

DNA synthesis in the imaginal wing discs of the American bollworm *Helicoverpa armigera* (Hübner)

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The effect of two insect growth regulators of plant origin viz. plumbagin and azadirachtin and the ecdysteroids 20-hydroxyecdysone, makisterone A and a phytoecdysteroid on DNA synthesis in imaginal wing discs of day 4 final instar *Helicoverpa armigera* larvae was studied. DNA synthesis increased with increase in time of incubation up to 8 h and decreased later without the addition of moulting hormone. Addition of 20-hydroxyecdysone supported long term acquisition of competence for DNA synthesis in the wing discs. Both DNA synthesis and protein content were drastically reduced in plumbagin and azadirachtin-treated insects. Under *in vitro* conditions, plumbagin had a more pronounced inhibitory effect than azadirachtin. All the ecdysteroids tested, viz. makisterone A, 20-hydroxyecdysone and the ecdysteroidal fraction from the silver fern *Cheilanthes farinosa* enhanced DNA synthesis.

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1. Introduction

Imaginal discs are invaginated epidermal sacs in which latent adult structures are carried as undifferentiated primordia within the feeding larvae (Oberlander 1983). At metamorphosis, imaginal discs complete their development and differentiate into the specific structures for which they have been destined. Extensive work has been done in the recent past on the imaginal discs of *Drosophila melanogaster*, which served as a tool for developmental biologists and geneticists (Oberlander 1985).

The rate of proliferation of imaginal wing discs increase markedly in the final stages of the last larval instar (Meyer *et al* 1980; Williams 1980). Increased growth rate, late in the larval stadium indicates hormonal involvement in the cell proliferation, tissue competence, tracheolar migration, evagination and cuticle deposition of the imaginal discs. Oberlander and Fulco (1967)

provided evidence that ecdysone alone was responsible for initiation of metamorphosis in the wing discs of *Galleria mellonella* under *in vitro* conditions. Postlethwait and Schneiderman (1968) observed a dose dependent effect of ecdysone on *D. melanogaster* imaginal discs cultured *in vitro*; low concentration stimulate growth and cell division whereas high concentrations stimulate cuticle deposition and block DNA synthesis, cell division and growth. The effects of 20-hydroxyecdysone given at the same dose caused stimulatory, inhibitory or no effects on the DNA synthesis in *Calliphora* discs depending upon the stage of the larvae at treatment (Vijverberg 1973). The development of wing discs of *Sarcophaga peregrina* was induced by ecdysterone, ponasterone A and cyasterone where as rubrosterone was inactive in inducing development (Ohmori and Ohtaki 1973). 20-Hydroxyecdysone, ponasterone A and the bisacylhydrazine based ecdysteroid agonists RH-5849 and RH-5992

Keywords. Azadirachtin; *Helicoverpa armigera*; 20-hydroxyecdysone; imaginal wing discs; plumbagin

Abbreviations used: EcR, Ecdysteroid receptor; ED₅₀, effective dose, 50%; DMSO, dimethyl sulfoxide; DPM, disintegration per minute; IGRs, insect growth regulators; TCA, tri-chloro acetic acid.

induced evagination of *in vitro* cultured imaginal wing discs from the last instar of beet armyworm, *Spodoptera exigua*. Though the response was qualitatively similar to that induced by 20-hydroxyecdysone, the concentrations required for inducing response varied widely (Smaghe and Degheele 1995). Two well known natural products, plumbagin from *Plumbago capensis* and azadirachtin from *Azadirachta indica* depleted the ecdysteroid titer in the American bollworm *Helicoverpa armigera* (Hübner), which in turn altered lysosomal enzyme activity causing overt morphological abnormalities during the metamorphic molt (Josephraj Kumar *et al* 1999).

The incorporation of tritiated thymidine by imaginal disc cells has been used as a parameter of DNA synthesis and consequently of cellular proliferation since the cells do not become polyploid or polytene (Vijverberg 1973). Therefore, an attempt has been made in this investigation to study DNA synthesis in the imaginal wing discs of *H. armigera*, as influenced by plumbagin and azadirachtin as well as a phytoecdysteroid recently purified on RP-HPLC by us from the silver fern, *Cheilanthes farinosa* (Josephraj Kumar *et al* 2000)

2. Methodology

2.1 Insect culture

A continuous colony of *H. armigera* was maintained on a chick pea based semi-synthetic diet (Singh and Rembold 1992) in an insectary at $25 \pm 1^\circ\text{C}$, 14 : 10 LD photoperiod and 55% RH. Plumbagin was topically applied on the final-instar larvae, 0–4 h after molting, at the effective dose, 50% (ED_{50}) dose of 100 mg g^{-1} . Azadirachtin, dissolved in a solution of ethanol and 0.7% NaCl (1 : 1) was injected (to avoid antifeedant effect) into one day final-instar larvae at the ED_{50} dose of $1.25 \text{ } \mu\text{g g}^{-1}$.

2.2 Dissection

The uniform sized and aged final-instar *H. armigera* larvae raised under constant density were opened mid-ventrally. The imaginal wing discs were located dorso-lateral to the mesothoracic leg after the overlying fat body was carefully cleared off. A large tracheolar mass present at the base of wing discs provides a marker in identification. Care was taken during dissection as the fat body and muscles tend to obscure the imaginal discs. Imaginal discs were distinctly seen under stereo binocular microscope, from one-day old final-instar larvae of *H. armigera*.

2.3 Incubation

A pair of mesothoracic wing discs was excised in lepidopteran saline (Bindokas and Adams 1988) from final-instar *H. armigera* and incubated immediately after

excision in a glass vial containing $100 \text{ } \mu\text{l}$ of sterile filtered insect tissue culture medium (0.1087 g ITC-199, 0.115 g Ficoll and 0.047 g HEPES diluted in 10 ml of distilled water). The medium ITC-199 was obtained from M/s Boehringer-Mannheim, Germany and hydrated before use. After one hour of pre-incubation, $1 \text{ } \mu\text{l}$ of ^3H thymidine (LCT-53) having specific activity 18 Ci/m mol obtained from BRIT, Mumbai was added into the insect tissue culture medium containing the wing discs. The experiments were performed at $26 \pm 0.5^\circ\text{C}$ in a shaker water bath (30 oscillations per minute). After the required period of incubation, the reaction was terminated by immersing the discs in $500 \text{ } \mu\text{l}$ of ice cold 5% tri-chloro acetic acid (TCA). The discs were then washed twice for 10 min each in $500 \text{ } \mu\text{l}$ of ice cold 5% TCA to eliminate non-specific binding if any (Egberts 1979). Thereafter, the discs were dried at 60°C in an eppendorf vial and solubilized in $50 \text{ } \mu\text{l}$ of 1 M benzethonium hydroxide (Sigma). To this, 1 ml of scintillation mixture (Riatron) was added and briefly vortexed. The incorporation of ^3H thymidine into the wing discs was measured directly as disintegration per minute (DPM) in a liquid scintillation analyzer (Packard, C1600 TR) at an efficiency of $> 65\%$.

2.4 Stage of insect and the incubation time

In order to ascertain the most appropriate stage and incubation time for the *in vitro* study, mesothoracic wing discs from three, four and five-day old final-instar larvae were excised and incubated as above. The experiment was terminated after 1 h incubation and the discs were processed and counted as described earlier.

For determination of the optimum incubation time, the mesothoracic wing discs of four-day old final instar larvae were excised and incubated for various time intervals (1–24 h) after addition of ^3H thymidine. On completion of the required period of incubation, the discs were processed and the radioactivity counted as above. Five to eleven replications were maintained for each stage and incubation time.

2.5 In vivo and in vitro effects of plumbagin and azadirachtin

Four-day old mesothoracic wing discs of plumbagin-treated, azadirachtin-treated and untreated control larvae were excised and incubated for 4 h and 8 h after addition of $1 \text{ } \mu\text{l}$ of ^3H thymidine to study the *in vitro* effect of the test compounds in the discs. The discs were processed for ^3H thymidine incorporation as detailed above. Six to seven replications were maintained for each treatment and protein content of control and treatments was determined by folin-phenol method, using BSA as a standard (Lowry *et al* 1951).

For *in vitro* studies, four-day old mesothoracic wing discs of untreated final-instar larvae were excised in lepidopteran saline. One pair of mesothoracic wing discs, constituting one replication, was transferred after a brief washing into a sterile glass vial containing 100 μ l of insect tissue culture medium to which different concentrations of test compounds [plumbagin dissolved in 40% acetone, azadirachtin and ecdysteroids dissolved in 50% dimethyl sulfoxide (DMSO)], were added and the discs were incubated as above. After 60 min of pre-incubation, 1 μ l of ^3H thymidine was added to the incubation medium followed by further incubation for 4 h or 24 h. For each treatment, data from a minimum of five replications were collected. Control discs were incubated in 50% DMSO alone. In a preliminary test it was found that there was no significant difference in ^3H thymidine incorporation between DMSO and acetone treated discs. At the end of incubation period, the discs were processed as described earlier. The various treatments involved in the study are shown below:

Compound	Concentration	Incubation period (h)
Plumbagin	10^{-8} to 10^{-3} M	4
Azadirachtin	10^{-7} to 10^{-3} M	4
20-Hydroxyecdysone	10 ppm	4 and 24
Makisterone A	5 ppm	4 and 24
Ecdysteroidal fraction of <i>C. farinosa</i>	10 and 100 ppm	4 and 24

Makisterone A, a C-28 steroidal molecule, was gifted by Prof. Rene Lafont, Ecole Normale Supérieure, Laboratoire de Zoologie, Paris Cedex, France.

Another *in vitro* experiment was conducted to ascertain if 20-hydroxyecdysone would influence DNA synthesis by wing discs of plumbagin or azadirachtin-treated insects. Four-day-old mesothoracic wing discs of plumbagin and azadirachtin-treated final-instar larvae were excised and incubated as above. 20-Hydroxyecdysone (10 ppm) was added 4 h after incubation and the discs were incubated further for 20 h. At the end of the incubation period (24 h), the discs were processed and counted as described earlier. Four replications for each treatment were performed. The data were statistically analysed as per Gomez and Gomez (1984). The significant differences between the mean responses under two different conditions were compared by Student's *t*-test. Comparisons of the responses under more than two different conditions were subjected to Analysis of Variance (ANOVA) and significance of the differences among the means was determined by *F*-test using critical difference (CD) values.

2.6 Photography

Thermal photographs of imaginal discs from temporary slide mounts were taken on an inverted microscope

equipped with a CCD camera to show the extent of tracheolar migration.

3. Results

3.1 Response of wing discs

It is obvious from table 1a that after one hour incubation the incorporation of radioactivity was significantly higher (almost double) in four and five-day old discs than in three-day old discs. Therefore, day 4 wing discs were used for further studies. For the determination of the optimum incubation time for *in vitro* studies, day 4 wing discs were incubated with ^3H thymidine for varying time intervals (1–24 h) and the results are presented in table 1b. It is evident that the incorporation of radioactivity increased with the time of incubation up to 8 h. After 8 h of incubation the levels of incorporation decreased. The lowest incorporation was observed in wing discs incubated 2 h, followed by 1 and 24 h which were on a par with each other.

3.2 Response of wing discs from plumbagin and azadirachtin-treated insects

There was significantly reduced incorporation of radioactivity in both treatments compared to the respective control treatment (table 2). At 4 h of incubation, plumbagin and azadirachtin-treated wing discs incorporated four and two times less ^3H thymidine respectively, compared to the control. However, at 8 h it was two times lower in plumbagin-treated discs and three times lower in case of azadirachtin-treated discs compared to their respective controls. Photographs of imaginal wing discs of controls, plumbagin and azadirachtin-treated insects are presented in figures 1–6.

3.3 In vitro effect of plumbagin, azadirachtin and ecdysteroids

Addition of plumbagin at concentrations ranging from 1×10^{-8} M to 1×10^{-4} M resulted in significant inhibition of radioactivity incorporation only at the higher concentrations of 1×10^{-5} M and 1×10^{-4} M (table 3). At lower

Table 1a. *In vitro* incorporation of ^3H thymidine by the wing discs of last instar *H. armigera*.

DPM/pair of wing discs 1 h after incubation			
Larval age (days)			
3	4	5	CD ($P = 0.05$)
1220 ^b \pm 334	2300 ^a \pm 319	2413 ^a \pm 1011	885

$N = 5$, mean(s) followed by the same alphabet are not significantly different (ANOVA).

concentrations (1×10^{-8} to 1×10^{-6} M), the incorporation of radioactivity was on par with that of the control. Addition of azadirachtin, at the same concentrations as above, could not evince any effect and the incorporation of radioactivity was on par with that of the control. At 1×10^{-3} M concentration, however, it was found to have a significant inhibitory effect on the incorporation of radioactivity. The effective concentrations of both these compounds are, therefore, found to be different under *in vitro* conditions. The concentration required for a 50% reduction in DNA synthesis was found to be 10^{-5} M for plumbagin and 10^{-3} M for azadirachtin. Moreover, plumbagin produced a more significant inhibitory effect than azadirachtin at the same concentrations.

From table 4, it is clear that the ecdysteroidal fraction from *C. farinosa* at both the concentrations used were found to enhance the incorporation of radioactivity significantly, followed by makisterone A and 20-hydroxyecdysone at 4 h of incubation. The extent of radioactivity incorporation in makisterone A and 20-hydroxyecdysone treatments was only on par with that of the control. At a prolonged incubation of 24 h, all three ecdysteroids stimulated the incorporation of thymidine into DNA. The ecdysteroidal fraction of *C. farinosa* was the most potent (nine-fold increase), whereas 20-hydroxyecdysone and makisterone A caused seven- and six-fold increase, respectively. 20-Hydroxyecdysone enhanced DNA synthesis on wing discs from plumbagin and azadirachtin-treated insects (table 5).

3.4 Protein content

Protein content of day 4 wing discs of plumbagin and azadirachtin-treated insects was significantly reduced compared to their respective control. Reduction was more pronounced in azadirachtin treated wing discs than those treated with plumbagin (table 6).

4. Discussion

Absence of polyploidy and polyteny in the larval imaginal disc cells and the results of DNAase treatments support the view that the incorporation of tritiated thymidine is a measure of DNA synthesis involved in cell proliferation (Vijverberg 1973).

Wing discs grow throughout the larval stage and differentiate into pupal and adult structures during larval-pupal and pupal-adult transformations (Oberlander 1985). In this investigation, the acquisition of competence for maximum incorporation of ^3H thymidine was recorded at day 4 of the last larval instar. This can be attributed to the ratio of 20-hydroxyecdysone to juvenile hormone titer, as day 4 coincides with the falling phase of the first critical ecdysteroid peak (Josephraj Kumar *et al* 1999). Cell programming in *Pieris brassicae* takes place at low ecdysone level and pupal characters become apparent after the main ecdysteroid peak when RNA and cuticle synthesis is stimulated (Lafont *et al* 1977).

Table 1b. *In vitro* incorporation of ^3H thymidine by the wing discs of *H. armigera* after various incubation times.

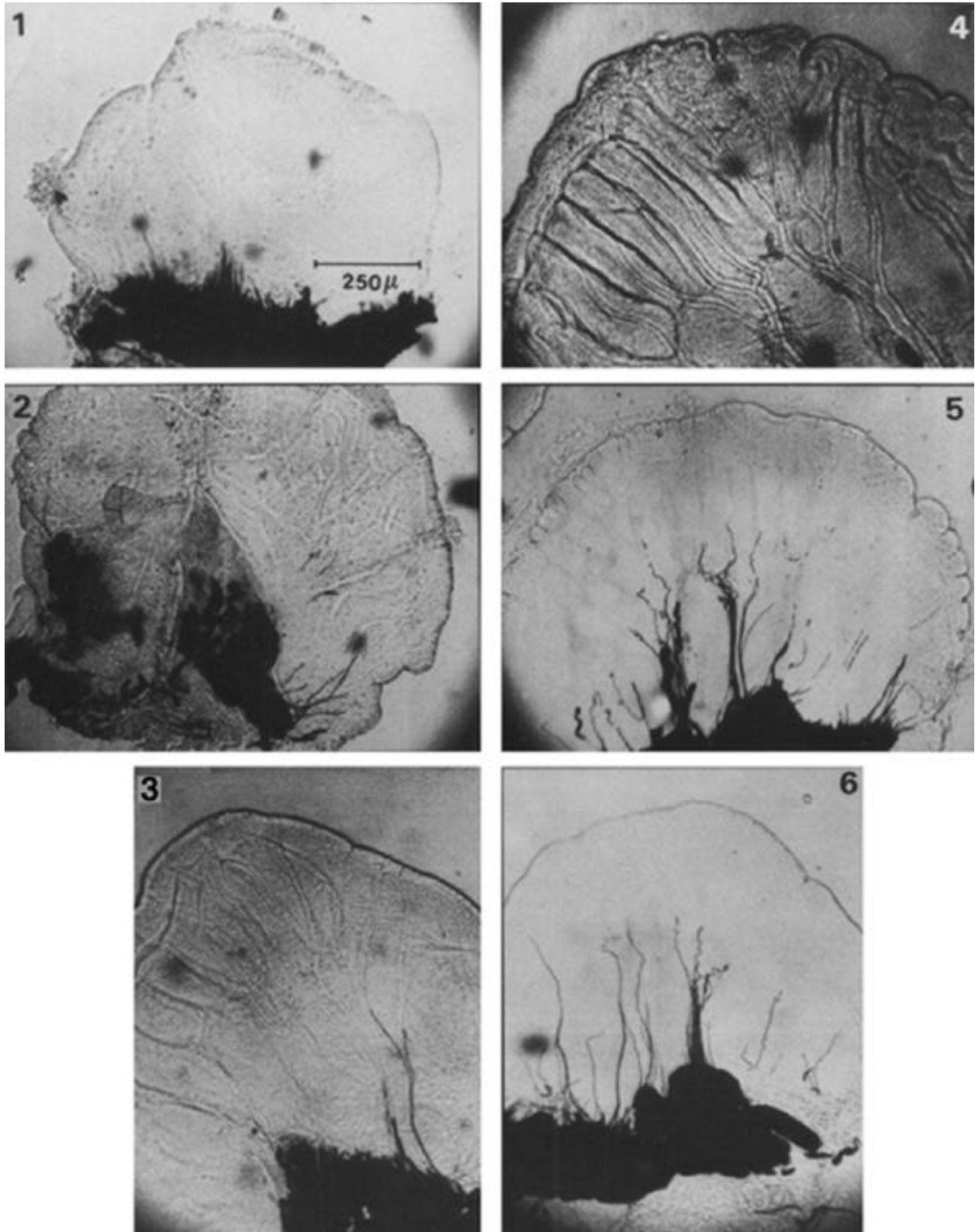
DPM/pair of day 4 wing discs of last instar								
Incubation time (h)								
1	2	4	6	8	12	18	24	CD ($P = 0.05$)
2300 ^d ± 319	1166 ^d ± 458	5972 ^c ± 1605	9319 ^b ± 1265	18146 ^a ± 5007	16816 ^a ± 2619	11183 ^b ± 2246	2440 ^d ± 1127	3379

$N = 4-11$, mean(s) followed by the same alphabet are not significantly different (ANOVA).

Table 2. *In vitro* incorporation of ^3H thymidine by the wing discs of plumbagin and azadirachtin-treated last instar larvae of *H. armigera*.

DPM/pair of day 4 wing discs				
Incubation time (h)	Control	Plumbagin (ED ₅₀)	Control	Azadirachtin (ED ₅₀)
4	6172 ± 1690	1401* ± 464	6055 ± 2115	2725* ± 720
8	17446 ± 5525	7375* ± 1973	18417 ± 4314	7193* ± 864

$N = 6$, *Significantly lower ($t = 0.05$) than the corresponding control values (Student's *t*-test).



Figures 1–6. (1–4) Wing discs of the last instar control larvae, days 1–4 respectively. Note the initiation of tracheolar migration into the terminal part of the discs on day 3 and their complete ramification on day 4. (5–6) Day 4 wing discs of the last instar larvae treated with plumbagin ED₅₀ (5) and azadirachtin ED₅₀ (6). Note the size of the wing disc that are comparable to day 2 control and tracheolar migration restricted to only a little more than half of the disc body. Same scale is maintained through out.

Due to the significant incorporation of radioactivity and the compactness of the wing discs on day 4 of the last instar, they were used in this study. Pre-incubation of discs for one hour was allowed uniformly to recover from dissection shock and avoid *in situ* influence of molting hormone. Day 4 wing discs incorporated ^3H thymidine up to 8 h of incubation. No appreciable incorporation of radioactivity was observed beyond 8 h of incubation in the absence of 20-hydroxyecdysone in the medium. In his pioneering work, Oberlander (1969a) found that wing discs synthesize DNA *in vitro* for no more than 24 h, unless they are cultured in the presence of ecdysone. The

Table 3. Effect of plumbagin and azadirachtin on the *in vitro* incorporation of ^3H thymidine by the wing discs of last instar *H. armigera*.

DPM/pair of day 4 wing discs in 4 h		
Molar concentration	Plumbagin	Azadirachtin
0	6089 ^a ± 1614	6274 ^a ± 1139
1 × 10 ⁻³	–	3174 ^b ± 1159
1 × 10 ⁻⁴	1244 ^b ± 281	5096 ^a ± 1441
1 × 10 ⁻⁵	2896 ^b ± 1336	6625 ^a ± 1814
1 × 10 ⁻⁶	4806 ^a ± 1385	5887 ^a ± 627
1 × 10 ⁻⁷	5173 ^a ± 2328	5965 ^a ± 1673
1 × 10 ⁻⁸	5404 ^a ± 635	–
CD (P = 0.05)	1863	1757

N = 5, Column mean(s) followed by the same alphabet are not significantly different (ANOVA).

continued presence of molting hormone was found to be essential to support the DNA synthesis by wing discs, as is also observed in this study. Further, the utilization of essential components in the medium or the appearance of some metabolites may be yet another possible reason for the reduced incorporation of radioactivity after a limited time of incubation.

In vitro studies on wing discs from plumbagin and azadirachtin-treated last instar *H. armigera* demonstrate significantly lower incorporation of radioactivity at both 4 and 8 h of incubation compared to control. The wing discs from the treated larvae were not influenced by the high *in situ* ecdysteroid titer. Further, the ratio of 20-hydroxyecdysone to juvenile hormone in the haemolymph has been drastically affected by both the treatments (Josephraj Kumar *et al* 1999). Addition of 1 × 10⁻⁵ M of plumbagin to the *in vitro* culture medium inhibited DNA synthesis by the imaginal wing discs. Azadirachtin could not cause such an effect at the same concentration (1 × 10⁻⁵ M) thereby showing the differences in the threshold levels of the two IGR compounds for influencing the DNA synthesis in the wing discs under *in vitro* condition. Such a variation may be ascribed to the difference in the entry of the two molecules having varied molecular structure and weight to the site of action viz. the nucleus.

At short-term incubation period of 4 h, the ecdysteroidal fraction of *C. farinosa* enhanced DNA synthesis *in vitro* followed by makisterone A and 20-hydroxy-

Table 4. Effect of ecdysteroids on the *in vitro* incorporation of ^3H thymidine by the wing discs of last instar larvae of *H. armigera*.

DPM/pair of day 4 wing discs						
Incubation time (h)	Control	20-hydroxy ecdysone (10 ppm)	Makisterone A (5 ppm)	Ecdysteroidal fraction of <i>Cheilanthus farinosa</i>		CD (P = 0.05)
				10 ppm	100 ppm	
4	6274 ^c ± 1139	7162 ^{bc} ± 886	7843 ^{abc} ± 2068	9300 ^{ab} ± 2394	9683 ^a ± 6220	2512
24	3724 ^d ± 446	26986 ^b ± 5257	21124 ^c ± 1855	26496 ^b ± 2544	32456 ^a ± 2690	4693

N = 5, row mean(s) followed by the same alphabet are not significantly different (ANOVA).

Table 5. Effect of 20-hydroxyecdysone on the *in vitro* incorporation of ^3H thymidine by the wing discs of plumbagin and azadirachtin-treated last instar larvae of *H. armigera*.

DPM/pair of day 4 wing discs in 24 h			
Plumbagin (ED ₅₀)	Plumbagin + 20-hydroxyecdysone**	Azadirachtin (ED ₅₀)	Azadirachtin + 20-hydroxyecdysone**
2209 ± 379	15367* ± 3609	1633 ± 1033	14467* ± 3368

*Significantly higher ($t = 0.05$) than the corresponding control values (Student's *t*-test).

N = 4, **20-hydroxyecdysone (10 ppm) was added 4 h after incubation.

Table 6. Protein content of the wing discs of last instar larvae of *H. armigera*.

µg per pair of day 4 wing discs			
Control	Plumbagin (ED ₅₀)	Control	Azadirachtin (ED ₅₀)
48.45 ± 1.73	36.93* ± 2.85	48.28 ± 2.24	33.37* ± 1.43

N = 5, *Significantly lower (*t* = 0.05) than the corresponding control values (Student's *t*-test).

ecdysone which are only on par with the control. At a prolonged incubation period of 24 h, all the three ecdysteroids caused significantly higher DNA synthesis than that of the control and the least incorporation among treatments was recorded with makisterone A. This evinces that makisterone A, a C-28 steroidal molecule could not simulate the effect of 20-hydroxyecdysone for a longer incubation period. For a shorter time of incubation, the competence of disc cells was well maintained with significant incorporation of radioactivity. So far, there are no such studies in the literature on makisterone A.

The structure and binding properties of ecdysteroid receptor (EcR) may vary among insect species and it was reported that imaginal discs evagination is a true approximation of the EcR binding affinity for ecdysteroids and non-steroidal agonists (Bidmon and Sliter 1990). The ecdysteroid-binding affinity of ponasterone A and RH-5992, an ecdysone agonist was higher than that of 20-hydroxyecdysone (Smagge and Degheele 1995). The enhanced synthesis of DNA due to ecdysteroidal fraction of *C. farinosa* compared to makisterone A may be attributed to the two-fold higher doses used and a preferential ecdysteroid-receptor binding effect of the *C. farinosa* ecdysteroid. Previous studies also suggest that ecdysterone and inokosterone could initiate metamorphosis of the wing discs of *G. mellonella* under *in vitro* conditions (Oberlander 1969b).

Stimulation of DNA synthesis was observed when 20-hydroxyecdysone was added to wing discs of treated insects with either plumbagin or azadirachtin suggesting that the two insect growth regulators (IGRs) could act on the transcription process. The fact IGRs act *in vitro* on disc cells after being explanted into saline suggest that they can interfere with the growth process controlled by ecdysteroid. The presence of 20-hydroxyecdysone has significantly enhanced tritiated thymidine incorporation as compared to those discs where no hormone was added. The delay in acquiring competence due to suppressed ecdysteroid titers by both the treatments, was counteracted by the continuous presence of 20-hydroxyecdysone in the medium. Protein content of the imaginal wing discs of insects subjected to both the treatments was significantly lower than their respective controls, due to the

physiological disturbances like suppressed ecdysteroid titres, reduced feeding and delayed gut-purge brought about by the treatments (Josephraj Kumar *et al* 1999). These results clearly indicated that wing discs incorporated more ³[H]thymidine into DNA after treatment with ecdysteroids and that IGRs such as plumbagin and azadirachtin suppressed incorporation.

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