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Validation of cytotoxicity and mutagenicity of Acetamiprid through Ames and root meristem study of *Trigonella foenum-graecum*. L

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Abstract: **The present investigation has been done to validate the mutagenic and cytotoxic effect of a neonicotinoid insecticide, Acetamiprid through ames test and root meristem study of *Trigonella foenum-graecum*. Presoaked seeds of *Trigonella foenum-graecum* were treated with various concentrations of Acetamiprid viz 15, 30, 45 and 60ppm for 6, 12 and 24 hrs. The treated seeds were washed thoroughly in running tap water and allowed to germinate on moist filter paper in petriplate at 25 ± 1°c. Cytogenetic damages were analyzed after 120 hrs. The result showed significant decrease ($p < 0.05$; $p < 0.01$) in mitotic index and increased chromosomal aberration and micronuclei. The ames test done at the same concentration to validate the mutagenicity also reflected similar results showing increased number of revertant colonies in TA100, TA 102 , TA 1535. The results were observed to be dose dependent for the selected tester strains. These findings help to validate that the insecticide Acetamiprid is a potential mutagen and also have cytotoxic effect on *Trigonella foenum-graecum*.**

Keywords: **Acetamiprid, Ames test, Chromosomal aberration, *Trigonella foenum-graecum*.**

1. INTRODUCTION

Acetamiprid, a neonicotinoid pesticide discovered in late 1980s. It has been used widely in plant protection, veterinary products, fish farming and as biocides to invertebrate pest control. It accounts for about one third of the world insecticide market. These types of pesticides are systemic in nature, thereby absorbed by roots or leaves and translocated to all parts of the plant, which in turn, makes them effectively toxic to herbivores insects [1]. The neonicotinoid pesticides mimic the action of neuro transmitters and they continuously stimulate neurons leading to death of target invertibrates. Majority of pesticides have been tested in a wide variety of mutagenicity assay chromosomal alteration, DNA damage and also its residual effects [2-6]. On account of its wide commercial usage, mode of action, the systemic effects in plants, consistent and environmental fate coupled with curbed information about the toxicity profiles of this compound, the present study was designed. Acetamiprid N–[(6-chloro-3-pyridyl) methyl]-N [1]- cyano-N-methyl-autamidine is a blue to tan crystalline solid, belonging to the chloropyridinyl group Fig (1).

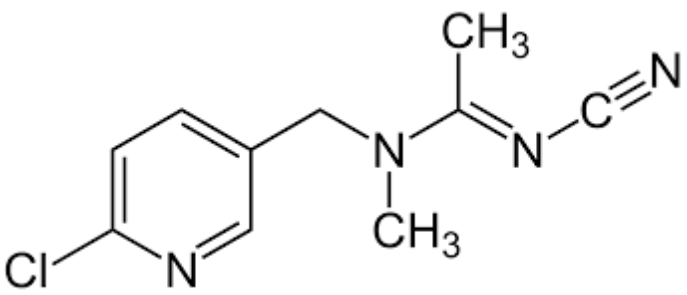

Fig.1 Acetamiprid

Recently European food safety authority (EFSA) published conclusion on the peer review of the pesticide risk assessment of the active substance Acetamiprid [7]. To the best of our knowledge, there is little information available so far about the impact of Acetamiprid on higher plants and its mutagenic potential. Therefore, the present study was undertaken to validate its cytotoxicity and mutagenicity by ames and root meristem examination of *Trigonella foenum-graecum*.

2. MATERIALS AND METHODS

Chemicals

A commercial formulation of Acetamiprid was purchased from local market as Assail (Acetamiprid-10% EC) other chemicals were purchased from E, merk. Co.India

Test system

Seeds of *Trigonella foenum-graecum* (2n= 16) were used for the present study

Cytogenetic assay

Presoaked (12hr) seeds were treated with above test concentrations of Acetamiprid for 6, 12 and 24 hrs. After the treatment, the seeds were thoroughly washed with running tap water for an hour and allowed to germinate on moist filter paper placed in petriplates at 25±1°c in dark. Ethyl methane sulfonate (EMS 10ppm) and tap water were also maintained simultaneously as positive and negative controls. After germination some of the roots (1-1.5cm length) were excised and fixed in acetic acid - ethanol (1:3) for cytogenetic assay. The frequencies of mitotic index (MI), chromosomal aberrations (CA), such as metaphase and anaphasic abnormalities and interphase cells with micronuclei (MN) were determined Fiskesjo last modified by Rank and Nelsen [8-9]. A minimum of 15000 cells from root tips were scored for each treatment and was analyzed.

Ames test

Salmonella tester strains were received from Krishgen, Bombay. The Acetamiprid was dissolved in sterile distilled water. Dose formulations were prepared on the day of use.

Dose Formulation Analysis

Dose formulation concentrations of 15, 30, 45 and 60ppm of samples were prepared by serial dilution for the study. Tester strains were exposed to the samples via plate incorporation methodology described by Maron and Ames [10].

Plating Procedures

These procedures were used in the dose range-finding and mutagenicity assays. Each plate was labeled with the samples, test phase, tester strain, activation condition, and dose. Treatments in the absence of S9 were performed by adding 100 µl tester strain and 100 µl test or control article to 2.5 ml molten diluted top agar (maintained at 45 ±2 ^{0}C). The mixtures were vortexed and overlaid onto the surface of bottom agar dishes. After the overlay solidifes; the plates were inverted and incubated for 72 hrs at 37 ±2 ^{0}C. After incubation the plates were evaluated for the condition of the background lawn for the evidence of cytotoxicity and samples precipitate in comparison with the control and the plates were evaluated for the number of revertant colonies.

Statistical analysis

All data values are expressed as mean ± SD and the level of significance between the control and treated groups were evaluated by student't'test.

3. RESULTS

The present investigation elucidated the cytotoxicity and mutagenicity by ames and root meristem examination of *Trigonella foenum-graecum*.

Effects on mitotic index (MI), chromosomal aberrations (CA) and micronuclei (MN)

The effects of Acetamiprid (APD) on mitotic index, chromosomal aberrations and micronuclei of *Trigonella foenum-graecum* root meristem cells are presented in Table 1. Mitotic index was significantly inhibited at 15, 30, 45 and 60ppm of Acetamiprid. Maximum inhibition of mitotic index was observed at 24hr of exposure when compared to 6 and 12 h (Table 1 and Fig. 2). Inhibition was dose and time dependent ($p<0.05$; $p<0.001$).

Table 2 showed the frequency and various types of chromosomal and mitotic aberrations. Dose - dependent increase of chromosomal aberrations were observed in 15, 30, 45 and 60ppm of Acetamiprid. Maximum chromosomal aberrations were recorded at 24 hr of exposure at 60ppm of Acetamiprid (Table 2 and Fig.3). Chromosomal aberrations were significantly increased at maximum hours of exposure ($p<0.05$; $p<0.001$). The frequencies of C-metaphase, sticky metaphase; anaphasic bridge, disturbed anaphase and metaphase were observed in all the concentrations of Acetamiprid (Plate 1). The frequency of cells with micronucleus was also significantly increased with respect to dose and duration of exposure (Table 2 and Fig.4). Positive control (EMS) exhibits significant ($P<0.05$; $p<0.001$) reduction in mitotic index and increases in chromosomal abberation and micronuclei.

Ames Test

The mutagenic effect of Acetamiprid in *S. typhimurium* strain TA100, TA102 and TA 1535 are presented in Table (Table 3). Treatment with various concentrations (15, 30, 45, 60 µg/ppm) of Acetamiprid significantly ($P<0.05$; $p<0.001$) increases the number of his^+ revertant colonies without metabolic activation when compared to positive control (Sodium azide) for the strain TA100, TA 102 and TA 1535. The increase of colonies was observed in dose-dependent manner and there was a considerable increase in his $^+$revertant colonies for all the three strains viz., TA 100, TA 102 and TA 1535. The maximum revertant colonies were observed at TA 100 at 30µg/ppm and TA102 and TA 1535 at the concentration of 60µg/ppm.

4. DISCUSSION

Acetamiprid is a neonicotinoid pesticide, used widely in plant protection is systemic in nature and was reported to have toxic effect both for plants and animals. This pesticide was examined for its cytotoxic and mutagenic effects through ames and root meristem study of *Trigonella foenum-graecum.* Chemicals than can induce mutations can potentially damage the germ line leading to fertility problems and to mutations in future generations. The *Salmonella typhimurium* microsome assay is a widely accepted short-term bacterial assay for identifying substances that can produce genetic damage that leads to gene mutations [11]. Hence this assay was used to detect the mutagenic potential of Acetamiprid.

A compound tested with the ames test was considered mutagenic if the number of his^+ revertant colonies was twice the value of the corresponding solvent control [12]. In this experiment, pre-incubation assay with different concentrations of Acetamiprid significantly increased the number of his+ revertant colonies in TA100, TA102 and TA 1535 of *S. typhimurium*. This has been reported that the pesticide was able to induce TAA (ochre) and G base pair mutations causing a transitions/transversions and base-pair substitution of the histine-dependant tester strain (TA100, TA102 and TA 1535) to the wild type (his+) proving the mutagenic potential ,such phenomenon was also reported by several workers in various pesticides [13- 15]. Wa et al [16] investigated the genotoxicity of dicratophas using ames test, significant change in the numbers of bacterial revertants in strains TA97a, TA98, TA100, TA102 and TA1535 was observed. Mutagenic assay done on several other pesticides like mebudipine, trichlorfon by ames showed a dose dependant increase in the number of revertant colonies for the strains TA100, TA 102 and TA1535 [17-18]. In our investigation the revertant colonies were formed to increase significantly in all the three strains TA100, TA102 and TA1535. Different kinds of chromosomal abnormalities such as gaps and fragments; laggard,c-metaphase and disturbed metaphase were observed. Among these sticky metaphase and disturbed metaphase was predominant and the induction of micronuclei by Acetamiprid indicates clastogenic potential of the test compound. Intermingling of chromatin fibres leads to stickiness of the chromosomes [19]. The stickiness of chromosomes will inturn lead to failure of chromosome movements [20-21]. The interaction of Acetamiprid with tubulin-SH group may lead to impairment of mitotic spindle resulting in C-mitosis and disturbed metaphase [22]. Lagging chromosomes or acentric fragments results in MN formation these chromosome fail to incorporate into either of the daughter nuclei during telophase of the cell cycle, which could cause cell death by the deletion of primary genes [23]. Our results were in akin to the previous reports showing various types of chromosomal aberrations. Apart from the above said cytotoxic effects Acetamiprid was reported to induce toxicity in mice [24-25] whose symptoms are observed through respiratory depression, decreased body weight and diarrhoea. Acetamiprid was also found to have toxic effects on human cells [26] and freshwater fishes [27]. Genotoxic evaluation of Acetamiprid using the mosquito genome showed various kinds of chromosomal aberrations [28]. Our results are in akin to these reports.

5. CONCLUSION

From this present study, the pesticide Acetamiprid may possess potential cytotoxic and mutagenic effects on *Trigonella foenum-graecum* and *Salmonella typhi*.

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APPENDICES - A

List of Table:

Table 1: Mitotic index in root meristem cells of *Trigonella foenum-graecum* treated with Acetamiprid (for each experimental variable 10 root tips)

Treatments (ppm)	Duration	Total number of cells analysed	Total number of dividing cells	Mitotic Index (%) (Mean ± SD)
Control	--	11786	8843	78.13 ± 9.90
EMS - 10	6 hr 12 hr 24 hr	11160 11260 10990	810 680 675	7.22 ± 0.33 6.18 ± 0.55 6.04 ± 0.46
APD – 15	6 hr 12 hr 24 hr	11222 10486 11204	5898 1590 1600	52.34 ± 6.51 14.12 ± 4.47 12.92 ± 1.85
APD – 30	6 hr 12 hr 24 hr	13004 11978 11764	8146 1452 1220	62.64 ± 5.23 12.20 ± 2.01 10.46 ± 1.59
APD – 45	6 hr 12 hr 24 hr	14154 12104 13596	1876 1282 1144	13.40 ± 3.31 11.96 ± 1.50 8.44 ± 1.13
APD – 60	6 hr 12 hr 24 hr	13334 12074 11492	1820 1226 1005	13.98 ±5.88 10.14 ± 1.72 8.01 ± 2.83

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Table 2: Chromosomal aberration and Micronuclei in root meristem cells of *Trigonella foenum-graecum* treated with Acetamiprid (for each experimental variable 10 root tips)

Treatments (ppm)	Duration	Total number of aberrant cells	Chromosomal aberration (%) (Mean ± SD)	Total number of cells with Micronuclei (MN)	MN/1000 (Mean ±SD)
Control	--	168	1.86 ± 0.44	2	0.18 ± 0.57
EMS - 10	6 hr	205	25.25 ± 1.24 **	18	1.61 ± 0.23
	12 hr	188	27.72 ± 2.05 **	39	3.44 ± 0.21
	24 hr	215	31.45 ± 2.01 **	45	4.07 ± 0.20
APD – 15	6 hr	116	1.98 ± 0.69	3	0.28 ± 0.82
	12 hr	392	24.75 ± 5.65 **	16	1.36 ± 1.32
	24 hr	430	26.85 ± 4.83 **	20	1.66 ± 1.12
APD – 30	6 hr	168	2.14 ± 1.04	3	0.22 ± 0.36
	12 hr	596	40.88 ± 8.06 **	26	2.07 ± 2.28
	24 hr	470	38.79 ± 6.76 **	28	2.41 ± 0.86
APD – 45	6 hr	469	26.43 ± 9.34 **	10	0.82 ± 0.68
	12 hr	360	26.22 ± 9.35 **	27	2.30 ± 1.30
	24 hr	581	51.47 ± 9.65 **	33	2.42 ± 1.05
APD – 60	6 hr	269	14.78 ± 4.35 **	25	1.79 ± 1.08
	12 hr	408	33.83 ± 9.13 **	34	2.53 ± 0.59
	24 hr	595	59.99 ± 11.46 **	32	3.15 ± 1.42

Table 3: Mean Colony Count - Strain TA 100, TA 102 and TA 1535- spontaneous mutation

Samples	Test Concentration (µg/plate) / (ppm)	Histidine revertant colonies (TA 100)	Histidine revertant colonies (TA 102)	Histidine revertant colonies (TA 1535)
Positive Control	Sodium azide (1.5 µg/plate)	1065 ± 62.52	447 ± 23.16	436 ± 21.12
Acetamiprid	15	560 ± 48.13	124 ± 32.19	109 ± 30.21
	30	TNTC	156 ± 28.15	123 ± 16.21
	45	TNTC	199 ± 32.10	162 ± 28.32
	60	TNTC	TNTC	TNTC
	Normal control	152 ± 24.42	328 ± 18.12	387 ± 31.20

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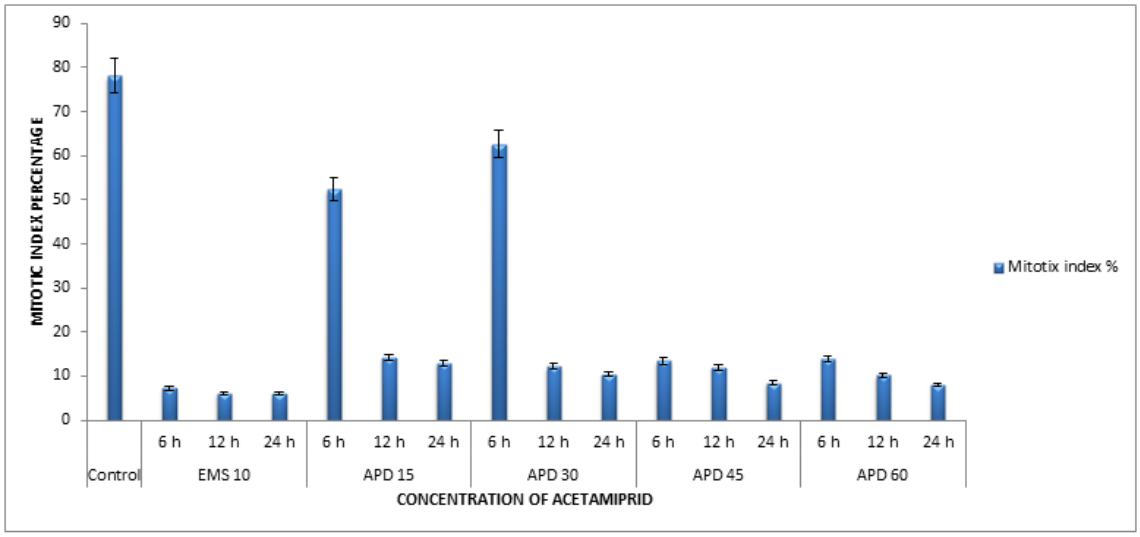

Fig. 2 Mitotic index in root meristem cells of *Trigonella foenum-graecum* treated with Acetamiprid

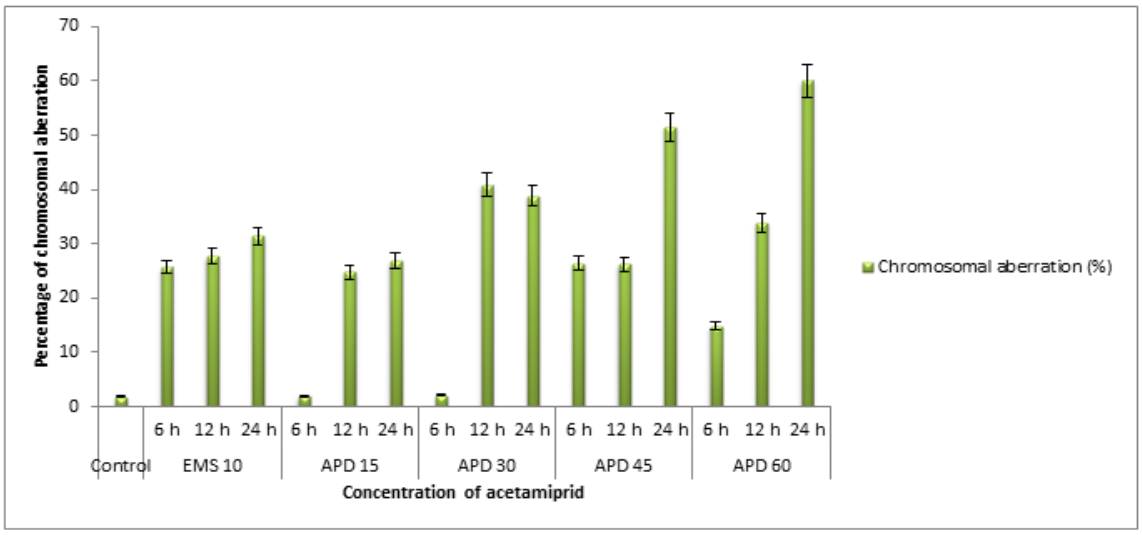

Fig.3 Chromosomal aberration in root meristem cells of *Trigonella foenum-graecum* treated with Acetamiprid (for each experimental variable 10 root tips)

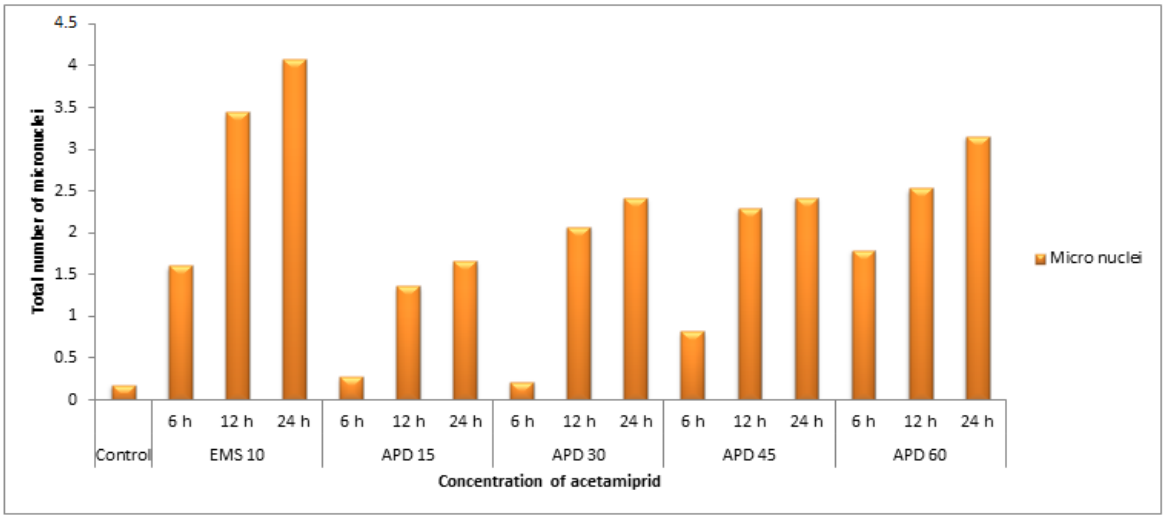

Fig.4 Micro nuclei in root meristem cells of *Trigonella foenum-graecum* treated with Acetamiprid (for each experimental variable 10 root tips)

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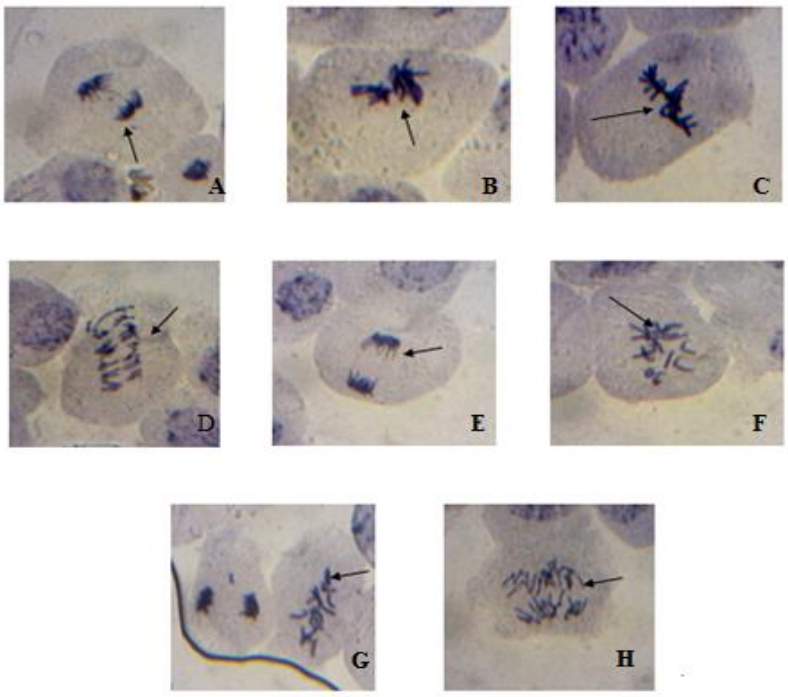


Plate 1 Chromosomal aberration in root meristem cells of *Trigonella foenum-graecum* treated with Acetamiprid

A. C-metaphase

B and C. Sticky chromosomes

D. Disturbed anaphase in a polyploid cell

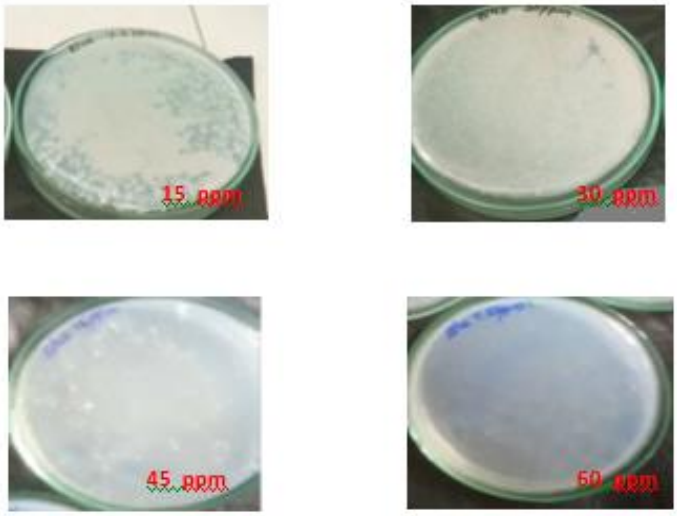
E. Broken bridge in anaphase

F. Chromosome laggards and spindle disturbance at metaphase

G. Vagrant chromosomes in anaphase

H. Multipolar anaphase and spindle disturbance

C-metaphase, sticky metaphase; anaphasic bridge, disturbed anaphase, chromosome laggards and spindle disturbance at metaphase, vagrant chromosomes in anaphase and multipolar anaphase and spindle disturbance



Spontaneous mutation - Strain TA 100

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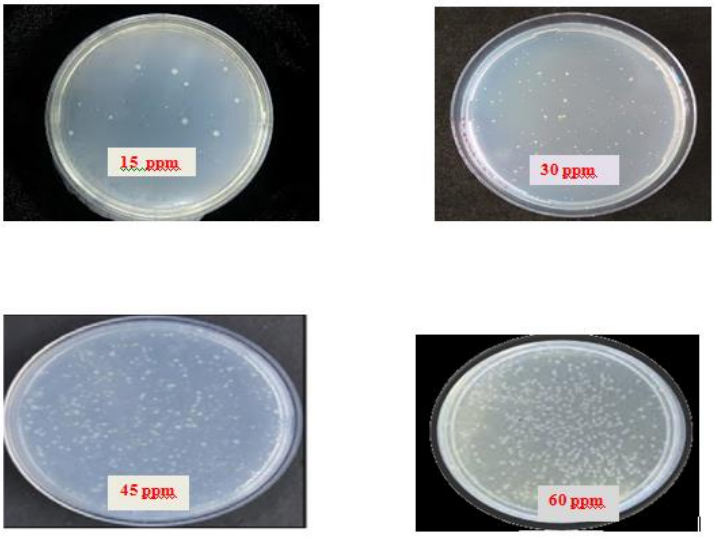
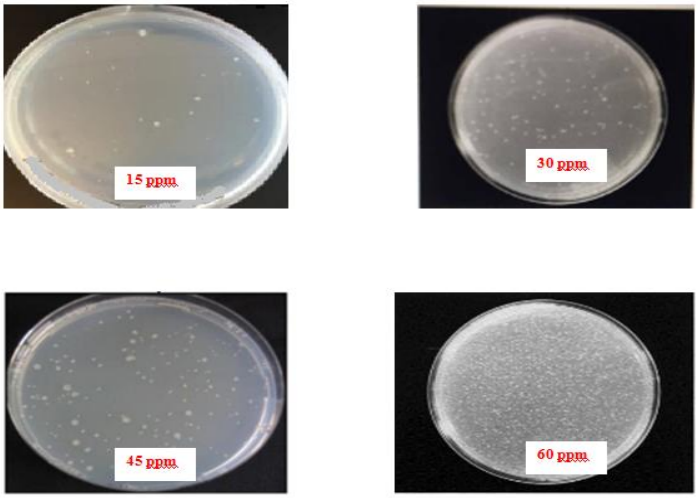


Spontaneous mutation - Strain TA 102



Spontaneous mutation - Strain TA 1535