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# Cytotoxic assay of 2,4–dichlorophenoxyacetic acid (2,4–D) by mitotic index profiles in *Culex pipiens fatigans*

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Neuroblast cells obtained from the brain tissues of larval stages of *Culex pipiens fatigans* were subjected to mitotic index analysis as part of an elaborate study of 2,4-dichlorophenoxyacetic acid (2,4–D) induced genotoxicity. Multiple applications in varying concentrations and durations were maintained for three consecutive generations. Mitotic activity was seriously hampered in all the experiments. A steady state of depression in dividing cells was observed in all generations, more conspicuously in $F_1$. The cell growth was inhibited and larval developmental time in treated series was affected and significantly delayed. 2,4-D was found to be extremely cyto-toxic in this organism.

Keywords: Cytotoxic assay, 2,4-dichlorophenoxyacetic acid (2,4–D), *Culex pipiens fatigans.*

## INTRODUCTION

Since the late 1940's, 2,4-dichlorophenoxyacetic acid (2,4–D) has been widely used for its dual properties in plants. At higher concentrations it is used as a plant killer (ALLARD *et al.*, 1946; HAMNER *et al.*, 1946; MOORE, 1974) and at lower concentrations it is known to promote growth in a manner apparently identical with that of indole-acetic acid (HSUEH & LOU, 1947; MOORE, 1974). The importance of 2,4–D for agriculture and forestry resulted in increased numbers of reports on its mode of action (BAMBERGER, 1970; ASHTON & CRAFTS, 1973; LOOS, 1975; WEGLER & EUE, 1977).

Recently 2,4–D has been so widely used that similar studies have been extended to animals as well.

JENSSEN & REMBERG (1976) reported the broad cytogenetic effects in mouse blood. In-vivo tests performed by YOURCHENKO (1977) confirmed these findings. Cytogenetic effects have also been studied in SV–40 transformed human fibroblasts (AHMED *et al.* 1977). However, all the reports are not positive. 2,4–D was not significantly able to influence DNA synthesis in mouse testes (SEILER, 1978). A less clear relationship is obained with 2,4–D in animals.

Derivatives of phenoxyacetic acid, notably pentachlorophenol (PCP), were discovered subsequently to have different properties. These include seriously hampering the cell growth and inhibition of DNA, RNA and ribosome synthesis (SIKKA & SHARMA, 1976; CHAND, 1980; EHRLICH *et al.*, 1987; DE MARINI *et al.*, 1990).

By that reckoning the toxicological evaluation of 2,4–D was not so high. A perusal of available literature indicated that barring reports of MOHANDAS & GRANT (1972) and BAYLISS (1973) – who studied 2,4–D induced mitotic abnormalities in *Drosophila* – not many studies have been undertaken relating to mitotic index, which is a convenient method of assessing the mutagenicity in any organism, especially in insects. Occasional studies by VOGEL & CHANDLER (1974), MAGNUSSON *et al.* (1977) and RASMUSSON (1977) were not conclusive.

An elaborate mutagenicity testing programme in filarial vector *Culex pipiens fatigans* was therefore undertaken. The present report is a part of it and deals specifically with the effects of 2,4–D on the mitotic activity in neuroblast cells and its overall affect on growth and development. The results are unique and assume a significant importance in the absence of any real answer to the problems arising out of indiscriminate use of 2,4–D from a genetic point of view.

## MATERIALS AND METHODS

Recently, indiscriminate use of 2,4–D is being debated. For these experiments, a border line dose, 100 parts per million (ppm) in between the herbicidal and hormonal properties, was selected. Lesser doses of 75, 50, 25 and 1 ppm were also tried. For various concentrations a stock solution of 1000 ppm in 2% alcoholic distilled water made on a volumetric basis was used and administered through whole body immersion of 4$^{th}$ instar larvae in 2 and 4 hrs exposures series. The experiment was initiated by taking 1000 larvae of uniform size and age in each concentration and duration. Consequently 100 larvae were sacrificed for brain squashes and the rest were inbred to obtain the next generation. Three subsequent exposures of 2,4–D were given in as many generations.

Serial exposure as adopted above is in contrast to single application so often used by the majority of authors (GREEN *et al.*, 1973; KHALATKAR & BHARGAVA, 1982; KUMARI & VAIDYANATH, 1989). This ensured extensive information about the nature and extent of damage to neuroblast cells, and facilitated reliable comparative studies in and among the progenies. Strict controls were maintained simultaneously in 2% alcoholic water. The experiments were replicated at least 3 times under identical conditions. Other details relating to feeding and rearing were similar to those described earlier (AHMAD *et al.*, 1983).

The cytological preparation were made using acetic acid-dissociated-air drying techniques (CROZIER, 1968). Dividing cells from over a total of 20,000 were thoroughly observed. Mitotic index (MI) was computed by the formula

$$MI = \frac{\text{Total no. of dividing cells}}{\text{Total no. of cells observed}} \times 100$$

In another study undertaken to observe the effects of 2,4–D on acquatic development, all the details pertaining to preparation, concentration and treatment of 2,4–D were uniform, though the stage of treatment was different – 1$^{st}$ instar in this case. A total of 10 batches each of 100 larvae were exposed and a separate control series was also maintained for comparison. Total larval duration and time to pupation, its range and mortality were carefully recorded at regular intervals for the entire aquatic duration.

## RESULTS AND DISCUSSION

Profile of mitotic indices, inhibitory effect of 2,4–D on dividing cells and delayed larval development have been shown in Tab. 1 and Figs. 1 and 2, respectively.

As recorded earlier most of the given concentrations of 2,4–D register serious consequences to the dividing cells from one generation to another, indicating a cumulative effect, consequently as a result of constant pressure of multiple exposure throughout. A steady rise in the frequency of injured cells with respect to chro-

mosomes is observed: There is corresponding increase in breaks, acentrics, dicentrics, gaps, ploidy and other aberrations (ALI & AHMAD, 1994a, 1994b). Changes studied at morphological level are equally significant; some of them already discovered, register increased frequencies while others were new (ALI & AHMAD, 1994c). These studies substantiated the cumulative effects. Incidentally the initial chemical damage induced by 2,4–D is subsequently manifested at the level of chromosome, some of which can be related to morphological mutations.

Conversely, changes in mitotic profiles are more due to higher concentrations rather than cumulative pressure. Incidentally serious impairment of divisional stages and low mitotic rates leave a reasonably high number of cells with impaired and injured chromosomes. Comparable effects achieved with higher concentration were mimicked by lower concentrations in a later generation (Fig. 1). Depression in the

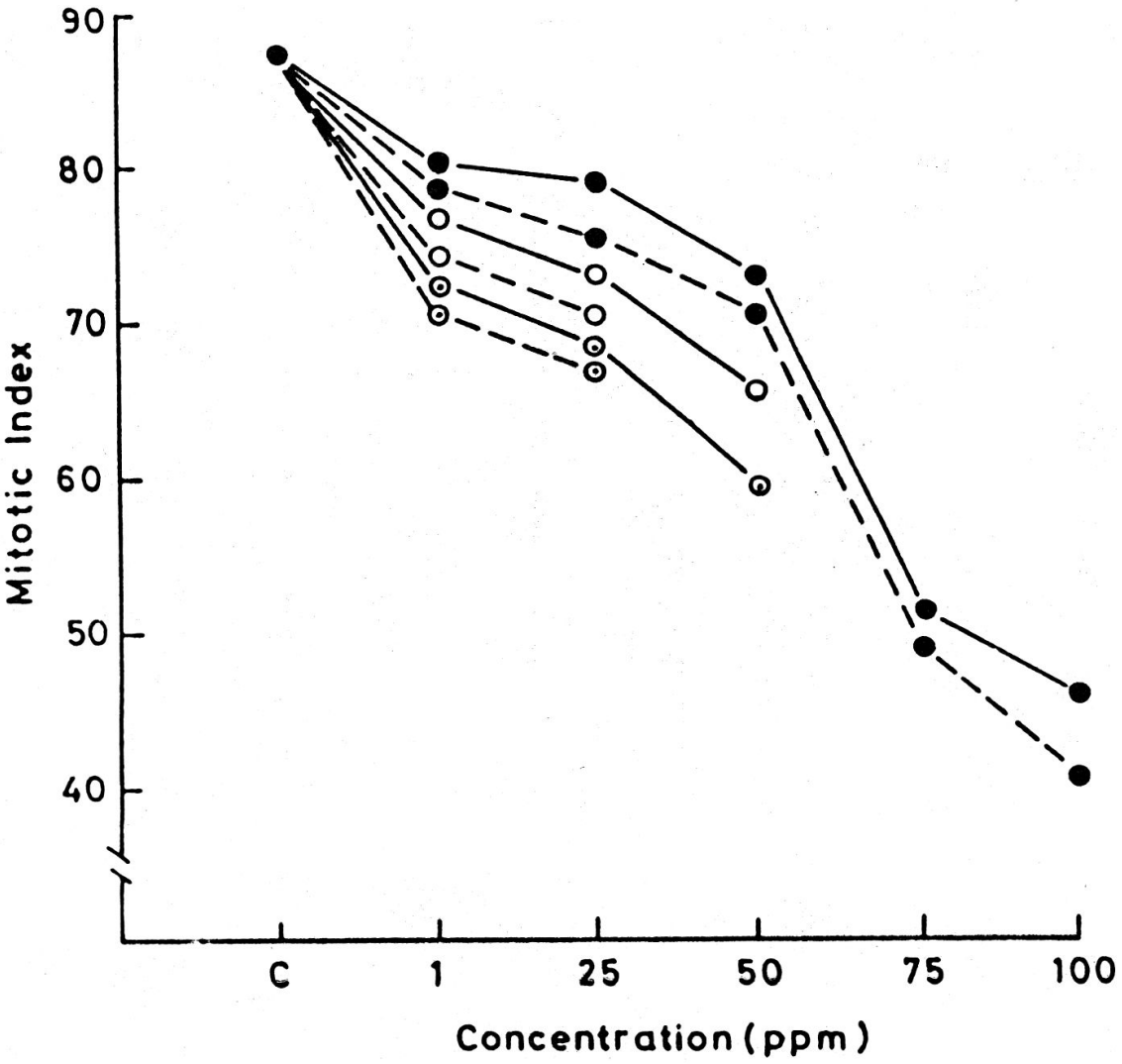


Fig. 1. Effect of various concentrations of 2,4–dichlorophenoxyacetic acid on mitotic activity in *Culex pipiens fatigans*. Exposure for each concentration was given for three successive generations. The decreasing profiles of the Mitotic Index are evident. – Symbols: ● = $F_1$; O = $F_2$; ʘ = $F_3$; full lines: 2hrs, hatched lines: 4 hrs exposure; C = control.

rate of division in exposed forms significantly increased the larval duration and further induced distorted growth in few cases. Time to pupation as a result of slow growth was increased by as much as 50 hrs in some replicates over and above the normal 183 hours. This has been depicted in Fig. 2.

A stable state of sterility induced by higher concentrations beyond 50 ppm is another striking effect of 2,4–D.

Inconclusive results indicate this phenomenon to be due to an impairment of spermatogenesis. Meiotic tissues are therefore under similar investigations. In the light of the above indications and also from the reports on pentachlorophenol (SIKKA & SHARMA, 1976), the potential of male sterility in genetic control of insect vectors of disease and pest management is fairly promising.

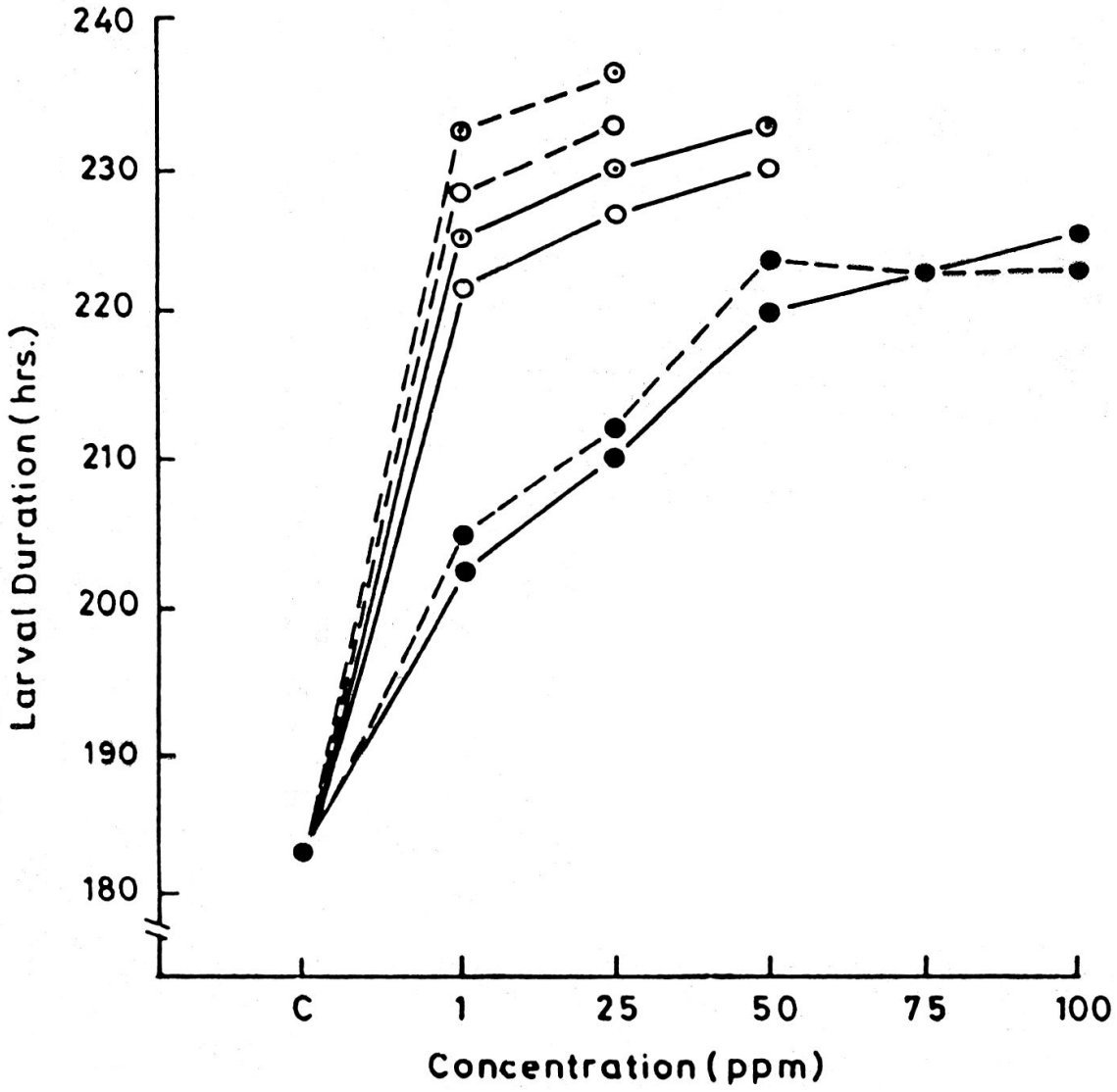


Fig. 2. Effect of various concentrations of 2,4–dichlorophenoxyacetic acid on larval development in *Culex pipiens fatigans*. Exposure for each concentration was given for three successive generations. There is a pronounced delayed development. – Symbols: ● = $F_1$; ○ = $F_2$; ⊙ = $F_3$; full lines: 2hrs, hatched lines: 4 hrs exposure; C = control.

Tab. 1. Mitotic index (MI) of 2,4-D treated neuroblast cells.

| Generation<br>Concentration<br>Duration | Cells screened | Cells in dividing stage | MI ± S.D. |
|---|---|---|---|
| P1: Control | 1130 | 985 | 87.15 ± 2.83 |
| F1: $C_1^2$ | 943 | 766 | 81.21 ± 3.46 |
| $C_1^4$ | 1234 | 982 | 79.56 ± 3.85 |
| $C_{25}^2$ | 756 | 610 | 80.63 ± 2.93 |
| $C_{25}^4$ | 1039 | 94 | 76.35 ± 4.62 |
| $C_{50}^2$ | 847 | 622 | 73.43 ± 4.08 |
| $C_{50}^4$ | 524 | 373 | 71.01 ± 2.76 |
| $C_{75}^2$ | 752 | 381 | 50.59 ± 2.30 |
| $C_{75}^4$ | 1665 | 836 | 50.17 ± 3.92 |
| $C_{100}^2$ | 425 | 199 | 46.53 ± 2.25 |
| $C_{100}^4$ | 621 | 258 | 41.49 ± 4.00 |
| F2: $C_1^2$ | 1132 | 889 | 78.51 ± 3.64 |
| $C_1^4$ | 1367 | 1023 | 74.80 ± 2.04 |
| $C_{25}^2$ | 692 | 493 | 71.94 ± 2.25 |
| $C_{25}^4$ | 963 | 657 | 70.27 ± 3.85 |
| $C_{50}^2$ | 715 | 426 | 64.19 ± 3.25 |
| $C_{50}^4$ / $C_{75}$ / $C_{100}$ | –* | –* | –* |
| F3: $C_1^2$ | 620 | 456 | 73.38 ± 3.25 |
| $C_1^4$ | 992 | 724 | 72.80 ± 3.85 |
| $C_{25}^2$ | 559 | 387 | 69.47 ± 4.49 |
| $C_{25}^4$ | 963 | 657 | 68.17 ± 4.85 |
| $C_{50}^2$ | 715 | 426 | 58.83 ± 4.75 |
| $C_{50}^4$ / $C_{75}$ / $C_{100}$ | –* | –* | –* |

C-subscripts refer to concentrations in 1, 25, 50, 75 & 100 ppm; superscripts refer to 2 or 4 hours treatment; * = sterility induced in these concentrations.

The low solubility of 2,4–D, as with most acids, makes it apparently less toxic on a molar basis. In the majority of mammals an accumulation of phenoxy acid is unlikely because of quick clearance (SEILER, 1978). This could not be confirmed in mosquitoes by any such report. It might, however, be possible that repeated exposures may have created a steady state of concentration capable of causing serious inhibition to mitotic cycle, inflicting injuries to chromosomes and delaying the development of various acquatic stages.

There are similar reports on 2,4–D in plant tissues – a comparison, however, had a limited validity (MELCHERS & BERGMANN, 1959; BAYLISS, 1973). In general it has been observed that phenoxy acids do influence the genetic material or the

mitotic cycle or both, but owing to the physiological activity of these substances on the plant cells, its significance for the present observations cannot be clearly assessed.

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