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韓應昆誌 32(2):225~235(1993)
Korean J. Appl. Entomol.

Comparative effects of nicotine and diazinon on larval mortality and activity of cytochrome *P*-450 monooxygenases in *Helicoverpa assulta* and *Spodoptera exigua*

담배나방과 파밤나방의 유충사망률과 cytochrome *P*-450 monooxygenases의 활성에 미치는 니코틴과 다이아지논의 영향

J.H. Lee and K.S. Boo[1]
이 정 호 · 부 경 생[1]

ABSTRACT This study was carried out to investigate effects of diazinon, an organophosphate, and nicotine, a plant-originated toxic chemical, on larval mortality and activity of cytochrome *P*-450 monooxygenases, a major detoxification enzyme system, in *Helicoverpa assulta* and *Spodoptera exigua*. Diazinon treatment gave a higher mortality to *H. assulta* larvae than *S. exigua* larvae. In contrast to the case of diazinon, nicotine caused a higher mortality to *S. exigua* than to *H. assulta* larvae. It was partly due to the fact that nicotine induced the activity of midgut cytochrome *P*-450 monooxygenases (MFO) more than diazinon did in *H assulta* larvae. When *H. assulta* larvae were reared on their host plant, *Nicotiana tabacum* leaves, other components were mostly metabolized with the exception of dietary nicotine.

KEY WORDS *Helicoverpa assulta*, *Spodoptera exigua*, cytochrome *P*-450 monooxygenases (MFO), nicotine, diazinon

초 록 담배나방(*Helicoverpa assulta*)과 파밤나방(*Spodoptera exigua*)유충에 대한 유기인계 살충제인 다이아지논과 담배의 산물인 니코틴의 영향을 조사하기 위하여 본 실험을 시행하였다. 다이아지논이 처리된 담배나방유충의 사망률은 파밤나방유충의 경우보다 훨씬 높았으며 니코틴 처리구에서는 이와 반대양상을 나타내었다. 담배나방유충 중장의 cytochrome *P*-450 monooxygenases (MFO)활성은 다이아지논 처리구에 비해서 니코틴 처리구에서 더욱 높게 나타났다. 담배나방유충을 기주식물인 담배잎으로 사육하였을 때 다른 화합물과는 달리 대부분의 니코틴은 변화없이 배설되었다.

검 색 어 담배나방, 파밤나방, cytochrome *P*-450 monooxygenases (MFO), 니코틴, 다이아지논

Cytochrome *P*-450 monooxy-genases system has an important role in synthesis and regulation of insect hormones and pheromones and in detoxification of allelochemicals and synthetic insecticides (Hodgson 1983). For example, cytochrome *P*-450 monooxygenases are implicated as a major factor in detoxification ofcarbamates, organophosphates and pyreth-

1 서울대학교 농생대 농생물학과 및 농업생물신소재연구센터 (Dept. of Agricultural Biology and the Research Center of New Bio-materials in Agriculture, College of Agriculture and Life Sciences, Seoul Natl. University, Suwon, 441-744, Korea)

roids. Consequently, cytochrome *P*-450 monooxygenase-mediated detoxification is one of the most important mechanisms by which insects become resistant to insecticides (Hodgson 1983, Brattsten *et al.* 1986). Riskallah *et al.* (1986) reported that the tobacco budworm, *Heliothis virescens*, larvae fed on wild tomato leaves had increased tolerance to the organophosphorous insecticide, diazinon. This was attributed to an increase in metabolism of the insecticide due to induction of the microsomal monooxygenases.

Papilionidae species display a broad range of feeding pattern, from oligophagy on a single host plant familily to polyphagy on over a dozen families. Accompanying this diversity of feeding strategies is a diversity of physiological mechanisms for processing host plant allelochemicals. Studies on members of this family as well as other Lepidoptera suggest that oligophagy is associated with high activity, in addition to high substrate specificity, of detoxicative enzymes (Berenbaum 1991)

The oriental tobacco budworm, *Helicoverpa assulta*, having an oligophagous feeding pattern, utilizes *Capsicum annuum* and *Nicotiana tabacum* leaves containing the poisonous dietary nicotine, as major host plants. On the contrary, the beet armyworm, *Spodoptera exigua*, having a polyphagous feeding pattern, shows the insecticide resistance. So, this study was carried out to investigate the comparative effects of nicotine and diazinon on larval mortality and cytochrome *P*-450 monooxygenases activity of *H. assulta* and *S. exigua*. And also, the metabolism of dietary nicotine in their host plant or artificial diets of *H. assulta* was examined.

MATERIALS AND METHODS

Insects

Helicoverpa assulta larvae were collected in the suburbs of Suwon and maintained on an artificial diet (Table 1). *Spodoptera exigua* having a resistance to several organophosphate insecticides were received from the Screening Research Center, Korea Research Institute of Chemical Technology. *H. assulta* and *S. exigua* larvae were reared first in groups of 20 until the 2nd instar in a 12 × 3 cm (D×H) petridish and then in a 4 × 5 cm petridish individually to the last instar. The insectary was maintained at 26 ± 0.5℃ and a photoperiod of 16L/8D.

Table 1. Composition of artificial diets for *Helicoverpa assulta* and *Spodoptera exigua*

Ingredients	*H. assulta*[a]	*S. exigua*[b]
Kidney bean(g)	–	95
Wheat germ(g)	–	95
Corn(g)	90	–
Soybean(g)	100	–
Brewers'yeast(g)	24	28
Milk(g)	6	–
Agar(g)	24	15
Vitamin mixture(g)	4.3	–
Ascorbic acid(g)	4.1	6.0
Sorbic acid(g)	2.2	2.2
Metyl-*p*-hydroxybenzoate(g)	2.4	3.0
Hot pepper seed oil(ml)	4.0	–
Formalin(35%)(ml)	2.0	2.0
Distilled water(ml)	1000	800

[a] Modified from Park (1991).
[b] Mochida and Miyahara (1974).

Effect of diazinon on development and induction of cytochrome *P*-450 monooxygenases activity

H. assulta larvae were placed in groups of 10 in a 12 × 3 cm (D × H) petridish and provided with the artificial diet containing 0, 0.0033, 0.0066 or 0.0133%(w/w) diazinon from the beginning of 2nd, 3rd, 4th or last instar to the end of their larval development. Each of the four treatments contained four replicates that consisted of 10 larvae. Larvae were maintained at 26 ± 0.5℃ and a photoperiod of 16L/8D. After the treatment, mortality, 5th instar larval weight, molting time and pupation time were recorded and larval period and eclosion rate were calculated for each replicate.

In last instar treatment, protein content and activity of cytochrome *P*-450 reductase of the midgut and the fat body in *H. assulta* and *S. exigua* were measured everyday until maturation.

Effect of diazinon and nicotine on mortality

Larvae of *H. assulta* and *S. exigua* were placed in groups of 10 in a 12 × 3 cm (D × H) petridish and provided with the artificial diet containing 0.0033, 0.0066, 0.0133, 0.0200 or 0.0250%(w/w) diazinon or 1.0, 2.0, 4.0, 5.5 or 7.7%(w/w) nicotine from the beginning of 1st, 2nd, 3rd, 4th or last instar to the end of their larval development. Each of the five treatments contained four replicated that consisted of 30 larvae. Larvae were maintained at 26 ± 0.5℃ and a photoperiod of 16L/8D. On the third day of the treatment, mortality was recorded and percentage of mortality was calculated for each replicate. In last instar larvae treated with 0.0033% diazinon or 2.0% nicotine, activites of midgut cytochrome *P*-450 reductase were measured veryday until pupation time.

Preparation of the enzyme (cytochrome *P*-450 monooxygenases) extracts

Midgut and fat body microsomes were prepared by the method of Feyereisen *et al.*(1985). Midgut tissue was dissected in ice-cold 0.1M sodium phosphate buffer (SPB)(PH 7.2) containing 10% glycerol, 1mM ethylenediaminetetracetic acid (EDTA), 0.1mM 4,4-dithio-DL-threitol(DTT), 1mM 1-phenyl-2-thiourea (PTU) dissolved in ethylene glycol monoethyl ether and 1mM phenylmethylsulfonyl fluoride (PMSF) dissolved in ethylene glycol monoethyl ether (Lee and Scott 1989a). Contents were removed in the dissected tissues and tissues of 10 midguts were homogenized thoroughly in 10ml ice-cold 0.1M SPB (PH 7.2) in a teflon homogenizer. The homogenates were centrifuged at 10,000g for 15 min. At once, the supernatant fraction was utilized for cytochrome *P*-450 reductase activity assay. In this paper, the activity of NADPH-cytochrome *P*-450 reductase expresses that of cytochrome *P*-450 monooxygenases.

Activity assay of NADPH-cytochrome *P*-450 monooxygenases

NADPH-cytochrome *P*-450 monooxygenases activity was measured at 30℃ by Pye Unicam UV/VIS Spectrophotometer at 550nm (Williams and Ksmin 1962). Compositions activity assay are in Table 2. Proteins were determined by the Lowry method (Lowry *et al.* 1951) using bovine serum albumin (BSA) as standard.

Table 2. Composition of the reaction mixture for testing the activity of NADPH-cytochrome *P*-450 monooxygenases

Cytochrome *c* 2mM	150μl
NADPH 30mM	150μl
NaCN 40mM	20μl
SPB* 0.1M, PH7.2	200μl
Enzyme Extracts	20μl

*Sodium Phosphate Buffer

Assay of nicotine

(1) Preparation of nicotine extract

H. assulta larvae were reared on their host plant *Nicotiana tabacum* or the artificial diet containing the nicotine. Larval excrements obtained in each treatment were homogenized thoroughly in a teflon homogenizer. After mixing with chloroform, 3° distilled water and 10% sodium hydroxide (NaOH) at the same proportion, the homogenates were put into the solution. After shakng the mixture for the ten minutes, it was filtered through the sodium sulfate (Na_2SO_4, 2g) laid on the filter paper. Chlorogorm layers uptaken from this procedure were utilized for gas chromatography analysis (Cundiff and Markunas 1955).

(2) Gas chromatography

The metabolites of dietary nicotine contained in excrements were analyzed by SHIMAZU GC -14A gas chromatograph possessing the flame ionization detector (FID). Coated (BP-10) capillary column (25m × 0.25 mm) was used. Column temperature 185℃ and detector (FID) temperature 210℃ were respectively maintained.

RESULTS

Effects of diazinon

Diazinon in the artificial diet significantly prolonged the larval developmental periods, reduced the weights of 5th instar larvae and the eclosion rate of *H. assulta* (Table 3). This negative effect of diazinon was more pronounced at higher concentrations and when treated at younger stage. When diazinon was given to the last instar larvae of *H. assulta*, the same trend was obeserved in their weight (Fig. 1). But the 5th instar larval period was significantly prolonged only at the highest concentration. The higher effect of diazinon at younger larval stage seemed to be due to their higher active metabolism, as seen in food assimilation and consequently their higher weight increase rate (Fig. 2).

Table 3. Effect of diazinon[a] on larval period and weight, and adult emergence rate in *H. assulta*

Concentration (%)	Larval period(days)			5th instar larval weitht (mg)	Eclosion rate (%)
	2nd	3rd	4th		
0.0000	2.0 ± 0.1	2.0 ± 0.2	3.0 ± 0.2	110 ± 15	96
0.0033					
2nd	2.0 ± 0.4	4.2 ± 2.1	7.4 ± 3.1	53 ± 18	52
3rd		3.3 ± 0.9	4.6 ± 1.3	63 ± 18	64
4th			3.1 ± 0.4	102 ± 17	76
0.0066					
2nd	3.2 ± 2.4	6.2 ± 3.1	10.1 ± 4.3	41 ± 18	40
3rd		4.5 ± 2.1	7.6 ± 3.2	45 ± 16	56
4th			5.3 ± 2.2	51 ± 15	65

Each value represents the mean ± SEM of 4 to 6 experiments using 8 to 10 insects per experiment.

[a] Diazinon was mixed in the artificial diet, at the dosage indicated, and the artificial diet was given to the larvae from beginning at 2nd, 3rd, or 4th instar to the end of larval development.

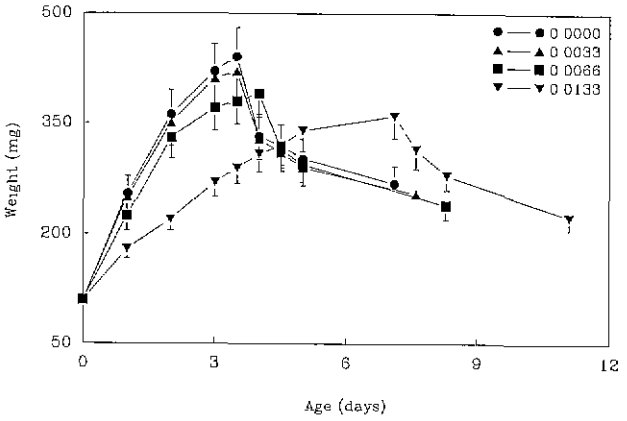

Fig. 1. Difference in developmental period and body weight in the last instar larvae of *H. assulta* fed on the artificial diet containing diazinon at the dosage (%) indicated. Each point represents the mean ± SEM of 4 to 5 experiments using 9 to 12 insects per experiment. The absence of a vertical bar indicates that the SEM is smaller than the size of the datum poing.

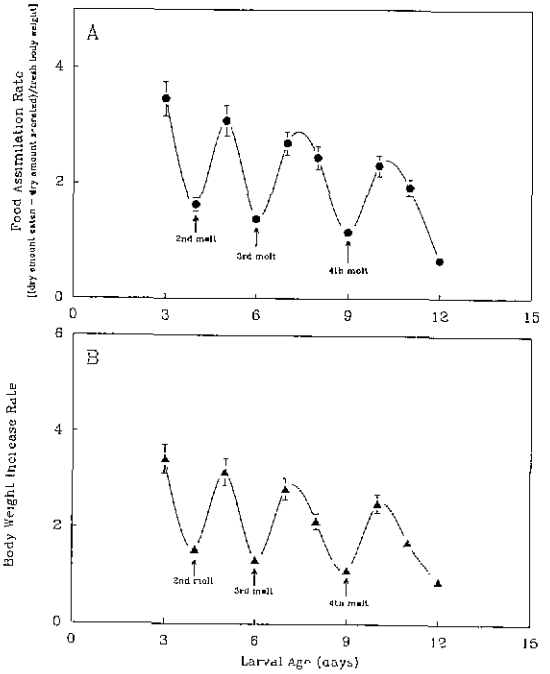

Fig. 2. Food assimilation rate (A) and body weight increase rate (B) from 2nd to 5th instar larval stage in *H. assulta*. Each point represents the mean ± SEM to 4 experiments using 9 to 40 insects per experiment. The absence of a vertical bar indicates that the SEM is smaller than the size of the datum poing.

The specific activity of cytochrome *P*-450 monooxygenases was significantly induced in the midgut of *H. assulta* larvae when treated with diazinon (Fig. 3A). Most of the enzyme induction occurred during the first 24 hour. But the enzyme activity in the fat body was not much different among treatments (Fig. 4B)/ On the other hand, protein content was inversely proportional to the treatment concentration in both tissues (Figs. 3C, D), which means that diazinon treatment specifically induced cytochrome *P*-450 monooxygenases activity only in the midgut.

Diazinon generally gave a much higher mortality to *H. assulta* larvae than *S. exigua* larvae (Fig. 4). In a given species, higher mortality was obtained at higher concentration or when treated at younger larval stages, as expected.

The lower mortality in *S. exigua* larvae treated at the same concentration, in comparison to *H. assulta* larvae, was incidentally correlated to the higer specific activity of midgut cytochrome *P*-450 monooxygenases. In *S. exigua* the specific activity was more pronounced in those larvae treated withhigher concentrations of diazinon (Fig. 5).

Effects of nicotine

In contrast to the case of diazinon, nicotine caused a higher mortality to *S. exigua* than to *H. assulta* larvae (Fig. 6). This difference in mortality can be partially explained by the difference in enzyme induction capacity of the two chemicals. Namely, nicotine induced the activities of midgut cytochrome *P* - 450

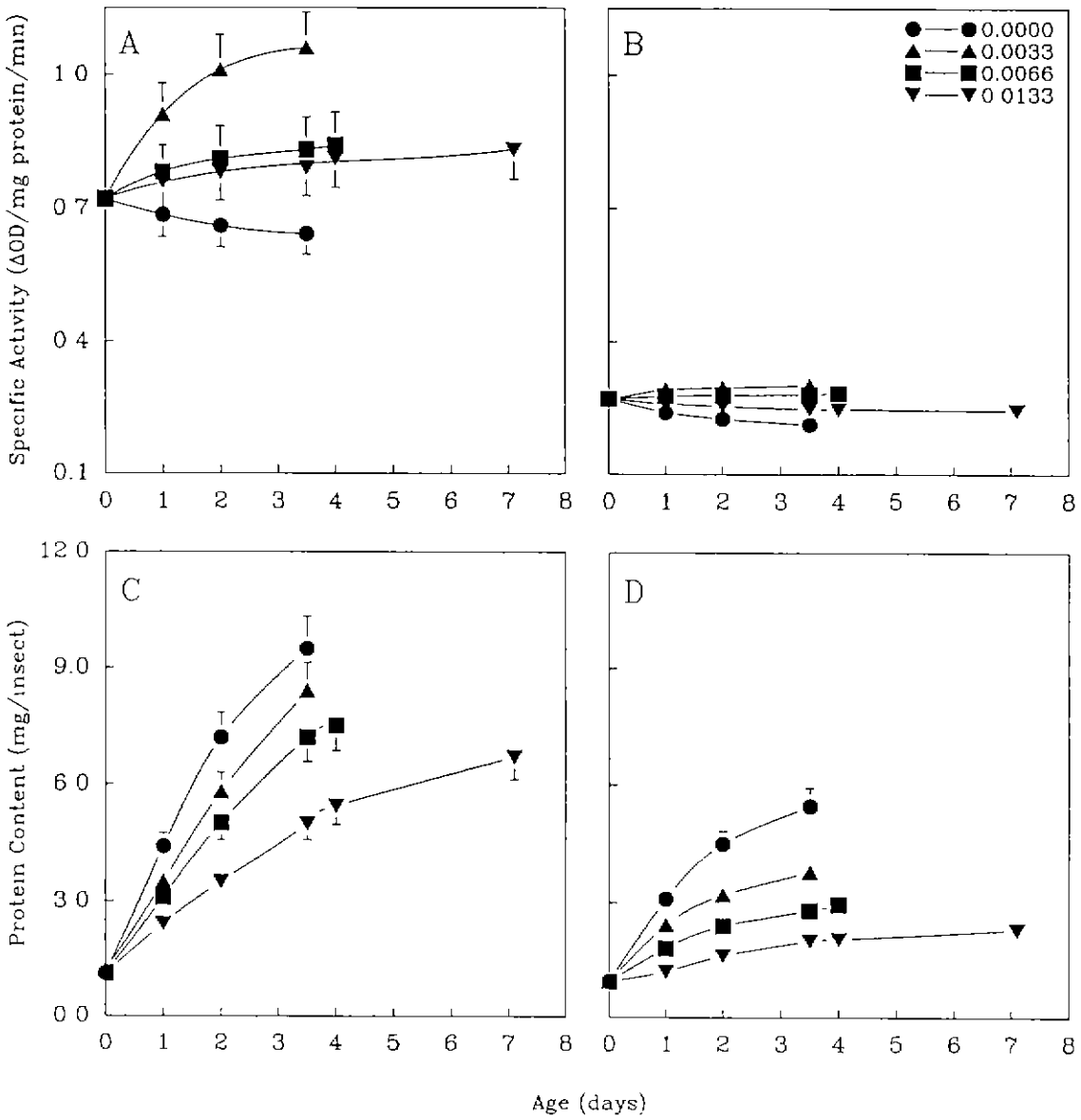

Fig. 3. Difference in specific activity of cyt. *P*-450 reductase (A, B) and protein content (C, D) in midgut (A, C) and fatbody (B, D) from the last instar larvae of *H. assulta* fed on the artificial diet containing diazinon at the dosage(%) indicated. Each point represents the mean ± SEM of 4 experiments using 9 insects per experiment. The absence of a vertical bar indicates that the SEM is smaller than the size of the datum poing.

monooxygenases more than diazinon did in *P*-450 monooxygenases larvae (Fig. 7). But, in *S. exigua* larvae, the enzyme induction efficiency of nicotine was less than that of diazinon.

Metabolism of nicotine

In nicotine treatment, mixed in artificial diets, *H. assulta* larvae excreted the dietary nicotine without metabolism, which would otherwise be very poisonous (Fig. 8). Also, when *H. assulta* larvae reared on their host plant, *Nicotiana*

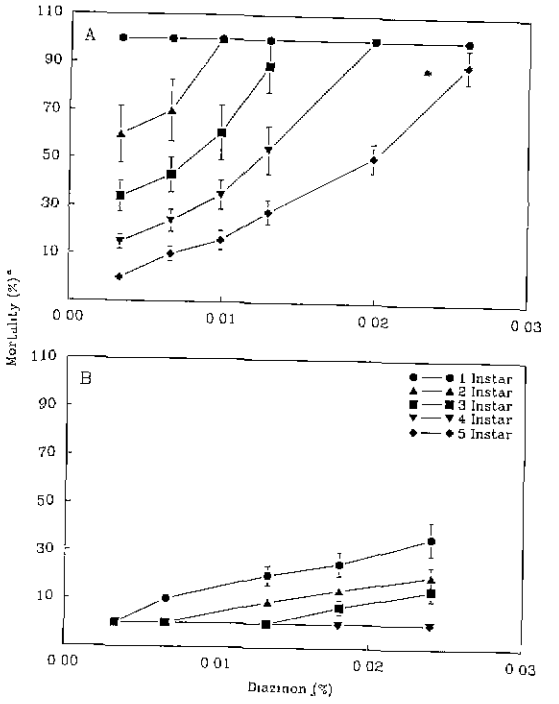

Fig. 4. Effect of diazinon, mixed in the artificial diet, on mortality of *H. assulta* (A) and *S. exigua* (B).

[a] Mortality was calculated 3 days after the start of the treatment.

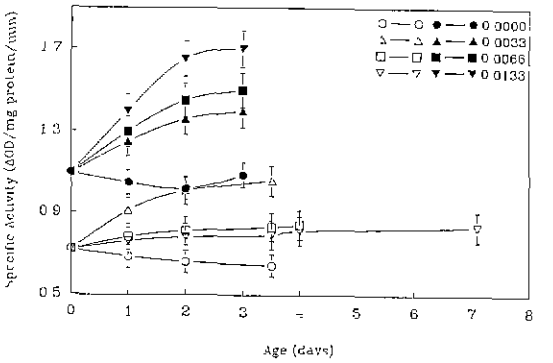

Fig. 5. Difference in specific activity of midgut cyt. *P*-450 reductase from the last instar larvae of *S. exigua* (filled symbols) and *H. assulta* (open symbols)developmental period and body weight in the last instar larvae of *H. assulta* fed on the artificial diet treated with diazinon at the dosage (%) indicated. Each point represents the mean ± SEM of 4 to 5 experiments using 10 insects per experiment. The absence of a vertical bar indicates that the SEM is smaller than the size of the datum poing.

tabacum, other components were mostly metabolized with the exception of dietary nicotine (Fig. 9).

DISCUSSION

Ehrlich and Raven (1964) were among the first to propose a new theory of biochemical coevolution, in which the synthesis of plant secondary substances as plant toxins is specifically related to patterns of host plant utilization by phytophagous insects. According to this theory, the production and accumulation of a particular toxin (e.g. an alkaloid) is followed by a reciprocal response in the insect to the toxin, e.g. adaptation by detoxification and excretion, so that it can feed on that plant. This coevolutionary theory was originally based on circumstantial evidence, chiefly the known feeding habits of phytophagous insects and the fact that the majority of such insects are monophagous or oligophagous rather than polyphagous.

S. exigua used in this experiment is known to be polyphagous and also to be a resistant strain to several organophosphate insecticides. The last point was also evident in this test, by showing a low mortality when treated with diazinon, which is much lower than *H. assulta*. This can be partially explained by higher specific activity of cytochrome *P*-450 monooxygenases in the midgut of *S. exigua*. This difference was maintained even when the enzyme was induced by diazinon treatment. Krieger *et al.* (1971) already reported that activity of midgut microsomal monooxygenases is higher, on average, in

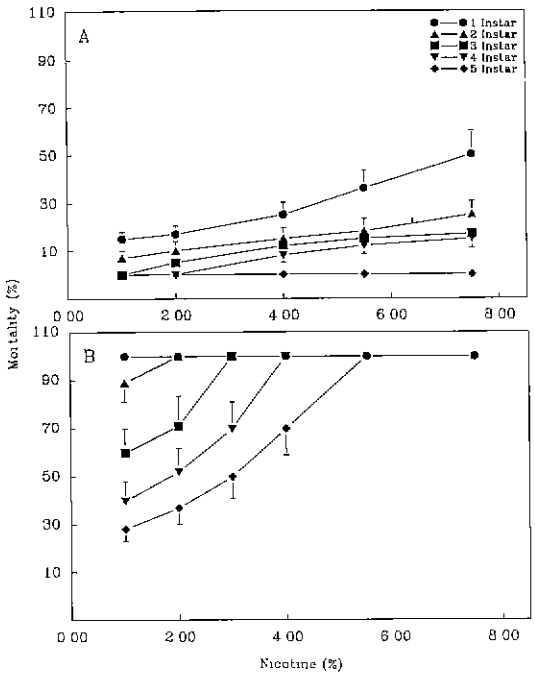

Fig. 6. Effect of nicotine, mixed in the artificial diet, on mortality of *H. assulta* (A) and *S. exigua* (B).

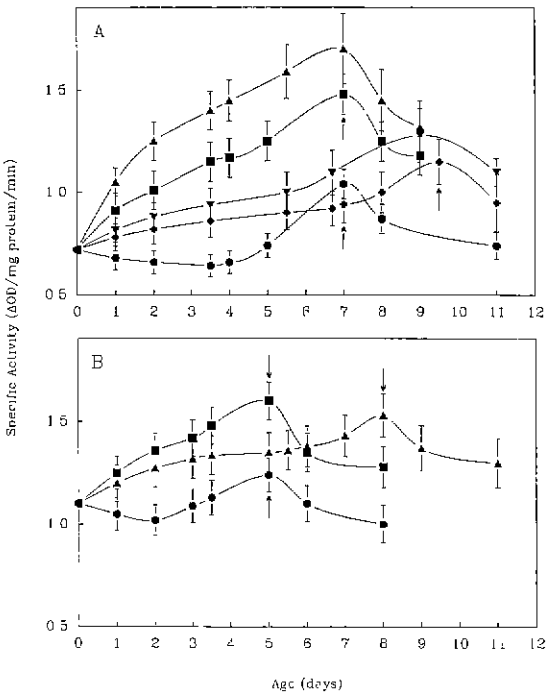

Fig. 7. Difference in specific activity of midgut cyt. *P*-450 reductase form the last instar larvae of *H. assulta* (A) and *S. exigua* (B) fed on the artificial diet + 0.0033% diazinon (■ —— ■), the artificial diet + 1.0% nicotine (▲ —— ▲), *Nicotiana tabacum* (▼ —— ▼), *Capsicum annum* (◆ —— ◆) and the artificial diet only (● —— ●) (Pupation time is indicated by an arrow).

polyphagous than in monophagous lepidopteran larvae. *H. assulta* larvae are known to feed only on a few species of plants, such as the tobacco and the hot pepper, while *S. exigua* larvae are polyphagous. In any species the enzyme was not induced in the fat body by diazinon treatment.

But nicotine treatment gave a totally different story. Almost every aspect is quite opposite from what happened when treated with diazinon. That is, nicotine gave a lower mortality to, and showed a high induction ability of cytochrome *P*-450 monooxygenases in *H. assulta* larvae. Adverse effects of nicotine was much higher to *S. exigua* larvae than *H. assulta* larvae. As in the case of *S. exigua* treated with diazinon, lower influence of nicotine on *H. assulta* larvae seems to be due to its high induction capacity of the detoxifying enzyme. But, more importantly, *H. assulta* larvae seems to absorb into their haemolymph no nicotine at all, but, instead, excrete nicotine right away after feeding, even without modifying it chemically. This is clearly shown in figs. 8 and 9.

This fact implies that *H. assulta*, an oligophagous species, has developed a high capacity to escape from toxic effects of nicotine produced in its host plant, while *S. exigua* does not have such an ability. Similar situation has been reported in swallotails. Swallowtail specialists appear to have substantially higher levels of enzymatic activities against characteristic host plant allelochemical than do nonspecialists. For

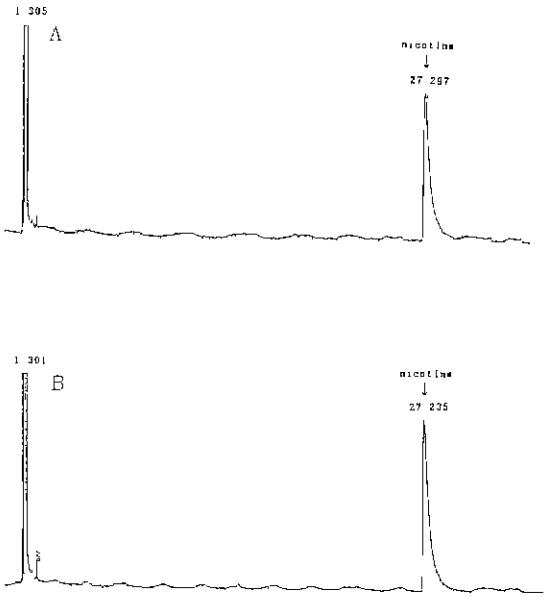

Fig. 8. Gas Chromatograph of nicotine from the artificial diet (A) and the excrement (B) of the last instar larvae of *H. assulta* .
*Condition: column temp. 100℃, injector temp. 230 ℃, detector temp. 230℃.

example, black swallowtails can metabolize furanocoumarins, which are characterstic host plant allelochemicals, 10 to 30 times faster than nonspecialists, and tiger swallotails can cleave glycosides 70 to 100 times faster than nonspecialists (Lindroth 1989). Indeed, a general survey of reports of enzymatic detoxification of a variety of substrates by a wide range of species shows a clear trend toward higher specific activities in specialists, particularly for substrates frequently encountered in host plants (Berenbaum 1991).

H. assulta larvae are known to take a longer development period when reared on tobacco leaves than artificial diets or hot pepper fruits. However, the present study suggested that *H. assulta* larvae should not be adversely affected by feeding on tobacco leaves. *Manduca sexta* is another species which feeds on tobacco leaves

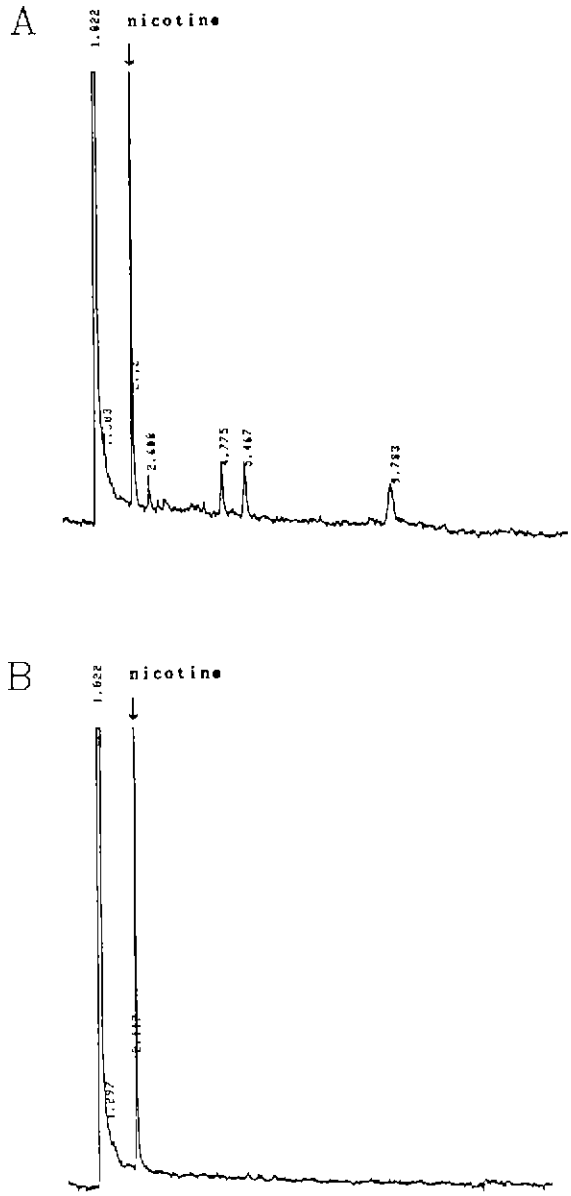

Fig. 9. Gas Chromatograph of nicotine from the leaves of *Nicotiana tabacum* (A) and the excrement (B) of the last instar larvae of *H. assulta* .
*Condition: column temp. 185℃, injector temp. 210 ℃, detector temp. 210℃.

with impunity. This is known simply due to the fact that the dietary nicotine is rapidly excreted without metabolism. And the species has a second line of defense in that the tissues surround-

ing the nerve tissues are impermeable to nicotine which may be absorbed in the blood (Blum 1983). Therefore, in *H. assulta* larvae, more studies are needed in explaining their relatively poor performance in development when reared on tobacco leaves. It could be due to poor nutritional values of tobacco leaves, due to presence and absorption of unknown allelochemicals in tobacco leaves, or partial absorption of nicotine. Allelochemicals can be also handled by enzymes other than cytochrome *P*-450 monooxygenases. In *H. assulta* we have studied only cytochrome *P*-450 monooxygenases and nicotine excretion, more studies are needed for a full understanding of the interactions.

All these allelochemicals are produced by plants in the first instance as protective devices aganist insect feeding. However, in almost every instance, insects have evolved defence or detoxifying mechanisms. Indeed, almost every type of toxin is utilized by a particular insect in a positive way as a feeding stimulant or attractant, even though it may be detrimental to most other insect species. This is true in case of alkaloids (sparteine), mustard oils (sinigrin), cardiac glycosides, and cyanogens (Ahmad 1983). The dietary nicotine must be a negative effector for the development of *H. assulta* larvae, since the larval development is prolonged when they are reared on *H. tabacum* leaves and artificial diets containing nicotine. But, though *H. assulta* were inferior to *M. sexta* which showed no delayed development at higher concentration treatment of nicotine. *H. assulta* larvae can sufficiently protect their own body from the poisonous dietary nicotine in the utilization of *N. tabacum* leaves as a food source. For that reason, it is possible for *H. assulta* larvae to reduce the competition of other insects on which dietary nicotine works as a repellent. Consequently, dietary nicotine may also work as a positive effector for the survival of *H. assulta* on tobacco plants.

REFERENCES

Ahmad, S. 1983. Herbivorous Insect: Host seeking *behaviour and mechanisms*. 257. *pp.* Academic Press. New York.

Berenbaum, M.R. 1991. Comparative processing of allelochemicals in the Papilionidae (Lepidoptera). Arch. Insect Biochem. Physiol. 17: 212~221.

Blum, M.S. 1983. Detoxification, deactivation and utilization of plant compounds by insects. *In Plant resistance to insects, eds. by* P. A. Hedin, pp. 265~278. Amer. Chem. Soc., Washington, D. C.

Brattsten, L.B., C.W. Holyoke, J.R. Leeper & D.F. Raffa. 1986. Insecticide resistance: Challenge to pest management and basic research. Science. 231: 1255~1260.

Cundiff, R.H. & D.C. *Markunas*. 1955. Determination of nicotine, nornicotine and total alkaloids in tobacco. Analy. Chem. 27: 1650.

Ehrlich, R.R. & P.H. Raven 1964. Butterflies and plants: A study in coevolution. Evolution 18: 586~608.

Feyereisen, R. & D.E. Farnsworth. 1985. Developmental changes of microsomal cytochrome *P*-450 monooxygenases in larval and adult *Diploptera punctata*. Insect Biochem. 15: 755~761.

Hodgson, E. 1983. The significance of cytochrome *P*-450 in insects. Insect Biochem. 13: 237~246.

Krieger, R.I., P.P. Feeny & C.F. Wilkinson. 1971. Detoxification enzymes is the gute of caterpillars: An evolutionary answer to plant defenses. Science 171: 579~581.

Lee, S. S. T. & J.S. Scott. 1989. An improved method for preparation, stabilization and storage of housefly (Diptera: Muscidae) microsomes. J. Econ. Ent. 82: 1559~1563.

Lindroth, R.L. 1989. Host plant alteration of detoxification activity in *Papilio glaucus*. Entomol. Exp. Appl. 50: 29.

Lowery, O.H., N.J. Rosebrough, A.L. Farr & R.J.

Randall. 1951. Protein measurement with the folin-phenol reagent. J. Biol. Chem. 193: 265~275.

Mochida, O. & Y. Miyahara. 1974. Effect of γ-radiation on male pupae and adults of *Spodoptera litura* (Lepidoptera; Noctudae). Appl. Ent. Zoo. 9: 41~47.

Park, K.C. 1991. Composition and activity of female sex pheromone in the oriental tobacco budworm, *Heliothis virenscens* (Guenee). A Ph. D. dissertation. Seoul. Natl. Univ. (in Korean).

Riskallah, M.R., W.C. Dauterman and E. Hodgson. 1986. Nutritional effects on the induction of cytochrome *P*-450 and glutathione transferase in larvae ot the tovacco budworm, *Heliothis virenscens*. Insect Biochem. 16: 491~499.

Wiliams, C.H. & H.Kamin. 1962. Microsomal triphosphopyridine nucleotide-cytochrome *c* reductase of liver. J. Biol. Chem. 237: 587~595.

(Received March 26, 1993)