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Glyphosate, Alachor and Maleic Hydrazide have Genotoxic Effect on *Trigonella foenum-graecum* L.

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Abstract In the present study effects of herbicides glyphosate (GP), alachlor (AL) and maleic hydrazide (MH) is studied on mitotic cells of *Trigonella foenum-graecum* L. Seeds of *T. foenum-graecum* L. treated with a series of concentrations ranging from 0.1%, 0.2%, 0.3%, 0.4% and 0.5% for 1, 2 and 6 h and their effect on mitotic index and chromosomal aberrations was studied. The results indicate that these herbicides reduced mitotic index in dose-dependent manner. In addition, increase in the percentage of abnormal mitotic plates was observed in herbicide treated groups which was both concentration and time dependent. Commonly observed abnormalities were c-mitosis, laggards, bridges, stickiness, c-anaphase, precocious separation, un-equal distribution and fragments. The result of the present investigation indicates that commonly used herbicides GP, AL and MH have significant genotoxic effect on *T. foenum-graecum* plant.

Keywords Genotoxicity · Herbicides · Mitotic index · Chromosomal Abnormalities · *Trigonella foenum-graecum* L

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Pesticides, which include herbicides, insecticides and fungicides, are used extensively to improve crop yield. More than 2.5 million tons of pesticides are applied every year to agricultural crops worldwide (Van der Werf 1996). Many reports have shown the genotoxic effect of several commonly used pesticides (Garrett et al. 1986; Sinha 1989) mainly through their action on crucial biomolecules such as DNA (Crosby 1982). Since many pesticides do not undergo quick physio-chemical and biological degradation due to their high stability, they can cause serious threat not only to animal kingdom but also to plant kingdom.

Glyphosate, which is probably the world's most commonly used pesticide, is an organophosphorus herbicide, containing glyphosate glycine as its active ingredient. Its mutagenic action on plants has been reported by an earlier study (Rank et al. 1993). Alachlor (AL) is an organochlorine (belonging to the α-halaoacetanilide series) commonly used for control of annual grasses and broad leaf weeds in cultivation of brassicas, soybeans, peanuts, cotton, sugarcane and corn (Garrett et al. 1986; Hackett et al. 2005; Kiely et al. 2004). Maleic hydrazide (MH) is a pyridazine which inhibits the synthesis of nucleic acid and proteins (De Marco et al. 1992). Mutagenic action of GP, AL and MH has been reported earlier (Rank et al. 1993; Swietlinska and Zuk 1978; Siddiqui et al. 2008). In the present investigation, we have compared the genotoxicity of these three herbicides on a common self-pollinated multipurpose cash crop of India, *Trigonella foenum-graecum,* commonly known as methi in India.

Materials and Methods

Healthy seeds of *T. foenum-graecum* were obtained from the Council of Scientific and Industrial Research (CSIR),

Bhopal, Madhya Pradesh, India. The pesticides were purchased from Sigma Chemicals Ltd, USA (Maleic hydrazide—CAS No.123-33-1, Alachlor—CAS No.159772-60-8, Glyphosate—CAS No. 6100-05-6).

Healthy *Trigonella* seeds of uniform size were selected and presoaked in distilled water for 12 h and divided into different groups with 50 seeds in each group. They seeds were then treated with different concentrations (0.1%, 0.2%, 0.3%, 0.4% and 0.5%) of pesticides by soaking them in 250 mL of Maleic hydrazide (MH), Alachlor (AL) and Glyphosate (GP) solutions for 1, 2 and 6 h. Seeds in control group were soaked in double distilled water. During the treatment period, the containers were shaken frequently in order to provide sufficient aeration to the seeds. After the treatment the seeds were thoroughly washed with double distilled water to remove trace amount of adherent pesticides and were placed in a Petri dishes on moistened sterile cotton. The Petri dishes were kept in plant growth cabinet for the next 72 h in dark maintained at 25 ± 2°C. When the newly emerged roots were 1–2 cm in length, they were used in the test. The entire experiment was repeated thrice under similar conditions.

The cytogenetic analysis was carried out in root tips of germinated seeds treated with different concentration of herbicides. Chromosome preparation was made from root tips using the method described by Qian (1998) with minor modification (Siddiqui et al. 2007). Briefly, the primary root of 10 seedlings per treatment were fixed in freshly prepared Carnoy's fixative (1 glacial acetic acid: 3 absolute alcohol) for 24 h, transferred in 70% alcohol and stored in a refrigerator until use. Root tips were hydrolyzed in 5 N HCl for 20 min at room temperature and then stained in 2% acetocarmine for 1 h. Chromosome spreads were prepared from squashes of root tips as described by Savaskan and Toker (1991). At least 500 cells per root tip were scored under light microscope. Mitotic index and chromosomal aberrations in metaphase and anaphase plates were studied using a light microscope under oil immersion. From each slide, a minimum of 500 cells were scored and the mitotic index was calculated. Chromosomal aberrations such as chromosome fragments, precocious separation, laggard chromosome, sticky chromosomes, bridge formation, c-mitosis and vagrants were studied in a minimum of 50 metaphase and anaphase plates per slide. The slides were coded and examined blind.

The data were analysed by analysis of variance (ANOVA) using SPSS software (version 16.0, SPSS Inc.,

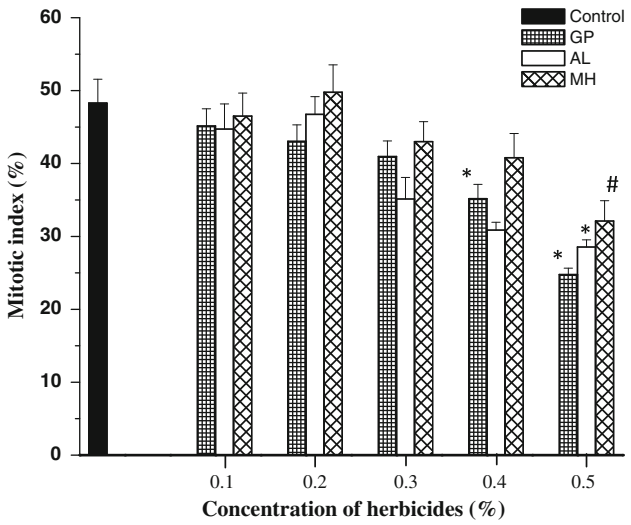

Fig. 1 Mitotic index in metaphase–anaphase plate of *Trigonella foenum-graecum* root tips exposed to different concentrations of GP, AL and MH for 1 h. #$p < 0.01$, *$p < 0.001$ V/s control

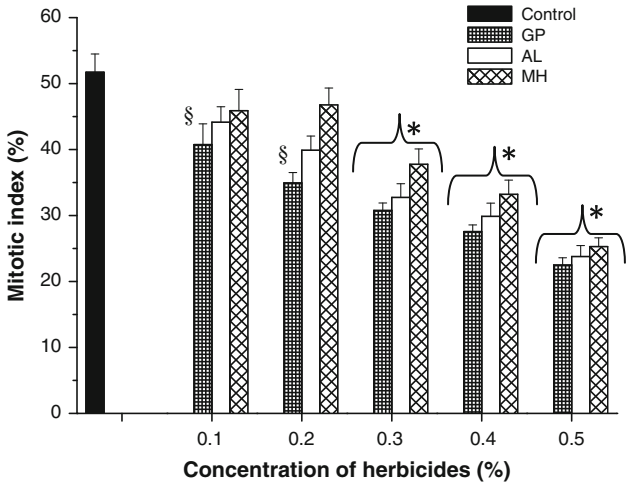

Fig. 2 Mitotic index in metaphase–anaphase plate of *Trigonella foenum-graecum* root tips exposed to different concentrations of GP, AL and MH for 2 h. §$p < 0.05$, *$p < 0.001$ V/s control

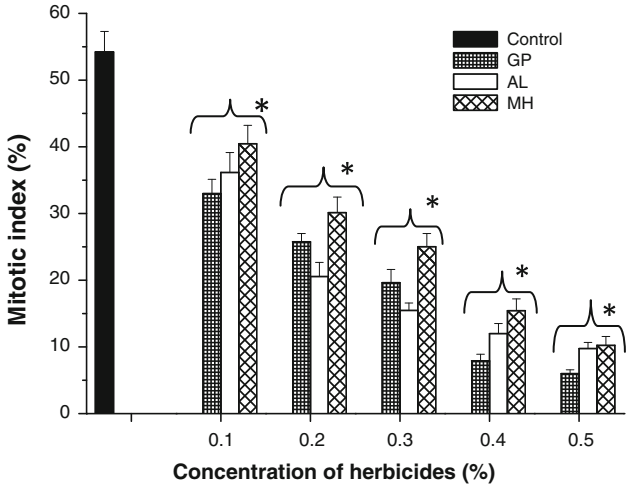

Fig. 3 Mitotic index in metaphase–anaphase plate of *Trigonella foenum-graecum* root tips exposed to different concentrations of GP, AL and MH for 6 h. *$p < 0.001$ V/s control

Table 1 Cytogenetic analysis in metaphase-anaphase plate of *Trigonella foenum-graecum* root tips exposed to different concentrations of GP, AL and MH for 1 h

Conc. of herbicides (%)	Aberrations (% mean ± SE)						
	Single bridge	Single bridge + fragment	Precocious separation	C-mitosis	Stickiness	Double bridge	Laggard
GP							
0.0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.001
0.1	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.15 ± 0.003*	0.25 ± 0.03
0.2	0.00 ± 0.00	0.38 ± 0.01*	0.34 ± 0.01*	0.00 ± 0.00	0.00 ± 0.00	0.26 ± 0.09*	0.35 ± 0.01
0.3	0.65 ± 0.06*	0.55 ± 0.05*	0.96 ± 0.05*	0.25 ± 0.03*	0.35 ± 0.03*	0.46 ± 0.03*	0.75 ± 0.03
0.4	0.97 ± 0.05*	0.76 ± 0.02*	1.66 ± 0.63*	0.98 ± 0.10*	0.68 ± 0.05*	0.66 ± 0.05*	0.98 ± 0.21♣
0.5	1.24 ± 0.20*	0.98 ± 0.10*	1.50 ± 0.99*	0.97 ± 0.23*	0.91 ± 0.23*	0.86 ± 0.11	1.10 ± 0.42@
AL							
0.0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00
0.1	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.000	0.40 ± 0.05*	0.46 ± 0.05
0.2	0.00 ± 0.00	0.00 ± 0.00	0.65 ± 0.30*	0.00 ± 0.00	0.00 ± 0.00	0.40 ± 0.05*	0.66 ± 0.05
0.3	0.67 ± 0.03*	0.34 ± 0.03*	1.53 ± 0.33*	0.65 ± 0.01*	0.46 ± 0.05*	0.61 ± 0.05*	1.10 ± 0.33
0.4	0.97 ± 0.10*	0.76 ± 0.02*	1.20 ± 0.66*	0.98 ± 0.10*	0.76 ± 0.15*	0.98 ± 0.05*	1.80 ± 0.94
0.5	1.27 ± 0.41*	0.96 ± 0.08*	1.30 ± 0.88*	1.02 ± 0.30*	1.10 ± 0.63*	1.23 ± 0.60*	1.48 ± 0.81
MH							
0.0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.001
0.1	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.53 ± 0.03*	0.76 ± 0.060
0.2	0.00 ± 0.00	0.56 ± 0.03*	0.87 ± 0.05*	0.00 ± 0.00	0.00 ± 0.00	0.59 ± 0.03*	0.87 ± 0.04
0.3	0.88 ± 0.05*	0.88 ± 0.05*	1.53 ± 0.33*	0.65 ± 0.01*	0.67 ± 0.04*	0.72 ± 0.13*	1.55 ± 0.32
0.4	1.17 ± 0.20*	1.09 ± 0.41*	1.66 ± 0.63*	1.56 ± 0.63*	0.98 ± 0.23*	1.80 ± 0.47*	1.80 ± 0.94
0.5	2.26 ± 0.90*	2.56 ± 0.88*	1.86 ± 0.71*	1.50 ± 0.77*	1.66 ± 0.76*	1.86 ± 0.43*	1.83 ± 0.89

♣ $p < 0.05$; @ $p < 0.01$; * $p < 0.001$ compared to control

Table 2 Cytogenetic analysis in metaphase-anaphase plate of *Trigonella foenum-graecum* root tips exposed to different concentrations of GP, AL and MH for 2 h

Conc. of herbicides (%)	Aberrations (% mean ± SE)						
	Single bridge	Single bridge + fragment	Precocious separation	C-mitosis	Stickiness	Double bridge	Laggard
GP							
0.0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.30 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.20 ± 0.01
0.1	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.66 ± 0.05	0.00 ± 0.00	0.00 ± 0.00	0.44 ± 0.03
0.2	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.87 ± 0.03	1.98 ± 0.57*	1.18 ± 0.33*	1.21 ± 0.88
0.3	1.10 ± 0.57*	0.98 ± 0.32*	0.51 ± 0.03*	1.65 ± 0.87	4.89 ± 0.65*	3.15 ± 0.90*	2.11 ± 0.99
0.4	3.66 ± 0.93*	1.16 ± 0.94*	2.75 ± 0.05*	3.20 ± 0.99	7.73 ± 1.33*	8.75 ± 1.14*	5.85 ± 1.21
0.5	7.83 ± 1.36*	1.60 ± 0.98*	4.21 ± 1.02*	7.75 ± 1.30*	11.36 ± 3.45*	10.66 ± 2.73*	10.85 ± 3.05*
AL							
0.0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.30 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.20 ± 0.01
0.1	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.83 ± 0.03	0.00 ± 0.00	0.98 ± 0.030*	0.61 ± 0.01
0.2	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.33 ± 0.88	1.98 ± 0.57*	2.94 ± 0.700*	1.87 ± 0.06
0.3	2.14 ± 0.80*	0.00 ± 0.00	0.68 ± 0.03*	2.80 ± 0.96	4.89 ± 0.65*	4.88 ± 1.52*	2.11 ± 0.99
0.4	4.64 ± 1.12*	1.26 ± 0.33*	3.95 ± 0.76*	4.26 ± 1.58	9.88 ± 2.10*	10.83 ± 3.15*	7.80 ± 1.07
0.5	9.70 ± 2.77*	1.86 ± 0.66*	5.23 ± 0.99*	9.13 ± 2.15*	12.87 ± 3.69*	12.05 ± 4.15*	13.76 ± 4.12*
MH							
0.0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	3.00 ± 0.99*	0.00 ± 0.00	0.00 ± 0.00	0.20 ± 0.01
0.1	2.05 ± 0.12*	0.00 ± 0.00	0.00 ± 0.00	1.55 ± 0.06	2.26 ± 0.66*	2.26 ± 0.21*	0.74 ± 0.03
0.2	4.56 ± 0.99*	0.00 ± 0.00	1.48 ± 0.31*	2.20 ± 0.05	3.14 ± 0.88*	4.67 ± 1.10*	0.74 ± 0.03
0.3	6.60 ± 1.23*	0.00 ± 0.00	1.76 ± 0.88*	4.33 ± 0.10	6.33 ± 1.03*	6.10 ± 1.45*	4.31 ± 1.70
0.4	8.59 ± 2.99*	1.85 ± 0.25*	4.22 ± 1.32*	6.3 ± 0.20	12.36 ± 3.6*	12.11 ± 2.16*	8.33 ± 2.75@
0.5	14.22 ± 3.15*	3.00 ± 0.99*	8.75 ± 1.41*	2.4 ± 0.22*	13.76 ± 4.9*	14.22 ± 4.15*	10.44 ± 1.64*

♣ $p < 0.05$; @ $p < 0.01$; * $p < 0.001$ compared to control

Chicago, USA). The differences were considered as statistically significant at $p < 0.05$. LSD stands for Fisher's least significant difference.

Results and Discussion

The effects of herbicides GP, AL and MH on the mitotic index (MI) of *T. foenum-graecum* root tip cells are presented in Figs. 1, 2 and 3. In the control group the mitotic index was approximately 48%, 52% and 55% in seeds treated with double distilled water for 1, 2 and 6 h respectively. Compared to control group, a marginal decline (non-significant) in mitotic index was observed in seeds treated with up to 0.3% of GP, AL or MH for 1 h. At 0.4% only AL showed a significant inhibitory effect on MI compared to control when treated for 1 h ($p < 0.001$). However, in seeds treated with 0.5% of herbicides mitotic index was significantly lower than control in all the groups (GP and, AL, $p < 0.001$, MH, $p < 0.01$). When the seeds were treated for 2 h with 0.1% of GP, AL and MH only GP had a significant inhibitory effect on mitosis compared to control ($p < 0.05$). However, at 0.2% concentration, both GP and AL resulted in significant decrease compared to control ($p < 0.001$ and $p < 0.05$ respectively). Above this concentration all the three herbicides significantly reduced the mitosis in a dose-dependent manner ($p < 0.001$). In group of seeds treated for 6 h with herbicides, at all the concentrations of GP, AL and MH inhibited the mitosis significantly ($p < 0.001$) compared to control, with increased inhibition occurring at each successively higher herbicide treatment. Overall, the herbicide GP had highest toxicity followed by AL and MH.

In the control group no aberrant metaphase plates were observed except laggard ($\sim$0.01%). In seeds exposed to GP for 1 h, the percentage of aberrant metaphase plates increased with increase in the concentration of GP. At the lowest concentration (0.1%) common aberrations observed were laggard and double bridge with a total aberration of $\sim$0.4%. Further increases in GP concentration induced single bridge, fragments, precocious separation, sticky chromosomes etc., in addition to double bridge and laggard. At 0.5% concentration, seeds treated with GP for 1 h had $\sim$8% aberrant metaphase plates. Similarly, seeds treated with 0.1% AL for 1 h had almost two times higher percent aberrant metaphase plates compared to the same concentration of GP. However, at the highest concentration of AL, the total aberrant metaphase plates were almost similar to that of GP group ($\sim$8%). The seeds treated with MH had highest percentage of aberrant metaphase plates compared to GP and AL at all the concentrations tested (Tables 1, 2, 3).

This study investigated the genotoxicity of GP, AL and MH, which are common ingredients of the commercial herbicide and pesticide formulations on fenugreek seeds. The doses of active ingredients used in the present study may not be similar to the amount of pesticides sprayed on the plants to control the weeds in an agricultural setup. However, it may give us an idea about the possible consequences of these active ingredients on seed germination potential and the cytological activities upon exposure to these agents.

Pisum sativum is the plant of choice to test the genotoxicity of various chemicals. Earlier studies have shown that GP, AL and MH have mutagenic action on *P. sativum* and *Allium cepa* (Rank et al. 1993; Swietlinska and Zuk 1978; Siddiqui et al. 2008). In the present investigation we used fenugreek seeds as the experimental model to test whether similar effects of GP, AL and MH are manifested in fenugreek. Our findings in the present investigation further confirm these reports. All the three active ingredients exhibited significant inhibitory action on mitosis in root tips germinated from fenugreek seeds. The effect on mitosis could be due to their inhibitory action on synthesis of DNA, RNA and proteins (De Marco et al. 1992; Barriuso et al. 2010; Kaymak 2005; Mike et al. 1983; Egli et al. 1985) and/or spindle fibers (Barbara et al. 1991; Grossmann et al. 2001; Claudio et al. 1992). In addition, Glyphosate is known to block the cell cycle in G2-M phase by inhibiting the CDK1/cyclin activation (Marc et al. 2002, 2004a, b). Similar effects of Alachlor and maleic hydrazide have also been reported by previous studies (Granby and Vahl 2001; Kevin and Stephan 1986; Ismail et al. 2009).

Cytological aberrations in plants can serve as an excellent monitoring system for the detection of environmental chemicals that may pose a genetic hazard. In the present investigation, we observed various types of chromosomal anomalies such as c-mitosis, laggard chromosomes, vagrants, bridges, stickiness, c-anaphase, precocious separations, unequal distribution and fragments in *T. foenum-graecum* after treatment with GP, AL and MH. These results indicate the potential of these agents to induce mitotic irregularities, as observed by few earlier studies (Abdelsalam et al. 1997; Siddiqui et al. 2009). The chromosomal aberration induced by these herbicides could be due to their inhibitory action on spindle proteins and also their ability to induce sister chromatid exchange (Barbara et al. 1991; Grossmann et al. 2001; Claudio et al. 1992; Tartar et al. 2006; Gichner et al. 2002).

Studies have shown that free radicals can cause genomic instability in cells. The reactive oxygen species are highly unstable and can cause disorder in the cytoskeleton, imbalance in energy metabolism, and DNA damage which can lead to chromosomal aberrations (Egorova et al. 2001; Lyu 2001; Ghamery et al. 2000). The genotoxic effect of the chemicals observed in the present investigation could be partly attributed to the

Table 3 Cytogenetic analysis in metaphase-anaphase plate of *Trigonella foenum-graecum* root tips exposed to different concentrations of GP, AL and MH for 6 h

Conc. of herbicides (%)	Aberrations (% mean ± SE)						
	Single bridge	Single bridge + fragment	Precocious separation	C-mitosis	Stickiness	Double bridge	Laggard
GP							
0.0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.10 ± 0.01	0.00 ± 0.00	0.20 ± 0.001
0.1	1.67 ± 0.21*	0.00 ± 0.000	0.35 ± 0.03*	0.32 ± 0.010	0.98 ± 0.02*	0.65 ± 0.03	0.47 ± 0.04*
0.2	3.74 ± 0.92*	1.4 ± 0.70*	2.20 ± 0.82*	2.13 ± 0.87	2.77 ± 0.28*	1.65 ± 0.44	3.40 ± 0.71*
0.3	4.79 ± 1.25*	2.75 ± 0.15*	3.03 ± 0.90*	2.65 ± 0.57	4.69 ± 0.99*	3.24 ± 0.97	4.20 ± 0.77*
0.4	6.68 ± 1.56*	3.84 ± 0.91*	4.21 ± 1.06*	3.13 ± 1.70	11.33 ± .2.73*	7.41 ± 2.56	9.88 ± 2.33*
0.5	11.5 ± 2.29*	6.66 ± 0.20*	9.77 ± 2.00*	11.43 ± 2.8*	13.47 ± 3.6*	14.72 ± 3.75*	12.25 ± 4.43*
AL							
0.0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.10 ± 0.01	0.00 ± 0.00	0.20 ± 0.001	0.00 ± 0.00
0.1	2.86 ± 0.66*	0.00 ± 0.000	0.00 ± 0.000	0.53 ± 0.01	1.57 ± 0.55*	0.98 ± 0.30	0.61 ± 0.05*
0.2	5.43 ± 0.99*	2.03 ± 0.99*	2.37 ± 0.42*	3.07 ± 0.37	4.00 ± 0.90*	2.65 ± 0.78	4.22 ± 0.99*
0.3	6.80 ± 1.09*	3.14 ± 1.10*	4.35 ± 0.88*	3.72 ± 0.94	6.75 ± 1.57*	6.27 ± 0.99	4.19 ± 1.12*
0.4	9.84 ± 2.15*	4.69 ± 1.56*	6.60 ± 1.34*	5.13 ± 1.99@	12.15 ± 3.19*	11.91 ± 2.11@	10.77 ± 3.33*
0.5	14.51 ± 3.09*	7.79 ± 2.15*	10.18 ± 2.21*	2.12 ± 0.66	15.40 ± 4.9*	15.51 ± 4.15*	12.47 ± 4.15*
MH							
0.0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.20 ± 0.001	0.00 ± 0.00
0.1	3.56 ± 0.55*	0.00 ± 0.000	0.00 ± 0.000	0.81 ± 0.05	3.57 ± 0.99*	1.67 ± 0.067	0.83 ± 0.05*
0.2	6.83 ± 0.99*	2.86 ± 0.66*	3.55 ± 0.77*	4.15 ± 0.91	5.13 ± 1.03*	3.60 ± 0.92	5.16 ± 0.98*
0.3	7.81 ± 1.02*	3.16 ± 0.99*	5.17 ± 0.99*	7.65 ± 1.32	7.46 ± 2.11*	8.59 ± 1.77	7.63 ± 1.03*
0.4	11.8 ± 2.01*	9.15 ± 2.31*	5.60 ± 1.23*	7.56 ± 1.34*	9.20 ± 2.75♣	10.44 ± 2.52*	13.76 ± 3.66*
0.5	13.76 ± 4.2*	9.51 ± 2.15*	12.11 ± 2.76*	12.11 ± 3.33*	15.40 ± 4.01*	10.44 ± 2.66♣	12.36 ± 4.15*

♣ $p < 0.05$; @ $p < 0.01$; * $p < 0.001$ compared to control

oxidative stress induced by these agents. To support this hypothesis, several studies in the past have proved that these agents alter the redox status in plant cells (Bowes et al. 1980; Burnet et al. 1993; Powles et al. 1994; Sathasivan et al. 1991; Miteva et al. 2010).

In conclusion, GP, AL and MH exhibited a strong genotoxic effect on the fenugreek plant under the experimental conditions used in the present study. It is necessary to further extend the research on the quality of the products derived from plants/seeds exposed to these agents in terms of their nutritional value, their susceptibility to adapt stress and diseases. In addition, it is important to note that the pesticide formulations containing these active ingredients may or may not bring about similar effect on plant cells. In this direction, further studies are necessary to assess their effect on biological systems.

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