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Karyomorphological effects of two new oil formulations on *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae)



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ABSTRACT

The cotton bollworm *Helicoverpa armigera* is a serious pest of many economically important crops. Since this pest has become resistant to the conventional synthetic insecticides, newer compounds and formulations are being developed against this insect pest. Many natural compounds isolated from the plants were tested against this pest. Among them *Hyptis suaveolens* and *Melochia corchorifolia* showed insecticidal properties against *H. armigera*. Based on bioefficacy studies, caryophyllene and β -sitosterol were isolated from *H. suaveolens* and *M. corchorifolia* respectively. The isolated natural compounds were further developed as formulations in various combinations with neem (*Azadirachta indica*) and karanj (*Pongamia pinnata*) oils. The present study indicated that the formulations influenced the karyomorphology of *H. armigera*.

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1. Introduction

The cotton bollworm *H. armigera* is a highly polyphagous pest and excessive feeder, finally affecting the crop yield. This pest attacks most of the cultivated crops in the Indian subcontinent; also it causes economic losses up to 50%. Most of the chemicals used to control this pest have different modes of action. Due to the continuous application of the synthetic chemical pesticides, the insects have developed resistance to insecticides (Tossou et al., 2019). Hence, more studies are conducted against this insect pest with biological products which are safer environmentally.

Many plant products showed larvicidal (Baskar et al., 2018a; Ahsaei et al., 2020; Pavela et al., 2020; El-Sayed et al., 2020), feeding deterrent and growth inhibitory activities (Baskar and Ignacimuthu, 2012; Baskar et al., 2014). The plant products altered the histology of *H. armigera* (Packiam et al., 2013) and *Spodoptera litura* (Baskar et al., 2015); they affected proteins and enzymes (Baskar et al., 2014; Baskar et al., 2018a).

Melochia corchorifolia showed antibacterial (Pullaiah, 2014) and feeding deterrent (Pavunraj et al., 2012) activities. Peerzada (1997) reported that 1, 8 cineole, caryophyllene and sabinene were the main constituents of this plant. Raja et al. (2005) identified two potential antifeedant compounds, namely 5-keto-pent-3, 4-enyl-2'phenol and 5-penty-methylene oxy-4, 4-dimethyl-cyclohexenol from *H. suaveolens*. Plant derived product Ponnem affected the DNA of *H. armigera* which was confirmed by comet assay (Packiam et al., 2015). Two new oil formulations, namely neem oil, karanj oil and caryophyllene isolated from ethyl acetate extract of *Hyptis suaveolens* and neem oil, karanj oil and β -sitosterol isolated from ethyl acetate extract of *M. Corchorifolia* showed pest control properties at the laboratory condition (Pavunraj et al., 2016).

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The aim of the present study was to evaluate the effects of the above two formulations on the karyomorphological changes in *H. armigera*.

2. Material and methods

2.1. Plant materials

The leaves of *H. suaveolens* and *M. corchorifolia* were collected in and around Chennai, India and identified by a botanist at Department of Botany, Loyola College, Chennai, India

2.2. Insect culture

The *Helicoverpa armigera* was obtained from cultivated crops from Thiruvalluar, India. They were brought into laboratory and reared on bhendi fruit up to larval period. Once the larvae became pupae, they were placed in the cage for adult emergence; the emerged adults were fed with 10% honey solution. The adults laid eggs and larvae emerged from them; the hatched larvae were fed with artificial diet. The insects were maintained for many generations at laboratory (Baskar and Ignacimuthu, 2012).

2.3. Isolation of compounds

The ethyl acetate extracts of *H. suaveolens* and *M. corchorifolia* were subjected to fractionation. 15 and 13 fractions were isolated from respective plants. Based on the feeding deterrent activity fraction 2 of *H. suaveolens* and fraction 9 of *M. corchorifolia* were found to be active against insects. From these active fractions caryophyllene and β -sitosterol were isolated from *H. suaveolens* and *M. corchorifolia* respectively (Pavunraj et al., 2016).

2.4. Oils

The neem (*Azadirachta indica*) and karanj (*Pongamia pinnata*) oils were purchased from oil mills in Dindigul district of Tamil Nadu.

2.5. Preparation of formulations

Various combinations of neem and karanj oils with the isolated compounds were prepared in different ratios individually; based on their bioefficacy two effective formulations were selected for this study. They were: Formulation 1 – (Neem oil 4.45 ml + karanj oil 4.45 ml, emulsifier (UNITOP chemicals) 1 ml, stabilizer (DMA-NE) 0.1 ml Caryophyllene (12.25 mg)), Formulation 2 (Neem oil 4.45 ml + karanj oil 4.45 ml, emulsifier 1 ml, stabilizer 0.1 ml and β -sitosterol (12.25 mg). Control (emulsifier 1 ml, stabilizer 0.1 ml and distilled water 8.9 ml) was also maintained (Pavunraj et al., 2016).

2.6. Treatments

Cotton leaf discs were dipped individually in 0.3% concentration of Formulations 1 and 2, along with control for about 30 s and then air dried. The treated leaf discs were placed into Petri dishes which contained pre wetted paper (filter) to avoid early drying of leaf disc. Pre starved (2 hrs) *H. armigera* larvae were released into control and treatment leaf discs. All the treatments were replicated 3 times ($10 \times 3 = 30$); each Petri dish contained single larva. After 24 hrs of treatment the larvae were subjected to karyomorphological observations.

2.7. Karyomorphology

After 24 hrs of treatment, the karyomorphology of the insect was carried out following the method of Murakami and Imai (1974) with slight modification. The gonads and brain tissues from male and female insects were dissected out in 0.7% sodium chloride (Insect saline) in which 0.1 ml of 0.05% Colchicine was added and left for three hours. The tissues were transferred to 1% potassium chloride solution for 15 min. Then the tissues were stored in refrigerator in acetic acid and methanol mixture (1:3) for 10 min. The tissues were then teased in 2 ml of acetic acid and methanol mixture (1:3) into suspension (Sample) in an embryo cup. Then tissue was dropped on to clean grease free pre-warmed glass slide at 45 °C and the excess sample was drawn off with a fine Drummond pipette. After 24 hrs of air-drying, the slides were stained with 3% Giemsa diluted in Sorrsenson's phosphate buffer (pH-6.8) for 30 mins. After staining, the slides were subjected to air drying for 24 hrs. Then the slides were screened for metaphase plates. Metaphase plates with good karyomorphology were microphotographed under high magnification oil lumens 100X.

3. Results

The karyomorphology of the larval cells of *H. armigera* revealed that a diploid number ($2n = 60$) was observed in both testicular and ovarian cells at mitotic division. Diverse karyomorphological variations such as condensation, short rods, small spheres or tots etc had been observed at the metaphase. The microscopic observation disclosed both smaller and larger contoured karyomorphology. The banding pattern was indistinguishable due to clustering.

An average of about 100 spermatogonial metaphase spreads were observed from larvae of *H. armigera* and it confirmed a diploid model number of 60 chromosomes ($2n = 60$) in 65% of the spreads. Besides, a few instances of varying diploid number were also observed. The metaphase spread apparently showed chromosomes with distinct configuration (Fig. 1A & B). Some chromosomes were characterized by elongated morphology with darkly stained heterochromatin region (HCR); besides secondary constriction (SC) was noticed in some chromosomes and there were Z chromosomes, common in lepidopterans in general. Most of the chromosomes were found to be highly condensed due to heterochromatinization. Furthermore the chromosomes observed from control larval sample appeared to have distinct configuration with respective arms and bands. Both in the mitotic and meiotic cells the diploid and haploid chromosomes resembled each other but for the number (Figs. 2A, 2B). By contrast the larvae treated with the two new botanical formulations showed great variation not only in the karyomorphology (Figs. 2A and 2B) but also endoreduplication. Between the two formulations, the first formulation (T1) seemed to have influenced more ploidy than the second formulation (T2) (Figs. 2A and 2B). Most gonadal tissues showed higher degree of polyploidy due to endomitosis or more endoreduplication. In majority of chromosomal spreads (Fig. 2A) some chromosomes with chromatids were found twisted; no display of primary constriction and no indication of localized centromere were noted. The treated chromosomes were also characterized by Nucleolar Organizer (NOR) region (Fig. 2B); the nucleoli in interface nuclei, however, were darkly stained. The diploid complement also contained two such (NOR) chromosomes. The metaphase spreading was surrounded by densely stained inner chromatids in the gamete cells as well. The chromomere pattern of the gametic chromosomes was not as clear as in the mitotic chromomeres. In some metaphase plates, cross shaped end to end configuration was observed.

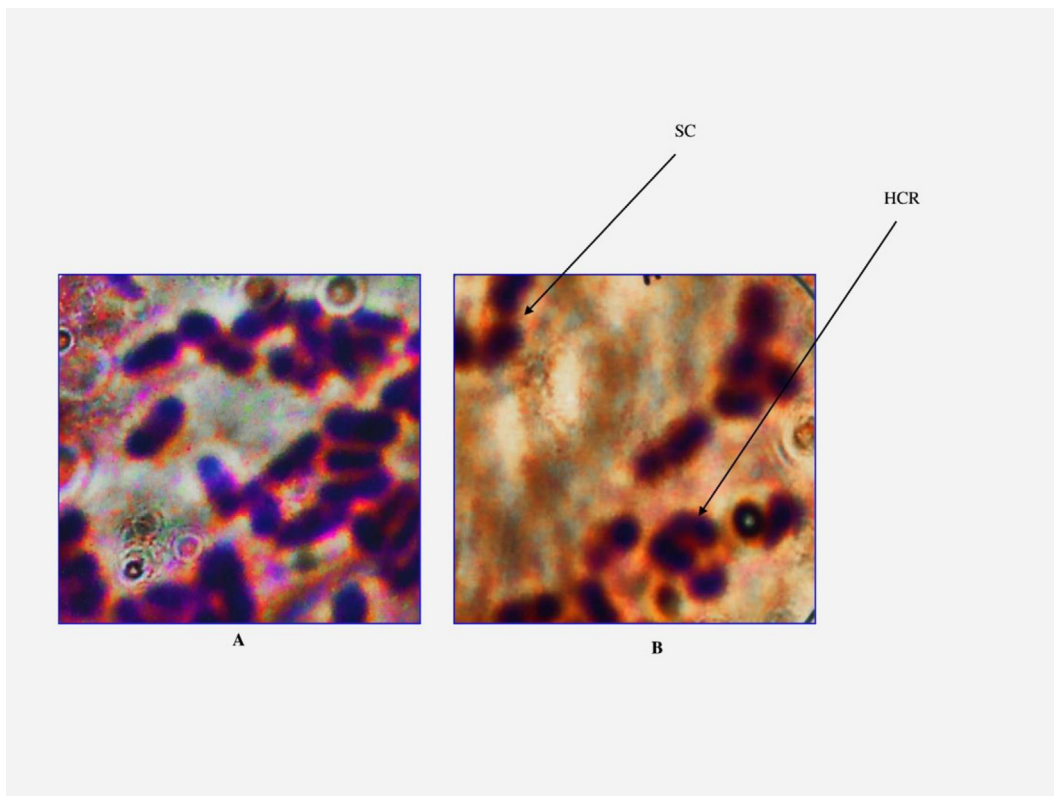


Fig. 1. (A) Diploidy of *H. armigera* obtained from other than sperm cells. (B) Haploid of *H. armigera* obtained from the male gamete. SC = Sister Chromosomes; HCR = Heterochromatin region.

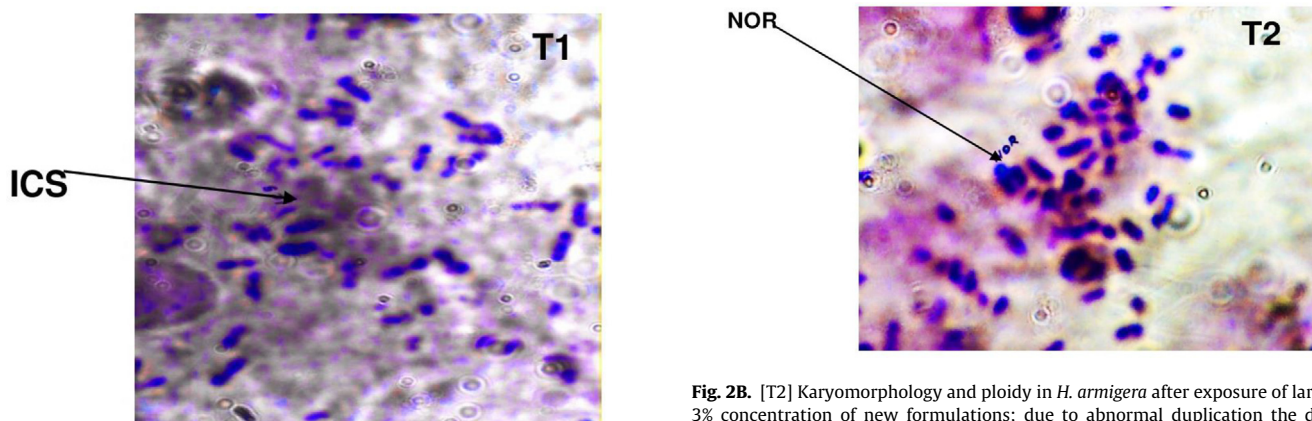


Fig. 2A. [T1] Karyomorphology and ploidy in *H. armigera* after exposure of larvae to 3% concentration of the new formulations; due to abnormal duplication the diploid number exceeds ($2n > 60$). ICS = Inner chromatid structure.

Fig. 2B. [T2] Karyomorphology and ploidy in *H. armigera* after exposure of larvae to 3% concentration of new formulations; due to abnormal duplication the diploid number exceeds ($2n > 60$). NOR = Nucleolus Organizer Region.

Male meiosis was ideal for counting the chromosome number in *H. armigera* because it showed maximally condensed to double ball structures during reduction division. The chromatid topology of such chromosomes showed variation in the treated larval samples. The kinetic organization of meiotic chromosomes appeared to be markedly different from the mitotic chromosomes. The discrepancy between the treated and untreated chromosomes could be resolved based on the mechanical stress applied to the anaphase-moving chromosome coupled with multiple insertion sites due to the treatment with the botanical formulation.

4. Discussion

Depicting a well spread metaphase chromosomes deserves due standardization. In the present investigation the chromosomology of *H. armigera* necessitated the modification of the method of Narang and Gupta (1970) in which the concentration of potassium chloride (5%) as hypertonic solution facilitated the swelling of the cells of the testis and gametes. However, incomplete metaphase spreads are also possible due to mincing procedure (Narang and Gupta, 1970) and centrifugation (Kawazoe, 1987). The above method coupled with 0.05% colchicine at 4 hrs exposure was helpful to get well spread chromosomes. Furthermore the

preparation made out of the follicular cells of testis gave better results than ovarian cells. Changes in the morphology of chromosomes in terms of deletion, duplication etc., can be reciprocal to external pressures (Banno et al., 1995). The characterization of the chromosomes in *H. armigera* has been difficult task due to larger number of chromosomes and smaller size with holocentric nature. A few chromosomes of *H. armigera* also appeared as short rods (or) spherical bodies or as tots. Even at the onset of anaphase, the two daughter chromosomes aligned parallel to one another or towards equatorial plane as the chromosomes moved towards poles. Such chromosomes were believed to have a diffused centromeric structure which is not localized but spread to the entire length of chromosomes. Hence they are called holocentric chromosomes (White, 1973). The centromere in the chromosomes is distinguished as primary constriction and the other stained region as secondary constriction and are associated with the nucleolus as reported in many groups of insects as Nucleolar Organizer Region (NOR). The localization on NOR is found to be relatively constant in many organisms. In many insects, the males normally have heteromorphic pairs (XX) in lepidopterans. In *H. armigera* the situation is reversed; the female is heterogametic or having heteromorphic chromosomes (ZW), whereas the male has monomorphic chromosomes (ZZ) (Bull, 1983). It has been suggested that in sex chromosomes heteromorphism is a result of accumulation of deleterious mutations. The most probable method of assessing the relationship and divergence of karyotype in species is direct comparison of the dimension and morphology of the species. A comparison on these different metaphase plates confirmed intra specific variation to the model diploid number which is $2n = 60$ and haploid number $n = 30$. Furthermore, a critical examination of 100 metaphases revealed the diversity in relative length indicating that *H. armigera* is conservative species in retaining the chromosome number with difference in the chromosome length. However, the karyotype conservatism could be more aberrant than the real number of chromosome rearrangements as well as considerable amount of interspecific chromosomes changes that could have occurred during the course of evolution, which could not be deciphered due to small size and holocentric nature of chromosomes. Butterflies and moths have small genome with high chromosome number whereas that of lepidopterans are small and lacking the landmarks such as local centromere or C bands, etc., and has been the bottleneck towards the cytogenetic analysis of lepidopterans (Bedo, 1984).

The chromosomes of *H. armigera* evidenced the details of organization towards the identification of nucleolar organization and ascertaining the presence or absence of primary constriction in mitotic chromosomes and presence and absence of local centromer. There is a report on the presence of primary constriction in mitotic in lepidopterans (Rishi and Rishi, 1990). It may be inferred that the botanical formulation did interfere with the chromosomal complement by inducing ploidy in the form of endoreduplication, endo-mitosis, endo-replication and nuclear organizing region. The exact mechanisms in binding of this botanical on the part of chromosomal complement demands further studies; such studies will certainly provide much evidence on the target binding and validation. Although the data have been interesting, the interpretation warrants further investigation.

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