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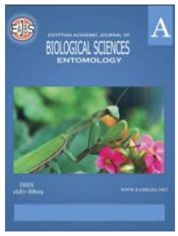

Low Dose Gamma Radiation Induced Chromosomal Aberrations For the Management of the Green Vegetable Stink Bug, *Nezara viridula* (Hemiptera: Pentatomidae)

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ABSTRACT

The impact of the low doses of ionizing radiation has been a matter of important consideration over the last few years. The present work investigates the chromosomal aberrations induced by two doses of radiations; a low dose as 10 Gy and a high dose as 60 Gy gamma radiation on the male germ cells of the green vegetable stink bug, *Nezara viridula*. The karyotype of the holocentric chromosomes at meiotic metaphase I for the non-irradiated and the irradiated adult males were examined by light microscope. Fourteen chromosomes were observed at the meiotic metaphase I of the non-irradiated spermatogonial cells. The typical diploid number of the 14 chromosomes was (12A + XY). Most of the cells showed six rod-like autosomal bivalent chromosomes and one pair of sex chromosomes. In some cells five to four bivalent rod-like with one or two ring chromosomes were recorded. Chromosomal aberrations were detected at meiotic metaphase I in the germ cells of the irradiated testes with 10 Gy dose. Translocation and aggregation (sticky) chromosomes with a tetravalent structure as well as B-Chromosomes were observed. Chromosomal fragmentations were also demonstrated in few gonial cells. At high dose as 60 Gy of gamma radiation, chromosomal fragmentations were only observed in the cells. The results confirm that low dose 10 Gy gamma radiation has induced chromosomal aberrations in the irradiated spermatogonial cells of the green vegetable stink bug, *N. viridula*. The present work provides part of the cytogenetic background necessary for the development of biological control protocols and future development of the sterile insect technique and support for the potential application of inherited partial sterility as an innovation in insect control against this economically important pest species.

INTRODUCTION

The southern green vegetable stink bug, *Nezara viridula* (L) is a very serious pest infesting vegetable crops and soybeans in Egypt, India and the Southeastern United States. It also attacks rice crops in East and Southeast Asia and in Australia, where they invade sunflowers (Hill, 1983). It feeds on a wide range of crops, developing fruits and seeds and also bore into the tender young growth of plants and trees, causing local necrosis and providing entry for pathogens and disperses to many crops through the seasons (Todd and Herzog, 1980). The lifespan of adults ranges from several weeks to half a year, depending on the season (Kiritani, 1963).

In temperate zones, they typically hibernate on weeds and the smallest population size appears in early spring. It is difficult to be controlled over wide areas because of the type of damage produced and the huge variety of crops on which it subsists (Hoffman, 1935). Effective pest management should drive *N. viridula* under the economic thresholds of diverse crops. The strategy of an area-wide control could present the best strategy for success because *N. viridula* is prolific, polyphagous bug that lives well on short-season crops. The autocidal methods and the genetic manipulation could be effective against low-density populations dispersed across wide ranges and against pests with high-density (Knipling, 1969, 1981). It is important to understand the genetics of this pest for developing environmentally friendly control strategies such as the sterile insect technique (SIT) and inherited sterility (IS).

The success of SIT for monitoring and eradication of many insects, has evoked further interest in the detailed effects of radiation on insect germ cells. Sterility has to be genetic in origin and based on aspermia, infecundity, physiologically compromised gametes or inability to mate (LaChance, 1967). Earlier work by Gatenby *et al*. (1929) showed that insect spermatogonia are radiosensitive in that they are destroyed by high doses of radiations but some of the cells show a relatively low mutagenicity. Non-repairable radiation damage to chromosomes, e.g. dominant lethal mutations, chromosome breakages or deletions may explain the total breakdown of cells. Casarett (1968) had documented that the chromosomes are the most sensitive to structural alteration during the mitotic S-stage. The structural aberrations in the chromosomes induced by radiation are the main cause of early death to the zygot (LaChance, 1967; Smith and von Borstel, 1972).

Cytological data on five species of the genus Nezara had been reported previously (Ueshima, 1979; Manna and Deb-Mallick, 1981; Satapathy and Patnaik, 1991). The genus possesses holocentric chromosomes which consisting of six autosome pairs plus a simple pair of sex chromosome (XY) in males and (XX) in females (Hughes-Schrader and Schrader, 1957; Camacho *et al*., 1985). All Heteropteran species possess holocentric chromosomes i.e. without localized centromere (Papeschi *et al*., 2003). The sterility by radiation of the species with holocentric chromosomes could be introduced through genetic mechanisms, including translocations, fragmentations, inherited sterility and hybrid incompatibility (Robinson, 2002). These radiation-induced aberrations might be expected to influence the insect fertility and reproductive potential. Generally, the ionizing radiation induces different chromosome abnormalities, simple aberrations which form only two chromosome breaks and complex aberrations which involve three or more breakages (Sachs *et al*., 2004). The broken chromosome ends may fuse in new combinations to produce dicentric chromosomes and acentric fragments. During cell division, the dicentric chromosomes delay cell division and acentric fragments are lost to make genetic imbalances. These changes lead to cell death (Hallman, 2004; Robinson, 2005; Li *et al*., 2016). In most insect species, the adults are highly radio-tolerant because the dividing cells proceed only in gonads, not in somatic tissues (LaChance, 1967). Hallman (2003) had suggested that, the development and the normal growth or the reproduction of the organism might be prevented by lower doses of radiation while high doses of irradiation could immediately kill insects.

The adult hemipterans may possess partial sterility after they are subjected to 30 to 100 Gy of ionizing radiation with low linear energy transfer. Exposure to lower radiation doses during a growth stage in an insect's life could have a greater impact on reproductive fitness, without serious side effects on mating behavior and survivorship (Mau *et al*., 1967; LaChance *et al*., 1970; LaChance and Riemann, 1973; Maudlin, 1976). Knipling (1969) had proposed that the release of semi sterile insects could have a deleterious effect on the target insect species. Releasing semi sterile stink bug insects could insert damaging genetic stress on the target population. Dyby and Sailer (1999) had exposed the fourth nymphal instar *N. viridula* to different doses of ionizing gamma radiation. They had recorded that the newly hatched females were partially sterile and laid nonviable eggs in high numbers compared to the control when irradiated with 9 Gy dose. Half of the nymphs had survived to the adult stage after irradiation with 20 Gy dose. While at 40 and 60 Gy doses, most of the mid-instar fourth nymphs became atrophied and had a delayed molt and died in the fifth instar. The majority of nymphs died in the fourth instar at 80 Gy dose. Žunič et al. (2002) had studied the effect of 5 Gy X-ray irradiation on the fifth nymphal instars of *N. viridula*. The mating behaviour of the emerged adults was not affected but the irradiation during growth stage had decreased the fertility and fecundity. The somatic effects such as the increase of nymphal mortality, prolongation of molting and increased adult mortality during the first day after the molt was reported. Decreased fecundity and fertility and increased progeny mortality was suggested that these changes could be under genetic effects. Recently, Ibrahim *et al.* (2015) and Ibrahim (2016) had documented several deformities in the ultrastructure of the spermatids and the spermatozoa in the adult male germ cells of *N. viridula.* Also, abnormal activities of acid phosphatase (ACP) and gloucose-6-phosphatase (G-6-PDH) enzymes were observed after irradiation the fourth nymphal instars with 40 Gy gamma radiation. These abnormalities might be produced as a consequence of chromosomal aberrations induced by irradiation.

A small amount of information is available about the radiobiological properties of holocentric chromosomes; although several authors had carried out radiation research on this type of chromosome in a variety of organisms. Neither comprehensive picture of the viability of these aberrations nor the relation between aberrations and lethality had emerged. (Tempelaar, 1979; LaChance and Graham, 1984; Makee *et al.*, 2011). Up to date, there is no published data demonstrating the influence of gamma radiation on the *N. viridula* chromosomes. For that, the aim of the present research was to investigate and analyze the karyotype of the holocentric chromosomes at meiotic metaphase I for non-irradiated and irradiated adult males. Also, to demonstrate the types of chromosomal aberrations induced in the male germ cells of *N. viridula* irradiated with a low dose as 10 Gy and a high dose as 60 Gy gamma radiation.

MATERIALS AND METHODS

Insect colony

N. viridula eggs were collected from the field and maintained in the laboratory at Entomology Department, Faculty of Science, Ain Shams University, Cairo, Egypt until hatching. The newly hatched nymphs were fed on fresh green beans (*Phaseolus vulgaris*) and raw peanuts (*Arachis hypogaea*) in an environmental conditions at 25 $\pm$ 2^{o}C, 50 $\pm$ 5% RH and 16:8 h (L:D) photoperiod.

Irradiation technique

Newly emerged fourth nymphal instars were collected and irradiated to 10 Gy and 60 Gy doses using ^{60}Co Indian gamma cell (Gy 4000 A), located at the National Center for Radiation Research and Technology, Nasr City, Cairo, Egypt. The dose rate was 0.713 Rad/ Sec.

Chromosomal assay

The testes of the newly hatched non-irradiated (normal) and irradiated males were used for the assay. Five testes from normal and three testes of 10 Gy and three testes of 60 Gy were removed and fixed in 4:3:1 (chloroform: ethanol: acetic acid) for 4 hours followed by storage in 80% ethanol. Individual testes were softened in PBS with 2% trypsin freshly added for 1-2 hours before squashing in 45% acetic acid and staining in 2% lacto-propionic orcein. Karyotype of meiotic metaphase I were obtained from 24 h males testes and then examined under light microscope.

RESULTS AND DISCUSSION

The development of an AW-IPM program that involve SIT or IS component is greatly promoted by extensive information on the genetics, biology, and ecology of the target species. The meiotic metaphase I karyotype of the non-irradiated testes showed fourteen chromosomes at the meiotic metaphase I of the spermatogonial cells (128 counted cells). The typical diploid number of 14 chromosomes (12A + XY) was counted. Most of the cells showed six rod-like bivalent autosomal chromosomes and one pair of rod-like sex chromosome. However, in some cells, five or four rod-like with one or two ring chromosomes with 92.86 % (range 0-2). Among the autosomal chromosomes, there is one large rod-like bivalent designated as A1 (100%), while the other autosomes decrease gradually in size. The X chromosome is medium sized (100%), while the Y is the smallest of the complement (100%) (Fig. 1). Tempelaar (1979); LaChance and Graham (1984); Makee *et al.*, (2011) were previously studied the chromosomal structure of *N. viridula* species. The standard chromosome number of Pentatomidae family is 14 as observed in more than 75% of the investigated species (Ueshima, 1979; Manna and Deb-Mallick, 1981; Nuamah, 1982; Satapathy and Patnaik, 1988; Surbhi *et al.*, 2015). Characteristics of the chromosomes in the family Pentatomidae are basically holocentric in nature, i.e. they have no localized centromere. This makes the microtubules to bind to the entire chromosomal surface during meiosis and the homologous chromosomes migrate in parallel to the cell poles at the metaphase I plate (Van Den Berg *et al.*, 2001; Surbhi *et al.*, 2015; Bardella *et al.*, 2016) and anaphase (Rebagliati *et al.*, 2001).

B-Chromosome was detected when the germ cells were irradiated with 10 Gy and it was 40% (range 0-1) at the first treated testes (75 counted cells), 7.69% (range 0-2) at the second treated testes (26 counted cells) and it was 32.65% (range 0-4) at the third treated testes (49 counted cells) (Fig. 2). B- Chromosome was recorded in the metaphase I karyotype of irradiated germ cells with relatively moderate percentage in the germ cells of two males and with a low percentage in the third male germ cells. Camacho *et al.* (2000) had reported that the B-Chromosomes are extra dispensable chromosomes and are present in some individuals from some populations in some insect species and do not recombine with the A-Chromosomes. It was proposed that B-Chromosomes could originate from A-Chromosomes or even driven from sex chromosomes both under favorable or unfavorable environmental condition and also under exposure to radiation. B-Chromosomes had been generated in *Nasonia sp*. (Hymenoptera: Pteromalidae) by using chemical mutagenesis, cytoplasmic incompatibility, and X-irradiation. B-Chromosomes may have potential as a biological control agent because they make a substantial reduction in population growth and lead to a much lower pest population density (Werren and Stouthamer, 2003).

Translocations of chromosomes with tetravalent structure were detected at meiotic metaphase I when the germ cells irradiated with 10 Gy (Fig. 3). The tetravalent structure of the chromosomes was represented by 41.33 % (range 0-1) from the first testes, and by 11.54 % (range 0-1) of the second testes, and by 4.08% (range 0-2) of the third testes. Aggregation or sticky chromosomes were also recorded (Fig. 4). Fragmentations of chromosomes were observed in few cells (Fig. 5). The chromosomes of all the gonial cells from the three testes irradiated with 60 Gy (51 cells from the first, 121 cells from the second and 62 cells from the third testes) had only demonstrated fragmentation at meiotic metaphase I. The breakage of the chromosomes were ranged from less to highly fragmented (Fig. 6).

All the hemipteran insects have holocentric chromosomes that allow them to maintain chromosome pieces at cell division. This is useful for preserving somatic tissue but must produce complications for reproductive fitness, if the damaged chromosomes have an equal chance of completing gametogenesis and fertilization. In pentatomid males, the kinetochore becomes restricted to a limited region of the chromosome ends during meiosis, but not during mitosis (Hughes-Schrader and Schrader, 1961). However, chromosome fragments in males can be transmitted through meiosis and can persist for three generations (Hughes-Schrader and Schrader, 1961; LaChance and Begrugillier, 1969). The kinetochore(s) on the fragments remains functional. Males that transmit chromosome fragments have reduced fertility (LaChance *et al.*, 1970). Chromosome fragments and translocations are correlated with arrested development and low fertility in *Oncopeltus fasciatus* (LaChance *et al.*, 1970; LaChance and Richard, 1973). The drastic reduction of fertility of the progeny bearing fragmented and translocated chromosomal complements adds more evidence that inherited partial sterility observed in the Lepidoptera, which also possess holokinetic chromosomes, is based on the continued transmission of aberrant chromosomal complements (LaChance and Degrugillier, 1969). Duby and Sailer (1999) had suggested that the dose which introduces sterilization for the wild type of *N. viridula* lie between 15 and 20 Gy. The wild types when irradiated as fourth nymphal instars with low dose gamma radiation, they had continuous reproductive loss could be caused by genetic damage that produces deformities. The males may have the recessive lethal trait or chromosome damage. Semi sterility may be more easily sustained if the nymphs had been subjected to lower levels of radiation.

Radiation or chemicals are useful at low doses but seriously hazardous at high doses (Sutou, 2017). In addition to that, the higher doses have a negative effect on the mating performance and the biological quality of the irradiated insects when competing with wild individuals in the field (Bloem and Carpenter, 2001; Paladino *et al.*, 2016). These parameters are less affected when the parental generation of insects which possess holocentric chromosomes are exposed to lower sub-sterilizing doses, resulting in a higher sterility in the first filial generation (F1) (North, 1975; La Chance, 1985). This phenomenon is called inherited sterility (IS) and had been proved to be highly effective for the control of several Lepidopteran species (Mastro, 1993; Bloem *et al.*, 1999, 2001, 2003). The F1 individuals carry chromosomal fragments and translocations that are responsible for the production of genetically unbalanced gametes (Carpenter *et al.*, 2005) and these male individuals also transfer less amount of fertile sperms (Koudelová and Cook, 2001). The chromosomal aberrations induced by radiation could be seen in changes in the number of chromosomes, and/or aberrant chromosome morphology and the quantity of sex chromatin bodies (Carpenter *et al.*, 2005; Makee and Tafesh, 2006).

The biological efficiency of low dose irradiation could be higher than the exposure to acute irradiation (Grosovsky, 1999; Ma *et al.*, 2010; Yushkova and Zainullin, 2016). The main effect of low doses is radiation-induced genetic instability which lasts during cell divisions after the cell has been irradiated (Holmberg *et al.*, 1998; McIlrath *et al.*, 2003). The genetic instability is favored by, divergences in cell cycle control, changes of repair activity and apoptosis (Murnane, 1996). Irradiation greatly destroy cells at early stages as embryos, larvae and pupal stages. It was proposed that in addition to the well- known direct effects of the low irradiation doses on target cells, they could also have an indirect effect on neighbors cells involving damage signaling molecules (Zaka *et al.*, 2002). These molecules would be transmitted from damaged cells, causing genetic instability, apoptosis, and micronucleus formation or mutation induction in neighbor non-irradiated or undamaged cells. This phenomenon, called "bystander effect", had been detected in many cell lines after exposure to very low and low doses. Weissenborn and Streffer (1988) had stated that the irradiated cells that have preserved their ability to divide, only a small proportion of chromosome aberrations occur during the first mitosis, the rest of the chromosomes being integrated into the genome and gradually expressed during successive cell divisions. The probablity that some of these abnormalities sustain through numerous generations. This phenomenon is called "genomic instability" and it is fully expressed at low doses like the bystander effect.

Radiation-induced chromosomal aberrations in some other hemipteran insects have been recorded previously. Cytogenetic studies on the adult males of large milkweed bug, *Oncopeltus fasciatus* (Hemiptera: Lygaeidae) irradiated with 9000 rad of X-rays, and crossed to untreated (control) females showed that a single chromosomal fragment could lower the fertility and the degree of sterility, depending on the size of the fragments and its pairing behaviors. The drastic reduction of fertility of the progeny bearing fragmented and translocated chromosomal complements adds further evidence that inherited partial sterility observed is based on the continued transmission of aberrant chromosomal complements (La Chance and Degrugillier, 1969; La Chance *et al.*, 1970). Lachance and Richard (1973) had reported that the *O. fasciatus* adults when irradiated with 8 and 20 Krad of gamma rays and 200 rad of X-rays, were less fertile than controls and none of the treated insects produced full sterility. The semi-sterility of the F1 males was correlated with chromosome translocation and fragmentation in the spermatocytes. Cytogenetic examinations of F1, F2 and F3 males of parent males of *Rhodnius prolixus* (Heteroptera, Tritomidae) irradiated with 6 Krad gamma rays indicated that the changes in fertility were correlated with the degree of chromosomal abnormalities recorded (Maudlin, 1976). Males of *Physopelta Schlanuschi* (Heteroptera: Pyrrhocoridae) were irradiated with different doses of X-ray. The frequency of total chromosome aberrations at different meiotic stages indicated that the chromosomes of spermatogonial metaphase and anaphase I were more sensitive to X-rays than that of other stages (Dey and Manna, 1983). Large differences in radiosensitivity were recorded in irradiated adult males of four species of insects, *Musca domestica* (Diptera), *Oncopeltus fasciatus* (Hemiptera), *Anagasta kuhniella* (Lepidoptera) and *Heliothis virescens* (Lepidoptera). These variations referred to the differences in repair mechanisms, oxygen concentration, monocentric versus holocentric chromosomes and intrinsic differences in mitotic cell cycles which influence the radiation-induced chromosome breaks (La Chance and Graham, 1984). Chromosomal aberrations in Grape phylloxera, *Daktulospharia vitifoliae* (Fitch) (Homoptera: Phylloxeridae) females had been induced by different gamma radiation doses. The chromosomes of all examined embryos of irradiated insects showed aberrations like sticky chromosomes, inter-chromosome translocations, and increases in the chromosomal number on the metaphase plate in some cells. When a break occurs in the holocentric chromosomes, the fragments usually not lost and can still be attached to the spindle and the number of chromosomes on the metaphase plate of some embryos' cells was increased. The chromosomal aberrations had influenced the mortality, longevity, and reproduction (Makee *et al.*, 2011).

On the other hand, there are many literatures had documented the effect of different types of radiation on many insect species, animals, and plants which possessing holocentric chromosomes. Translocations, aggregation, and fragmentations had been induced in some orders of insects such as Lepidoptera (Traut *et al.*, 1986; Makee and Tafesh, 2006, 2007; Makee *et al.*, 2008), Hymenoptera (Verma, 1994), and Diptera (Franz and Kerremans, 1993; Gunderina 1997; Gunderina and Aimanova, 1998, Beliakova and Anisimov, 2000). Aberration of chromosomes induced by irradiation in the two-spotted red spider mite, *Tetranycbus urticae* (Acari: Tetranychidae) was documented by Tempelaar (1979). Jankowska *et al.* (2015) had recorded translocations and fragmentations induced by radiations in some plant species possess holocentric chromosomes.

In conclusion, the recorded results may offer genetic evidence of using low dose gamma radiation as 10 Gy for irradiation of *N. viridula* before their release. Also, it provides part of the genetic background necessary for the development of quality control protocols and the future development of an IS program against this economically important pest species.

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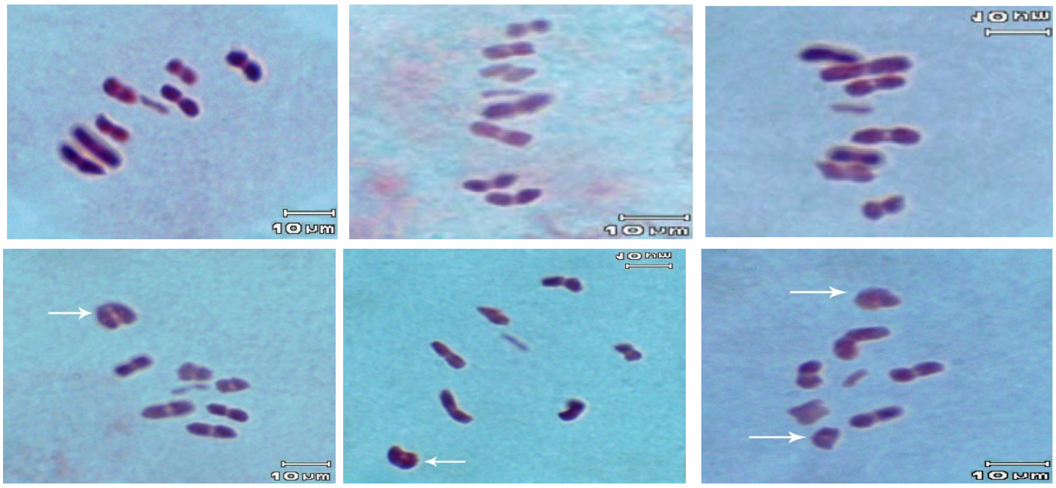

Fig. 1: Meiotic metaphase I karyotype of the non-irradiated (normal) germ cells of the green vegetable stink bug males, *Nezara viridula*. Six autosomal rod like bivalent chromosomes and one pair of sex chromosome (XY). In some cells, one or two ring chromosome (arrow). Bars: 10 µm.

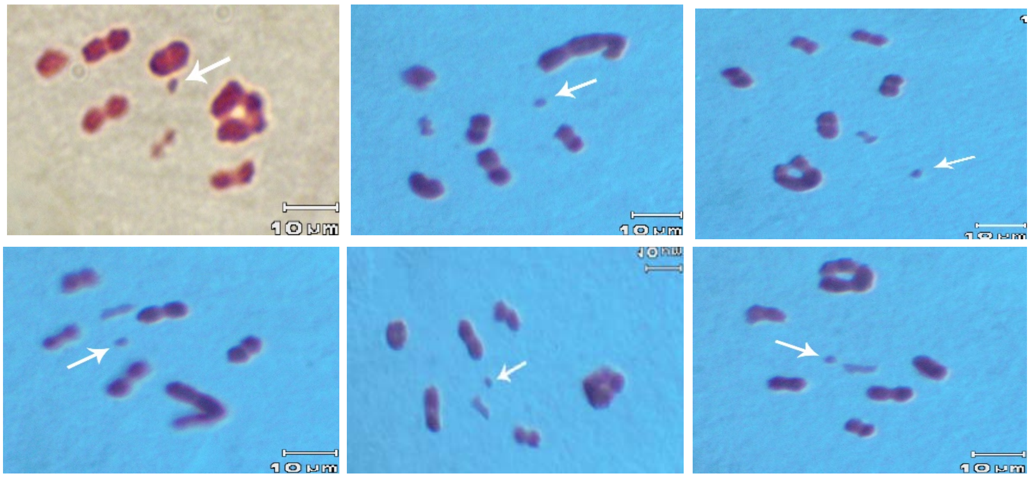

Fig. 2: Meiotic metaphase I karyotype of irradiated germ cells of green vegetable stink bug males, *Nezara viridula* with 10 Gy dose of gamma radiations showing. B- Chromosome (arrow). Bars: 10 µm.

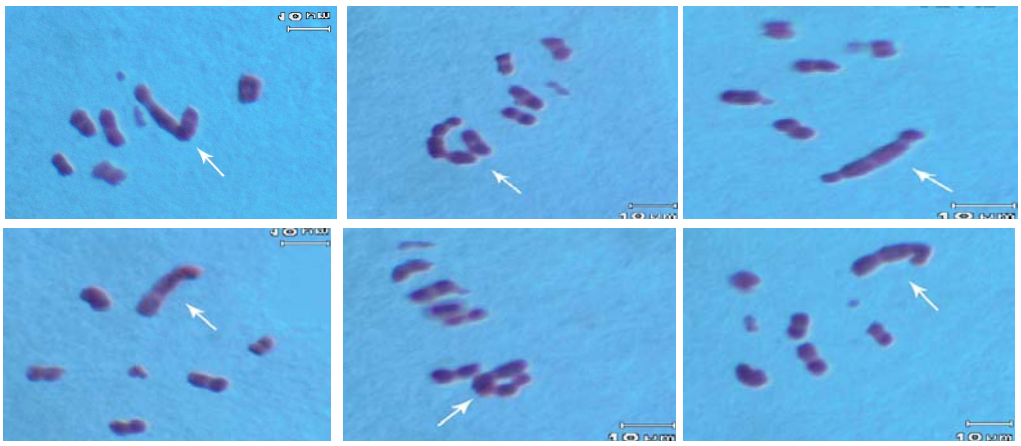

Fig. 3: Meiotic metaphase I karyotype germ cells of irradiated green vegetable stink bug males, *Nezara viridula* with 10 Gy dose of gamma radiations showing translocated chromosomes (arrow). Bars: 10 µm.

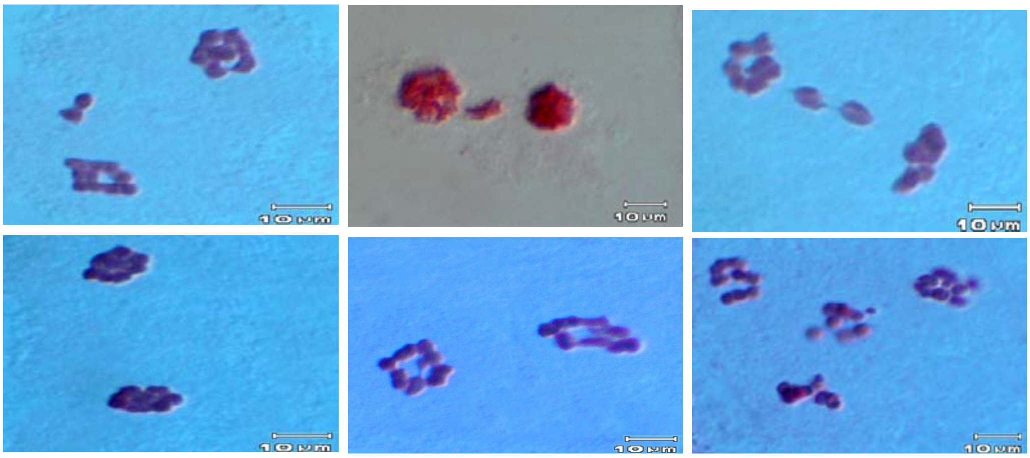

Fig. 4: Meiotic metaphase I karyotype germ cells of irradiated green vegetable stink bug males, *Nezara viridula* with 10 Gy dose of gamma radiations showing aggregated (sticky) chromosomes. Bars: 10 µm.

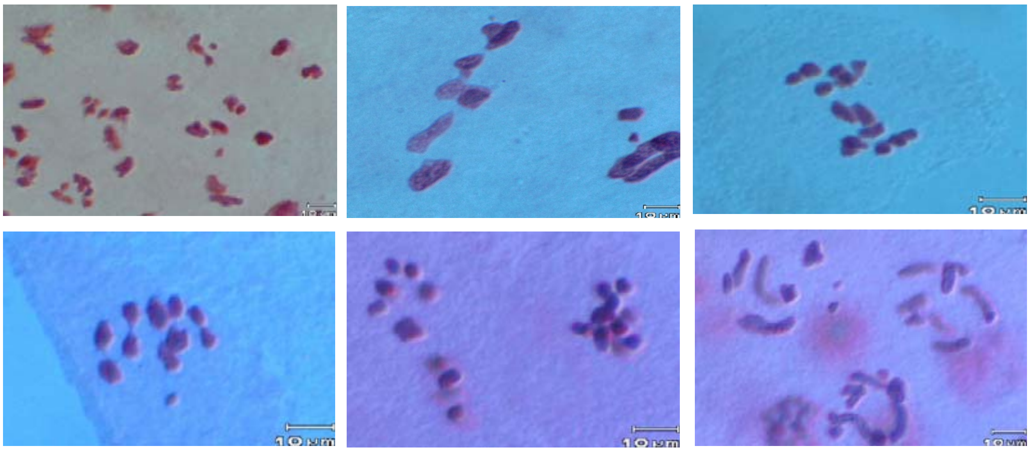

Fig. 5: Meiotic metaphase I karyotype germ cells of irradiated green vegetable stink bug males, *Nezara viridula* with 10 Gy dose of gamma radiations showing the fragmented chromosomes. Bars: 10 µm

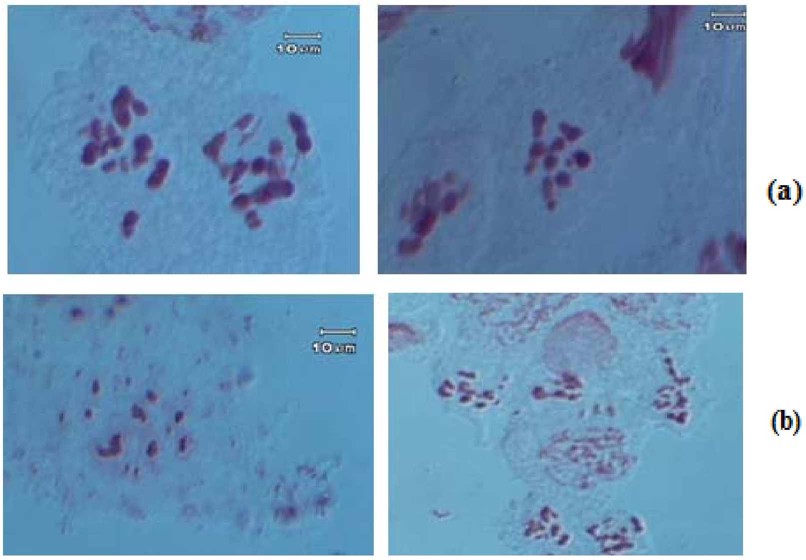

Fig. 6 Meiotic metaphase I karyotype germ cells of irradiated green vegetable stink bug males, *Nezara viridula* with 60 Gy dose of gamma radiations showing less fragmented (a) and highly fragmented chromosomes. Bars: 10 µm.

ARABIC SUMMARY

الجرعة الصغيرة من اشعة جاما تحدث تغيرات كروموسومية لمكافحة بقة الخضروات الخضراء ذات الرائحة العفنة *Nezara viridula* (رتبة نصفية الاجنحة: بنتاتوميدي)
(Hemiptera: Pentatomidae)

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إن تأثير الجرعات الصغيرة من الاشعاع كان محل اهتمام كبير علي مدار السنوات القليلة الماضية. بحثت الدراسة الانية التغيرات الحادثة في الكرموسومات نتيجة تأثيرجرعتين من الاشعاع، جرعة صغيرة مثل ١٠ (جري) وجرعة كبيرة مثل ٦٠ (جري) علي الخلايا الجرثومية الجنسية لذكور بقة الخضراوات الخضراء ذات الرائحة العفنة. لقد تم دراسة الشكل الكروموسومي للذكور غير المشععة في مرحلة الميتافيز ١ باستخدام الميكروسكوب الضوئي، ووجد ان ١٤ كروموسوم في مرحلة الميتافيز ١، وان العدد الطبيعي كان متمثلا ب ١٢ كروموسوم جسدي وزوج كروموسوم جنسي. وكان أغلب الخلايا تظهر٦ ازواج كروموسومات جسدية عصوية ثنائية الشكل وزوج وحيد من الكروموسومات الجنسية. وفي بعض الخلايا كان يوجد ٥ او ٤ ازواج من الكروموسومات عصوية الشكل وزوج او زوجين من الكروموسومات حلقي الشكل.

التغيرات الكروموسومية قد تحددت في مرحلة الميتافيز١ في الخلايا الجنسية الذكرية المشععة بجرعة ١٠ جري وقد كانت انتقال والتصاق وتفتيت الكروموسومات بشكل رباعي الاجزاء مع وجود وظهور ال بي كروموسوم. أما عند الجرعة ٦٠ جري لم يلاحظ الا تفتيت للكروموسومات فقط.

ان النتائج أكدت ان الجرعة الصغيرة ١٠ جري احدثت تغيرات في كروموسومات الخلايا الجنسية لذكور البقة الخضراء وهذة النتائج تعطي خلفية جينية مهمة لتفعيل برامج المكافحة الحيوية في المستقبل باستخدام طرق التعقيم الذكوري والتعقيم المورث بجرعة صغيرة لمكافحة هذة الافة الخطيرة للافات الزراعية.