Turk J Zool
26 (2002) 121-126

Research Article

Effects of Some DNA Gyrase Inhibitors on the Survival and Development of *Pimpla turionellae* (Hymenoptera: Ichneumonidae) Larvae Reared on an Artificial Diet

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Received: 07.06.2000

Abstract: The effects of novobiocin, nalidixic and oxolinic acids, which are potent antibacterial agents, on the survival and development of the hymenopterous endoparasitoid *Pimpla turionellae* L. were investigated alone by rearing the larvae aseptically on chemically defined synthetic diets. The percentage of fifth instar larvae was not significantly affected by the diets with different levels of the antibiotics used in the study. A diet containing the lowest level of novobiocin significantly increased the yields of pupae and adults. This level also significantly shortened the developmental time for fifth instar, but had no significant effect on the complete development of the larvae up to adult emergence. Oxolinic acid at the lowest level had no effect on survival, but prolonged the development of the insect. In general, the rate of development was significantly increased and survival was decreased with high levels of the antibiotics used in the study. However, nalidixic acid at all tested levels caused a striking decrease in survival. These results suggest the dietary compatibility of novobiocin and, to some extent, oxolinic acid at the lowest tested level with the artificial rearing of the insect for biological control.

Key Words: *Pimpla turionellae*, novobiocin, nutrition, survival, development

Bazı DNA Cayraz İnhibitörlerinin Sentetik Besinde Yetiştirilen *Pimpla turionellae* (Hymenoptera: Ichneumonidae) Larvalarının Yaşama ve Gelişmesine Etkileri

Özet: Endoparazitoid bir zarkanatlı türü, *Pimpla turionellae* L., kimyasal yapısı bilinen sentetik besin ortamlarında aseptik şartlarda beslenerek, güçlü antibakteriyel ajanlar olan novobiyosin, nalidiksik ve oksolinik asitin böceğin yaşama ve gelişmesine ayrı ayrı etkileri inclendi. Beşinci evredeki larvaların yaşama yüzdesi antibiyotiklerin farklı miktalarını içeren besinler tarafından önemli derecede etkilenmemiştir. Novobiyosinin en düşük miktarını içeren besin pup ve ergin yüzdesini önemli derecede artırmıştır. Bu miktar aynı zamanda beşinci evreye ulaşmak için gereken süreyi kısaltmış, ancak larvaların ergin evreye kadar gelişmesi üzerinde önemli bir etki yapmamıştır. Oksolinik asitin en düşük miktarı yaşama üzerinde etkili olmazken böceğin gelişmesini geçiktirmiştir. Antibiyotiklerin yüksek miktarları genellikle gelişme oranını artırmış, yaşamayı düşürmüştür. Nalidiksik asitin denenen bütün miktarları ise yaşamayı dikkate değer bir şekilde düşürmüştür. Bu sonuçlar novobiyosinin ve bir dereceye kadar oksolinik asitin denenen en düşük miktarının böceğin biyolojik kontrol amacıyla yapay ortamlarda yetiştirilmesinde kullanılan sentetik besinlere ilave edilebileceğini göstermektedir.

Anahtar Sözcükler: *Pimpla turionellae*, novobiyosin, beslenme, yaşama, gelişme

Introduction

Throughout development to remain protected in the natural host, the larvae of *Pimpla turionellae* discharge an anal secretion which has antibiotic effects against bacteria and fungi (1). However, microbial contaminations are a serious problem during their artificial mass-rearing outside of natural hosts under laboratory conditions. These contaminations make the rearing of *Pimpla* larvae unsatisfactory and sometimes impossible, and eventually do not allow the normal development of these larvae on an artificial diet. These undesirable effects have inspired considerable efforts toward the addition of some antimicrobial agents alone or in combination into the diet for the control of microorganisms (2,3). These efforts showed that these agents at high dietary levels had adverse effects on the survival and development of the larvae up to adult emergence. On the other hand, the physical texture of the diets was also observed to be affected when they were used at high levels. For example, high levels of antimicrobial agents, especially antibiotics, imparted a more slurry-like or, conversely, a more gelled form to the diets. These levels also caused the formation of some aggregations and destroyed the homogeneity of the diet. This situation may interfere with the nutritional value of the diet and the food intake of the larvae. These larvae may be prevented from feeding or obtaining a

nutrient (or nutrients) in suitable proportions. For this reason, the present work has been directed towards the use of antibacterially more potent antibiotics to control bacterial contaminations at much lower levels in the artificial diet. It is possible for these low levels of antibiotics to minimize the apparent changes in the physical properties of the diet and the contribution of these changes to the main effects of antibiotics on the insect. Therefore, this work deals mainly with the effects of some bacterial DNA gyrase inhibitors such as novobiocin, nalidixic and oxolinic acids, on the survival and development of *P. turionellae* larvae reared on an artificial diet.

Materials and Methods

Stock culture of *P. turionellae* was obtained at 23±1ºC, 75±5% relative humidity, and at a 16 hour illumination period per day, by the parasitization of its host pupae, the greater wax moth *Galleria mellonellae* L. Adult individuals were fed with 50% filtered honey solution every day, and with host pupae every other day for the sucking of haemolymph to provide sufficient protein.

In the artificial rearing experiments, the synthetic diet described by Yazgan (4) was used for the larvae of *P. turionellae*. Detailed accounts of the composition and preparation of the diet and the equipment and techniques for handling and rearing the larvae aseptically for nutritional experiments are described elsewhere (2,3).

Antibiotics used in these rearing experiments, such as novobiocin (Sodium Salt, micronized form, 907 µg/mg) and oxolinic acid (99%, micronized form) which are produced by Upjohn and Warner-Lambert Research Institute respectively, were donated by the Eczacıbaşı Medicine Company Ltd., Turkey. Nalidixic acid (99%) was purchased from Sigma Chemical Co. The concentration of the antibiotics was expressed as mg/100 ml of the diet. The required amounts of the agents were dissolved in a certain volume of bidistilled water, then these solutions were sterilized by filtering with a 0.22 µm pore-sized membrane filter and then predetermined volumes of the antibiotic solutions were added to the diets shortly before gel formation. The total volume of the diet was established by adjusting the volume of water in the diet. These completed diets were dispensed into test-tubes (10 x 7.5 mm) to provide about 0.5 ml of diet. This volume of diet is apparently excessive for the nutritional requirements of the larvae with regards their normal development. This excessive amount of diet, in addition to providing nutrients required for normal development of the larvae, ws used to ensure the larvae had a larger feeding area than that in general feeding schemes. For this purpose, this amount of diet was dispensed in a large lump on the lower wall of the reclined test tubes. This was done to ensure that the initial escaping of the larvae from the diet because of the possible repellent effects of the antibiotics was minimized. One newly hatched larva was inoculated into each test-tube containing 0.5 ml of diet. Ten larvae were used for each diet and each experiment was replicated three times. The feeding experiments were done under the same laboratory conditions as mentioned for the stock culture.

The antibiotics were tested for their individual effects on the survival and development of the insect by the addition of these agents alone into the diets. Four diets containing graded levels of each antibiotic were tested and compared with a control diet (without antibiotic). Novobiocin at levels of 1.5, 3.0, 4.5 and 6.0 mg; nalidixic acid at levels of 1.0, 2.0, 3.0 and 4.0 mg; and Oxolinic acid at levels of 0.75, 1.50, 2.25 and 3.00 mg/100 ml of diet were tested for their effects on the insect.

The effects of the tested diets with antimicrobial agents on the insect were measured by determining the rate of development (average time to reach fifth instar, pupal and adult instars) and survival (number of survivors in these stages) of the larvae. Data on the rate of development were evaluated by analysis of the variance (5). To determine significant differences between means, Duncan's (6) multiple range test was used. Data on survival were compared by X^2 test (5). When F and X^2 exceeded their 0.05 value, the differences were considered significant.

Results

Before starting the experiments dealing with the effects of the antibiotics on the survival and development of *P. turionellae*, some preliminary experiments were carried out to determine the range of these antibiotics to be tested. First, novobiocin, nalidixic and oxolinic acids alone at levels of 5, 10, 15 and 20 mg were added to the diets for their effects on the insect. On the diets with levels higher than 10 mg of antibiotics none of the larvae completed their development to pupal instar and they stayed at the fifth instar for a long period. A few larvae

were able to produce only prepupae. The initial slurry or viscous texture of these diets was observed to become increasingly diluted during the active feeding period of the larvae, and poor gel formation was also observed in the diets having the highest levels of these antibiotics. Some of the newly inoculated larvae on such diets submerged and could not moult to subsequent larval instars. However, the larvae reaching the fifth larval stage were very small and their development was slow. At 10 mg of novobiocin and 5 mg of nalidixic and oxolinic acids, most of the larvae reached pupal stage, but few pupae were able to emerge to adult stage. Consequently, based on these pre-experiments, levels lower than 10 mg of novobiocin and 5 mg of nalidixic and oxolinic acids were added to the diets their effects on the insect. The effects of different levels of novobiocin, nalidixic and oxolinic acids on the survival and development of *P. turionellae* larvae reared on the artificial diet are shown in Table 1.

Although the percentage of survivors in the diet with 4.5 mg of novobiocin was low, none of the tested levels of this antibiotic had significant effects on the survival in the fifth instar. Larval developmental time in the diet with 6 mg of the agents was extended an additional day when compared to the control diet. However, this time was shortened by one day with the diet containing 1.5 mg of novobiocin. The percentages of the pupal and adult yields were significantly lower with high novobiocin levels. An approximate a 50% reduction in pupation and adult emergence was recorded with levels of 3 mg and above. However, the yields were significantly increased in the diet with 1.5 mg of novobiocin. This diet produced a pupal yield of 64.3% and an adult yield of 62.2%. Post-larval developmental time was similar for levels up to 4.5 mg of novobiocin, but at the highest level (6 mg) a significant prolongation was observed when compared to the control diets (Table 1A).

Larval survival and development of the insect were not significantly affected by all tested levels of nalidixic acid. The diets with 1.0 mg and 3.0 mg of nalidixic acid alone decreased the percentage of survivors in the fifth instar. Nevertheless, the differences between the diets with all tested levels of the antibiotic and the control diet were not significant. However, pupation and adult yields were significantly decreased by all tested levels of nalidixic acid. The higher levels of this agent markedly decreased

Table 1. The effects of some DNA gyrase inhibitors alone on the survival and development of the *P. turionellae* larvae.

Levels of antibiotics (mg of 100 ml of diet)	Initial No. of larvae	Survival to fifth instar (%)	Time to fifth instar, days (mean* ± S.D.)#	Survival to pupal instar (%)	Time to pupal instar, days (mean* ± S.D.)#	Survival to adult instar (%)	Time to adult instar, days (mean* ± S.D.)#
A- Novobiocin							
Control	28	92.9a	11.3 ± 0.2a,b	46.4a	24.4 ± 0.5a	38.7a	32.7 ± 0.8a
1.5	27	88.9a	10.3 ± 0.5c	64.3b	25.0 ± 0.4a	62.2b	33.0 ± 0.0a
3.0	29	86.2a	10.8 ± 0.7ac	20.7c	25.2 ± 0.6a	17.2c	33.3 ± 0.7a
4.5	26	63.2a	11.8 ± 0.4bd	15.4c	25.0 ± 0.0a	15.4c	33.3 ± 0.7a
6.0	28	78.6a	12.3 ± 0.5d	10.7c	26.3 ± 0.6b	10.7c	35.3 ± 0.7b
B- Nalidixic acid							
Control	24	88.3a	10.7 ± 0.5a	44.6a	25.0 ± 0.8a	44.6a	33.7 ± 0.6a
1.0	25	66.0a	11.1 ± 0.3a	20.0b	24.7 ± 0.7a	20.0b	34.2 ± 0.7a
2.0	27	81.5a	11.0 ± 0.9a	25.9b	25.2 ± 0.6a	25.9b	34.2 ± 0.7a
3.0	23	65.2a	11.6 ± 0.4a	13.7c	24.5 ± §	13.7c	34.5 ± §
4.0	23	74.0a	11.3 ± 0.4a	13.0c	25.3 ± §	13.0c	34.5 ± §
C-Oxolinic acid							
Control	26	88.4a	8.1 ± 0.1a	46.1a	21.1 ± 0.1a	46.1a	29.7 ± 1.8a
0.75	25	88.0a	8.0 ± 0.0a	44.0a	23.0 ± 0.0b	43.0a	32.5 ± 1.9b
1.50	26	92.3a	8.0 ± 0.0a	19.2b	23.0 ± 0.0b	19.2b	33.1 ± 2.1b
2.25	26	88.4a	8.1 ± 0.1a	15.3b	23.0 ± 0.0b	15.3b	33.0 ± 0.0b
3.00	20	95.0a	9.1 ± 0.1b	15.0b	23.0 ± §	15.0b	33.0 ± §

*: Average of 3 replicates, 10 larvae per replicate

#: Values followed by the same letter are not significantly different from each other, P > 0.05

§: From two replications

the adult yields to percentages as low as 13.7% and 13.0% respectively (Table 1B). The low levels of nalidixic acid appeared to have no effect on the complete development of the insect. Since the pupae and adults were obtained from only two replications of the diets with high levels of the antibiotic, the data on the development could not be statistically evaluated.

All tested levels of oxolinic acid had not significant effect on the percentages of survivors in the fifth instar. However, the average time to reach this stage was similar for levels up to 2.25 mg, but it was significantly prolonged by the highest level of this agent when compared to the control. The percentages of survival in the pupal and adult stages were significantly lower for levels above 1.50 mg of oxolinic acid. The higher levels (1.5, 2.25, 3.0) resulted in a significant and striking decrease in the yields of pupae and adults. However, there was no statistically significant difference between the diet with 0.75 mg of this agent and the control diet. Pupa and adult yields from the diet with the lowest level of oxolinic acid were about equal to those from the control diet. The development of the larvae to adult emergence was also significantly retarded by all tested levels of oxolinic acid (Table 1C).

Discussion

This work showed that the presence of an antibiotic alone in the diet has a significant positive effect on the survival of a parasitic hymenopterous species, *P. turionellae*. The lowest dietary level of novobiocin produced a yield of 64.3% pupae and 62.2% adults. This level also had no significant effect on the complete development of the insect. This antibiotic could be used at much lower levels in the larval diet of *P. turionellae* and possibly other pupal parasitoid to enhance the survival and thus obtain adult individuals with high yields for biological control purposes. Novobiocin at this level may have played a role in improving the nutritional value or quality of the diet. This may provide an efficient utilization of the diet by the larvae. As suggested by Zucoloto (7), if a diet is nutritionally better or of good quality it would be normal to obtain a high yield of adults. A question arises from the work reported here about whether such low levels are also capable of controlling microbial contamination. Levels of both the antibiotics may be unable to control microorganisms or their effectiveness may be dependent on the concentration of contamination. Further works are necessary to investigate this important question. There have been no works about the positive effects of antimicrobial agents on parasitoid hymenopterous insects. However, a work with a parasitoid dipterous species, *Hypoderma lineatum* (Villers), showed that a low level of chloramphenicol in the artificial diet significantly increased the survival of young larvae to pupation by approximately 70.0%(8). It was demonstrated that novobiocin sodium, at the lowest tested level, had no significant effects and even caused a slight increase in the pupal and adult yields of another parasitoid dipterous insect, *Agria affinis* auct. nec Fallen. These yields were decreased inversely, but the rate of development was increased directly with the increasing levels of this antibiotic (9). Novobiocin had similar effects on both of these entomophagous insects, but it was tolerated at much lower levels by larvae of the insect used in our experiments compared with those tested on A. affinis. This difference in tolerance to levels of the antibiotic may be attributed to the unconnected or blind gut of *Pimpla* larvae (2). Since the larvae are not able to permanently defecate contents of the gut during the active feeding period, they could not consume more diet than that required for their normal development. Thus, this may consequently determine the intake of an antimicrobial agent.

The negative effects of the antibiotics at high levels on the survival of *P. turionellae* were similar. This indicates that these effects may be dependent on the level of antimicrobial agents in the diet. These antibiotics, especially nalidixic and oxolinic acids, share more or less a similar chemical structure. Novobiocin also brings a novoise sugar moiety (10). The negative effects of these agents at high concentrations may be a result of their structural properties. On the other hand, the sugar moiety of novobiocin might be responsible for its beneficial and detrimental effects on the insect. In the presence of the antibiotic at the lowest level, this sugar may meet a deficiency in the diet from the nutritional or physical standpoint, whereas at higher levels it may have impaired the nutritional balance and consequent quality of the diet. This may be supported by the observation that a macrolacton antibiotic, abamectin with a sugar moiety, was detrimental at high levels to the gypsy moth, *Lymantria dispar* L. (11). In the preliminary work carried out on *P. turionellae*, it was demonstrated that streptomycin, which is an aminoglycoside antibiotic, at high levels followed the same trend on the survival and

development of *P. turionellae* (2). It is reasonable to suggest that increased levels of the antibiotics may cause a change in the nutritional balance of the diet. For example, with increasing levels of streptomycin in the larval diet of *A. affinis* the rates of growth, development and survival were decreased; however, these effects were reduced following an increase in the levels of nutrients (12).

It has been observed that diets with high levels of antibiotics have become increasingly more slurry-like during the active feeding period of the larvae when compared to the control diet. These diets produced significantly lower yields of adults. The larvae fed on these diets were smaller in size and, consequently, the adults were feebler than those of the control diet. Similarly, Singh and House (13) demonstrated that most of the antimicrobial agents in the diet of *A. affinis* notably resulted in abnormally small larvae at increased levels. It is well known that novobiocin could combine with magnesium ions (14). Another work suggests that the negative effects of tetracycline on a parasitic protozoan, *Plasmodium falciparum*, may be due to its complexes with calcium ions in the diet (15). It is possible for the physical texture of the diet to be affected by such interactions. A change in the physical properties of the diet may interfere with its nutritional composition and with the food intake of the larvae. The physical properties of the diet are of great importance in the artificial rearing of insects (16). It has been reported that a semi-liquid form is necessary to support the larvae on the surface and to provide a suitable environment for the feeding activity of endoparasitoids *Itoplectis conquisitor* (Say) (17) and *Pimpla turionellae* (4) larvae. In artificial rearing, the diets are not only a food but they are also an environment for the larvae (18).

The lowest level of oxolinic acid had no significant effects on the yields of pupae and adults, but prolonged the average time to reach these stages when compared to the control. It would be expected that a non-nutritive dietary additives influence the feeding activity of the larvae. Thus, such a diet may be utilized in different ways by the larvae. Singh and House (13) suggested that antimicrobial agents had an effect on the feeding activity of insects because of possible repellent effects. Therefore, dietetics confirms that although they have no effects on survival, the use of antimicrobial agents is often undesirable for the control of microbial contamination since even at low levels there is usually an effect on the rate of development in the mass rearing of insects (2,9,19,20).

One possible explanation of the beneficial and detrimental effects of antibiotics on the insect is that these effects could be due mostly to their dietary implication and the consequent nutritional advantage or impairment of the larvae rather than their direct effects. Nevertheless, it would be interesting to determine the biochemical basis for these exceptional effects of the antibiotics tested at much lower levels as well. It has been reported that these antibiotics inhibit many of the enzymes involved in the replicative process of DNA in eukaryotes (21-23). However, some antibiotics are known to have physiologically positive effects on some nutrients in economically important higher animals (24).

This work demonstrates two discrepant effects of the antibiotic alone added at different levels into the larval diet of *P. turionellae*. Although a positive effect was obtained with the lowest level of novobiocin, nevertheless this partly indicates that antimicrobial agents might be of practical importance in the artificial mass rearing of the insect for biological control. The significant positive effects of such a potent antibacterial agent on the survival of the insect confirms the suggestion of Slansky (25) that most of the responses of an insect throughout its life occur within a nutritional context. Therefore, it may be that the detrimental effects of high novobiocin levels or nalidixic and oxolinic acids are decreased with increased nutrient levels in the diet, as it is in *A. affinis* for streptomycin (12). However, there is direct evidence that such detrimental effects on the insect may be completely eliminated by the addition of these antibiotics at different combinational levels into the diet. A previous work with antifungal agents showed that a combinational level of methyl p-hydroxybenzoate and nystatin caused a significant increase in the yields of pupae and adults, and decreased the rate of development according to their individual levels, and that combinational levels were apparently effective in keeping the initial physical texture of the diet throughout the feeding period of the larvae (3).

Acknowledgements

I gratefully acknowledge the help of Professor Dr. Ş. Yazgan for his supervision and criticism throughout this work.

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