EFFECTS OF ETHYL METHANESULFONATE ON MEIOTIC CHROMOSOMES OF SILKWORM BOMBYX MORI L.

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The effects of ethyl methanesulfonate (EMS), on meiotic chromosomes in general and translocations in particular was studied during spermatogenesis in two races of silkworm Bombyx mori L. Different concentrations of EMS like 2.5, 5, 10, 20 and 40 mM in 0.75 % NaCl was employed by ‘oral injection’. The results indicated that increase in concentration of EMS would enhance the frequency of aberrations in total as well as individual types. They include loops, partially paired bivalents, fragments, stickiness and, stickiness and clumping. This basic knowledge could be useful in silkworm breeding programme.

Keywords: Ethyl methanesulfonate, meiotic chromosomes, chromosomal aberrations, translocation, auto-sexing breeds.

INTRODUCTION

The silkworm Bombyx mori L. though found to be economically important still appears to be an untapped insect in cytological aspects. The reason attributed for the lack of information in this line is mainly due to the presence of large number of smaller chromosomes (2n=56). As in the other lepidoptera, silkworm chromosomes are almost round or rod shaped except in prophase I stages, which renders the identification of individual chromosomes difficult. Although several scientists succeeded in the induction of mutations like inversions (Tazima, 1938; Tanaka, 1934), fragmentations (Kawaguchi, 1936), and translocations (Kawaguchi, 1936; Aruga, 1943; Tazima, 1943) (quoted from Tazima, 1964) in the silkworm, cytological evidence is very scanty. The nature of effects of mutagens on the structure and behavior of silkworm chromosomes has not been elucidated. Such a line of investigation is needed to understand the cytological features of the genetic material. This paper presents the effects of ethyl methanesulfonate on silkworm chromosomes in general and on translocations in particular, as the translocation between autosomes and the ‘W’ chromosome has an important role in the evolution of auto-sexing breeds.

MATERIALS AND METHODS

Two silkworm races, namely Pure Mysore (multivoltine) and NB4D2 (bivoltine), were selected as an experimental system in the present investigation. Disease free layings of both races were obtained from the germplasm of the Department of Sericultural Science, University of Mysore, and the rearing was conducted following the method described by Krishnaswami (1978, 1979).

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Silkworm larvae at the age of the first day fifth instar were used for treatment. During treatment, the average weight of Pure Mysore and NB4D2 larvae was calculated to be 0.57 and 1.05 g, respectively.

The alkylating agent, ethyl methanesulfonate (EMS) (Loba Chemie), was used for experiments. Various concentrations of EMS like 2.5, 5, 10, 20 and 40 mM were prepared in 0.75 % NaCl solution just before treatment. Forty μl of each concentration was administered separately to each worm into the gut through the mouth by ‘oral injection’. The control larvae received 40 μl of 0.75 % NaCl solution. For each concentration 60 larvae were treated in triplicate. After the treatment, the larvae were allowed to continue further development.

Male larvae of both EMS and control batches from 24 h of treatment until the day of spinning were used for the preparation of meiotic chromosomes from the testis. The testes were dissected out and dipped immediately into freshly prepared 1% potassium chloride (KCl) solution. Just before treatment with hypotonic solution (1% KCl), the cells were squeezed out by pressing with grooved forceps. After treating for 10 min in hypotonic solution, the cells were centrifuged at about 1500 rpm for 10 min at room temperature, and the supernatant was discarded. The pellet was dispersed in fresh hypotonic solution and incubated at 37° C for 25 min. Mixing of the pellet with hypotonic solution, incubation at 37° C for 25 min, and centrifugation were repeated twice. Thereafter, the pellet was fixed by 3 successive treatments with freshly prepared fixative (acetic acid: methanol, 1:3; v/v) for 30 min, centrifuged at 1000 rpm for 10 min and the supernatant discarded. After the last change, the pellet was mixed in the required quantity of fixative and dropped over a clean slide from a distance of 30 cm, air dried and subsequently stained for 60 min in 10% Giemsa stain in Sorenson’s phosphate buffer (pH 6.8). The slides were then washed in distilled water, dried on a slide warmer and mounted in DPX with a coverglass.

The slides were screened for chromosomal aberrations at various stages of meiosis using a light microscope (Leitz photomicroscope equipped with 35 mm Leica camera) under 100×10X magnification. Frequencies of individual aberrations were tabulated by scoring fragments, loops, partially paired bivalents, translocations, stickiness and, stickiness and clumping.

One way ANOVA was employed to determine the level of significance between control and treated batches as well as among treated batches (Fisher and Yates 1953). Regression analysis was employed to determine the dose-effect relationships between the doses of EMS used and the yield of chromosomal aberrations using the equation $Y = a + bx$ (Snedecor and Cochran, 1967).

**RESULTS AND DISCUSSION**

The ethyl methanesulfonate induced aberrations in the silkworm chromosomes were of gross as well as individual types. The gross type of aberrations included stickiness, clumping and pulverisation. Individual types represented so-called point mutations including fragmentation, loops, partially paired bivalents and reciprocal translocation. Different types of aberrations and frequencies are presented in plates I, II, III and IV, and tables I, II, III, IV, V and VI respectively. The regression analysis revealed that there was a linear increase in the percentage of total chromosomal aberrations in relation to the dose of EMS tested (figures 1,2,3,4,5 and 6).

Of the tested races, Pure Mysore larvae were more sensitive to EMS than those of NB4D2 race. Total aberrations at 40 mM concentration in the Pure Mysore race at pachytene, metaphase I and metaphase II were 50.28 ± 0.2, 84.58 ± 0.37 and 86.18 ± 0.3 percent, respectively. On the other hand, in the NB4D2 race the total aberrations at pachytene, metaphase I and metaphase II were 46.30 ± 0.14, 73.03 ± 0.39 and 76.93 ± 0.23 percent, respectively. A similar type of variation was observed in all individual types of aberrations considered as well as at all doses of EMS tested (Tables I-VI).
Plate 1. Sequence of meiosis in the testes of control silkworms of Pure Mysore race.
*Planche 1. Séquence de la méiose dans les testicules des vers à soie témoins de la race Pure Mysore.*
Plate II. Sequence of meiosis in the testes of control silkworms of NB4 D2 race.
*Planche II. Séquence de la méiose dans les testicules des vers à soie témoins de la race NB4D2.*
a. Pachytene showing a fragment (arrow).

b. Pachytene showing two loops (arrow A & B) and a unpaired bivalent (arrow C).

c. Aberrant chromosomes loops (arrow A & B) and unpaired bivalent (arrow C) [Figure b. magnified].

Plate III. Sequence of chromosomal aberrations in the testes of EMS treated Pure Mysore silkworms.

Planche III. Séquence des aberrations chromosomiques dans les testicules de vers à soie de Pure Mysore traités à l'EMS.
Plate III. Sequence of chromosomal aberrations in the testes of EMS treated Pure Mysore silkworms.

Planche III. Séquence des aberrations chromosomiques dans les testicules de vers à soie de Pure Mysore traités à l'EMS.
h. Metaphase I showing clumping.

h. Métaphase I présentant un rétrécissement.

i. Metaphase II showing a minute fragment (arrow) and stickiness.

i. Métaphase II présentant un fragment minuscule (flèche) et un attachement.

j. Metaphase II showing clumping.

j. Métaphase II présentant un rétrécissement.

The increased level of aberrations in Pure Mysore larvae compared to NB4D2 worms might be due to the smaller biomass of the former race compared to the later.

Among different types of aberrations observed, chromosome stickiness and clumping (plate IIId, g, h, i, & j, and plate IV g, h, i & j) were prominent with very high frequencies. In stickiness and clumping, the chromosome complements stuck together and formed an irregular mass. In extreme clumping, the individuality of the chromosomes was lost. Various biochemical views for this phenomenon have been put forth by many workers. Stickiness results from the breakdown of the nucleic acid into nuclear sap (Darlington, 1942; De Roberties et al., 1948). According to Leuchtenberger, it is due to high proteolytic activity (Kumaraswamy, 1977). Mc Gill et al., (1974), reported that the stickiness is due to formation of submicroscopic chromatin strands between unrelated chromosomes. It is also possible that the protein component of the chromosomes might get depolymerised and increases stickiness upon treatment with EMS. Most of the reports suggest that metaphases show highest susceptibility to treatment with mutagens, perhaps due to the maximum condensation of the chromosomes at this stage, with metaphase II being more sensitive. The present results confirm this observation. Mohamood and Vasudev (1993) made a similar observation in the grasshopper Poicilocerus pictus using EMS.
Plate IV. Sequence of chromosomal aberrations in the testes of EMS treated NB$_4$D$_2$ silkworms.

Planche IV. Séquence d’aberrations chromosomiques dans les testicules de vers à soie NB$_4$D$_2$ traités à l’EMS.
d. Metaphase I showing two minute fragments (arrows).

\[ d.\text{ Métaphase I présentant deux fragments minuscules (flèches).} \]

e. Metaphase I showing translocated quadrivalent.

\[ e.\text{ Métaphase I présentant un quadrivalent transloqué.} \]

f. Translocated quadrivalent (arrow) [figure (e) magnified].

\[ f.\text{ Quadrivalent transloqué (flèche) [figure (e) agrandie].} \]

Plate IV. Sequence of chromosomal aberrations in the testes of EMS treated NB₄D₂ silkworms.

\[ \text{Planche IV. Séquence d'aberrations chromosomiques dans les testicules de vers à soie \textit{NB₄D₂} traités à l'EMS.} \]
Plate IV. Sequence of chromosomal aberrations in the testes of EMS treated NB₄D₂ silkworms.

Table I. Frequencies (in percentage) of Aberration in the Pachytene stage in *Bombyx mori* induced by EMS. Race: Pure Mysore.

<table>
<thead>
<tr>
<th>Types</th>
<th>EMS Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td>Loops / Boucles</td>
<td>0.69 ± 0.12</td>
</tr>
<tr>
<td>Fragments</td>
<td>2.88 ± 0.14</td>
</tr>
<tr>
<td>Unpaired chromosomes</td>
<td>0.39 ± 0.08</td>
</tr>
<tr>
<td>(Y-shaped) Chromosomes non</td>
<td></td>
</tr>
<tr>
<td>appariés (en forme de Y)</td>
<td></td>
</tr>
<tr>
<td>Total aberration</td>
<td>3.96 ± 0.18</td>
</tr>
</tbody>
</table>

500 sets of chromosomes were screened per dose. / 500 lots de chromosomes analysés par dose. All the values are significant at 1% level compared to controls and among different doses. / Toutes les valeurs sont significatives à un seuil de 1 % par comparaison aux témoins et entre les différentes doses. ± SEM. / Intervalle de confiance de la moyenne.
Table II. Frequencies (in percentage) of Aberration in the Pachytene stage in Bombyx mori induced by EMS, Race; NB₄D₂.

Tableau II. Fréquences (en pourcentage) d’aberrations au stade pachytène chez Bombyx mori induites par EMS, Race NB₄D₂.

<table>
<thead>
<tr>
<th>Types</th>
<th>EMS Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td>Loops/ Boucles</td>
<td>0.72 ± 0.08</td>
</tr>
<tr>
<td>Fragments</td>
<td>2.18 ± 0.15</td>
</tr>
<tr>
<td>Unpaired chromosomes (Y-shaped)</td>
<td>0.36 ± 0.08</td>
</tr>
<tr>
<td>Chromosomes non appariés (en forme de Y)</td>
<td></td>
</tr>
<tr>
<td>Total aberration</td>
<td>3.26 ± 0.21</td>
</tr>
</tbody>
</table>

500 sets of chromosomes were screened per dose. / 500 lots de chromosomes analysés par dose.
All the values are significant at 1% level compared to controls and among different doses. / Toutes les valeurs sont significatives à un seuil de 1 % par rapport aux témoins et entre les différentes doses.
± SEM. / Intervalle de confiance de la moyenne. * Not significant. / Non significatif.
** Significant at 5% level (CD- 0.753). / Significatif au seuil de 5 % (DC - 0.753).

Figure 1. Dose effect relationships of chromosomal aberrations induced by EMS during Pachytene of Pure Mysore silkworms.
Figure 1. Relations entre les doses et les aberrations chromosomiques induites par EMS pendant le stade Pachytène des vers à soie Pure Mysore.
Table III. Frequencies (in percentage) of Aberration in the Metaphase I stage in *Bombyx mori* induced by EMS, Race; Pure Mysore.

Tableau III. Fréquences (en pourcentage) d’aberrations au stade de la métaphase I chez *Bombyx mori* induites par EMS.

<table>
<thead>
<tr>
<th>Types</th>
<th>EMS Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td>Translocations</td>
<td>0.00 ± 0.0</td>
</tr>
<tr>
<td>Fragments</td>
<td>0.00 ± 0.0</td>
</tr>
<tr>
<td>Stickiness Attachment</td>
<td>1.29 ± 0.12</td>
</tr>
<tr>
<td>Stickiness and Clumping</td>
<td>1.44 ± 0.12</td>
</tr>
<tr>
<td>Total Total</td>
<td>2.73 ± 0.21</td>
</tr>
</tbody>
</table>

500 sets of chromosomes were screened per dose. / 500 lots de chromosomes analysés par dose.

All the values are significant at 1% level compared to controls and among different doses. / Toutes les valeurs sont significatives au seuil de 1 % par comparaison aux témoins et entre les différentes doses.

± SEM. / Intervalle de confiance de la moyenne. * Not significant. / Non significatif.

+ Significant at 5% level (CD- 0.471). / Significatif au seuil de 5 %. (DC - 0.471).

Figure 2. Dose-effect relationships of chromosomal aberrations induced by EMS during Pachytene of NB₄D₂ silkworms.

Figure 2. Relations entre la dose et les aberrations chromosomiques induites par l’EMS au cours du Pachytène chez les vers à soie NB₄D₂.
Table IV. Frequencies (in percentage) of Aberration in the Metaphase I stage in *Bombyx mori* induced by EMS. Race; NB4D2.

*Tableau IV. Fréquences (en pourcentage) d’aberrations au stade de la métaphase I chez Bombyx mori induites par EMS. Race. : NB4D2.*

<table>
<thead>
<tr>
<th>Types</th>
<th>EMS Concentration (mM)</th>
<th>CD at 1%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0</td>
<td>2.5</td>
</tr>
<tr>
<td>Translocations</td>
<td>0.00 ± 0.0</td>
<td>1.82 ± 0.07</td>
</tr>
<tr>
<td>Fragments</td>
<td>0.00 ± 0.0</td>
<td>2.50 ± 0.13</td>
</tr>
<tr>
<td>Stickiness</td>
<td>1.22 ± 0.11</td>
<td>12.18 ± 0.43</td>
</tr>
<tr>
<td>Attachement</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stickiness and Clumping</td>
<td>1.32 ± 0.13</td>
<td>13.87 ± 0.43</td>
</tr>
<tr>
<td>Attachement et rapprochement</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>2.54 ± 0.13</td>
<td>30.37 ± 0.84</td>
</tr>
</tbody>
</table>

500 sets of chromosomes were screened per dose. / *500 lots de chromosomes sont analysés par dose.*

All the values are significant at 1% level compared to controls and among different doses. / *Toutes les valeurs sont significatives au seuil de 1% par comparaison aux témoins et entre les différentes doses.*

± SEM / *Intervalle de confiance de la moyenne.* *Not significant / Non significatif.*

** Significant at 5% level (CD- 0.4065). / *Significatif au seuil de 5 % (DC – 0.4065).*

+ Significant at 5% level (CD- 0.539). / *Significatif au seuil de 5 % (DC – 0.539).*

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Figure 3. Dose-effect relationships of chromosomal aberrations induced by EMS during Metaphase-I of Pure Mysore silkworms.

*Figure 3. Relations entre la dose et les aberrations chromosomiques induites par l’EMS au cours de la Métaphase-I chez des vers à soie Pure Mysore.*

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Table V. Frequencies (in percentage) of Aberration in the Metaphase II stage in Bombyx mori induced by EMS. Race: Pure Mysore.

Tableau V. Fréquences (en pourcentage) d’aberrations au stade de la métaphase II chez Bombyx mori induites par EMS. Race: Pure Mysore.

<table>
<thead>
<tr>
<th>Types</th>
<th>EMS Concentration (mM)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0</td>
<td>2.5</td>
<td>5.0</td>
<td>10.0</td>
<td>20.0</td>
<td>40.0</td>
<td>CD at 1%</td>
</tr>
<tr>
<td>Fragments</td>
<td>0.00 ± 0.0</td>
<td>1.56* ± 0.10</td>
<td>2.36* ± 0.09</td>
<td>4.08 ± 0.11</td>
<td>6.04± 0.29</td>
<td>7.36 ± 0.16</td>
<td>0.9760</td>
</tr>
<tr>
<td>StickinessAttachment</td>
<td>1.08 ± 0.07</td>
<td>19.34 ± 0.10</td>
<td>23.54± 0.18</td>
<td>31.54 ± 0.10</td>
<td>33.32 ± 0.16</td>
<td>38.26 ± 0.20</td>
<td>0.9636</td>
</tr>
<tr>
<td>Stickiness and ClumpingAttachment et rétrécissement</td>
<td>1.24 ± 0.07</td>
<td>22.07 ± 0.20</td>
<td>29.43 ± 0.12</td>
<td>35.09 ± 0.11</td>
<td>38.07 ± 0.10</td>
<td>40.56 ± 0.17</td>
<td>0.9475</td>
</tr>
<tr>
<td>Total aberrationTotal</td>
<td>2.32 ± 0.10</td>
<td>42.97 ± 0.16</td>
<td>55.33 ± 0.29</td>
<td>70.71 ± 0.15</td>
<td>77.43 ± 0.36</td>
<td>86.18 ± 0.30</td>
<td>1.6970</td>
</tr>
</tbody>
</table>

500 sets of chromosomes were screened per dose. / 500 lots de chromosomes sont analysés par dose.
All the values are significant at 1% level compared to controls and among different doses. / Toutes les valeurs sont significatives au seuil de 1 % par comparaison aux témoins et entre les différentes doses.
± SEM. / Intervalle de confiance de la moyenne.
* Significant at 5% level (CD-0.656). / Significatif au seuil de 5 %.

Figure 4. Dose-effect relationships of chromosomal aberrations induced by EMS during Metaphase-I of NB₄D₂ silkworms.
Figure 4. Relations entre les doses et les aberrations chromosomiques induites par l’EMS au cours de la Métaphase-I chez des vers à soie NB₄D₂.
Table VI. Frequencies (in percentage) of Aberration in the Metaphase II stage in *Bombyx mori* induced by EMS, Race : NB4D2.

*Tableau VI. Fréquences (en pourcentage) d’aberrations au stade de la métaphase II chez *Bombyx mori* induites par EMS. Race : NB4D2.*

<table>
<thead>
<tr>
<th>Types</th>
<th>EMS Concentration (mM)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0</td>
<td>2.5</td>
<td>5.0</td>
<td>10.0</td>
<td>20.0</td>
<td>40.0</td>
</tr>
<tr>
<td>Fragments</td>
<td>0.00 ± 0.0</td>
<td>0.98* ± 0.04</td>
<td>1.06* ± 0.02</td>
<td>2.40 ± 0.08</td>
<td>4.50 ± 0.17</td>
<td>6.91 ± 0.14</td>
</tr>
<tr>
<td>Stickiness Attached</td>
<td>0.54 ± 0.03</td>
<td>12.01 ± 0.30</td>
<td>4.50 ± 0.0</td>
<td>19.77 ± 0.12</td>
<td>22.49 ± 0.09</td>
<td>32.04 ± 0.24</td>
</tr>
<tr>
<td>Stickiness and Clumping</td>
<td>1.25 ± 0.07</td>
<td>13.98 ± 0.16</td>
<td>18.35 ± 0.28</td>
<td>29.72 ± 0.11</td>
<td>34.38 ± 0.13</td>
<td>37.98 ± 0.09</td>
</tr>
<tr>
<td>Total</td>
<td>1.79 ± 0.08</td>
<td>26.97 ± 0.26</td>
<td>39.18 ± 0.33</td>
<td>54.61 ± 0.20</td>
<td>68.31 ± 0.15</td>
<td>76.93 ± 0.23</td>
</tr>
</tbody>
</table>

500 sets of chromosomes were screened per dose. / *500 lots de chromosomes sont analysés par dose.*

All the values are significant at 1% level compared to controls and among different doses. / *Toutes les valeurs sont significatives au seuil de 1 % par comparaison aux témoins et entre les différentes doses.*

± SEM. / *Intervalle de confiance de la moyenne.*

* Not significant. / *Non significatif.*

Figure 5. Dose-effect relationships of chromosomal aberrations induced by EMS during Metaphase-II of Pure Mysore silkworms.

*Figure 5. Relations entre les doses et les aberrations chromosomiques induites par EMS au cours de la métaphase-II chez des vers à soie de Pure Mysore.*
Figure 6. Dose effect relationships of chromosomal aberrations induced by EMS during Metaphase-II of NB4D2 silkworms.

Figure 6. Relations entre les doses et les aberrations chromosomiques induites par EMS au cours de la métaphase II chez des vers à soie NB4D2.

Fragmentation represents the second major type of aberration, which is identified by the presence of minute chromosome(s) (plate IIIa, d & i, and plate IVa, d and i). However, mechanisms involved are not well understood. The hypothesis often cited is that DNA bases are ethylated by EMS and gets gradually hydrolyzed from the deoxyribose, leaving behind an apurinic or possibly an apyrimidine site, which leads to single strand breakage of DNA (Sega, 1984). Eventually this might lead to fragmentation.

Loops and partially paired bivalents occur due to the unpairing of some chromosomal segments (plate IIIb & c, and plate IVb and c); it might be due to the presence of non-homologous segment in the homologous chromosomes. Both these types of aberrations might be due to either inversion or translocation or duplication or deletion. As shown in the plate III, the loop formed by chromosome labelled A1 bulged out over the chromosome labelled A2. This might be due to either deletion of interstitial segment of lower chromosome (A2) or interstitial duplication of the upper chromosome (A1).

Reciprocal translocation (plate IIIe & f, and plate IVe and f) might occur either between the autosomes and sex chromosomes or between autosomes only. As there are no marker chromosomes nor morphologically identifiable sex chromosomes in Bombyx mori, the identification of sex chromosomes in spermatogenic cells under light microscope is difficult (Tazima, 1964); therefore, translocations in general were considered. However, it is necessary to study the frequency of translocations between autosomes and sex chromosomes with the influence of EMS as it helps in the evolution of sex-limited races.

Since the silkworm is an economically important insect, induction of sterility or lethality is not a good criterion; unlike in other insects which are pests to agricultural crops, animals and human beings. According to Mahesha, 1997 the lower doses like 2.5 and 5 mM do not cause any sterility or lethality in Bombyx mori. Hence these lower doses of EMS can be used in sericulture industry to
induce beneficial mutations, which go a long way to improve the industry and at the same time the higher doses of EMS, which cause sterility can be used to overcome the menace caused by other lepidopteron to mankind.

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REFERENCES