CHOLESTEROL OXIDASE EFFECTS ON REPRODUCTION AND EMBRYONIC DEVELOPMENT OF THE COTTON BOLL WEEVIL, *Anthonomus grandis* BOHEMAN (COLEOPTERA: CURCULIONIDAE)

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ABSTRACT: Effects of cholesterol oxidase on the fecundity and embryonic development of the cotton boll weevil (*Anthonomus grandis*) were studied. Newly emerged and mated females were exposed to different enzyme concentrations. An initial feeding during ten days with a diet containing cholesterol oxidase showed reduced fecundity and the hatching of larvae was also influenced. Histological preparations of ovaries from controls and from enzyme-fed individuals showed no apparent morphological differences of the oocytes, but chorion and vitelline membrane were partially degraded in ovules and eggs at the beginning of the embryonic development in COase-treated females.

Index terms: Cotton, insect, control, enzyme.

EFEITOS DA COLESTEROL OXIDASE SOBRE A REPRODUÇÃO E DESENVOLVIMENTO EMBRIONÁRIO DO BICUDO DO ALGODOEIRO, *Anthonomus grandis* BOHEMAN (COLEOPTERA: CURCULIONIDAE)

RESUMO: Estudaram-se os efeitos da colesterol oxidase (COase) sobre a fecundidade e desenvolvimento embrionário do bicudo do algodoeiro (*Anthonomus grandis*). Fêmeas recém emergidas foram colocadas para acasalamento e alimentação durante 10 dias com dieta contendo diferentes concentrações da enzima. A dieta com colesterol oxidase causou redução na fecundidade e eclosão das larvas. A partir de preparações histológicas dos ovários, nenhuma alteração aparente foi observada na morfologia dos oócitos nas fêmeas alimentadas com colesterol oxidase, contudo o córion e a membrana vitelina foram parcialmente degradados nos óvulos e ovos no início do desenvolvimento embrionário de fêmeas tratadas com COase.

Termos para indexação: Algodão, inseto, controle, enzima.

INTRODUCTION

The boll weevil (*Anthonomus grandis* Boheman, 1843) is one of the most serious pests of cotton (*Gossypium hirsutum* L.) and is usually controlled through the use of insecticides. Although the use of pesticides can reduces boll weevil infestations below the economic threshold level, it brings secondary undesirables consequences and has some limitations (ALMEIDA, 2000). Besides undesired environmental effects, costs of the insecticides are high and there is a relatively narrow window during the crop's development when insecticide application is effective since the eggs are laid within the cotton squares and bolls and the larvae develop entirely inside these structures.

A promising control approach is the use of proteins showing insecticidal effects, such as lectins and/or enzymes which could affect physiological process such as reproduction. Purcel et al. (1993) reported that low doses of the cholesterol oxidase (COase) caused disruption of midgut epithelium cells of boll weevil larvae whereas higher concentrations would lead to complete cell lysis. Later, it was shown that enzyme supplemented-diet interfered with boll weevil female fecundity (GREENPLATE et al., 1995) and larvae eclosion when eggs were directly treated with COase (SANTOS et al., 2002).

Although Greenplate et al. (1995) carried out substantial work on the effect of COase on adults and larvae, no detailed study of its action on oogenesis has been published so far. However, such knowledge is urgently needed since the most substantial damage *in vivo*, i.e. bud abscission, occurs after hatching. Consequently if COase would interfere with egg development and reduce larval eclosion, one might expect a significant reduction on bud and fruit abscission, a primary goal in boll weevil control. The natural abscission rate of cotton flower buds is about 20% (BARREIRO NETO et al., 1983; VIEIRA et al., 1999), but, under insect attack this rate increases significantly, becoming the major factor responsible for yield reduction (PARAJULEE and SLOSSER, 2001) .

The objective of this paper is to drawn attention to the effect of COase on fecundity and embryonic development of cotton boll weevil.

MATERIALS AND METHODS

Enzyme

Streptomyces cholesterol oxidase (EC 1.1.3.6) (Sigma 8649), was used throughout the experiments.

Feeding assays

Natural and artificial diets were used in the feeding experiment. Artificial diet (tablet form) was prepared with soybean meal, wheat germen, glucose, vitamins and mineral salts, as suggested by Monnerat et al. (2000). Fresh, young cotton buds (cv. 'Embrapa Precoce 1') were daily collected, as a source of natural diet. Cholesterol oxidase was incorporated into these diets as described later at different concentrations (Table 1). Bioassays were carried out under constant laboratory conditions (14:10 light/dark, 28 ± 1.2 °C and 65 ± 2.0% relative humidity).

Oviposition

Newly emerged adults (twenty four) caged in containers (8 cm diameter) were used in this bioassay and were fed on artificial diet (tablets, 10 x 10 x 3 mm) or young buds (10 mm diameter). Twelve were used as control and the others were fed on tablets or buds soaked in 100 mL solutions of cholesterol oxidase at 41, 53 and 59 mg/mL. These doses were established based on previous work carried out by Santos et al. (2002) related to investigations of the concentration-dependent effects of COase on hatching and mortality of boll weevil larvae. Diets were replaced every two-days over a 10 days period and the number of laid eggs was counted daily. Afterwards, the adults were fed on untreated artificial or natural diet until the end of the oviposition cycle. Containers were randomly distributed in the laboratory.

Histology

Eggs collected from ovarioles of the control and females fed for 10 days on artificial diet containing COase at 53 mg/mL, a dose corresponding to the LD_{50} established in previous study (SANTOS et al., 2002), were dissected in 100 mM sodium cacodylate buffer (pH 7.3). Ten females were used for each treatment. Furthermore, newly laid eggs and eggs at the beginning of the embryonic development were collected, water-washed and floated in 500 mL of water or 500 mL of COase at the same

TABLE 1. Effects of COase on reproduction of boll weevil female.

Doses of COase on diet (μg.mL^{-1})	Fecundity (nº eggs/cycles)		Eclosion (nº eggs/cycles)		Reproductive period (days)		nº eggs/day	
	AD	ND	AD	ND	AD	ND	AD	ND
Control	379 ± 48*	266 ± 30	128 ± 14	117 ± 17	57 ± 4	56 ± 5	6.5 ± 1.0	5.0 ± 0.4
41	254 ± 36	200 ± 8	98 ± 9	58 ± 11	63 ± 6	57 ± 7	4.1 ± 1.2	3.5 ± 0.4
53	198 ± 28	156 ± 10	68 ± 4	48 ± 5	50 ± 3	46 ± 7	4 .0 ± 1.3	3.0 ± 0.3
59	244 ± 13	101 ± 6	54 ± 6	39 ± 6	44 ± 3	42 ± 2	5.6 ± 0.9	3.0 ± 0.4
CV (%)	5.6	3.5	4.7	9.0	17.7	28.9	22.4	36.9

*Mean ± standard error. AD- artificial diet; ND- natural diet; CV- Coefficient of variation.

concentration above for 30 min and then rinsed with water. After this, eggs were fixed in 0.1 mM sodium cacodylate buffer (pH 7.3) containing 2% glutaraldehyde, 4% paraformaldehyde and 5 mM calcium chloride and post fixed in a solution of 2% osmium tetroxide + 1.6% potassium ferricyanide. The samples were then dehydrated in an acetone gradient and embedded in Spurr. Semi-thin 500 nm sections were obtained on a Leica Ultracut microtome and stained with toluidine blue for observations under light microscopy. Twenty five eggs were used for each treatment.

Statistic Analysis

The data obtained from both the artificial and natural diet conditions were submitted to variance analyses using the PROC GLM from SAS (2000). The analyses of the fecundity and eclosion data were made using a logarithmic scale in order to obtain homogeneity of the residual variance. The estimates of the differences and their confidence intervals (CI), between the control and other doses, were back transformed to original scale, thus giving the estimates and their CI in percentages.

RESULTS AND DISCUSSION

Influence of COase on the fecundity

When cholesterol oxidase was supplied in the artificial or natural diet to adults, egg production, hatching and reproductive period were significantly affected (Table 1). In all instances, a dose-dependent effect was observed due to COase action.

On average, fecundity was reduced to 43% ($CL_{95\%}$: 37; 67) and 39% ($CL_{0.95}$: 16; 55) in the females fed on enzyme-treated buds and artificial diet, respectively, in relation to the control. Fecundity decreased linearly in the interval from 0 (control) to 59 mg.mL^{-1} of COase in females fed on artificial ($F_{(1,19)}$ = 11.2 , P = 0.0034) and natural diet ($F_{(1,22)}$ = 35.7, P < 0.0001). In addition, eclosion from these eggs also decreased linearly in the interval from 41 to 59 mg.mL^{-1} of the enzyme in artificial ($F_{(1,13)}$ = 23.8 , P = 0.0003) and natural ($F_{(1,22)}$ = 34.4 , P < 0.0001) diets and eclosion was reduced by 43% ($CL_{0{\cdot}95}$: 28; 57), and 59% ($CL_{0{\cdot}95}$: 44; 70) respectively, indicating that this enzyme affected both fecundity and egg viability (Table 1).

The reproductive period was linearly decreased ($F_{(1,13)}$ = 9.7 , P = 0.0082) in the interval from 41 to 59 mg.mL^{-1} of COase in treatments with artificial diet but no significant difference was verified in treatments with natural diet, ($F_{(3,20)}$ = 1.57, P = 0.2278).

In relation to the daily number of eggs laid, it was verified that the control females laid approximately 2.0 eggs (CL $_{0{\cdot}95}$: 0.8 : 3) more than those submitted to the artificial diet with COase. In the natural diet, a similar behavior was observed, with approximately 1.9 eggs (CL $_{0{\cdot}95}$: 0.6 : 3).

The effect of COase on the boll weevil fecundity is shown in Fig. 1. Control and treatment displayed curves with similar shape, but the initial number of eggs laid by the enzyme-treated group was about half of that of the control and the reproductive period was two weeks shorter in treated females.

Since the insects ingested COase orally, the first symptoms are expected and have been reported to occur within the digestive system, where the cholesterol of the membranes of the midgut epithelium is oxidized by COase (PURCELL et al., 1993, SANTOS et al., 2002). In addition, the enzyme acts also on the female reproductive organs and egg viability (VANDERZANT and RICHARDSON, 1964; EARLE et al.; 1965; GREENPLATE et al.; 1995; SANTOS et al.; 2002). Since cholesterol is an important component of the cellular membranes and a precursor of insect ecdysteroids which are essential for the insect reproduction, it is quite possible that oxidation of some essential steroids by cholesterol oxidase is responsible for the effects observed in this work. However, the dramatic morphological alterations of ovary size, ovarioles and fat body might simply be associated with the inability to absorb enough nutrients, according to Greenplate et al. (1995).

The alterations described here are not permanent and certainly do not have any effect on the genomic level since offspring (F_1) resulting from females fed on artificial diet containing 59 mg/mL of COase do not show altered physiological and morphological characteristics (data not shown). Also, production and eclosion rate of eggs laid by these F_1 females did not differ from that of offspring from control females.

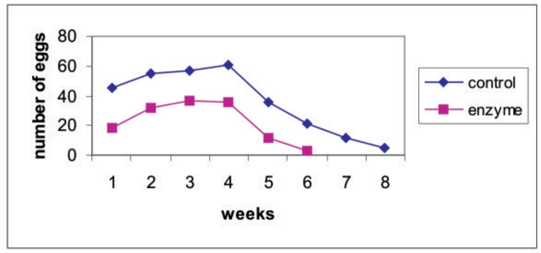

FIG. 1. Fecundity of boll weevil females fed on cotton buds (control) and artificial diet (53 mg/mL of COase *ad libitum*).

Histological observations

Besides reduced ovary development in COase-fed females (GREENPLATE et al., 1995), no apparent histopathological changes were observed between control and COase-fed female oocytes (Fig. 2 A and B). On the other hand, pronounced differences were verified in the chorion and vitelline membrane of enzyme-treated eggs (Fig. 2D and F) in relation to control (2C and E). Both exochorion and endochorion were condensed in eggs treated with COase (2D); the space between the vitelline membrane and chorion was also irregular and reduced. Rupture of vitelline membrane was verified in c.a. 60% of eggs at beginning of embryonic development (Fig. 2F).

Insects are unable to synthesize steroids and the insect moulting hormone ecdysone, a steroid, is probably derived from cholesterol or some related steroid ingested with the diet (CHAPMAN, 1982). This prothoracic hormone that controls apolysis as well as ecdysis has been shown, at least for Diptera, to influence the development of the vitelline membrane (RAIKHEL and LEA, 1982). Although in Coleoptera an ecdysone-dependence of the vitelline membrane genes has not been reported, a similar dependency might exist in this group since the rupture of the vitelline membrane reported here, could be associated to a probable access of COase to ecdysone and thus to

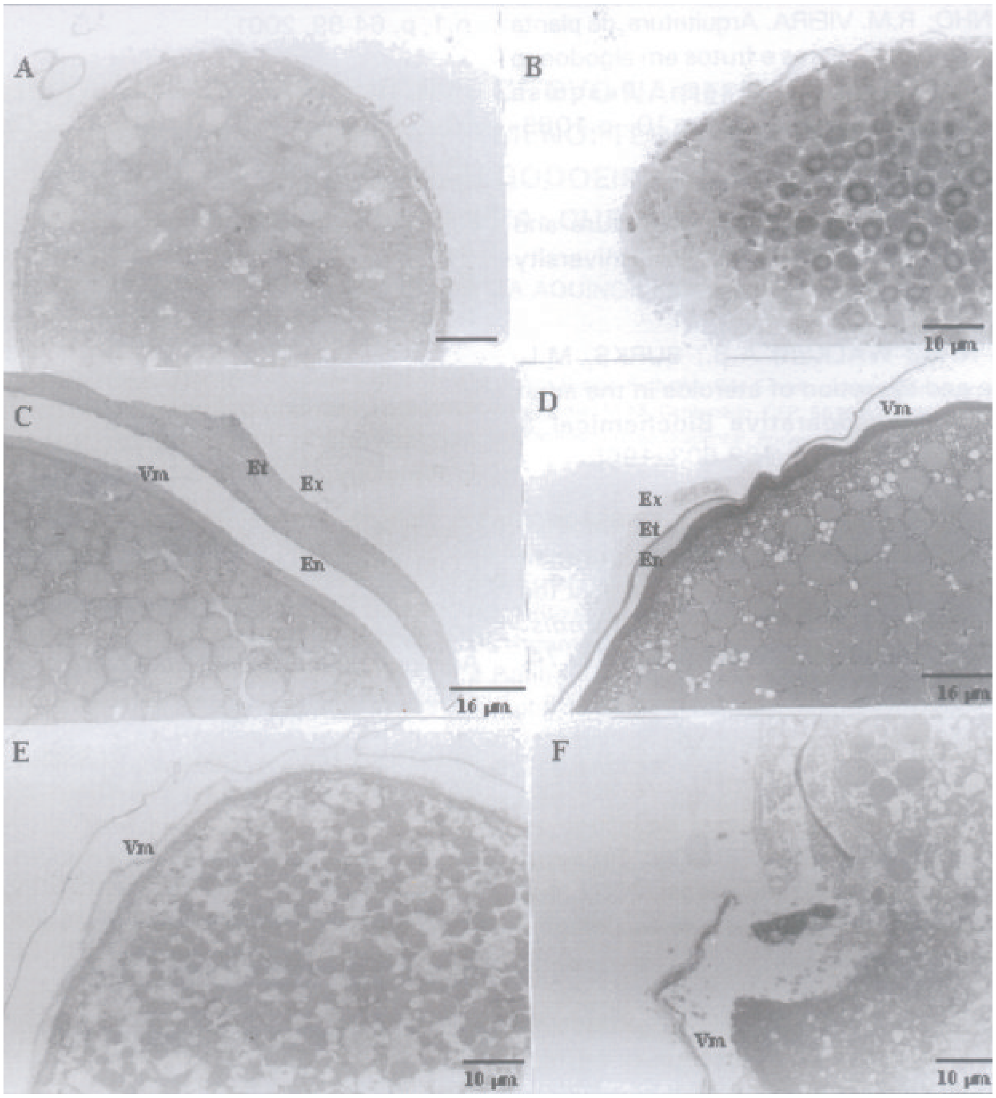

FIG. 2. Semi thin section of oocytes, ovules and eggs at beginning of embryonic treated with COase. A, C and E: controls of oocyte, ovule and eggs; B, D and F: the same material after enzyme treatment. Coloration: Toluidine Blue. Vm: vitelline membrane; En: endochorion, Ex- exochorion, Et- extrachorion.

incomplete or disturbed vitellogenesis. However, further investigations are necessary to clarify this point.

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