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Authors: Carabajal Paladino, Leonela Z., Ferrari, María E., Lauría, Juan P., Cagnotti, Cynthia L., Šíchová, Jindra, et al.

Source: Florida Entomologist, 99(sp1) : 43-53

Published By: Florida Entomological Society

URL: <https://doi.org/10.1653/024.099.sp107>

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The effect of X-rays on cytological traits of *Tuta absoluta* (Lepidoptera: Gelechiidae)

Leonela Z. Carabajal Paladino^{1,*}, María E. Ferrari², Juan P. Lauría³, Cynthia L. Cagnotti⁴, Jindra Šíchová^{1,5} and Silvia N. López⁴

Abstract

The tomato leafminer, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is one of the most important pests of tomato. With the purpose of developing environmentally friendly control tactics such as the inherited sterility (IS) technique against this species, it is essential to understand its genetics and biology. In this paper we analyzed the karyotype, sperm morphology and sperm ratio in wild-type and X-ray irradiated individuals of *T. absoluta*. The diploid chromosome number of *T. absoluta* was $2n = 58$ including the pair of sex chromosomes: ZZ in males and WZ in females, which were the largest elements of the complement. Irradiation of pupae in an X-ray machine with a dose of 200 Gy generated various types of chromosomal rearrangements including translocations and fragmentations, resulting in altered chromosome numbers. The analysis of spermatozoa in *T. absoluta* revealed a significant morphological difference between apyrene and eupyrene sperm bundles. Irradiation with X-ray doses of 100, 150, 200 and 250 Gy did not have a significant effect on the apyrene to eupyrene sperm ratio. However, males irradiated with 300 Gy produced significantly more apyrene sperm than non-irradiated males. All the doses applied influenced the morphology of eupyrene sperm bundles. The modified eupyrene sperm bundles could be used as a bioindicator during the monitoring of an IS program after the release of irradiated males. We found that the modified eupyrene spermatozoa were transferred to the bursae copulatrixes of the females. Males treated with 200 Gy transferred a greater proportion of modified eupyrene sperm than untreated males. The results presented herein provide essential information on the cytology of *T. absoluta*, which is required to evaluate the quality of the released insects, and for better understanding and application of IS against this economically important pest.

Key Words: karyotype; chromosomal aberrations; apyrene sperm; eupyrene sperm; inherited sterility; tomato leaf miner

Resumen

La polilla del tomate *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) es una de las plagas más importantes del cultivo de tomate. Con el objeto de desarrollar estrategias para su control que sean respetuosas con el ambiente, como por ejemplo la técnica de esterilidad heredada (EH), es indispensable entender su genética y su biología. En este trabajo analizamos el cariotipo, la morfología espermática y la proporción de espermatozoides apirenos e irradiados con rayos X. El número cromosómico diploide de *T. absoluta* es $2n = 58$, incluyendo el par de cromosomas sexuales: ZZ en machos y WZ en hembras, que constituyen los elementos de mayor tamaño en el complemento. La irradiación de pupas en un irradiador de rayos X con una dosis de 200 Gy generó diferentes reordenamientos cromosómicos incluyendo translocaciones y fragmentaciones, resultando en alteraciones en el número cromosómico. El análisis de los paquetes de espermatozoides de *T. absoluta* reveló un marcado polimorfismo entre los paquetes de espermatozoides apireno y eupireno. La irradiación de los individuos con dosis de 100, 150, 200 y 250 Gy no tuvo un efecto significativo sobre la proporción de espermatozoides apireno:eupireno. Sin embargo, los machos irradiados con 300 Gy produjeron una cantidad significativamente mayor de espermatozoides apirenos que los no irradiados. Todas las dosis de radiación influenciaron la morfología de los paquetes de espermatozoides eupireno. Estos paquetes deformes podrían ser utilizados como bioindicadores durante el proceso de monitoreo llevado a cabo en los programas de EH luego de la liberación de machos irradiados. Los espermatozoides eupirenos deformes fueron transferidos a las bursae copulatrixes de las hembras durante la cópula. Los machos tratados con 200 Gy transfirieron una cantidad mayor de espermatozoides deformes que los individuos no irradiados. Los resultados aquí presentados proveen información esencial sobre la citología de *T. absoluta*, la cual es necesaria para evaluar la calidad de los insectos liberados y para un mejor entendimiento y aplicación de EH contra esta especie de importancia económica mundial.

Palabras Clave: cariotipo; espermatozoides apireno; espermatozoides eupireno; esterilidad heredada; plaga de tomate

The tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is a neo-tropical species which is considered to be one of the key pests of tomato (*Solanum lycopersicum* L.). It is native to South America, but following its first detection in Spain in 2006 (Urbaneja et al. 2007), it spread very rapidly into Europe, northern Af-

rica and the Middle East (Desneux et al. 2010, 2011; Tropea Garzia et al. 2012). *Tuta absoluta* larvae feed on the mesophyll of the tomato plant, which reduces its photosynthesis, alters its structure, and creates holes that facilitate infections with fungi and bacteria (Colomo & Berta 1995; Pereyra & Sánchez 2006; Botto et al. 2000). As a result

¹Institute of Entomology, Biology Centre CAS, Branisovska 31/1160, 370 05, Ceske Budejovice, Czech Republic

²Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Argentina

³Instituto de Genética "Ewald A Favret", Instituto Nacional de Tecnología Agropecuaria, Argentina

⁴Instituto de Microbiología y Zoología Agrícola, Instituto Nacional de Tecnología Agropecuaria, Argentina

⁵Faculty of Science, University of South Bohemia, Czech Republic

*Corresponding author; E-mail: carabajal@entu.cas.cz

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of direct and indirect damage to the plants, severe yield losses may reach 90–100% (Desneux et al. 2010, 2011). Although the main host plant of *T. absoluta* is the tomato, their larvae can feed on a variety of cultivated and wild Solanaceae species (Mallea et al. 1972; García & Espul 1982; Galarza 1984; Notz 1992; CIP 1996; FERA 2009; OEPP/EPPO 2005, 2009; García Marí & Vercher 2010). The ability to colonize a wide range of non-cultivated species further highlights the pest status of *T. absoluta*, as these plants facilitate its introduction and establishment in new cultivated areas, and act as its reservoirs (Cifuentes et al. 2011).

One control tactic that is very friendly to the environment, and that can be used to reduce the damage of pest species to tolerable levels is the sterile insect technique (SIT). The SIT involves the use of ionizing radiation to sterilize male insects, which are then released into a target area where they will mate and prevent the reproduction of wild females (Knippling 1955). In Lepidoptera, the dose of radiation required to achieve full sterilization is notably higher than in other insect orders (LaChance 1967) due to the holokinetic nature of their chromosomes (LaChance 1967; Murakami & Imai 1974; Wolf et al. 1997; Tothová & Marec 2001). In holokinetic chromosomes, the kinetochore plate covers a significant portion of the chromosome length (Wolf 1996). Hence, most radiation-induced fragments are stable during mitosis and meiosis and can be transmitted through the germ cells to the next generation (Marec et al. 2001; Carpenter et al. 2005).

Higher doses, however, negatively affect the biological quality and mating performance of the irradiated insects when competing with wild individuals in the field (Bloem & Carpenter 2001). These parameters are less affected when the parental generation of moths is exposed to lower sub-sterilizing doses, resulting in a higher sterility in the first filial generation (F_1) (North 1975; LaChance 1985). This phenomenon is called inherited sterility (IS), and has been proved highly efficient for the control of several Lepidoptera species (North & Holt 1969; Proverbs et al. 1978; Carpenter et al. 1987; Carpenter & Gross 1993; Mastro 1993; Bloem et al. 1999, 2001). The F_1 individuals carry chromosomal fragments and translocations that are responsible for the production of genetically unbalanced gametes (Tothová & Marec 2001; Carpenter et al. 2005), and these male individuals also transfer less amount of fertile sperm (Koudelová & Cook 2001).

Most moths and butterflies have a modal chromosome number $n = 31$, which is considered to be the ancestral number for Lepidoptera (Lukhtanov 2000; Van't Hoff et al. 2013; Ahola et al. 2014). Females are heterogametic (ZO or WZ) and males are homogametic (ZZ), although sporadic rearrangements created multiple sex chromosome systems in some species (Traut et al. 2007). Lepidopteran females display a heterochromatic body in the interphase nuclei (reviewed in Traut & Marec 1996) also called sex chromatin body, which is derived from the W chromosome (Traut et al. 2007). The chromosomal aberrations induced by radiation can be seen in changes in the number of chromosomes, and/or aberrant chromosome morphology and quantity of sex chromatin bodies (Tothová & Marec 2001; Carpenter et al. 2005; Makee & Tafesh 2006).

Spermatogenesis in Lepidoptera is dichotomous and produces 2 types of sperm: eupyrene and apyrene (Meves 1903; Friedländer 1983; Kristensen 1984; Hamon & Chauvin 1992; Swallow & Wilkinson 2002; Till-Bottraud et al. 2005). Both types of sperm originate from synchronously dividing testicular cysts and at the end of spermatogenesis the spermatozoa are grouped in bundles. The apyrene sperm bundles dissociate and become motile prior to male ejaculation, whereas eupyrene sperm remain in bundles (Koudelová & Cook 2001) and dissociates immediately after the completion of mating (Seth et al. 2002). Although both types of sperm reach the spermatheca, only the nucleated eupyrene sperm fertilizes the eggs (Friedländer & Gitay 1972;

Drummond 1984; Osanai et al. 1989; Friedländer 1997). The function of the anucleated apyrene sperm is still not clear, and although it is not involved in fertilization, it constitutes 50–90% of the total amount of the transferred sperm (Silberglied et al. 1984). Koudelová & Cook (2001) showed that the apyrene to eupyrene sperm ratio in F_1 males of the Mediterranean flour moth, *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) that were descendants of parental individuals irradiated with various doses of gamma-rays fluctuated between values similar to those observed in wild-type males (9.5:1), and reached up to 100:1 with most frequent ratios around 30:1.

The IS technique may be a suitable control tactic to include in an area-wide (AW) integrated pest management (IPM) approach of *T. absoluta* both in the field and in greenhouses. Cagnotti et al. (2012) studied the impact of different doses of X-rays on the emergence, fecundity, and fertility of *T. absoluta* to determine the dose that would sterilize the female moths but at which the male moths would be partially sterile. However, there are no data on the karyotype of *T. absoluta*, its sperm morphology and the apyrene to eupyrene sperm ratio, and the effect of radiation on these traits.

In this study, we analyzed the karyotype of non-irradiated male and female *T. absoluta*, the impact of X-rays on the chromosome number of the F_1 generation of irradiated males, the morphology and quantity of eupyrene and apyrene sperm bundles in testes of irradiated and non-irradiated males, and the morphology of eupyrene sperm in the bursae copulatrix of non-irradiated females mated with irradiated and non-irradiated males. This work provided some basic knowledge for better understanding the effects of X-ray radiation on *T. absoluta*, the evaluation of the quality of the released insects, and the genetic basis of IS in this species.

Materials and Methods

EXPERIMENTAL INSECTS

The *T. absoluta* moths used in this work originated from the colony maintained at the Insectario de Investigaciones para Lucha Biológica (IILB), IMYZA, INTA (Hurlingham, Buenos Aires, Argentina). The insects were reared on tomato plants for about 32 generations, and the colony was periodically refreshed with wild insects collected in local greenhouses. Experimental moths were kept in a room with controlled environmental conditions at 25–27 °C, 60–85% RH, and 14:10 h L:D photoperiod.

Female and male *T. absoluta* pupae were sexed according to the position of the genital opening (Coelho & Franca 1987). The individuals were irradiated 48–72 h before adult emergence. Pupae were placed in a Petri dish (1.5 cm high and 5 cm in diam) and irradiated with doses of 100, 150, 200, 250 or 300 Gy in a Constant Potential X-Ray System MG 160 Philips (Philips Industrial X-Ray, Hamburg, Germany) at a dose rate of 39.11 Gy/min. Untreated pupae were used as a control group.

CYTOGENETIC ANALYSIS OF NON-IRRADIATED INDIVIDUALS

Spread chromosome preparations were obtained from wing imaginal discs, testes or ovaries of 3rd and 4th (last) instar larvae of *T. absoluta* following the methods of Traut (1976) with slight modifications detailed in Bressa et al. (2009). The preparations were dehydrated in an ethanol series (70%, 80%, and 100%, 30 s each) and stored at –20 °C. They were directly stained with 0.5 mg/mL DAPI (4',6-diamidino-2-phenylindole; Sigma-Aldrich, St. Louis, Missouri, USA) in antifade based on DABCO (1,4-diazabicyclo[2.2.2]octane; Sigma-Aldrich). The preparations were observed under an epifluorescent microscope Leica

DMLB equipped with a Leica DFC350FX CCD camera and Leica IM50 Software, version 4.0 (Leica Microsystems Imaging Solutions Ltd., Cambridge, United Kingdom). In the case of non-irradiated larvae, relative sizes of chromosome pairs in pachytene cells were measured using the computer application MicroMeasure version 3.3 (available at www.colostate.edu/Depts/Biology/Micromasure) (Reeves & Tear 2000).

To analyze the presence of sex chromatin in non-irradiated individuals, Malpighian tubules from male and female *T. absoluta* larvae were dissected in physiological solution (Glaser 1917 cited in Lockwood 1961), briefly fixed in Carnoy fixative (ethanol, chloroform and acetic acid, 6:3:1) and stained in 1.5% lacto acetic orcein. The preparations were inspected under a Zeiss Axioplan 2 microscope (Carl Zeiss, Jena, Germany). Black and white images were recorded with a cooled F-View CCD camera and captured with AnalySIS software, version 3.2 (Soft Imaging System GmbH, Münster, Germany).

CYTOGENETIC ANALYSIS OF THE F_1 GENERATION OF IRRADIATED MALES

According to Cagnotti et al. (2012) 200 Gy is the most appropriate dose for an IS program against *T. absoluta*. Hence, F_1 larvae that were descendants from males irradiated at this dose were used for the karyotype analysis. The larvae were obtained by crossing irradiated males with non-irradiated counterparts. Between 2 to 8 adult couples were placed in each of 15 acetate cylindrical cages each containing a tomato plant following the methods described by Cagnotti et al. (2012). Larvae were randomly picked from each cage and chromosome preparations were made from gonads and wing imaginal discs following the procedure described above for non-irradiated specimens. The preparations were directly stained with DAPI in DABCO, analyzed under a Zeiss Axioplan 2 microscope, and documented using an Olympus CCD monochrome camera XM10 (Olympus Corporation, Tokyo, Japan). A total of 108 chromosome preparations were made (92 from 32 F_1 males, 16 from 6 F_1 females).

APYRENE TO EUPYRENE SPERM RATIO IN IRRADIATED AND NON-IRRADIATED MALES

Immediately after emergence, adults emerging from untreated pupae and pupae irradiated with 100, 150, 200, 250 and 300 Gy were transferred to 70% ethanol in 1.5 mL microtubes and kept at 4 °C. Nine males per dose were studied. Testes were dissected out in physiological solution, transferred into 10 μ L of 60% acetic acid, and carefully ruptured to free the sperm bundles. The cell suspension was stained with 5 μ L of lacto-acetic orcein, covered with a cover slide and sealed with glue. The preparations were checked and photographed using an Olympus BX40 microscope equipped with an Olympus E330 camera (Olympus Corporation). The sperm bundles were classified according to their morphology (identification of organized nuclei and/or presence of micro-nuclei) into normal eupyrene, deformed eupyrene, and apyrene categories. Only those bundles with clear morphology were quantified, each slide was analyzed twice, and the mean values were used for the statistical analysis. The variables, ratio of apyrene to eupyrene sperm bundles, ratio of normal to total sperm bundles, and ratio of deformed to total sperm bundles were analyzed with 1-way ANOVA with one factor (dose) with 6 levels (0, 100, 150, 200, 250 and 300 Gy) in Statistica for Windows (StatSoft 2000). The data for the variable ratio of apyrene to total eupyrene sperm bundles were transformed according to $y = \ln(x)$, and as $y = \ln(x + 0.05)$ for proportion of deformed to total sperm bundles, in order to fulfil the assumption of homoscedasticity. When statistically significant differences were found, the treatments were compared by Fisher's LSD test.

EUPYRENE SPERMATOZOA IN THE BURSAE COPULATRICES OF FEMALES MATED WITH IRRADIATED AND NON-IRRADIATED MALES

Male *T. absoluta* pupae were irradiated with 200 Gy and emerged males were mated with virgin non-irradiated females. After completion of the mating, each couple was frozen at -20 °C. The bursae copulatrices of 8 females that had mated with non-irradiated males and those of 5 females that had mated with irradiated males were dissected in physiological solution, individually transferred into a drop of 60% acetic acid, and dispersed on a hot plate. The slides were directly stained with 0.5 mg/mL DAPI in DABCO, and checked and photographed using an Olympus BX40 microscope and an Olympus E330 camera. The morphologies of eupyrene spermatozoa were recorded, and each spermatozoon was classified as normal, deformed, or uncertain (when the spermatozoon was not well spread and it was not possible to accurately assess its shape). The ratios of abnormal eupyrene spermatozoa, and of eupyrene spermatozoa with an uncertain morphology, with respect to the total amount of transferred spermatozoa were statistically compared by means of 1-way ANOVA with one factor (dose) with 2 levels (0 and 200 Gy) in Statistica for Windows (StatSoft 2000).

Results

CYTOGENETIC ANALYSIS OF NON-IRRADIATED INDIVIDUALS

Mitotic metaphases obtained from wing imaginal discs of non-irradiated male and female *T. absoluta* contained $2n = 58$ chromosomes (Fig. 1a, b). Most of the chromosomes were small sized, except for 2 chromosomes that were noticeably larger (Fig. 1a, b). In oocytes and spermatocytes, the analysis of pachytene nuclei revealed 29 chromosome pairs in both sexes (Fig. 1c, d). In female pachytene complements, the WZ sex chromosome pair was easily identified due to the heterochromatic nature of the W chromosome (Fig. 1c). When the relative size of the chromosome bivalents was compared in females, the 2 largest elements of the diploid complement of *T. absoluta* clearly corresponded to sex chromosomes. This bivalent represented 7% of the complement, while each of the autosomes represented between 2% and 4.5% of the complement. Interphase cell nuclei of Malpighian tubules of *T. absoluta* females contained a heteropycnotic body corresponding to the sex chromatin, which was absent in males (Fig. 1e, f).

CYTOGENETIC ANALYSIS OF THE F_1 GENERATION OF IRRADIATED MALES

The analysis of mitotic complements (Fig. 2a), pachytenes (Fig. 2b) and meiotic metaphases I (Fig. 2c) revealed that most of the F_1 males showed different karyotypic variations, i.e., differences in number and/or size of chromosomes (Fig. 2a–c), and abnormal meiotic configurations, such as multivalents (Fig. 2b, c) due to the occurrence of a variety of radiation-induced chromosomal aberrations including chromosomal fragmentations (Fig. 2a), fusions (Fig. 2a), and translocations (Fig. 2b, c). Female descendants of irradiated parents did not show clear evidence of chromosomal rearrangements.

APYRENE TO EUPYRENE SPERM RATIO IN IRRADIATED AND NON-IRRADIATED MALES

Spermatogenesis in *T. absoluta*, as well as in other lepidopterans, occurs in testicular cysts and hence eupyrene and apyrene sperm bundles were observed (Fig. 3a, b). Both types of sperm bundles showed morphological dimorphism. The eupyrene sperm bundles (Fig. 3a)

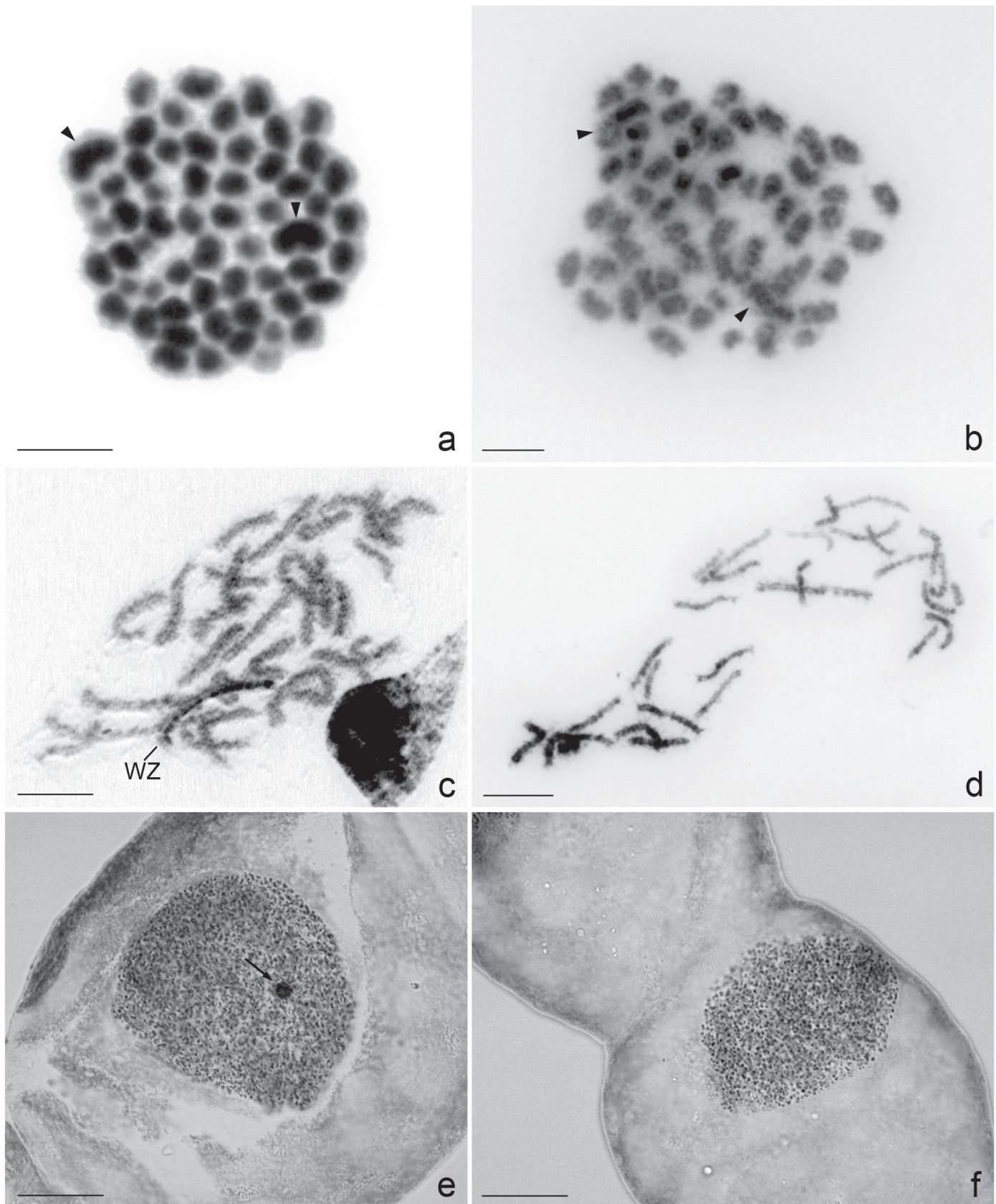


Fig. 1. Cytogenetic analysis of non-irradiated *T. absoluta* individuals. (a) Female mitotic metaphase; (b) male mitotic metaphase; (c) female pachytene complement with a WZ bivalent identified according to W-chromosome heterochromatin; (d) male pachytene complement; (e) a highly polyloid female nucleus of Malpighian tubule cells with a large sex chromatin body; (f) a male nucleus of Malpighian tubule cells without W chromatin. Bar = 5 μ m (a,b); 10 μ m (c,d); 20 μ m (e,f). Arrow-heads indicate largest chromosomes of the complement, i.e., W and Z sex chromosomes; and arrow indicates sex chromatin.

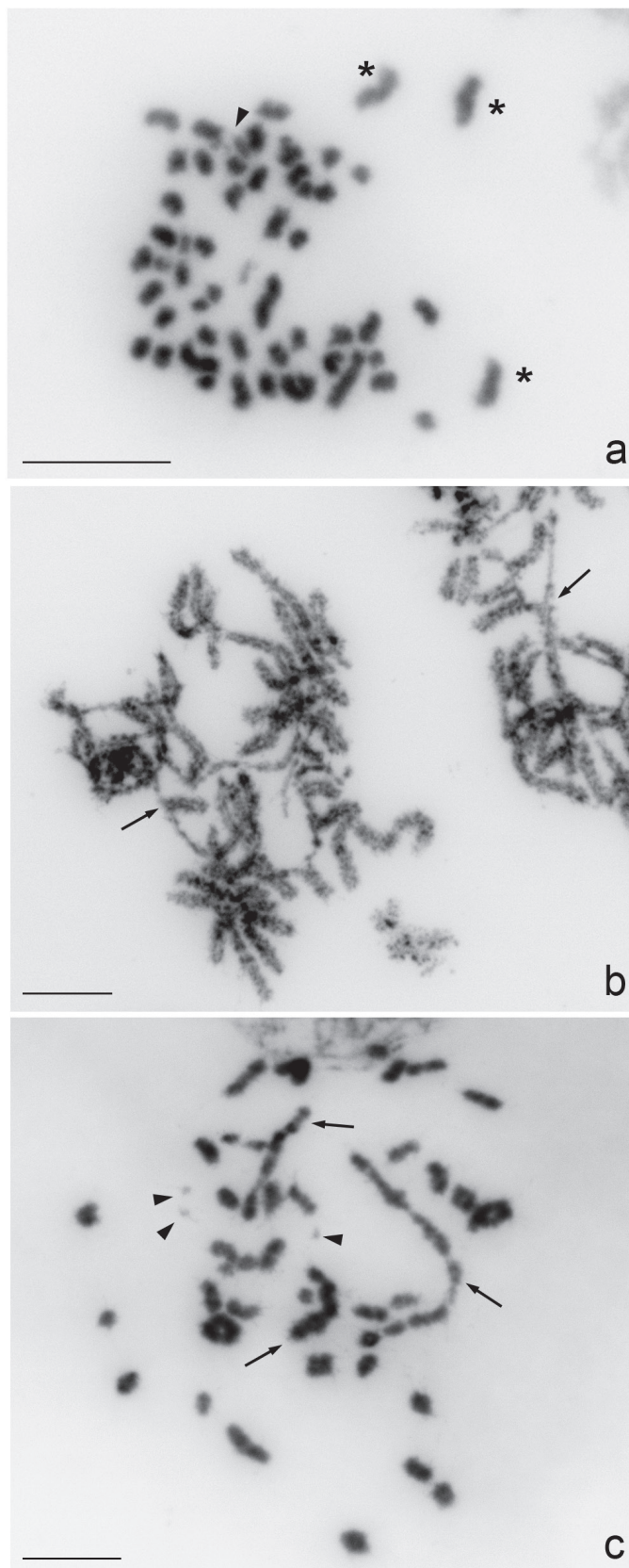


Fig. 2. Cytogenetic analysis of the F₁ generation of irradiated males. (a) Male mitotic metaphase with chromosomal fragments and fusions; (b) male pachytene complement with chromosomal multivalents; (c) male metaphase I with multivalents and chromosomal fragments. Bar = 10 µm. Arrowheads indicate chromosomal fragments; arrow indicates multivalents; and asterisks indicate chromosome fusion.

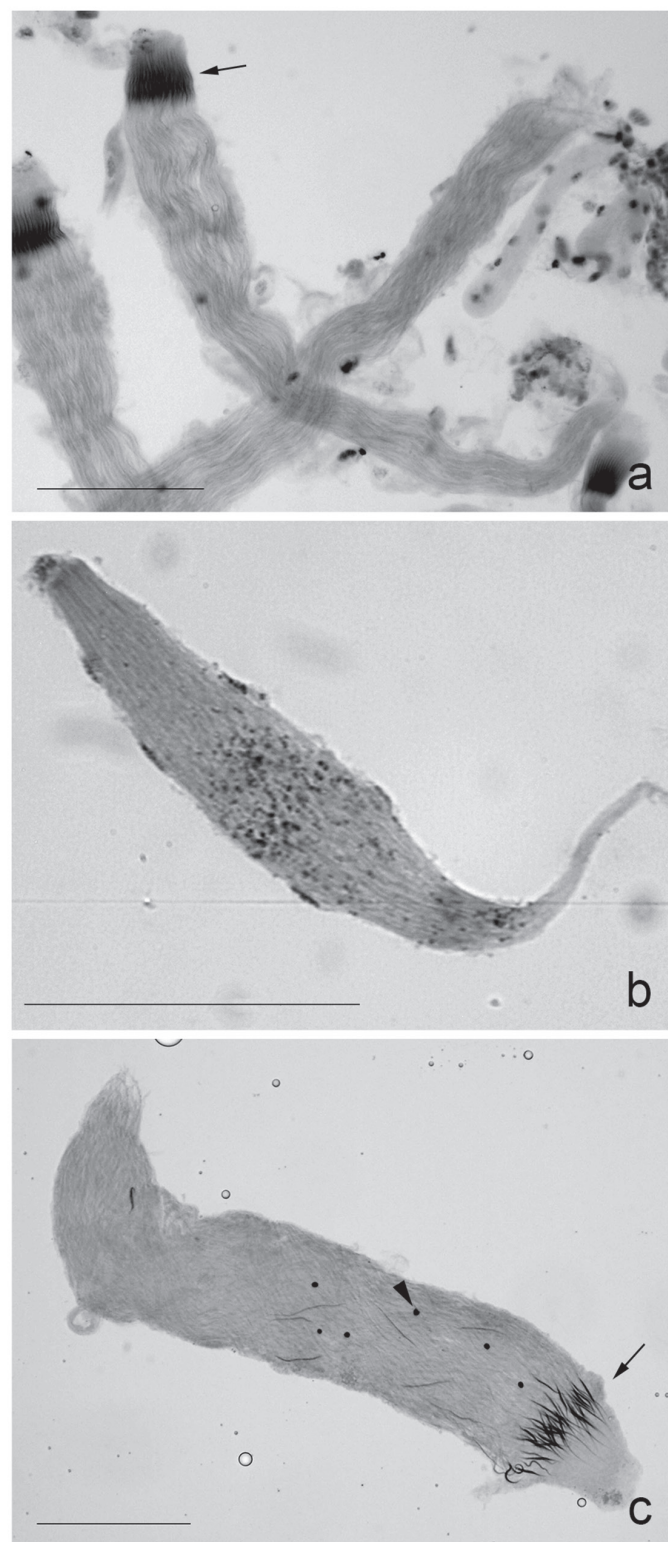


Fig. 3. Cytogenetic analysis of eupyrene and apyrene sperm in *Tuta absoluta*. (a) Normal eupyrene sperm bundle; (b) apyrene sperm bundle; (c) deformed eupyrene sperm bundle. Bar = 100 µm. Arrow indicates eupyrene sperm nuclei; arrowhead indicates micronuclei.

were 400–450 µm long, with the filiform nuclei all well organized and ordered on one side. The width of the bundle was uniform along the whole length, and they acquired a wavy appearance when matured. In contrast, apyrene sperm bundles (Fig. 3b) were 200 µm long, and

had a spindle like shape that was wide in the middle and tapered at both ends. No nuclei were visible at either end, but dot like nuclear fragments were distributed in the middle section. In irradiated males, many eupyrene bundles had abnormal morphologies (Fig. 3c), with disorganized nuclei on one side of the bundle and nuclear fragments, i.e., micronuclei, in the tails.

The apyrene to eupyrene sperm bundle ratio in non-irradiated individuals was 1.44 ± 0.08 (SE). In irradiated males, this variable ranged from 1.43 ± 0.06 (SE) in individuals irradiated with 100 Gy to 1.79 ± 0.15 (SE) in males irradiated with 300 Gy (Fig. 4a). ANOVA revealed that the radiation treatment had a significant effect on this variable ($F = 2.58$; $df = 5,54$; $P = 0.04$). Males irradiated with 300 Gy had a greater apyrene to eupyrene sperm ratio compared with males treated with all the other doses ($P < 0.05$), except those irradiated with 250 Gy ($P > 0.05$). The apyrene to eupyrene sperm ratios of untreated males and males irradiated with doses from 100 to 250 Gy were not significantly different ($P > 0.05$).

The ratio of normal eupyrene sperm to total sperm bundles varied from 0.34 ± 0.02 (SE) in males irradiated with 300 Gy to 0.41 ± 0.01 (SE) in untreated individuals (Fig. 4b). Statistical analysis revealed a significant effect of radiation on this variable ($F = 4.74$; $df = 5,54$; $P = 0.001$). Males irradiated with 300 Gy produced less eupyrene sperm of normal morphology than all the other irradiation doses ($P < 0.05$), except for males irradiated with 250 Gy ($P > 0.05$). Doses higher than or equal to 200 Gy differed significantly from the untreated control group ($P < 0.05$).

When the ratio of deformed eupyrene bundles to the total amount of sperm bundles was analyzed in untreated males, no deformed eupyrene sperm bundle was observed, with the exception of one male, whereas in all the irradiated individuals this ratio varied from 0.02 ± 0.003 (SE) to 0.03 ± 0.005 (SE) (Fig. 4c). A statistically significant effect of radiation was detected ($F = 14.20$; $df = 5,54$; $P < 0.005$) when irradiated individuals were compared with the untreated control group ($P < 0.05$), but no differences amongst the different doses were observed ($P > 0.05$).

EUPYRENE SPERMATOZOA IN THE BURSAE COPULATRICES OF FEMALES MATED WITH IRRADIATED AND NON-IRRADIATED MALES

Normal and abnormal eupyrene spermatozoa were found in the bursa copulatrix of females after copulation with treated (200 Gy) and untreated males. The heads of normal eupyrene spermatozoon of *T. absoluta* were filiform (Fig. 5a) as previously observed when eupyrene bundles were analyzed (Fig. 3a). Abnormal eupyrene spermatozoon heads showed a slight modification with a peculiar angle in the middle section (Fig. 5b). The proportions of abnormal spermatozoa and spermatozoa with uncertain morphology were greater in the bursae copulatrices of females mated with irradiated males compared with females mated with wild-type specimens ($F = 16.02$; $df = 1,13$; $P = 0.002$ and $F = 19.06$; $df = 1,13$; $P = 0.001$, respectively) (Fig. 6).

Discussion

The development of an AW-IPM program that contains a SIT or an IS component is greatly facilitated by extensive information on the biology, ecology and genetics of the target species. In the present work we analyzed the karyotype and the effect of X-ray radiation on the cytology of *T. absoluta*, one of the most important pests of tomatoes.

Most previous studies regarding IS in Lepidoptera have been carried out using gamma radiation instead of X-rays (Carpenter et al. 2005).

Arthur (2004) studied the effect of several doses of gamma radiation on different stages of *T. absoluta*, and found that treating pupae with 300 Gy was lethal, and that a treatment with 200 Gy generated sterile adults. Taking into account that nowadays gamma irradiators are difficult to purchase and to transport (Mastrangelo et al. 2010; Mehta & Parker 2011), X-ray irradiators constitute a potential alternative. They do not depend on a radioactive source, and are easier and safer to obtain, although issues of reliability remain important. Although the effects of X-rays and gamma-rays on insects are generally similar, these types of radiation differ in their energy spectra (Mehta 2009), which might explain the difference between the results obtained by Arthur (2004) and those of Cagnotti et al. (2012) and the present work.

The haploid chromosome number of *T. absoluta* was $n = 29$, which is in agreement with previously reported modal haploid chromosome numbers found in Gelechiidae (Lukhtanov & Pupliesiene 1999). The presence of a very large bivalent in the diploid complement is also a typical trait of this lepidopteran family (Beliajeff 1930; Ennis 1976; LaChance & Ruud 1979; Bedo 1984; Lukhtanov & Kuznetsova 1988; Bartlett & Del Fosse 1991; Lukhtanov & Pupliesiene 1999). In *T. absoluta*, this large pair of chromosomes proved to be the WZ sex chromosome bivalent (present work). Such a size difference between sex chromosomes and autosomes is unusual in Lepidoptera, and was described in detail only in some species of the family Tortricidae (Šíchová et al. 2013, and references therein). In the codling moth, *Cydia pomonella* L. (Lepidoptera: Tortricidae), the European grapevine moth, *Lobesia botrana* Den. & Schiff. (Lepidoptera: Tortricidae), and the vine moth, *Eupoecilia ambiguella* (Hübner) (Lepidoptera: Tortricidae), these large sex chromosomes arose from a fusion between an autosomal pair—chromosome 15 of the silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae) reference genome—and the ancestral sex chromosomes (Nguyen et al. 2013). The implications of this neo-sex chromosome system on the adaptive potential of tortricid moths, and on their speciation, were studied by Nguyen et al. (2013). A detailed description of the chromosomal fusion that originated the sex chromosomes in *T. absoluta*, the nature of the large chromosomal pair observed in other species of Gelechiidae, and the potential role of this chromosomal rearrangement on the irradiation of gelechiids deserves further research.

The detailed karyotype analysis of non-irradiated *T. absoluta* larvae allowed us to identify radiation-induced chromosomal aberrations in F_1 individuals, as was previously done in other Lepidoptera species (Saifutdinov 1989; Al-Taweel et al. 1990; Carpenter 1991; Carpenter et al. 1997; Makee & Tafesh 2006). The presence of sex chromatin in this species is a useful parameter for monitoring the effect of radiation, as its morphology and quantity can be used as a marker to identify sex chromosome aberrations (Makee & Tafesh 2006).

The male progeny of irradiated males carried different types of chromosomal rearrangements including chromosome fusions, fissions and translocations. Due to the method applied for making the chromosome preparations, it was not possible to identify interstitial deletions and inversions. The karyotype alterations presented here such as fragmentations and translocations correspond to those described in F_1 descendants of irradiated *E. kuehniella* individuals (Tothová & Marec 2001). The small number of F_1 females available for chromosomal analysis could be explained by the sex ratio bias towards males in the descendants of irradiated males (Carpenter et al. 2005). The inability to detect any chromosomal rearrangements did not allow us to describe alterations in the karyotype of F_1 females, although it is assumed that these females carry at least a small amount of chromosome breaks (Tothová & Marec 2001). Further research including sex chromatin analysis, fluorescent in situ hybridization (FISH) with the (TTAGG) $_n$ telomeric probe, and synaptonemal complex detection is needed to better understand the impact of X-rays on the karyotype of *T. absoluta*.

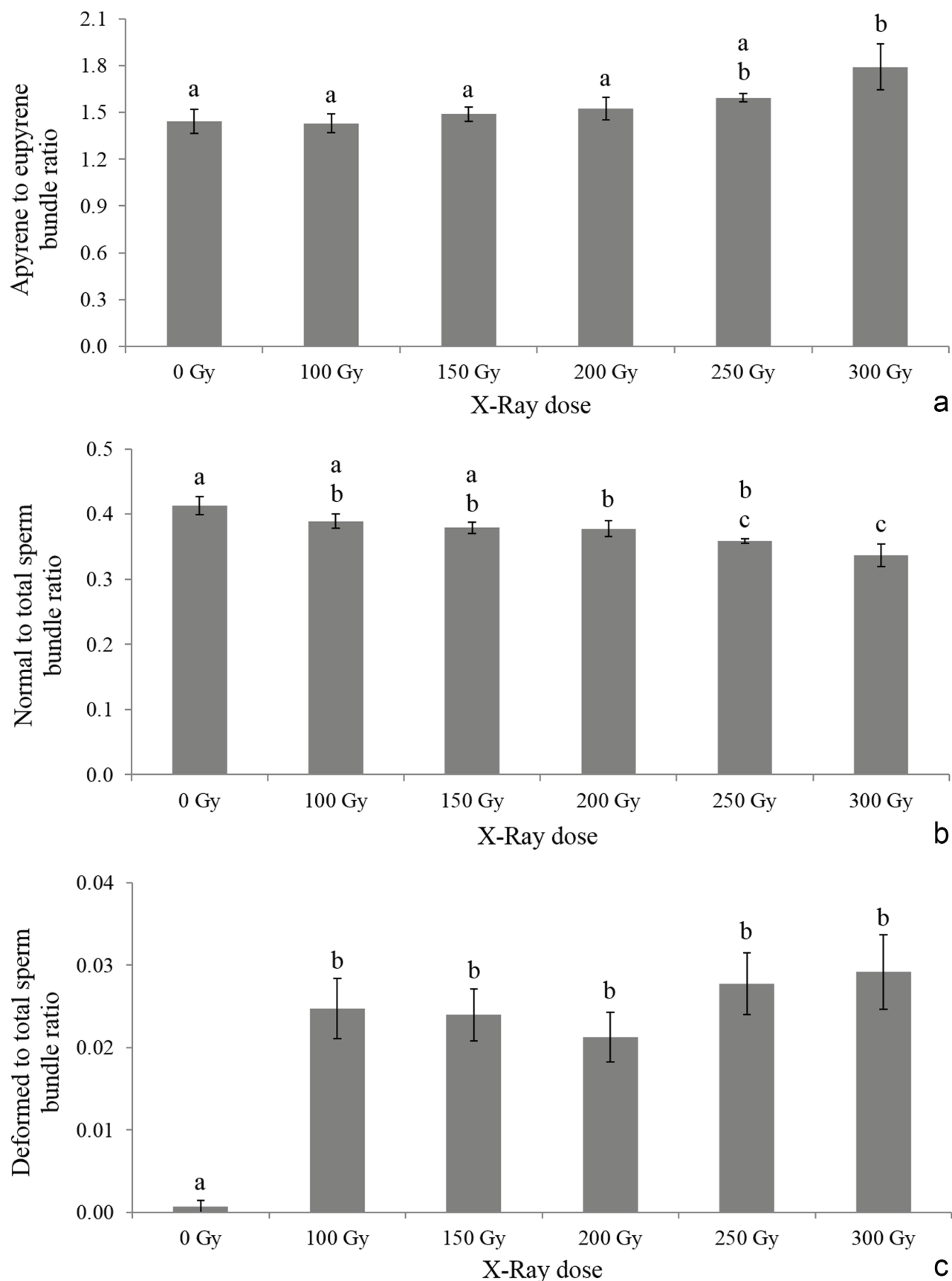


Fig. 4. Apyrene to eupyrene sperm ratios in irradiated and non-irradiated males. (a) Apyrene to eupyrene sperm ratio in males irradiated at different X-ray doses (mean ± SE); (b) ratio of normal eupyrene bundles relative to total sperm bundles (mean ± SE); (c) ratio of deformed eupyrene bundles relative to total sperm bundles (mean ± SE). Columns with different letters in graph are statistically different ($P < 0.05$).

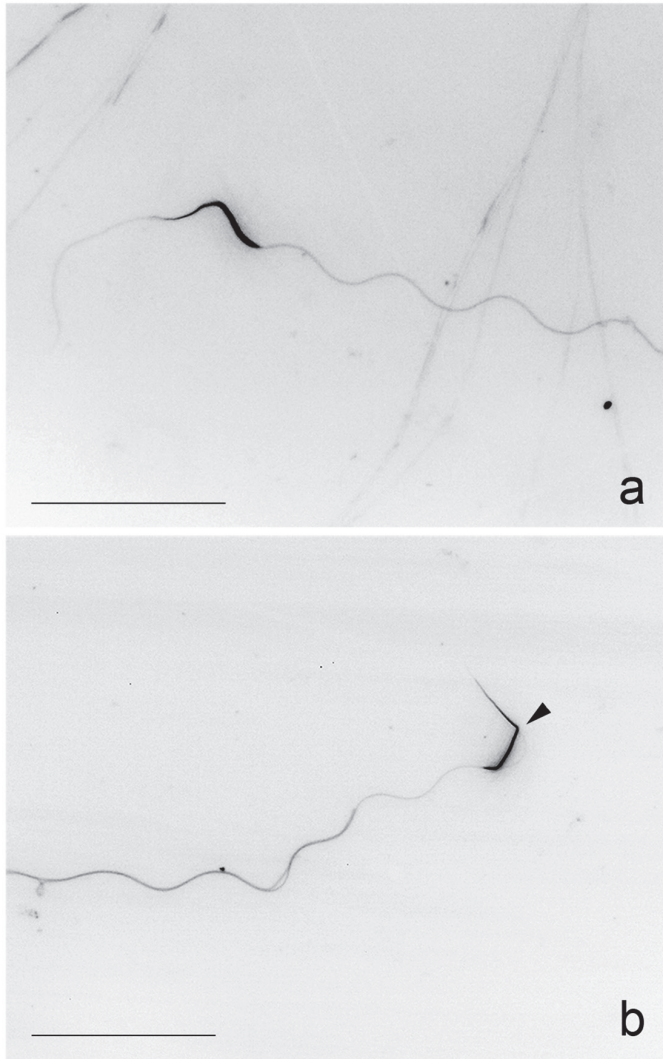


Fig. 5. Eupyrene spermatozoa in the bursae copulatrices of females mated either with irradiated or with non-irradiated males. (a) Normal eupyrene spermatozoon; (b) abnormal eupyrene spermatozoon. Bar = 100 μ m. Arrowhead indicates distinctive angle observed in abnormal eupyrene spermatozoa.

The description of eupyrene and apyrene sperm bundles in non-irradiated *T. absoluta* males provided basic information for the further in depth analysis of the impact of X-rays on these structures. The basic morphologies of *T. absoluta* eupyrene and apyrene bundles were similar to those previously observed in other Lepidoptera, with orderly grouped nuclei in the fertile sperm (Carpenter et al. 2009) and micronuclei in the tails of the infertile sperm (Lai-Fook 1982a). The eupyrene sperm of *T. absoluta* was 2 times longer than the apyrene sperm. The spermatozoa length is very variable amongst moths and butterflies, and ranges between 100 and 12,675 μ m for eupyrene sperm, and between 106 and 883 μ m for apyrene sperm (Morrow & Gage 2000). Generally, the length of the apyrene sperm is between one third to one half of that of the eupyrene sperm (Gage & Cook 1994), and *T. absoluta* is no exception in this respect. The obvious heteromorphism observed in both types of sperm bundles in *T. absoluta* is similar to that found in many other lepidopteran males (Friedländer 1997; Lai-Fook 1982a; Friedländer et al. 2005; Carpenter et al. 2009).

The apyrene to eupyrene sperm bundle ratio in non-irradiated *T. absoluta* males was 1.44:1. This ratio is very small compared with that of other Lepidoptera species such as *E. kuehniella* (9.5:1)

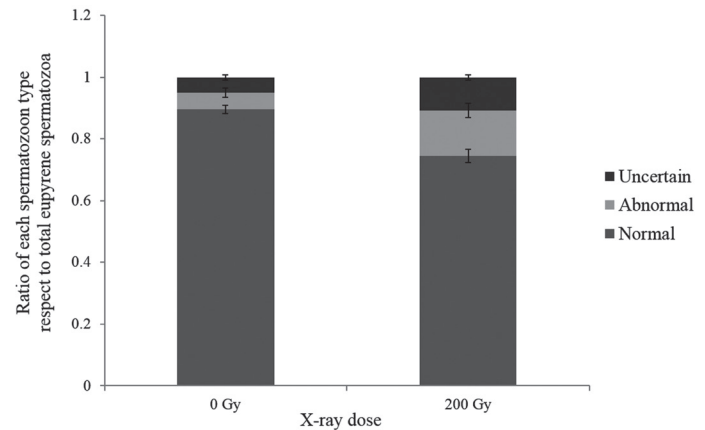


Fig. 6. Ratio of normal, abnormal, and uncertain eupyrene spermatozoa relative to total eupyrene spermatozoa (mean \pm SE) measured after completion of copula.

(Koudelová & Cook 2001), *B. mori* (7:3) (He et al. 1996; Kawamura & Sahara 2002), the oriental leafworm moth, *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) (5:1) (Etman & Hooper 1979), the tobacco hornworm, *Manduca sexta* (L.) (Lepidoptera: Sphingidae) (9.6:1) (Shepherd, unpublished data in Silberglied et al. 1984), and the Indian meal moth, *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) (9:1) (Gage & Cook 1994). As only well identified mature sperm bundles were included in our analysis, it is possible that the obtained ratio is an underestimate. Radiation treatment had no significant effect on this ratio, except for males treated with 300 Gy. Similar results were obtained for *E. kuehniella* by Koudelová & Cook (2001), and the authors concluded that the impact of the radiation treatment is higher in the progeny of the irradiated individuals. In fact, the IS technique presumes that the alterations in fertility traits will be mainly observed in the F_1 .

The detection of eupyrene bundles of abnormal morphology may be explained by nuclear fragmentations, cellular necrosis, and chromosome breaks, which commonly result from X-radiation treatments (Coggins 1973). In Lepidoptera, the spermatogenesis of the eupyrene sperm is regular and highly sensitive to genetic and experimental manipulation (Friedländer 1997). Hence, one expects to find nuclear rearrangements in eupyrene sperm after radiation treatment. Indeed, Carpenter et al. (2009) described these alterations in several lepidopteran species. These morphological variations in individual spermatozoa may lead to the development of abnormal eupyrene bundles with disorganized nuclei and micronuclei in the tails, as observed in *T. absoluta*. The production of eupyrene sperm takes place during the larval stage which constitutes the most sensitive stage for irradiation (Hayakawa 2007). However, the production of euspermatids continues to a lesser extent in pupae and adults (Lai-Fook 1982b). The deformed eupyrene bundles detected in irradiated *T. absoluta* males may have been produced by random damage of the DNA of the euspermatids already present during the radiation treatment, or of the nuclei of the few remaining euspermatogonia and euspermatocytes.

The detection of deformed eupyrene sperm bundles in irradiated males and the low prevalence of this kind of bundle in wild-type individuals (only one bundle was observed in one of the 9 analyzed individuals) allows us to propose the analysis of testis of males caught in the field as a method for monitoring irradiated and non-irradiated males during an IS project. Numerous techniques have been developed to mark insects to study their abundance, dispersal and survival (Schellhorn et al. 2004). External dye marking has frequently been used in

AW-IPM programs, yet it is not 100% effective in Lepidoptera. The dust only attaches to the scales and can be quickly groomed off, it may affect insect quality, and dust particles can be transferred to unmarked insects in the field or in traps and sweep nets used for sampling (Miller 1993); besides the dyes can pose a health hazard (Hagler & Jackson 2001; Parker 2005). Internal dyes fed through the larval diet have been used extensively for marking Lepidoptera, e.g., the pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae), and *C. pomonella* (Parker 2005). However, a dye suitable for one species is not necessarily appropriate for even a closely related species, and the dyes can be toxic or cause behavioral or other changes (Schroeder et al. 1974). The technique presented in this paper is relatively simple, does not require sophisticated equipment, and provides results almost immediately. The detection of deformed eupyrene bundles may complement the detection of internal dyes during the monitoring of irradiated males after being released in the field in small and medium scale IS projects.

Cagnotti et al. (2012) observed a significant decrease in the fecundity of *T. absoluta* females when mated with males irradiated with increasing X-ray doses (50, 100, 150, 200, 250, and 300 Gy). However, when the 300 Gy dose was excluded from the analysis, the relationship between fecundity and dose was not significant, although the dose affected significantly the fertility. These results can be explained by the distortion of the apyrene to eupyrene ratio detected in males irradiated at 300 Gy in our experiments, with a greater amount of apyrene sperm than that recorded in the untreated control. It may be possible that there is an apyrene to eupyrene ratio threshold that, if exceeded, compromises male fecundity. In addition, the production of abnormal eupyrene spermatozoa in irradiated males and the decrease in the production of normal eupyrene spermatozoa in males treated with doses greater than 200 Gy, can account for the decrease in fertility observed by Cagnotti et al. (2012) in *T. absoluta* females crossed with irradiated males. This explanation is also supported by the detection of deformed eupyrene sperm in the bursae copulatrices of females mated with males irradiated at 200 Gy. This suggests that eupyrene sperm bearing genetic damage are normally transferred during mating, and that no sperm selection may occur at this point. The production of abnormal eupyrene sperm and their impact on fertility and fecundity of Lepidoptera males have been extensively described in the literature (Carpenter et al. 1986; Henneberry & Clayton 1988; Carpenter et al. 2001; Nguyen & Nguyen 2001; Bloem et al. 2003; Ayvaz et al. 2007; Boshra 2007). It is worth pointing out that at the moment mating couples of *S. litura* separate, some of the eupyrene sperm have already migrated to the spermatheca (Seth et al. 2002), meaning that the proportions studied in this work may be over- or under-estimates of the proportion of deformed spermatozoa that are actually transferred to the bursa copulatrix of the female; in addition the proportions of the 2 types of spermatozoa that effectively reach the spermatheca, may be affected by differences in motility of the 2 types of sperm (normal and abnormal). Further studies on sperm transfer and mating duration in *T. absoluta* are warranted in order to allow a better interpretation of the results obtained herein.

The cytogenetic studies, analysis of sperm cells, and analysis of the effect of X-rays on the karyotype and sperm of *T. absoluta* presented here, are the first report on these traits in this species. This study offers genetic evidence that explains the results obtained by Cagnotti et al. (2012) and supports the suggestion of using a 200 Gy dose of X-rays for the treatment of *T. absoluta* pupae before their release. The present work provides part of the genetic background necessary for the development of quality control protocols for *T. absoluta*, and the future development of an IS program against this economically important pest species.

Acknowledgments

We would like to thank María José Bressa for allowing us to use a Leica microscope for cytogenetic analyses, Diego Segura for performing the irradiation procedure, Petr Nguyen for his comments on the manuscript, and to the anonymous reviewers for their valuable remarks. M.E.F. and C.C. acknowledge the support from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET); S.L. from Instituto Nacional de Tecnología Agropecuaria (INTA). This work was part of the FAO/IAEA Coordinated Research Project on Increasing the Efficiency of Lepidoptera SIT by Enhanced Quality Control. This work was funded by grant PICT-2007-00486 of ANPCyT, and by Research Contract 16332 of the IAEA. L.Z.C.P. is supported by project Postdoc BIOGLOBE (CZ.1.07/2.3.00/30.0032), realized through EU Education for Competitiveness Operational Programme and funded by European Social Fund and Czech State Budget. Mention of trade names or commercial products in this publication are solely for the purpose of providing specific information and do not imply recommendation or endorsement by the institutions that employ the authors.

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