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Histological and Histochemical Changes by Clove Essential Oil Upon the Gonads of *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae)

Alteraciones Histológicas e Histoquímicas Provocadas por el Aceite Esencial de Clavo de Olor en las Gónadas de *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae)

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SUMMARY: *Spodoptera frugiperda* is a polyphagous insect that causes economic losses to several crops in Brazil and is the major obstacle to corn production. Researches focusing on alternative control, e.g. botanical products are expanding to offer a wide variety of molecules that interfere with different biological parameters of insect pests. Thus, this study tested the hypothesis that clove essential oil affects the spermatogenesis, ovarioles histochemistry and the fertility of *S. frugiperda.* The results showed that clove essential oil affects the gametogenesis of *S. frugiperda* ovarioles, reflecting negatively on its reproduction, proving to be a promising tool for controlling this pest.

KEY WORDS: Fall armyworm; *Spodoptera frugiperda*; Reproduction; Histochemistry; Histology; Syzygium aromaticum.

INTRODUCTION

The importance of the fall armyworm, *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae), is not only due to its damage, but mainly to the difficulties in its control, which demands high number of synthetic insecticides applications, such as pyrethroids, organophosphates and urea derivatives (Cruz *et al*., 1999). The continued use of such products, besides several side effects, including biological imbalances, death of pollinating insects and food residues; is leading this pest to develop a pre-adaptive and hereditary resistance, increasing the need to investigate promising alternatives control that minimize the adverse effects of synthetic insecticides (Lima *et al*., 2009).

The search for new control methods includes the use of secondary plant metabolites, as several substances derived from plants have shown efficacy in pest control, exertingdifferent biological effects, such as repellency, feeding and growth inhibition, changes in the hormonal system, morphogenetic changes, disturbances in sexual behavior, sterilization, mortality in immature or adult stage, reduced fecundity and fertility, among others (Isman, 2006; Gallegos & & Maroneze, 2009).

Several essential oils with insecticidal properties have been evaluated on biological, physiological and behavioral parameters upon moths of the genre Spodoptera demonstrating the potential of these substances on pest control. Among the essential oils investigated, the clove, Syzygium aromaticum (Myrtaceae), is majorly constituted of Eugenol, an aromatic compound exhibiting proven antibacterial, antimycotic, antiinflammatory, anesthetic, antiseptic, antioxidant, allelopathic, repellent and insecticide activities (Oliveira *et al*., 2007; Silvestri *et al*., 2010). However, researches that focus on histopathological changes in the gonads of insects are quite restricted to synthetic insecticides (Shehata *et al*., 2006; Habluetzel *et al*., 2007; Senthil Nathan *et al*., 2008), and when addressing to essential oils, are restricted to the use of azadirachtin and Piper hispidinervum (Abdel-Rahman *et al*., 2004; Alves *et al*., 2014).

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Therefore, this study aimed to evaluate the effects of clove essential oil, in sublethal concentrations, on the spermatogenesis and histochemistry of *S. frugiperda* ovarioles, thereby determining potential interferences upon its fertility, which may lead to reduced population and consequently, reduced damage to the target crop.

MATERIAL AND METHOD

Our study was conducted at the Histology Laboratory of the Department of Animal Morphology and Physiology and the Agricultural Entomology Laboratory in the Department of Agronomy at the Rural Federal University of Pernambuco (UFRPE).

Insect Rearing. Larvae of *S. frugiperda* were obtained from the rearing stock at the Agricultural Entomology laboratory and maintained at 25.2±1.4 °C, relative humidity 67±0.7% and a photoperiod of 12 h. The larvae were fed with leaves of AG 1051 double hybrid corn. The corn plants were cultivated in a greenhouse, two plants/5l pot with soil mixed with worm humus in the proportion of 2:1 plus 12.13g of NPK (4:14:8 formulation).

Obtaining Essential Oil. The clove was purchased from commercial houses of spices in the city of Recife, PE, and at the Laboratory of Bioactive Natural Products, Chemistry Department (UFRPE), was submitted to hydrodistillation for 2 h. The essential oil was extracted using a Clevenger type apparatus. After draining the water, the extract was dried using anhydrous Na_2SO_4 and stored at low temperature in a dark, hermetically sealed container.

Phytotoxicity Test. A preliminary trial was carried out to test the phytotoxicity of clove oil, using concentrations of 1000, 500, 250, 125, 60, 50 and 30 mg/100 ml distilled water. Ten pieces of corn leaf, approximately 8.0 X 4.5 cm, were used for each concentration; each piece represented a repetition. The leaves were dipped for 10 sec in the test emulsions, then dried on a paper towel. The phytotoxicity level for each piece of leaf was assessed according to the size of burned areas, and classified as mild, moderate or severe using a percentage scale for phytotoxicity adapted from Frans *et al*. (1986). The mild phytotoxicity level was used for testing the bioactivity of clove oil.

Bioassays. Pieces of leaves of 20–40-day-old corn, approximately 6.0 X 4.5 cm, were immersed in either the 30 or 50 mg/L clove oil. The control leaves were immersed in DMSO only, 2 ml in 98 ml distilled water. After soaking for 10sec, each leaf piece was allowed to dry and offered to 10-day-old *S. frugiperda* larvae (3rd instar; average weight of 78.15 mg) for 48 h, long enough for them to consume the entire leaf. These larvae subsequently were fed with pieces of untreated leaves, replaced daily, until the pupa stage. Each treatment consisted of 150 individual larvae in 80 plastic containers with threaded lids. The testes from 10 male insects were collected at the last larval instar stage. Ovarioles were collected from 10 females 24 h of emerged larvae. The remaining adults were fed with a 10% honey solution, replaced daily, and the total number of eggs and viable eggs were counted. All treatments were carried out in a chamber at 25.2±1.4 °C, relative humidity 67±0.7% and a photoperiod of 12 h. Bioassays were carried out in duplicate.

Gonad histology and histochemistry. The gonads were fixed in 10% formalin for 24 h after collection. They subsequently were dehydrated through an ethanol series (70, 80 and 95%) for 10 min each, and impregnated with and embedded in Historesin. The blocks were sectioned using a Minot microtome (LEICA RM 2035) and sections were stained with toluidine blue and Mallory trichrome to detect connective tissue, periodic acid-Schiff (PAS) to detect neutral carbohydrates, and bromophenol blue to detect proteins. Stained sections were examined using a light microscope (Olympus BX-49). Photos were taken with a camera (Olympus BX-51) mounted on the microscope.

Total number of eggs and number of viable eggs. Each replication consisted of a pair of adult insects kept in PVC

Fig. 1. Phytotoxicity test with corn leaf pieces with clove oil from India in the concentrations of 1000; 500; 250; 125; 60; 50 e 30 mg/L the DMSO. Classification: 1000 e 500 – phytotoxicity severe; 250 e 125 - phytotoxicity moderate; 60, 50 e 30 - phytotoxicity mild.

cages 10 X 15 cm (diameter and height) lined internally with paper for oviposition. Ten repetitions were performed for each treatment. The moths (adult stage of the armyworm) were fed 10% honey solution and kept in an acclimated chamber at 25.2±1.4 °C, relative humidity 67±0.7% and a photoperiod of 12 h. Eggs were collected daily until the end of oviposition and placed in 10 cm diameter Petri dishes; the eggs subsequently were kept under the conditions described above. The total number of eggs and the number of viable eggs were counted. The data were subjected to analysis of variance and the averages were compared at a confidence interval of 95% probability ($p \leq 0.05$) using SAS software (SAS Institute, 2001).

RESULTS

The testes of control larvae were coated with connective tissue with invaginations that formed septa and divisions that delimited four testicular follicles (Fig. 2A). In the apical region of the follicle (germarium) numerous cysts were observed just below the germarium, which is characteristic of the growth zone. In the division and reduction zones (posterior to the growth zone) spermatids whose morphology ranged from oval to spherical were observed, and in the basal region of the follicle, transformation zone, numerous spermatozoids were observed (Figs. 2B–E).

After application of 50 mg/ml of clove oil, the number of cysts in the follicle was reduced, however, the lining and septa of the connective tissue of the testis did not differ

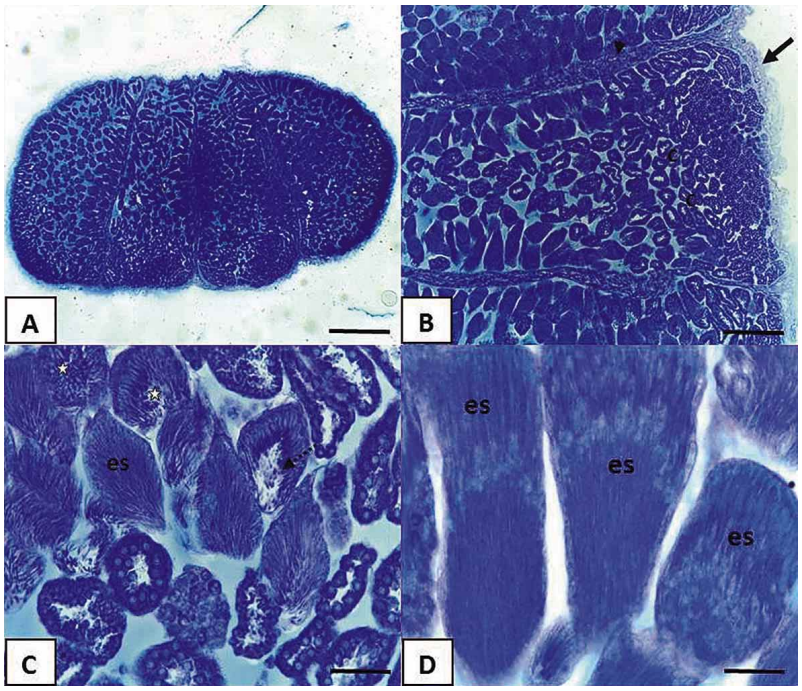

Fig. 2. Testes from *S. frugiperda* larva without treatment. A) Overview of the testes. Toluidine blue, Bar= 200 µm. B) Detail testicular coating; growth zone, reduction, division the transformation, Bar= 100 µm, Toluidine blue. C) Presence of spermatids, Bar= 25 µm, Toluidine blue. D) Bundles of spermatozoids, Bar= 25 µm, Toluidine blue. Arrow - connective tissue, c- cysts; t- tracheoles, arrowhead – connective tissue septum, star – oval spermatids, dashed arrow- spherical spermatids, es – spermatozoids.

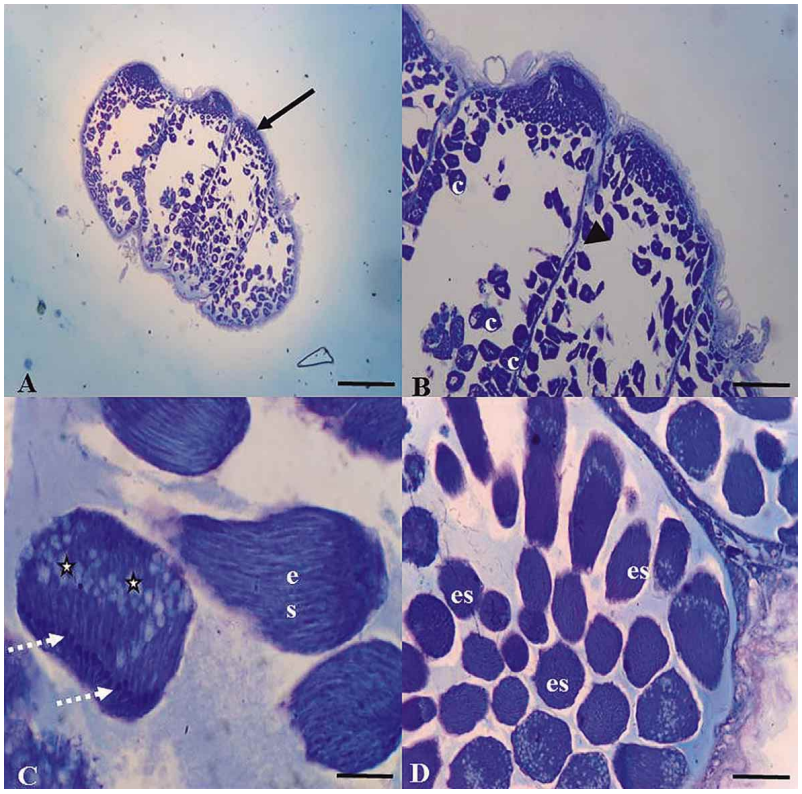

Fig. 3. Testis of *S. frugiperda* larva treated with 30 mg/L clove oil. A) Overview of the testis, Bar= 200 µm, Toluidine blue. B) Testicular coating detail, Bar= 100 µm, Toluidine blue. C) spermatids, Bar= 25 µm, Toluidine blue. D) Spermatozoid bundles, Bar= 100 µm, Toluidine blue. Arrow- connective tissue, arrowhead– connective tissue septum, c – cyst, star- oval spermatids, dashed arrow- spherical spermatids, es – spermatozoids.

from control (Fig. 3A–B). Several spermatids and spermatozoids were also observed (Figs. 3C–D).

The testes of control adult insects were coated externally by connective tissue, but it does not penetrate the organ to form septa, and consequently, did not present the follicles filled with spermatozoid bundles. Tracheoles and fat body were associated with the testes (Figs. 4A–B). There was a reduction in the numbers and dimensions of spermatozoid bundles in testes after treatment with either concentration of clove oil (Figs. 4 C–D).

Regardless of treatments, the ovarioles were coated by a thin sheath of connective tissue that covered the follicular cells. The vitellarium region was well developed in all of the treatments with clove oil. Nurse cells, characteristic of a polytrophic ovariole, were also observed among the oocytes in the ovariole (Figs. 5A–D).

The histochemical analyses by bromophenol blue staining revealed reduced protein content in the yolk of oocytes of females treated with the essential oil compared to controls (Figs. 6A and 6C). The PAS reaction also

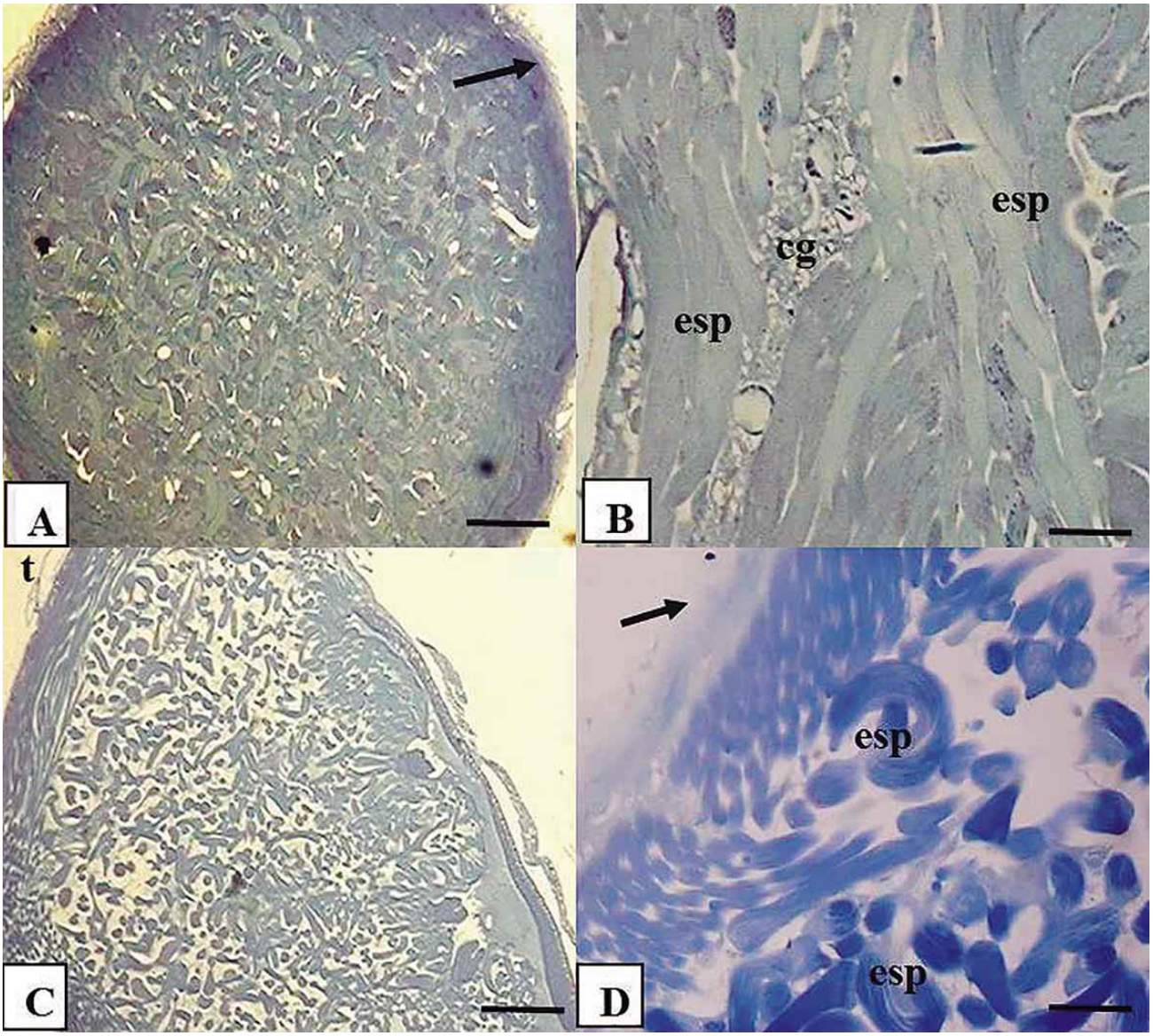

Fig. 4. Testicular *S. frugiperda* adult treated and control. A) General view of the testis without treatment, Bar= 200 µm, Toluidine blue. B) Control spermatozoid bundles, Bar= 100 µm, Toluidine blue. C) Note the abundance of traqueolas testicular surface control, Bar= 200 µm, Toluidine blue. D) fat body and Spermatozoid bundles control. E) testis clover oil treatments with 50 mg/L, Bar= 25 µm, Toluidine blue. Arrow - connective tissue, esp – spermatozoids, t – tracheoles, cg– fat body.

showed a reduction in the neutral carbohydrate content in the yolk of the larvae treated with 50mg/L of clove oil, when compared to the control (Figs. 7D–F).

It was verified that in the tested concentrations, clove oil caused a significant reduction in the total number of eggs compared to the control, significantly affecting viability and the number of hatched larvae (Table I).

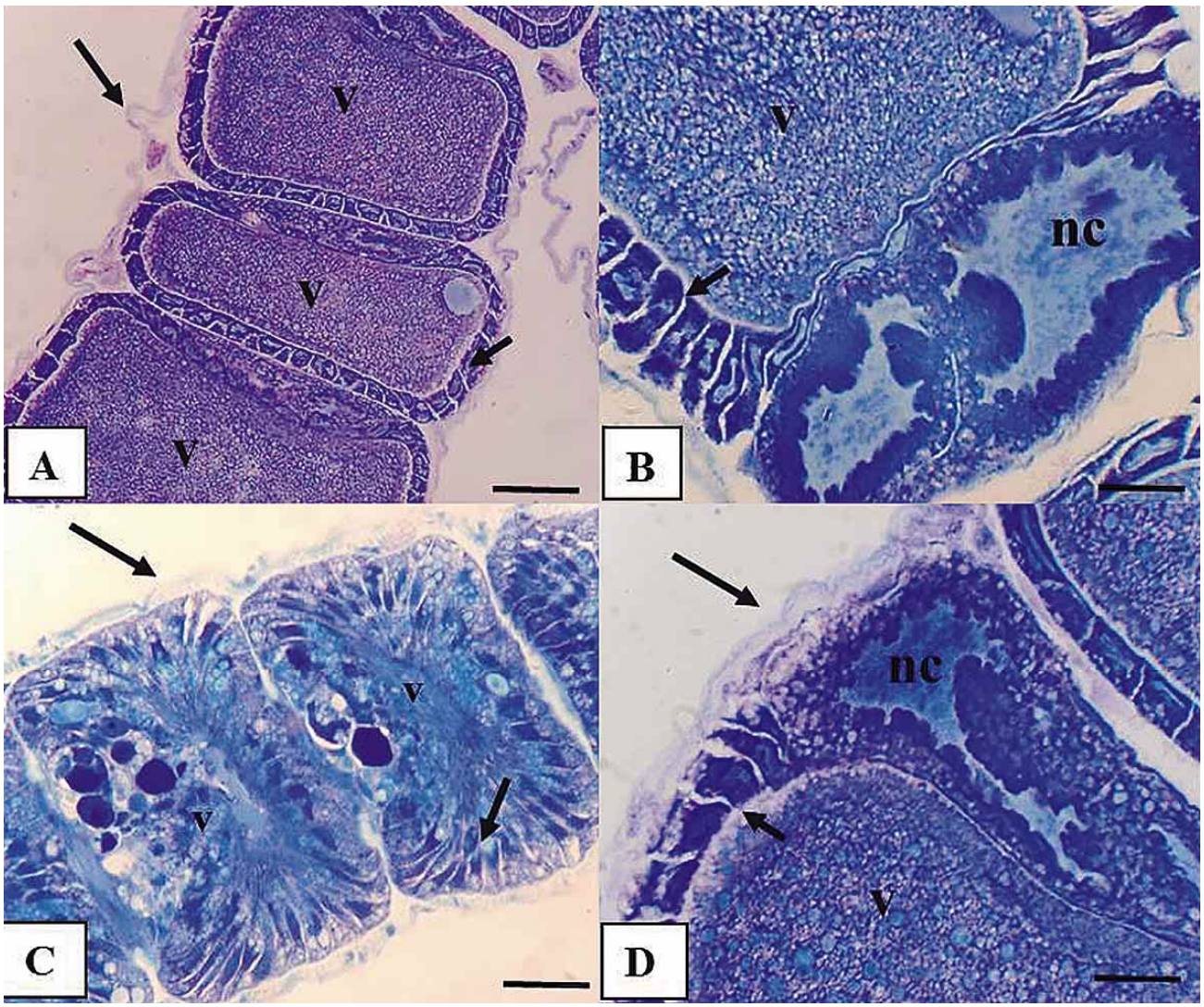

Fig. 5. Ovariole of a *S. frugiperda* adul: A and B) control, Bar= 100 µm and 25 µm, respectively, Toluidine blue. C and D) Treated with clover oil concentration of 50 mg/L. Bar= 100 µm, Toluidine blue. Arrow - sheath of connective tissue, short arrow – follicular cells, v – calf, cn – nurse cell.

Table I. Average total numbers of eggs and hatched eggs of *Spodoptera frugiperda* 3rd instar larvae treated with clove essential oil. Temp: 25.2±1.4 °C, RH: 67±0.7% and 12 h photophase.

Treatment (n= 3)[1]	Eggs (±SE)[2]	Hatched (±SE)[2]
Control	1604.8±235.63 [a]	97.2±0.37 [a]
Clove 50 mg/L	790.4±113.14 [b]	87.4±1.43 [b]
Statistics [P]	13.38 [0.0001]	24.38 [0.0001]

1N = replication numbers.
2Averages followed by the same letter do not differ by t test at 5% probability.

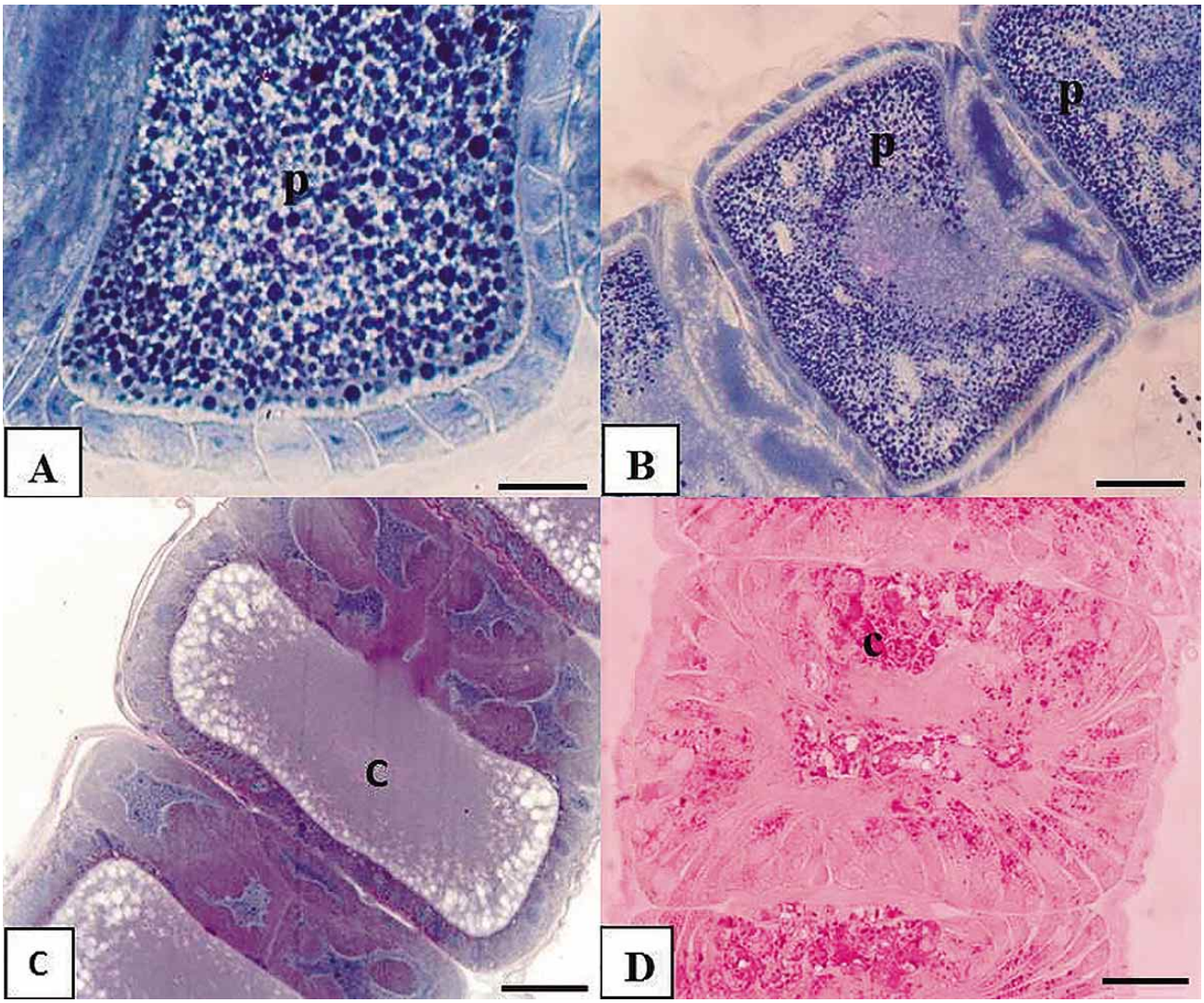

Fig. 6. Terminal portion of ovariole in *S. frugiperda* adults: A) Control, Bar= 100 µm, Bromophenol blue. B) Clover with dosages 50 mg/L. C) Ovariole control submitted to the P.A.S., Bar= 100 µm. D) P.A.S. ovarioles the treatment with India clove oil on concentration 50 mg/L. p- protein and c – carbohydrate.

DISCUSSION

The use of plants with insecticidal properties have been applied as a viable and attractive alternative in integrated pest management programs, mainly, due to the range of effects over several biological, physiological and behavioral parameters of the target insects (Isman; Gallegos & Maroneze).

The influence of the major compounds of clove oil with insecticidal properties (Raina *et al*., 2001), have also shown secondary effects in *S. frugiperda*, characterized by the interference in the spermatogenesis process by reducing the number of eggs laid. Similar results were found by Birah *et al*. (2010) and Alves *et al*. when using the extract of clove and long pepper, exhibiting a juvenoid action capable of affect the fertility and fecundity of Spodoptera litura (Lepidoptera: Noctuidae) and *S. frugiperda*, respectively. Moreover, similar effects were observed for the concentration of 50 mg/L of clove oil in this study. The reduced number of eggs and their viability in insects submitted to the clove oil, is probably associated with eating disorders, leading to negative effects on reproduction (Milano *et al*., 2010).

According to Al-Jahdali & Bisher (2007), Sayim (2007) the presence of vacuoles in cyst cells of testicular follicles in the larvae treated with clove oil suggests degeneration and reported that changes in membrane physiology can cause cytoplasmic vacuoles. As for the histochemical changes observed in females, concerning the protein content and neutral carbohydrates in ovarioles, it directly affects negatively the vitelogenesis for reproduction (Gillott, 2005; Sharma *et al*., 2011).

Physiological changes found in the reproductive system of the treated insects presumably are related to the deterrent property of clove, related to chemoreceptor substances, capable of blocking the phagostimulants, thus inhibiting feeding (Mordue & Nisbet, 2000). Furthermore, the deterrence effect may result in morphological and physiological changes on the reproductive system, thus compromising the reproductive activities, since its development is exclusively dependent on the nutrients acquired in the immature stage for *S. frugiperda* (Milano *et al*.).

According to Costa *et al*. (2004) and Milano *et al*., the reduced number of eggs and their viability are important effects of essential oils on the reproduction of insects, because decreased reproduction rates usually are associated with eating disorders and nutritional deficiency. Engelman (1998) stated that the number of ovarioles could be altered by the quantity and quality of nutrients and secondary metabolites available to the ovarioles during differentiation, which would cause changes in vitellogenesis, ova maturation and egg production.

The results demonstrate that the use of clove essential oil in the 50 mg/L concentration affects spermatogenesis and the histochemistry of *S. frugiperda* ovarioles, reflecting in its reproduction, proving to be a promising option in controlling this pest, since reproduction is an important tool in population dynamics, being a success factor modulator of insect pests in crops.

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RESUMEN: *Spodoptera frugiperda* es un insecto polífago que causa pérdidas económicas a varias cosechas en Brasil y es el mayor obstáculo para la producción de maíz. Este estudio está centrado en el control alternativo, con productos botánicos que se están expandiendo y ofrecen una amplia variedad de moléculas que interfieren con diferentes parámetros biológicos de plagas de insectos. Por tanto, se puso a prueba la hipótesis de que el aceite esencial de clavo de olor afecta la espermatogénesis. La histoquímica de los ovarioles y la fertilidad de *S. frugiperda*. Los resultados mostraron que el aceite esencial de clavo de olor afecta la gametogénesis de los ovarioles en *S. frugiperda*, lo que incide negativamente en su reproducción, demostrando ser una herramienta prometedora para el control de esta plaga.

PALABRAS CLAVE: Gusano cogollero; *Spodoptera frugiperda*; Reproducción; Histoquímica; Histologia; Syzygium aromaticum.

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