CROP PROTECTION

Growth Disruption, Abnormalities and Mortality of *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) Caused by Azadirachtin

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Neotropical Entomology 30(1): 113-125 (2001)

Redução do Crescimento, Deformidades e Mortalidade de *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) Causadas por Azadiractina

RESUMO - A azadiractina é o composto mais eficiente extraído da planta *Azadirachta indica* A. Juss, com propriedades insecticidas e que afetam o crescimento de diversas espécies de insetos. Seus efeitos no desenvolvimento e na sobrevivência de *Spodoptora littoralis* (Boisduval) foram estudados em condições de laboratório. Larvas de terceiro ínstar foram aliementada individualmente em dieta artificial tratada com azadiractina (de 0,01 ppm a 1 ppm p/v), por dois dias, e transferidas para dieta pura. A sobrevivência, a duração dos estágios e deformidades foram observados diariamente. Em um segundo experimento, os efeitos da azadiractina sobre lagartas de terceiro ínstar e lagartas de último ínstar foram comparados, após alimentar as lagartas por dois dias, utilizando-se 0,3 ppm e 0,6 ppm do composto e uma testemunha (água). O período de dois dias de alimentação prolongou os ínstares larvais, reduziu a taxa média de crescimento relativo (MRGR), impediu a ecdise, provocou anormalidades morfológicas e causou mortalidade em *S. littoralis*, de forma dependente da dose. Quando as concentrações mais altas foram empregadas, os efeitos aparecerem logo após o tratamento e a mortalidade foi mais elevada. Muitos insetos morreram após permanecerem inativos por vários dias ou durante ecdises prolongadas. Em concentrações mais baixas, se a ecdise foi completada, alterações no crescimento e anormalidades geralmente ocorreram nos estádios subseqüêntes. Quando a azadiractina foi oferecida às lagartas de último ínstar, intermediários "larva-pupa" foram produzidos e a mortalidade foi mais alta do que para as lagartas de terceiro ínstar. A azadiractina se mostrou promissora para o controle de *S. littoralis* não apenas por causar mortalidade, mas também por afetar o desenvolvimento e por causar deformidades envolvidas em atividades vitais dos insetos como alimentar-se, caminhar ou voar, o que pode torná-los vulneráveis a diversos tipos de agentes de mortalidade ou evitar que os mesmos causem dano à cultura.

PALAVRAS-CHAVE: Insecta, *Azadirachta indica*, nim, inseticida botânico, intermediário larva-pupa.

ABSTRACT - Azadirachtin is the most effective compound of the neem tree, *Azadirachta indica* A. Juss, with insecticidal and growth disruption properties on several species of insects. Its effects on the development and survival of *Spodoptora littoralis* (Boisduval) were studied under laboratory conditions. Third-instar larvae were individually fed on artificial diet treated with azadirachtin (from 0.01 ppm to 1 ppm wt/v), for two days, and transferred to plain diet. Survival, duration of stages and deformities were recorded daily. In a second experiment, the effects of azadirachtin on 3rd instar larvae and on last instar larvae were compared, by feeding the larvae with treated diet (0.3 ppm, 0.6 ppm and a control group), during the same period. A two-day feeding period promoted prolongation of the larval instars, reduction in the Mean Relative Growth Rate (MRGR), moulting disruption, morphological anomalies and mortality of *S. littoralis* in a dose-dependent manner. When higher concentrations were applied, the effects appeared shortly after treatment and mortality was higher. Many insects died after remaining inactive for several days or during prolonged moulting. At lower concentrations, if moulting was achieved, growth disruption and abnormalities were then likely to occur at the next moulting cycles. When azadirachtin was fed to last-instar larvae, larva-pupa intermediates were formed and the mortality was higher then for 3rd-instar larvae. Azadirachtin showed promissing for the control of the

species not only for causing mortality but also for disrupting the development and for causing deformities involved in vital activities like feeding, walking or flying, making the insect vulnerable to several sorts of mortality agents or prevent them from causing damage to the crop.

KEY WORDS: Insecta, neem, *Azadirachta indica*, botanical insecticide, larva-pupa intermediate.

The increasing number of investigations on plant-insect chemical interactions in the last few decades unveiled the potential of utilising secondary plant metabolites, or allelochemicals, as pest control agents. This interest in botanical pesticides resulted from the need to provide an alternative in IPM programmes to the synthetic insecticides, whose adverse effects on agroecological systems are well known (van Emden 1989, Whitten 1992) .

About 11 species of the family Meliaceae are referred to as possessing terpenoids with insect antifeedant properties (Simmonds *et al*. 1992). *Azadirachta indica* A. Juss, the neem tree, is the most promising species since chemicals extracted from it, besides showing antifeedant effects, can also cause growth disruption and mortality of insects. Azadirachtin is the main compound isolated from the neem tree and it can cause antifeedancy, growth reduction, moulting inhibition, anatomical abnormalities as well as mortality, in a vast range of insect species, many of them belonging to the Order Lepidoptera. Its action depends on the insect species and on the concentration applied.

The growth regulatory effects of azadirachtin are mostly concerned with its interference in the neuroendocrine system of the insects (Mordue (Luntz) & Nisbet 2000). The main hormones involved in growth regulation in insects are ecdysone and 20-hydroxy-ecdysone (moulting hormones) and juvenile hormone (JH). They are respectively produced in the prothoracic glands and *corpora allata*, through stimulation of hormones secreted in the brain (Wigglesworth 1972).

Apart from the moulting process, many other different physiological functions in insects are controlled by neurohormonal action. These include protein synthesis, reproduction, diapause and behaviour; all could be therefore affected indirectly by azadirachtin. Direct toxic effects observed on different tissues and cells (Hori *et al*. 1984, Schluter *et al*. 1985, Nasiruddin & Mordue (Luntz), 1993) add complexity to the variety of physiological events azadirachtin could affect.

The genus *Spodoptera* includes many species which constitute pests of economic importance for different crops, in different countries. In Brazil, the main species is *Spodoptera frugiperda* (J.E. Smith), which causes about 38% to 60% loss in maize production (Bianco 1991). *Spodoptera littoralis* (Boisduval) is an important pest of cotton in Southern Europe, Africa and the Middle East (Hosny *et al*. 1986). S. *littoralis* larvae feed mainly on leaves and stems and can seriously retard growth or reduce production of the cotton crop. During heavy infestations, however, they can also penetrate flowers and bolls.

There is little literature on the effects of azadirachtin on *S. littoralis* development and most of the studies refer to the use of neem (El-Sayed 1982, Meisner *et al*. 1981, Meisner & Ascher 1984) with no analyses of azadirachtin content included. These values can vary markedly according to the origin of the neem tree, method of extraction, temperature and humidity (Ermel *et al*. 1987), which can compromise the accuracy of the results. In addition other compounds present in neem extracts also show antifeedant or growth-disruption activity on *S. littoralis* (Simmonds *et al*. 1990) and other species (Aerts & Mordue (Luntz) 1997). Consequently although it is known that azadirachtin is the major constituent in neem seeds, the effects observed from applications of such extracts could result from a summation or synergism of azadirachtin and other terpenoids present in the mixture.

The present work was intended to evaluate the effects of the compound azadirachtin on the survival and development of *S. littoralis*, in order to better understand the action of the compound on lepidopterous larvae. Some aspects not yet studied were included, such as the effects on the length of the larval instars and other life stages and possible physical anomalies on the insects, as reported for locusts by Langwald & Schmutterer (1992) and Nicol *et al*. (1995) and for *M. sexta* by Schluter *et al*. (1985). These effects of azadirachtin when applied on 3rd instar larvae and on last instar larvae were also compared. The higher mortality rates obtained with azadirachtin on older larvae of *S. littoralis* (170-200 mg) when compared with effects on younger larvae (2-4 mg and 30-50 mg) (Ascher e*t al.* 1984) indicate a different action of the compound at different periods of development of the insect. This study could also contribute to assess the possibility of enlarging the period of time during which azadirachtin could be effectively applied in the field.

Material and Methods

The experiments were carried out in the School of Plant Sciences, The University of Reading, UK. Insect culturing and bioassays were run in the same experimental conditions, in a room kept at 26 (± 1) °C and long-day photoperiod (16h light: 8h dark). A strain of *S. littoralis* obtained from the University of Southampton was used throughout the study.

The chemical used was AZA POWDER which purported to have 10% (wt/wt) azadirachtin in a mixture of water soluble inert ingredients and was kindly supplied by Cardiff Chemicals Ltd, Cardiff. The content of azadirachtin in the samples was quantified by means of HPLC, according to (Ermel *et al.* 1987).

The larvae were fed on an artificial diet based on dry beans, wheatgerm and alfalfa. Azadirachtin was added to the artificial diet at a temperature below 40°C, before the diet solidified. Five concentrations a.i. were tested: 0.01 ppm, 0.05 ppm, 0.1 ppm, 0.5 ppm and 1 ppm (all wt/v). The highest concentration applied here was the lowest which had caused

100% larval mortality in a previous experiment (Carvalho 1996).

The larvae were treated at the 3rd instar to minimise handling effects and to increase the accuracy of measurements. A feeding period of two days was chosen to ensure that all larvae fed, but still to be shorter than the period neem extracts can be active in the field, which is about seven days according to Stokes & Redfern (1982) and Schmutterer (1990). After the feeding period the larvae were transferred to plain diet. Ten larvae were kept individually in plastic pots in four replicates (i.e. 40 insects) in each treatment.

Changes in life stages, physical anomalies and mortality were recorded daily. Duration of life stage and the mean Relative Growth Rate (MRGR) were determined. The larval weights for MRGR determination were taken before and after treatment.

The MRGR of the insects is the mean weight increment per initial weight per day of the insect over the period between the weighings. It was assessed by means of the formula:

$$M_{RGR\ (mg/mg/day)} = \frac{\log N \text{ final weight (mg)} - \log N \text{ initial weight (mg)}}{\text{time (days)}}$$

Data on length of the different stages were transformed to square roots. Subsequently ANOVA and Tukey tests were performed. Logistic regression analysis was used to evaluate statistically the mortality caused by the different concentrations of azadirachtin. The significance of correlations between mortality and length of life stages or MRGR was analysed by means of Probit analysis.

In a second experiment the effects of azadirachtin fed to 3rd-instar larvae and to last- instar ones were compared. Two concentrations of azadirachtin (0.3 ppm and 0.6 ppm) within the range used in the previous experiment were tested. Larvae were fed on the treated diet for two days and then transferred to plain diet, at the beginning of the 3rd instar or at the beginning of the last larval instar. Three replicates with 20 larvae were used in each treatment. A control group was fed on plain diet and kept under the same conditions.

The length of development stages, anomalies and mortality were recorded. The means of the length of stages were transformed to square roots and subjected to ANOVA and the Tukey test. Factorial logistic regression analysis was performed on mortality rates.

Results and Discussion

Growth Disruption Effects. The action of azadirachtin on the development of *S. littoralis* could be observed at the highest concentration (1 ppm) soon after treatment. The 3rd-instar larvae had the length of that instar significantly prolonged (Table 1). During larval growth a delay in development was also observed at the lower concentrations, and a dose-dependent response was seen at all concentrations (Table 1).

Only concentrations below 0.1 ppm allowed the larvae to complete the pre-pupal stage and pupate. Although these two stages appeared to be prolonged in a dose-dependent manner, their durations were not significantly different from the 0.01 ppm control duration ($P<0.05$) (Table 2). The length of the total life stage after treatment reflected the dose-dependent effect observed for the larval stage.

An effect of azadirachtin on the length of the larval instars of *S. littoralis* has not been previously reported. However, similar results relating to the whole larval stage of *S. frugiperda* were obtained by Redfern *et al.* (1981) and *S. litura* by Behera & Satapathy (1997). Larvae kept on treated diet had their pupal ecdysis delayed in comparison with the control insects.

The rate of growth, indicated by MRGR, calculated for the period when insects were feeding on treated diet, showed no clear evidence of any direct antifeedant effect at the concentrations tested (Table 3). Although a significant reduction on MRGR was observed for larvae fed on diet treated with 0.5 ppm azadirachtin, the reduction observed at 1ppm was not statistically different from the 0.01ppm control group ($P>0.05$). Such results agree with a similar lack of feeding inhibition obtained for *S. litura* during a two-days feeding period on castor leaves treated with 50 ppm azadirachtin and above (Ramachandran *et al*. 1989)

MRGR calculated for the whole larval stage after treatment was also dose-dependent, higher concentrations

Table 1. Mean length (days) of the larval instars of *S. littoralis* after 3rd-instar larvae fed for two days on diet treated with different concentrations of azadirachtin.

Azadirachtin	Larval instars				
(ppm)	3rd	4th	5th	6th	7th
0.01 (used as control)	2.7a	1.6a	1.1a	3.6a	4.0a
0.05	2.8a	1.7a	1.2ab	3.8a	4.4ab
0.1	2.9a	1.7ab	1.2ab	3.8ab	5.1 b
0.5	2.9a	2.0ab	1.9 b	4.9 b	7.4 c
1	3.8 b	2.3 b	2.2 b	-	-
LSD[1]	0.2	0.2	0.3	0.3	0.3

[1]Due to reduction in replicate numbers from mortality higher than 10%, the means were transformed to square roots before ANOVA. LSD values are given in transformed values, but the means are presented untransformed.
Means followed by the same letter within columns are not significantly different according to the Tukey test at P=0.05.

Table 2. Mean length (days) of the different life stages of *S. littoralis* after 3rd-instar larvae fed for two days on diet treated with different concentrations of azadirachtin.

Azadirachtin	Life stages			
(ppm)	Larva	Pre-pupa	Pupa	Total
0.01 (used as control)	12.9a	2.0a	9.6a	24.5a
0.05	13.7ab	2.0a	10.1a	25.3ab
0.1	14.7 b	2.3a	10.3a	27.2 b
0.5	17.7 c	-	-	-
1	-	-	-	-
LSD[1]	0.2	0.3	0.2	0.2

[1]Due to reduction in replicate numbers from mortality higher than 10%, the means were transformed to square roots before ANOVA. LSD values are given in transformed values, but the length means are presented untransformed.
Means followed by the same letter within columns are not significantly different according to the Tukey test at P=0.05.

causing lower MRGR (Table 3). This indicates the deleterious action of azadirachtin on insect growth even after the feeding period has ceased. This pos-ingestive action could be related to the reduction of food intake and to the hability of converting food into biomass, reported for *S. littoralis* (Martinez & van Emden 1999). MRGR could not be evaluated for 0.5 ppm and 1 ppm azadirachtin after treatment because 100% of the insects were killed during the period.

Table 3. MRGR of *S. littoralis* during treatment (two days feeding on diet treated with different concentrations of azadirachtin) and during the whole larval stage after treatment (transferred to plain diet).

Azadirachtin (ppm)	During treatment	After treatment
0.01 (used as control)	0.7508 b	0.2470 b
0.05	0.7404 b	0.2320ab
0.1	0.7160ab	0.2240a
0.5	0.6424a	-
1	0.7126ab	-
LSD	0.0887	0.0211

Means followed by the same letter within columns are not significantly different according to the Tukey test at P=0.05.

The MRGR of *S. exempta*, calculated for five days after treatment using the method of Waldbauer (1968), was reduced when 5th-instar larvae were treated topically with azadirachtin (Tanzubil & McCaffery 1990). Other authors have used larval weight gain rather than MRGR to measure the effect of azadirachtin or neem extracts on larval growth. Meisner & Nemny (1992) reported a lower weight gain of *S. littoralis* larvae which were treated topically or fed for two days on cotton leaves treated with Margosan-O (azadirachtin formulation) compared with control insects. The same effect was obtained by El-Sayed (1982) after 2nd- and 3rd-instar larvae of *S. littoralis* were fed on castor leaves treated with neem extract for three days.

A significant positive correlation between length of the life stages and mortality as affected by azadirachtin was clearly seen in this experiment (Fig. 1). Likewise Nicol *et al.* (1995), who tested the effect of neem-oil sprays on 3rd-instar nymphs of *S. gregaria* obtained a positive correlation between mortality rate and length of the nymphal period in most cases evaluated. The larval growth (MRGR) was also closely correlated with the mortality rates but in a negative way, with the treatments that produced lower MRGR in the larvae producing higher mortality rates (Fig. 2). However, Simmonds *et al.* (1990) reported no correlation between mortality and reduction in weight gain at the second day after last-instar *S. littoralis* larvae were treated with azadirachtin and Simmonds & Blaney (1984) obtained a positive correlation between mortality and weight gain of larvae of the same species after feeding them on diet treated with azadirachtin for seven days.

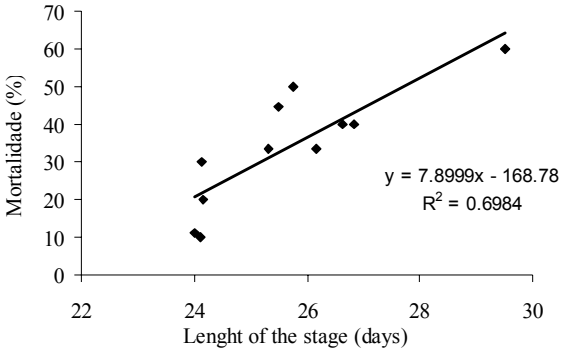

Figure 1. Correlation between length of larval stage and mortality of *S. littoralis* caused by azadirachtin after 3rd-instar larvae fed on treated diet for two days ($\chi^2 = 7.30$; $P<0.007$).

Anatomical Abnormalities. Azadirachtin affected moulting and induced a wide range of anatomical abnormalities in *S.*

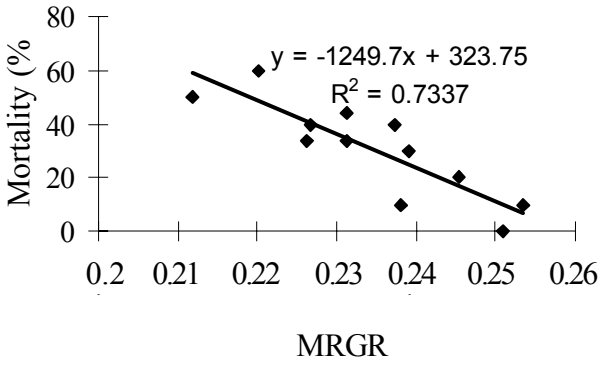

Figure 2. Correlation between MRGR and mortality of larval stage caused by azadirachtin after 3rd-instar larvae fed on treated diet for two days ($\chi^2 = 6.19$; $P<0.013$).

littoralis. The higher the concentration added to diet the more the insects were affected, as also reported for locusts by Nicol *et al.* (1995), and the alterations were shown earlier during the development of the insects. The observed abnormalities were categorized and are presented arranged by life stage in Tables 4 to 7. Each insect could show one or more than one of the described characteristics.

Only at 0.1 ppm azadirachtin and above did *S. littoralis* show anomalies during the larval and pre-pupal stages (Tables 4 and 5). The moulting period was prolonged in a dose-dependent manner. At 0.1 ppm azadirachtin, 2.5% of the larvae died during moult. With 0.5 ppm azadirachtin added to the diet, 25.0% of the larvae went through moult which was extended by between one day to more than one week, leading to 22.5% mortality during the process. At 1 ppm azadirachtin, prolonged moulting occurred in 92.5% of the insects and was followed by death in 75.0% of the cases. In an analogous study, *S. frugiperda* larvae had 95% ecdysis inhibition after feeding on diet treated with 1 ppm azadirachtin for ten days (Kubo & Klocke 1984).

Those larvae that did moult did not complete the process properly, and the defects intensified with the sequence of moults into the later instars, as observed on locusts (Nicol *et al.* 1995). This indicates that the two days feeding on azadirachtin in the diet resulted in irreversible damage to physiological processes essential to the development of *S. littoralis*.

Several anomalies, possibly related to defective moulting, were observed at 0.1 ppm azadirachtin, but particularly at 0.5 ppm and 1 ppm (Table 4). Parts of the old cuticle remained adhering to the larval cuticle. Body setae and structures located at the extremities of the legs and mouth parts were lost. The cuticle colour turned to a uniform dark grey, losing the spotted pattern characteristic of the species. *S. littoralis*

Table 4. Frequency (%) of moulting disruption and anomalies observed in larvae of *S. littoralis* after 3rd-instar larvae fed for two days on diet treated with different concentrations of azadirachtin.

Description	Azadirachtin (ppm)		
(n=40)	0.1	0.5	1
General			
Larva died during moulting	2.5	22.5	75.0
Prolonged moulting period	0	25.0	92.5
Same head capsule size after moulting	0	5.0	0
Wrinkled body	2.5	5.0	2.5
Swollen body	0	35.0	0
Swollen dorsal segments	0	20.0	2.5
Smaller body (compared to the same treat)	0	5.0	12.5
Total	4.0	65.0	100
Cuticle			
Different pattern of colour/ spots	100	65.0	7.5
Almost uniform grey cuticle	0	42.5	2.5
Dark cuticle	25.0	52.5	5.0
Parts of the old cuticle remaining on the new	0	15.0	10.0
Total	100	62.5	20.0
Appendages			
Defective back legs extremities	0	2.5	7.5
Defective front legs tarsi	0	0	2.5
Deformed mouthparts	2.5	0	0
Total	2.5	2.5	7.5
Total of affected larvae	100	97.5	100

No malformations were registered at 0.01 ppm and 0.05 ppm azadirachtin.

Table 5. Frequency (%) of anomalies observed in pre-pupae of *S. littoralis* after 3rd-instar larvae fed for two days on diet treated with different concentrations of azadirachtin.

Description	Azadirachtin (ppm)	
	0.1 (n = 35)	0.5 (n = 7)
General		
Deformed body	2.9	0
Stretched body	0	42.9
Swollen body	2.9	57.1
Swollen segments	5.7	0
Total	8.6	100
Cuticle		
Different pattern of colour and spots	5.7	57.1
Dark cuticle	0	14.3
Greenish cuticle	5.7	42.9
Parts of the old cuticle remaining on the new 2.9	14.3	
Total	8.6	57.1
Total of affected pre-pupae	8.6	100

No malformations were registered at 0.01 ppm and 0.05 ppm azadirachtin. All the insects fed on diet treated with 1 ppm azadirachtin died prior to this stage.

Table 6. Frequency (%) of moulting disruption and anomalies observed in pupae of *S. littoralis* after 3rd-instar larvae fed for two days on diet treated with different concentrations of azadirachtin.

Description	Azadirachtin (ppm)		
	0.01 (n=36)	0.05 (n=36)	0.1 (n=32)
General			
Pupa died during moulting period	0	2.8	0
Imperfect metamorphosis	0	0	3.1
Wrinkled body	0	0	3.1
Smaller body (comparing to the same treat)	0	0	6.3
Deformed body	2.8	2.8	9.4
Total	2.8	5.6	12.5
Cuticle			
Areas with thin cuticle	0	11.1	31.3
Dark spots on the cuticle	11.1	16.7	15.6
Total	11.1	22.2	31.3
Wings			
Deformed wing	0	8.3	0
Wrinkled wing	5.6	0	6.3
Thin cuticle on the wing region	0	5.6	21.9
Swollen wing	0	5.6	6.3
Leaking through wing	0	5.6	6.3
Dark spot	8.3	8.3	0
Total	15.6	19.4	28.1
Appendages			
Defective leg	0	2.8	0
Defective antenna	5.6	5.6	0
Defective mouthparts	8.3	8.3	0
Total	12.5	8.3	0
Total of affected pupae	22.2	30.6	37.5

All the insects fed on diet treated with 0.5 ppm and 1 ppm azadirachtin died prior to this stage.

Table 7. Frequency (%) of anomalies in adults of *S. littoralis* after 3rd-instar larvae fed for two days on diet treated with different concentrations of azadirachtin.

Description	Azadirachtin (ppm)		
	0.01 (n=34)	0.05 (n=25)	0.1 (n=21)
Deformed body	2.9	0	0
Incomplete wing base	14.7	16.0	42.9
Twisted wing	2.9	0	9.5
Shortened antenna	2.9	0	0
Shortened mouth parts	2.9	0	0
Stretched out mouth parts	2.9	0	0
Twisted genitalia	0	0	4.8
Total of affected adults	26.5	16.0	47.6

All the insects fed on diet treated with 0.5 ppm and 1 ppm azadirachtin died prior to this stage.

1st-instar larvae fed on castor leaves treated with 0.2% neem seed aqueous suspension for three days also became darker and remained in the pre-pupal stage longer than control larvae (El-Sayed 1982).

A larval mortality of only 2.5% was observed at 0.1 ppm azadirachtin. However, 100% of the larvae showed an aberrant pattern of colour or spots on the cuticle in the later larval instars, which suggests that some physiological activity was impaired. This anomaly was the only one present at the highest frequency at the lowest azadirachtin concentration (Table 4), and seems to be connected with the moult. Mortality of larvae fed on diet treated with 0.1 ppm azadirachtin was lower than of those fed on 0.5 ppm and 1 ppm. Hence, a higher number of larvae fed on the lowest concentration experienced more moults, thus increasing the possibility of an aberrant pattern of colour to be expressed.

At 0.5 ppm and 1 ppm azadirachtin, the larvae in the last three instars either had their body or at least several thoracic segments dorsally swollen. Mortality reached 80% at 0.5 ppm by the end of the larval stage. All surviving insects showed some sort of damage during the period and were reduced in size as compared with the control larvae. All larvae at the higher of these two concentrations were smaller than the control larvae, and showed some morphological alteration (Table 4) and died before the last larval instar.

Although all the insects fed on diet treated with 0.1 ppm showed an aberrant pattern of cuticle colour during the larval stage, only 8.6% of the pre-pupae showed any kind of azadirachtin effect (Table 5). All pre-pupae fed on diet treated with 0.5 ppm azadirachtin presented generalised symptoms of the action of azadirachtin. They had swollen or stretched bodies and failed to pupate. Also most of them showed different patterns of cuticle colour and spots.

At the pupal stage, all insects fed on diet treated with 0.5 ppm azadirachtin and above were killed and all remaining insects showed some kind of abnormality (Table 6).

Several pupae at 0.05 ppm azadirachtin, but particularly at 0.1 ppm, had areas with abnormally thin cuticle. Pupae from all the treatments, including 0.01 ppm, taken as the control, showed some kind of alteration in the pigmentation of the cuticle or black spots resulting from cuticular melanization. Such black spots were also observed in *Bombyx mori* (L.) larvae treated with azadirachtin (Koul *et al*. 1987) and were associated with low levels of ecdysteroids and juvenile hormone (Hori *et al*. 1984). Again alterations like imperfect metamorphosis, wrinkled, deformed or even smaller bodies were more frequent at the highest concentration with surviving insects (0.1 ppm azadirachtin).

The most common pupal defect, especially in insects fed on the highest concentration of azadirachtin, was deformed or swollen wings. Often the wings had a very thin cuticle which swelled up, ruptured and allowed the internal fluid of the pupae to leak out. Some deformities in the legs and head appendages were observed at the lower concentrations. Schluter *et al*. (1985) also observed deformed wings and head appendages of pupae of *M. sexta* after 5th-instar larvae had been injected with azadirachtin.

Adults had deformed wings or incomplete wing bases (Table 7). Deformities of the front legs and antennae were observed once. Similarly the main defects caused in *S. gregaria* adults by azadirachtin treatment were related to the wings, antennae and legs (Nicol *et al*. 1995). Azadirachtin treatment of last larval instar of *S. litura* caused abnormalities in adults which included attachment of the exuvium to the abdomen, deformed wings and a prolapsed anal brush in the females (Rao & Subrahmanyam 1987).

When these abnormalities were put together with the mortality rates obtained in the various treatments, the deleterious effects of azadirachtin on *S. littoralis* could be clearly seen by the reduced number of normal adults ($P<0.001$ - by means of logistic regression) (Fig. 3).

Mortality. Azadirachtin caused a significant increase on mortality of *S. littoralis* ($P<0.001$) which intensified during the development of the insects (Table 8). Comparison of the mean mortality at each concentration with 0.01 ppm

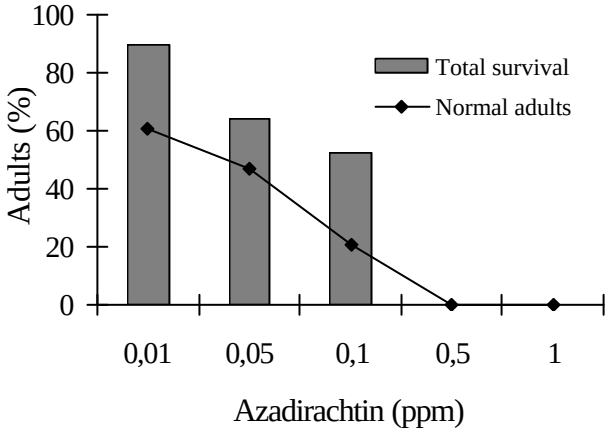

Figure 3. Accumulated mortality of larvae of *S. littoralis* fed for two days at the 3rd instar on diet treated with different concentrations (ppm) of azadirachtin.

Table 8. Mean mortality of *S. littoralis* caused by feeding 3rd instar larvae for two days on diet treated with different concentrations of azadirachtin.

Azadirachtin	Life stages						
(ppm)	3rd Instar	4th Instar	5th Instar	6th Instar	7th Instar	Pre-pupa	Pupa
0.01	7.5	7.5	7.5	7.5	7.5	7.5	10.3
0.05	5.6	5.6	5.6	5.6	5.6	5.6	***36.1***[1]
0.1	10.0	12.5	12.5	12.5	12.5	***20.6***	***47.5***
0.5	7.5	10.0	21.4	***64.5***	***80.6***	***100***	***100***
1	***52.5***	***72.5***	***90.0***	***100***	***100***	***100***	***100***
χ^2 statistic	22.42	38.45	62.35	29.77	46.99	2.89	7.40
P<	0.001	0.001	0.001	0.001	0.001	0.10	0.05

[1]Values in bold italic differ statistically from 0.01 ppm treatment (taken as control) in each stage by paired comparison provided by logistic regression analysis. The values of the χ^2 statistic refer to the lowest other concentration to differ from 0.01 ppm (within columns).

concentration (taken as the control) showed a dose-dependent effect which started to be evident at the 6th instar.

Azadirachtin concentrations of 0.1 ppm and below did not cause any statistically significant mortality during the larval stage.

Higher rates of mortality were obtained when higher concentrations of the compound were used, as reported for other insect species of *Spodoptera* (Tanzubil & McCaffery 1990, Koul *et al*. 1995). From 0.5 ppm and above, azadirachtin prevented *S. littoralis* larvae from pupating. At 0.5 ppm azadirachtin, 20% of the insects were able to complete the larval period, but none were able to pupate. Azadirachtin also caused 100% mortality of the larvae at 1 ppm, confirming results previously obtained (Carvalho 1996). The time taken to reach about 100% was clearly shorter at 1 ppm than at 0.5 ppm (10 versus 26 days respectively) (Fig. 4). Mortality was associated mainly with failure to moult. Most of the larvae died after the moult was prolonged for several days.

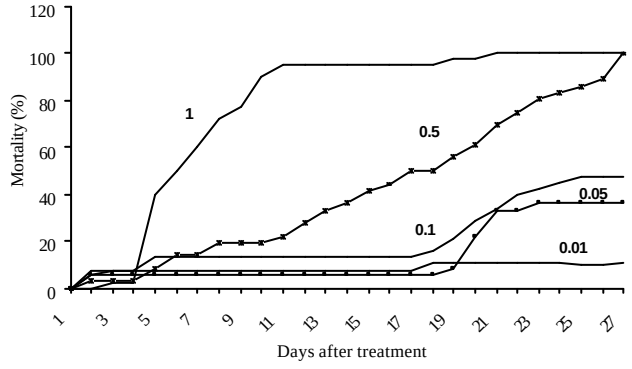

Figure 4. Percentage survival and normal adults of *S. littoralis* after 40 3rd-instar larvae fed on diet treated with different concentrations of azadirachtin (ppm).

The effect of azadirachtin on the development of *S. littoralis* when the larvae were treated at the last larval instar in comparison with treatment at the 3rd instar.

Length of Life Stages. Larvae exposed to azadirachtin at the 3rd instar took longer to reach the pre-pupa in comparison with the control insects (Table 9). This confirms the results obtained in the former experiment. When the larvae were treated at the beginning of the last instar, the effects were not expressed immediately. However, at the next stage (pre-pupa), these insects appeared more affected then those treated at the 3rd larval instar and showed both a prolonged pre-pupal stage and higher mortality. It is remarkable that, even though the larvae treated at the 3rd instar showed the effect of azadirachtin during the larval stage, the subsequent effects on the pre-pupa and pupa were not as pronounced as those observed when last-instar larvae were treated.

Mortality. As in the previous experiments, azadirachtin caused higher mortality at the higher concentration (Table 10). By the pre-pupal stage, the insects treated at the last larval instar showed 75% and 95% mortality at 0.3 ppm and 0.6 ppm azadirachtin, respectively. At the pupal stage, a dose-dependent effect could not be observed for these treatments since the lower concentration tested already caused almost 100% mortality. However, the insects treated in the 3rd larval instar had mortality rates statistically different from the control group and also a rate that was significantly higher at the higher concentration (P<0.001).

The compound was drasticaly more lethal when offered to the larvae during the last larval instar than during the 3rd one (P<0.001). A very high mortality was obtained already at the pre-pupal stage at both concentrations (75% at 0.3 ppm and 95% at 0.6 ppm) as compared to the later mortality of the larvae treated at the 3rd larval instar (only 5% mortality in both concentrations). The same trend was obtained for larvae of the same species treated with neem seed extracts (Ascher *et al*. 1984), although the age of the larvae was not stated.

Abnormalities. As observed in the previous experiment, 3rd-

Table 9. Mean length (days) of different life stages of *S. littoralis* when the larvae were fed for two days on diet treated with two concentrations of azadirachtin during the 3rd and the last larval instar.

Instar when treated	Azadirachtin (ppm)	Larva	Pre-pupa	Pupa	Total
Control	0	10.9a	2.1a	9.8a	11.9a
3rd instar	0.3	12.5 b	2.1a	10.6 b	12.7 b
	0.6	13.0 b	2.1ab	11.4 c	13.6 c
Last instar[1]	0.3	10.9a	2.7 b	12.0	14.7
	0.6	10.9a	2.8 b	-	-
LSD[2]		1.5	0.2	0.1	0.1

[1]Data on pupa and total period were not included in the statistical analysis due to the reduced number of insects alive.
[2]Due to reduced replicate numbers from mortality higher than 10%, the means were transformed to square roots before ANOVA. LSD values are given in transformed values, but the means are presented untransformed.

instar larvae which fed on azadirachtin developed several anomalies (Fig. 5). Again these were deformities of the pupae, deformities of the wings in both pupae and adults and death resulting from inability to moult properly. Some pupae and moult occurred, and the body was still covered by the larval exuvium. None were able to emerge. Such intermediates were not found when azadirachtin was applied to the earlier stage of development.

Table 10. Accumulated mortality (%) of *S. littoralis* at different stages after feeding on diet treated with two different concentrations of azadirachtin for two days at the 3rd and the last larval instar.

Instar when treated	Azadirachtin (ppm)	Larva	Pre-pupa	Pupa
Control	0	0.0	1.7	3.3
3rd Instar	0.3	1.7	5.0	26.7
	0.6	0.0	5.0	41.7
Last instar	0.3	0.0	75.0	96.7
	0.6	0.0	95.0	98.3
χ^2 statistic	Effect of concentration:		233.92	214.85
	Effect of instar:		181.21	126.06

In both life stages, both effects of concentration and instar were statistically significant at $P<0.001$.

one adult did not shed their head capsule during the moult and therefore died. Umeh (1988) described similar anomalies for *Spilosoma maculus* (Wlk.) when 2nd-instar larvae were fed on cowpea leaves brushed with neem extract.

Azadirachtin treatment to the last-instar larvae affected the insects more severely than earlier treatment and led to rapid appearance of anomalies in pre-pupae (Fig. 5). Similarly *S. littoralis* last-instar larvae fed on 0.5% neem aqueous suspension presented 53% malformation in adults (El-Sayed 1982). Besides the very high mortality of pre-pupae, many insects were not able to pupate properly and formed larva-pupa intermediates (Fig. 6).

Larva-pupa intermediates had a pupal cuticle which was usually tanned on the abdomen, and on the dorsal region of the head and thorax. The remaining parts became darker and finally black when the insect was close to death. However no

Similar intermediates were obtained when last-instar larvae of *S. litura, S. mauritia, Ephestia kuehniella* Zell. and *M. sexta* were subjected to azadirachtin action (Gujar & Mehrotra 1983, Jagannadh & Nair 1992). Larva-pupa intermediates could also be produced in *M. sexta* by injecting high doses of moulting hormone (20hydroxyecdysone) into pre-pupae shortly before pupating (Schluter *et al*. 1985).

These results in combination reinforce the theory that azadirachtin affects the neurosecretory system and is more likely to kill the insect by disturbing the ecdysteroid regulation than by being toxic to the insect. During pupation and the pupa-adult moult, the insects undergo transformations that involve complex neuroendocrine processes that do not occur at the same intensity during larva to larva moults (Nijhout 1994). This different level of neuroendocrine activity at the larva to pupa moult could explain the lower growth

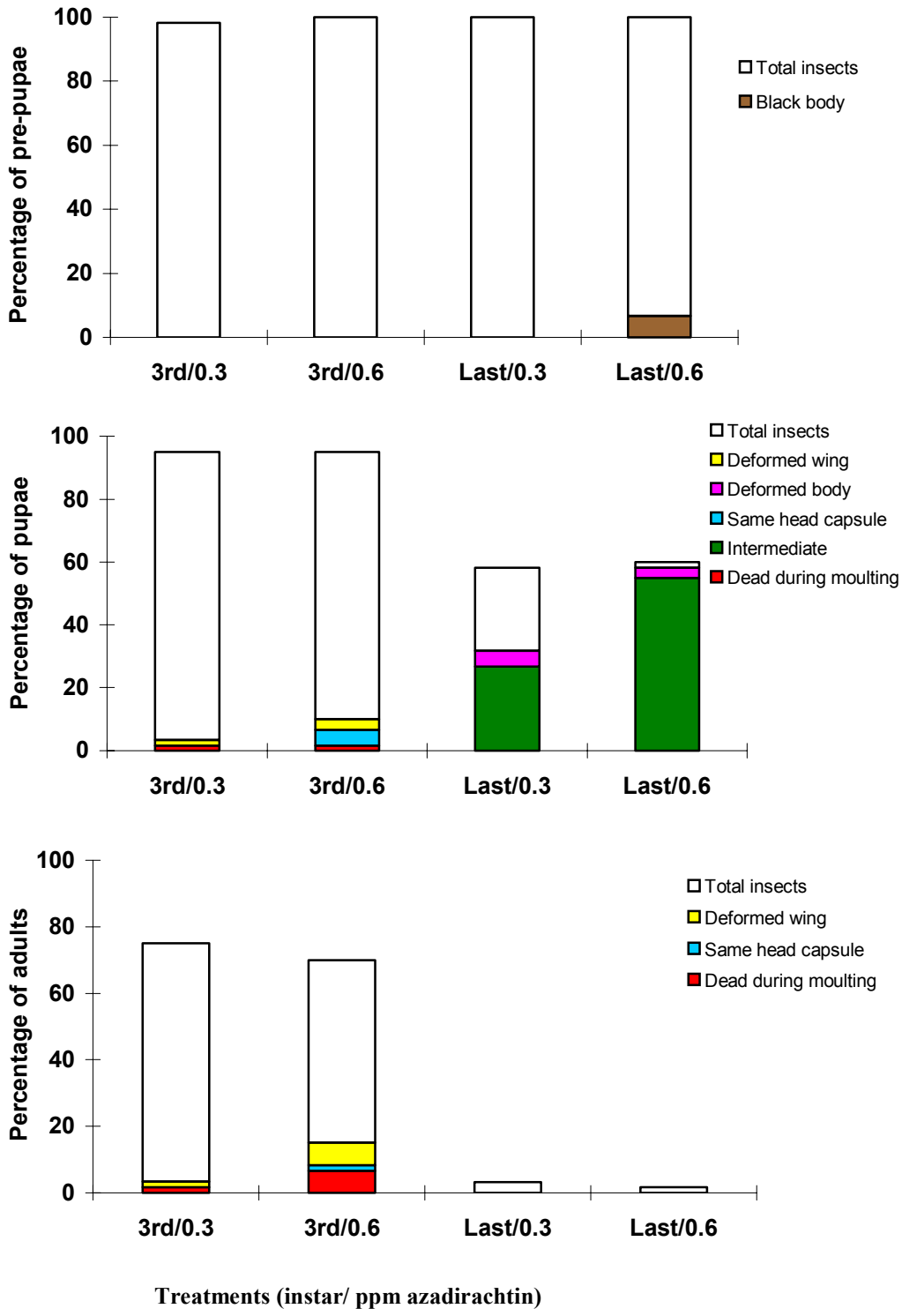

Figure 5. Anomalies observed in *S. littoralis* after the larvae were fed at the third and the last larval instar for two days on diet treated with different concentrations of azadirachtin (Total insects is the percentage of surviving insects at the beginning of each stage).

Figure 6. Larva-pupa intermediates formed by *S. littoralis* when the larvae fed on diet treated with azadirachtin in the last larval instar.

disruption effects of azadirachtin on 3rd-instar larvae. In addition, that insects excrete around 90% of the compound within seven to 24h has been reported for locusts (Rembold *et al*. 1988) and for *Rhodnius* (Garcia *et al*. 1989). Therefore a lower amount of azadirachtin would remain in the body of these insects at the pre-pupa and pupa stage in comparison with insects treated at the last larval instar.

The results could not be attributed to different rates of food intake, since *S. littoralis* is able to eat 11.8 times its weight per day during the 4th larval instar and only 2.5 times during the last instar (Martinez & van Emden 1999). Therefore, larvae treated at the 3rd larval instar would have ingested higher amounts of azadirachtin per body weight than those at the last instar. However the mortality rates and anomalies obtained in this experiment do not reflect these findings. They indicate that the action of azadirachtin is not only related to the absolute amount ingested (dose-dependent effect) but depends even more on the stage of development in which the compound is applied.

The results obtained here are promissing for the control of *S. littoralis*. A two-day feeding period was enough to promote prolongation of the larval instars, reduction in MRGR, moulting disruption, morphological anomalies and mortality of the species.

When higher concentrations were applied, the effects appeared shortly after treatment and the moulting disruption led to mortality. Many insects died after remaining inactive for several days or during prolonged moulting. At lower concentrations, if moulting was achieved despite treatment, moult disruption was then likely to occur at the next moulting cycle.

The high mortality observed during the moulting process and the higher mortality obtained with insects treated at the last larval instar, with production of larva-pupa intermediates, support the affirmation that the action of azadirachtin is connected with endocrine events in the insect. Several anomalies and deformities reported here can result from these kinds of disruption, some of them involved in vital activities like feeding, walking or flying, what can make the insect vulnerable to several sorts of mortality agents or prevent them from causing damage to the crop.

Acknowledgements

To Dr. A.J. Mordue and Dr. Allan R. McCaffery for sugestions and to CNPq for financial support.

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Received 12/I/2000. Accepted 12/12/2000.