1997). Our results refer to a possible utility of combining benzoylphenylurea and ecdysone agonists for greater effectiveness in controlling *S. littoralis* and that is environmentally acceptable

3.2- Tebufenozide plus Pyriproxyfen:

It is well known that juvenile hormone (JH) and 20-hydroxyecdysone (20E) regulate insect molting and metamorphosis. The major restriction in the use of JH as an insect control agent is that it is mainly effectiveness at a relatively narrow temporal window (*i.e.* at the end of the final larval instar). JH is normally present at a high level during the earlier larval stages and exogenous JH has little or no disruptive effect during that time. Unfortunately, it is during these larval stages that many insect pests do much of their damage (Nijhout, 1994). On the other hand, ecdysone agonists are active against catterpillars and subsequently induce lethal larval molt (Wing *et al.*, 1988; Heller *et al.*, 1992 and Dhadialla *et al.*, 1998).

The effectiveness of simultaneous treatment of 4th instar larvae with pyriproxyfen and tebufenozide had been studied. Subsequently, combined leaf application at various concentrations (in the range between LC_{10} and LC_{70}) of tebufenozide plus 75 ppm pyriproxyfen, (the recommended concentration). It is clear that the larvae in this study have been stressed and in opposing directions.

Results in Table (2) show that LC_{50} of combination was 4.76 ppm compared with 9.76 ppm for tebufenozide alone. LC_{90} of the combination was 480.7 ppm vs. 18.07 for tebufenozide alone. Pyriproxyfen seemed to act as synergist at low concentrations of tebufenozide but as antagonist at high concentrations of tebufenozide. The slope of LCP-line of tebufenozide alone was 4.79±0.457. It decreased in the combination to 0.64±0.44. This low value indicated that pyriproxyfen countered the lethal effect of tebufenozide and consequently heterogenicity in the sensitivity of insect population toward this combination. This result indicated that no advantage would be gained by simultaneous application of tebufenozide with pyriproxyfen. Juvenile hormones counteract some deleterious effects of dibenzoylhydrrazine (Oberlander *et al.*, 1995). Silhacek *et al.* (1990) and Smagghe & Degheele (1994b) described a similar situation. In the presence of JH, RH-5849 may stimulate larval molts and delayed pupation of *Tribolium freemani* Hinton, since early pupation caused by RH-5849 was antagonized by JH I (Hirashima *et al.*, 1995).

A number of studies have been performed which given some indirect hints as to the mechanisms of interaction between JH and 20-hydroxyecdysone. In most cases, JH in some way counteract or ameliorates the effects of 20-hydroxyecdysone.

Early work on *Plodia interpunctella* imaginal discs in organ culture showed that JH-I prevented ecdysteroid-stimulated cuticle formation in cultured wing

imaginal discs (Oberlander & Tombin, 1972). Additionally, Chihara *et al.*, (1972) worked on *Drosophila* imaginal wing discs and found about 100 mg/ml JH inhibited 20E-induced evagination of discs. In support to this direction, Longom *et al.*, (1975) found that juvenile hormone blocked that ecdysone stimulation of DNA synthesis and proposed that juvenile hormone and ecdysone act in a balanced fashion to regulate DNA synthesis imaginal disks. On the contrary, Becker (1978) found that a combination of two hormones (various concentrations of JH, constant concentration of 5ug 20E/ml) showed no compensation for the detrimental effect of JH on *Calliphara erythrocephola* peritrophic membrane but rather intensification.

Willis (1997) showed that as little as 0.01 nM of JHI would prevent 20E induced detachment of *P. interpunctella* cells from the culture dish surface. Moreover, Cottam & Milner (1998) found that JH moderated the effect of 20E on *Drosophila* cell line (C18+) when added simultaneously or with 4 h pre-incubation before 20E addition.

Using chitinase DNA clone as a hybridization probe, Kramer *et al.*, (1993) found that topical application of juvenile hormone mimic (fenoxycarb) into isolated abdomens of fifth instar of *Manduca sexta* larvae could abolish the increase in the expression of chitinase gene caused by 20E.

3.3- Chlorfluazuron plus Pyriproxyfen:

Based on LC_{50} (Table 3), results indicated that pyriproxyfen synergized (LC_{50}= 0.018ppm) chlorfluazuron compared to chlorfluazuron alone (LC_{50}= 0.13). The remarkable observation is that the LC_{90} of the mixture increased to 1.46 ppm compared to 0.77 ppm to chlorfluazuron alone. Moreover, the steepenst of the LCP-line of the mixture decreased to 0.673±0.164 compared to chlorfluazuron alone, 1.711±0.127.

The increasing in LC_{90} and the flatter LCP-line of the mixture may be due to the antagonistic effect of pyriproxyfen toward chlorfluazuron at high concentrations. Accordingly, no advantage would be gained from this mixing fashion.

This result is implicit in Fahmy (1993) who assessed the susceptibility of *P. xylostella* to combination of chlorfluazuron and pyriproxyfen. At LC50, the combination is more effective but at LC90, chlorfluazuron was more effective. Additionally, Rebertson *et al.* (1984) reported that methoprene (JHA) acted as antagonist to diflubenzuron and SAY SIR 5814.

SUMMARY

The biological activity of insect growth regulators (IGR's) *i.e.*, Chlorfluazuron, Pyriproxyfen and Tebufenozide was assessed against newly molted 4th instar *Spodoptera littoralis* larvae using leaf-dipping technique. The accumulative toxicity was estimated up to pupation. Results showed that chlorfluazuron and tebufenozide exhibited toxic action. Based on LC_{50} values, chlorfluazuron more effective than tebufenozide. However, pyriproxyfen had no insecticidal activity against the 4th instar larvae.

The effect of LC_{50} of chlorfluazuron and tebufenozide on 4th instar larvae indicated that tebufenozide caused its main mortality percent during the next moult 46.3% (from total mortality up to pupation). The effectiveness was decreased during next instars but re-increasing during pupation to 30.6%. The lethal median concentration (LC_{50}) of chlorfluazuron caused high mortality percent during later developmental stages (36% during 5th instar and 25% during pupation).

The joint action of the binary mixture of tested compounds was studied. Chlorfluazuron seemed to be potentiate the effectiveness of tebufenozide and *vice versa*. On the other hand, Pyriproxyfen seemed to act as synergist at low concentration of tebufenozide or chlorfluazuron but showed antagonistic action at high concentrations. Therefore, no advantage would be gained by simultaneous application of pyriproxyfen with tebufenozide or chlorfluazuron.

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