

CYTOLOGICAL CHANGES INDUCED BY CLETHODIM IN *PISUM SATIVUM* PLANT

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Abstract

Effects of clethodim treatment on mitotic cells of *P. sativum* were studied in the present investigation. *Pisum sativum* seeds are treated with different concentrations of clethodim varying from 0.01, 0.02, 0.03 and 0.04% for 1 and 2 hrs and their effect on seed germination, radicle length, mitotic index and chromosomal anomalies were investigated. The outcomes reveal that treatment of clethodim on *P. sativum* seeds diminishes seed germination, radicle length, and mitotic index in dose-dependent manner. Also, in clethodim treated seeds which are time and concentration dependent, an escalation in the proportion of abnormal mitotic phases was observed. C-mitosis, fragments, precocious separation, stickiness and bridges were the most frequently observed deviations. The results revealed that frequently used herbicide clethodim has a substantial cytotoxic effect on *P. sativum*.

Introduction

To enhance the yield of crops, pesticides that include insecticides, fungicides and herbicides are used widely. Each year, greater than 0.0025 billion tons of pesticides are used in agrarian crops globally (Van der Werf 1996). Previously reported results have shown the genotoxic effect of various frequently applied pesticides (Garrett *et al.* 1986, Siddiqui *et al.* 2009) primarily through their application on vital biomolecules for example DNA (Crosby 1982). Due to their highly stable nature, various pesticides do not undergo rapid biological and physio-chemical degradation, so they are a grave threat to both plants and animals.

Herbicide clethodim falls into the cyclohexanedione oxime class. From post-emergence, clethodim is active as an herbicide against various species of grass weed mostly in sunflower, soybean, cotton, and other broad-leaved plants (Edwards 2005). Maleic hydrazide (MH) is one of the few herbicides which prevents the synthesis of proteins and nucleic acids (Siddiqui *et al.* 2008). Mutagenic action of glyphosate, alachor and maleic hydrazide was reported by (Siddiqui *et al.* 2012), diclofop-methyl and lindane has been stated by (Anila and Ditika 2013) and Anilofos was described by Arzu *et al.* (2015) on root tip cells of plant. *P. sativum* is used in the manufacturing of ayurvedic medicines, fertilizer and vegetable (Davies *et al.* 1985). It is the most frequently used legume, which is an important source of dietary protein. Although *P. sativum* is a prospective versatile crop, very few studies are available on the effect of clethodim on *P. sativum* plant especially in Asir region (Abha) of Kingdom of Saudi Arabia. In the present analysis an effort was made to examine the toxic effect of clethodim on *P. sativum*.

Materials and Methods

Herbicide clethodim falls into the cyclohexanedione oxime class. It is procured from the Delta chemical company of Saudi Arabia in Riyadh. *P. sativum* seeds, variety ARKIL were obtained from the Indian Council of Agricultural Research - Bhoj Krishi Vigyan Kendra, Near Village Naktara, P. O. Bankhedi, NH-86 Ext., Raisen Sagar Road, Bhopal.

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Uniform sized and wholesome seeds of *P. sativum* were taken and soaked in double distilled water for six hrs at a pH of 6.7. *P. sativum* seeds were soaked for one and two hrs in different concentrations (0.01, 0.02, 0.03 and 0.04%) of 150 mL clethodim solutions in 250 ml glass beaker. For supplying adequate air to the seeds, they were shaken repeatedly. The seeds were then suitably washed with double distilled water in order to remove any residual quantity of clethodim, if present. In control group, the seeds were treated with double distilled water. After 1 and 2 hrs, on sterilized filter paper 30 seeds from various concentrations kept in Petri dishes of 15 cm in diameter and incubated in a plant growth cabinet in dark for 72 hrs having a temperature of $20 \pm 2^{\circ}\text{C}$. Germination of seeds was defined by perceiving the radicle development. By means of a millimeter ruler, at every 24 hrs up to 72 hrs, radicle length was assessed of the seeds germinated (Siddiqui 2016, Siddiqui *et al.* 2018). The whole experiment was done thrice under same requirements.

As stated by Qian (1998), root tips of different treatment groups were treated for mitotic preparation. Root tips were incised and fixed for 24 hrs in Carnoy's fixative solution and then shifted in 70% alcohol and kept in a fridge for further use. At room temperature, root tips were hydrolyzed for 20 minutes in 5 N HCl and stained for 1 hour in 2% acetocarmine. As stated by Savaskan and Toker (1991), chromosome spreads were prepared by means of squash method and slight amendments were made by Siddiqui *et al.* 2007. Entire slides were examined blind and the groups were coded. Using light microscope under oil immersion, in metaphase and anaphase plates, mitotic index and chromosomal anomalies were examined. At least 500 cells were scored from each slide and mitotic index was calculated. In at least 50 metaphase or anaphase plates in each slide, chromosomal anomalies such as; c-mitosis, fragments, precocious separations, sticky chromosomes, and bridges formation were calculated.

The data of seed germination, root length, mitotic index and mitotic phases were analyzed statistically using one-way ANOVA test using GPIS software (1.13) (Graphpad, California, USA). Turkey test was performed in order to evaluate the significant differences between each treatment group and untreated group. The level of statistical significance was in all cases $p \leq 0.05$. Three independent experiments were carried out and all values are stated as mean \pm SE.

Results and Discussion

In control group, after 1 hour clethodim treatment (0.88 ± 0.37) of seeds germinated at 24 hrs, which increased to (1.00 ± 0.00) at 48 and 72 hrs and at 2 hrs (0.66 ± 0.03) of seed germinated at 24 hrs which increased to (1.0 ± 0.00) at 48 and 72 hrs (Table 1).

At 24 hrs, after 1 hour clethodim treatment at 0.01% concentration, approximately (0.27 ± 0.02) seed germination was reported and (0.13 ± 0.01) seed germination was recorded for 0.02 to 0.04% concentration, which were significantly ($p \leq 0.001$) lower than control. After 1 hour treatment with clethodim at 0.01% concentration, at 48 hrs approximately (0.66 ± 0.04) seed germination was recorded and (0.47 ± 0.02), (0.53 ± 0.03) and (0.40 ± 0.02) seed germination were listed for 0.02, 0.03, 0.04% concentration respectively, which were significantly ($p \leq 0.001$) lesser than control. A similar pattern was recorded for 72 hrs. At 24 hrs, after 2 hrs clethodim treatment at 0.01 and 0.02% concentration, (13 ± 0.01) seed germination was reported and no seed germination was recorded in 0.03 to 0.04% concentration, which were significantly ($p \leq 0.001$) lesser than control. A similar pattern was recorded for 48 and 72 hrs.

Effect of treatment of clethodim for 1 and 2 hrs on radicle length of *P. sativum* is shown Table 2. The radicle length increased with surge in time in both 1 and 2 hrs treatment with clethodim. In control, after one-hour clethodim treatment, at 24 hrs the radicle length was 2.03 ± 0.65 cm which increased to 3.25 ± 1.03 cm at 48 hrs and 3.96 ± 0.97 cm at 72 hrs. Reduction in radicle length was

observed in dose-dependent manner with rise in concentrations. There was a significant ($p \leq 0.001$) reduction in radicle length when compared with control. In control group, after two hrs clethodim treatment, the radicle length increased with enhancement in time which was 1.54 ± 0.11 cm at 24 hrs, 1.9 ± 0.4 cm at 48 hrs and 2.5 ± 1.0 cm at 72 hrs which was significantly ($p \leq 0.001$) lesser than control. In treated seeds at lower doses (0.01 to 0.02%), clethodim have significant ($p \leq 0.001$) effect on radicle length when compared with control and at

Table 1. Seed germination of *Pisum sativum* seeds treated with various concentration of clethodim for 1 and 2 hrs.

Concentration (%)	Treatment Time	Seed germination (%)		
		24 hrs	48 hrs	72 hrs
CN	1 hr	0.88±0.37	1.00±0.00	1.00±0.00
0.01%		0.27 ± 0.02 [¥]	0.66±0.04 [¥]	0.93±0.25
0.02%		0.13±0.01 ^{¥µ}	0.47±0.02 [¥]	0.87±0.36
0.03%		0.13±0.01 ^{¥µ}	0.53±0.03 ^{¥a}	0.80 ±0.40 ^µ
0.04%		0.13±0.01 ^{¥µ}	0.40±0.02 [¥]	0.53±0.03 [¥]
CN	2 hrs	0.66 ±0.03	1.0±0.00	1.00±0.00
0.01%		0.13±0.01 [¥]	0.53±0.02 [¥]	0.88±0.34
0.02%		0.13±0.01 [¥]	0.53±0.02 [¥]	0.73±0.44 [£]
0.03%		0.00±0.00 [¥]	0.13±0.01 [¥]	0.66±0.27 ^{¥µ}
0.04%		0.00±0.00 [¥]	0.13±0.01 [¥]	0.33±0.02 ^{¥£}

^µp ≤ 0.05; [£]p ≤ 0.01; [¥]p ≤ 0.001 compared to control. Data are mean of three replicates ± SEM. CN = Untreated group.

Table 2. Radicle length of *Pisum sativum* seeds treated with various concentration of clethodim for 1 and 2 hrs.

Concentration (%)	Treatment Time	Radicle length (CM)		
		24 hrs	48 hrs	72 hrs
CN	1 hr	2.03±0.65	3.25 ±1.03	3.96±0.97
0.01%		0.50±0.29 [¥]	2.44±1.37 [£]	2.91±1.70 [¥]
0.02%		0.50±0.38 [¥]	1.92±0.78 [¥]	2.50±1.20 [¥]
0.03%		0.27±0.07 [¥]	1.52±0.61 [¥]	1.90±0.70 ^{¥£}
0.04%		0.23±0.061 ^{¥µ}	1.07±0.23 ^{¥£}	1.50±0.32 ^{¥£}
CN	2 hrs	1.54±0.11	1.90 ±0.40	2.5±1.00
0.01%		0.58±0.07 [¥]	1.00±0.02 [¥]	1.3±0.05 [¥]
0.02%		0.36±0.02 ^{¥£}	0.63±0.03 [¥]	1.3±0.02 [¥]
0.03%		0.00±0.00 [¥]	0.44±0.02 ^{¥µ}	0.9±0.03 ^{¥£}
0.04%		0.00±0.00 ^{¥£}	0.32±0.01 [¥]	0.5±0.02 ^{¥£}

^µp ≤ 0.05; [£]p ≤ 0.01; [¥]p ≤ 0.001 compared to control. Data are mean of three replicates ± SEM. CN = Untreated group

higher concentration (0.03 to 0.04%), no radicle length was observed at 24 hrs after 2 hrs clethodim treatment. However, at 48 and 72 hrs, reduction in radicle length was reported in dose-dependent manner from lower to higher concentrations. It resulted in a significant ($p \leq 0.001$)

reduction in radicle length as compared to control. Higher inhibitory effect of clethodim treatment is reported in 2 hrs as compared to one hour.

Mitotic index indicates the rate of cell division or proliferation. Effect of 1 and 2 hrs treatment of clethodim on the mitotic index of *P. sativum* are summarized in Fig. 1. The untreated seeds showed a mitotic index 14.54 ± 2.0 for 1 hr and 18.0 ± 2.15 for 2 hrs, respectively. For 1 and 2 hrs clethodim treated seeds, the mitotic index was found to decrease with rise in concentrations. Even at the lowest dose (0.01%), clethodim caused a significant reduction in mitotic index as compared to untreated group ($p \leq 0.001$). With further surge in concentrations, there was a steady decline in mitotic index, which was significantly lesser than control ($p \leq 0.001$) at all concentrations. At 0.04% concentration, the mitotic index was nearly 3.5 times less than untreated group 4.25 ± 0.8 at 1 hr and 5.5 ± 0.91 at 2 hrs, respectively. Two hrs clethodim treatment has higher inhibitory effect on mitotic index as compared to one-hour treatment.

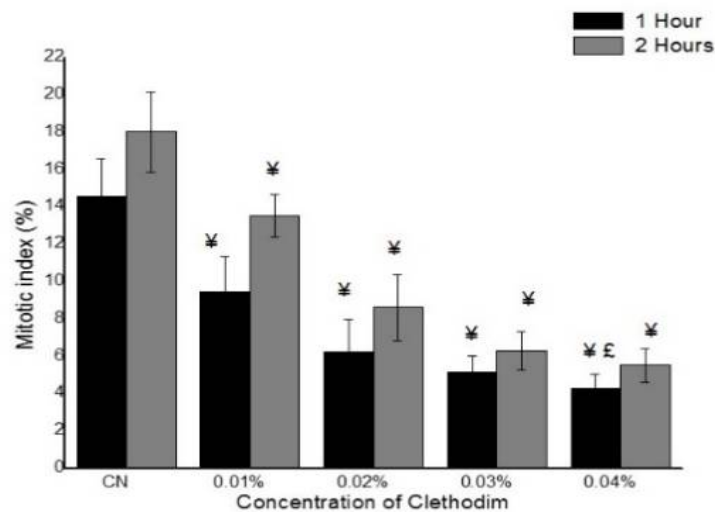


Fig. 1. Mitotic index in metaphase–anaphase plate of *P. sativum* root tips exposed to different concentrations of clethodim for 1 hr and 2 hrs.

For different time durations of 1 and 2 hrs, the mitotic anomalies in root tips of *P. sativum* subjected to various clethodim doses are illustrated in Table 3 and Fig. 2. The chromosomal arrangement from root tips of clethodim treated seeds revealed numerous mitotic anomalies for example c-mitosis (C-M), fragments (Fr), precocious separations (PS), sticky chromosomes (STC) and bridges (Br) in 1 and 2 hrs. In untreated group, in metaphase-anaphase plate no chromosomal abnormalities were detected. Augmented occurrences of chromosomal irregularities such as c-mitosis, fragments, precocious separation, sticky chromosome, and bridges were stated in 1 and 2 hrs clethodim treated seeds of *P. sativum*, which were significantly higher ($P \leq 0.001$) as compared to control. But increased frequencies of chromosomal anomalies such as c- mitosis, fragments, precocious separation, sticky chromosome, and bridges were found to be higher in 2 hrs as compared to one-hour clethodim treated seeds. Among these aberrations sticky chromosomes was the most detected chromosomal aberration (4.5%). Additionally, the incidence of remaining irregularities were sticky chromosomes > c-mitosis > bridges > fragments > precocious separations in one-hour clethodim treated seeds of *P. sativum*. Even at lower concentrations of clethodim (0.01 and 0.02%) all the above stated anomalies were found in the mitotic preparations,

PS, SC and C- mitosis being the most frequent. Analogous trend was found in clethodim seeds treated at higher concentrations (0.03 and 0.04%). A significantly higher ($p \leq 0.001$) incidence of all these abnormalities were obviously evident. Analogous trend was found in chromosomal abnormality of 2 hrs clethodim treated seeds. Moreover, the occurrence of remaining irregularities were sticky chromosomes > c-mitosis > precocious separations > fragments > bridges in 2 hrs clethodim treated seeds. Among these anomalies sticky chromosomes was (4.2%), c-mitosis (3.2%), precocious separations (2.32%) fragments (2.0%) and bridges (1.7%) in 2 hrs.

Table 3. Cytogenetic analysis in metaphase-anaphase plate of *P. sativum* root tips exposed to different concentrations of clethodim for 1 and 2 hrs.

Concentration (%)	Treatment time	Metaphase - Anaphase anomalies				
		C-mitosis	Fragments	P. Sep.	Sticky Chr.	Bridges
CN	1 hr	0.00±0.0	0.0±0.0	0.0±0.00	0.0±0.0	0.0±0.00
0.01%		0.80±0.2 ^μ	1.8±0.3 [¥]	1.5±0.35 [¥]	2.5±0.8 [¥]	1.5±0.30 [¥]
0.02%		1.91±0.9 ^{¥£}	1.6±0.2 [¥]	1.9±0.20 ^{¥£}	1.9±0.9 [¥]	1.7±0.75 [¥]
0.03%		3.00±1.0 ^{¥£}	1.7±0.2 [¥]	1.6±0.30 [¥]	2.5±1.0 [¥]	2.0±0.20 [¥]
0.04%		2.9±0.9 ^{¥£}	2.0±0.4 ^{μ¥}	1.9±0.90 [¥]	4.5±1 [¥]	3.2±0.21 [¥]
CN	2 hrs	0.0 ± 0.0	0.0±0.00	0.0 ± 0.00	0.0±0.0	0.0±0.00
0.01%		1.0±0.2 [£]	2.0±0.30 [¥]	1.2±0.20 [¥]	2.1 ±0.4 [¥]	1.3±0.30 [¥]
0.02%		2.1±0.9 ^{¥£}	1.4±0.70 [¥]	1.8±0.20 [¥]	2.2±0.4 [¥]	1.0±0.34 [¥]
0.03%		3.3±1.1 [¥]	1.4±0.20 [¥]	1.3±0.70 [¥]	3.3±1.0 [¥]	1.4±0.45 ^{¥£}
0.04%		3.2±1.2 ^{¥£}	2.0 ± 0.45 ^{μ¥}	2.3±0.32 [¥]	4.2±0.21	1.7±0.21 [¥]

^μ $p \leq 0.05$; [£] $p \leq 0.01$; [¥] $p \leq 0.001$ compared to control. Data are mean of three replicates ± SEM. CN = Untreated group

Throughout growth and sprouting progressions in seeds, the metabolic reactions regarding synthesis, molecular dilapidation are inspiring to regulate, as metabolism and cell integrity are related with a variability of enzymes roles that are specific to all species (Vieira *et al.* 2006). Seed germination is affected by light, humidity level, incubation temperature and oxygen concentration (Seregin and Kozhevnikova 2006). After clethodim treatment, seeds planted instantly exhibited a postponement and inhibition in the seed germination on *P. sativum*. The study divulges the fact that delay in germination of *P. sativum* seeds are associated with the doses of clethodim treatment. There might be a possibility that the effect of clethodim is similar to the effect of glyphosate on indole-3-acetic acid which is an endogenous auxin that diminishes the percentage of germination and development in plants (Clay and Griffin 2000, Siddiqui *et al.* 2012).

Delay and inhibition in the radicle length of *P. sativum* is caused by clethodim treatment. This delay was mostly correlated to cell elongation throughout activity of apical meristematic cells, differentiation and the delay and inhibition in protein synthesis (Webster and MacLeod 1996, Seth *et al.* 2007).

To evaluate the incidence of cellular division, the mitotic index is a consistent parameter (Fernandes *et al.* 2007). The cytotoxic potential of clethodim in *P. sativum* is illustrated by concentration and time dependent inhibition of mitotic index. In the present investigation, due to decline in mitotic index, clethodim showed a cytotoxic effect. Analogous actions on mitotic index were described by former study in *A. cepa* (Andrioli *et al.* 2012). Due to the mitodepressive effect of compound, there might be a substantial decrease in MI as clethodim hinders the normal cell

cycle consequently leading to a reduction in number of dividing cells (Arzu *et al.* 2015). The decrease in mitotic index may also be attributed to blocking of G₁ phase or subduing or prevention of DNA synthesis or effect of substance at G₂ phase of cell cycle (Anila and Ditika 2013). Hence, reduction in mitotic index in the present investigation could be due to the above-mentioned causes. C-mitosis, fragments, precocious separation, sticky chromosome, and bridges are the most found chromosomal abnormalities. They are caused either due to spindle failure (c-mitosis) or dysfunction of chromatin (stickiness, bridges, and fragments). Chromosome bridges are formed due to fusion and breaking of chromosome or chromatid (Luo *et al.* 2004). Stickiness is generally of irreversible nature and it possibly results in cell death (Liman *et al.* 2010). C-mitosis are formed by disruptions in the formation of spindle fibers (Haliem 1990) or unstable microtubules (Odeigah *et al.* 1997). Chromosome fragments are also formed by breakdown of double stranded DNA caused by reactive oxygen. Fragments results from chromosomes stickiness and their inability of parting of chromatids to poles (Fusconi *et al.* 2007, Siddiqui 2018). Precocious separation results from the restraining effect of herbicides on spindle proteins and their capability to make exchange of sister chromatids (Gichner *et al.* 2002). It is reported in many studies that in cells, genomic variability is caused by free radicals.

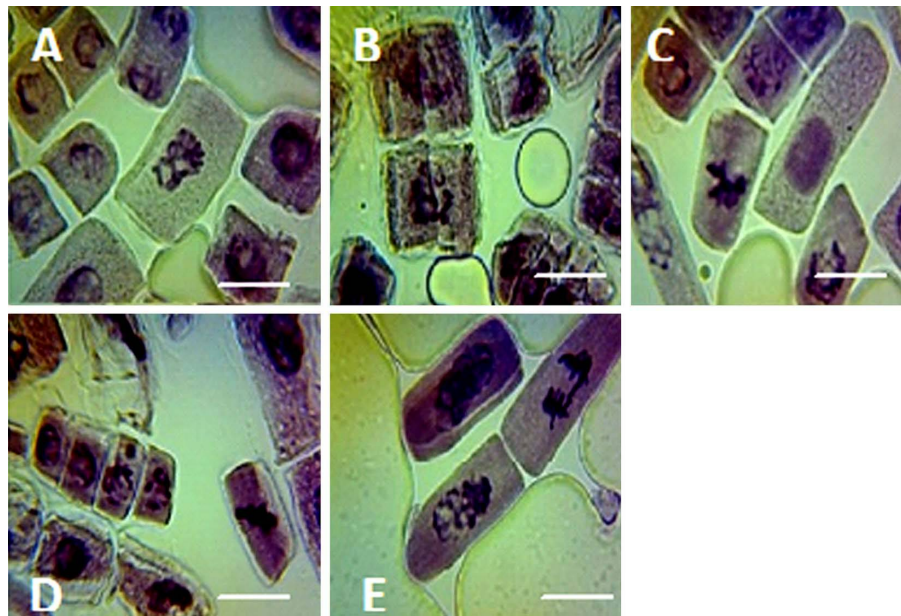


Fig. 2. The effect of clethodim on chromosomal aberrations of *P. sativum*. A= C-mitosis, B= Fragments, C= Precocious Separation, D= Stickiness, E= Bridges, Scale bars=10 μ m.

Present study shows that clethodim herbicide which is generally used in Saudi Arabia for agricultural crops can cause cytotoxic effects and can pose a serious threat to plants, animals, and environment. The present investigation is of great importance as it is useful in deciding the proper dosages of herbicide clethodim which can be used for agricultural purposes so that it maintains its beneficial functions but have inconsequential cytotoxic effects on plants. It is important to extend the research work on the goods developed from seeds/plants subjected to these herbicides regarding their vulnerability to acclimate strain and fight diseases and nutritive value.

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