



Assessment of Cytotoxic and Genotoxic Effects of Clodinafop-propargyl Commercial Formulation on *Allium cepa* L.

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Abstract

Pesticides constitute a heterogeneous category of chemicals specifically designed for the control of pests, weeds or plant diseases. Pesticides have been considered potential chemical mutagens. In the present study, the cytotoxic and genotoxic potential of the commercial herbicide Topik (containing 80g/L clodinafop-propargyl and 20 g/l cloquintocet-mexyl) was determined using *Allium cepa* root tip cells. The EC₅₀ value was determined as 126.51 ppm using a root growth inhibition test and then the roots were treated with 30, 60, 120, 150 and 240 ppm concentrations for 48 h. The results indicated that topik significantly decreased the mitotic index (MI) in all treatments when compared with the control group in a dose-dependent manner. On the other hand, topik affected the percentage of mitotic stages. For the genotoxic potential, topik significantly increased the frequency of abnormal cells. The observed abnormalities types were breaks which seem to be the most frequent type, bridges, C-mitosis, laggards and stickiness. This study indicates that topik decreased the mitotic index and produced clastogenic and aneugenic types of abnormalities in *Allium cepa* tip cells. The data obtained in this study showed that plant bioassays is a useful and an important test battery to detect possible genotoxicity of chemicals.

Key words: Topik, Clodinafop-propargyl, Cloquintocet-mexyl, Cytotoxicity, Genotoxicity, *Allium cepa*

1. Introduction

Topik (80 EC) is a selective herbicide, comprising two active ingredients; clodinafop-propargyl (80 g/l) and cloquintocet-mexyl (20 g/l). Clodinafop-propargyl is an aryl phenoxy-propionate herbicide which acts by inhibiting fatty acid synthesis [1]. Clodinafop-propargyl is the proposed common name for 2-propynyl-(R)-2-[4-(5-chloro-3-fluoro-2-pyridinyloxy)-phenoxy] propionate [2] It is intended for use as a systematic post-emergence herbicide on wheat, rye, triticale, and durum wheat [3]. Cloquintocet-mexyl is the proposed common name for 5-chloro-8-quinolinoxyacetic acid-1-methyl hexylester. It is a safener compound intended for use with clodinafop-propargyl to prevent its phytotoxic activity by accelerating the herbicide metabolism. It is known to increase the rate of hydroxylation, ether cleavage and glucosylation of the clodinafop propargyl [4] The two compounds of topik were separately subjected to many toxicological studies. Clodinafop-propargyl and cloquintocet-mexyl mammalian toxicity was tested using different experimental models and routes [5] However, there is a great lack of data on the toxicity of clodinafop-propargyl and cloquintocet-mexyl combination. Just few studies investigated the toxicity of this combination as a commercial formulation. In fact, cloquintocet-mexyl is less toxicologically active than clodinafop-propargyl and is present in the formulation at a much lower concentration. However, a subcut toxicological study in rats with the combination of active

ingredient and safener in the dose range of LD 33 to LD 50 appeared to show some potentiation of the safener toxicity by the active ingredient [6]. Hence, further investigations are needed to clarify the effect of clodinafop-propargyl and cloquintocet-mexyl combination.

Genotoxicity is one of the serious side effects of pesticides exposure. Cloquintocet-mexyl was negative in a battery of *in vitro* mutagenicity tests. Therefore, it is considered devoid of any genotoxic potential at the levels of specific genes, chromosomes or DNA primary structure [7,8]. Previous studies indicate that clodinafop-propargyl was negative in *Salmonella typhimurium* reverse mutation assays at the concentration ranging from 0 to 5000 µg/plate with and without S9 activation. *In vivo* mouse bone marrow micronucleus assay showed negative response at 1667 to 5000 mg/kg bw. The Chinese hamster V79 forward mutation assay was also negative with and without S9 activation at a concentrations range of 0 to 50 µg/ml and 0 to 500 µg/ml respectively. The *in vitro* unscheduled DNA synthesis assay in primary rat hepatocytes and cultured human fibroblasts showed negative results at the range of 0 to 70 µg/ml [5]. Inconclusive results obtained in the *in vitro* test chromosomal aberrations in cultured human lymphocytes [7, 9, 10]. Positive effects were seen using *in vitro* chromosomal aberrations in Chinese hamster ovary cells at cytotoxic concentrations (50 µg/ml) [5] and the *in vitro* interaction with DNA by intercalation [11]. An increase in DNA damage was observed in silkworm exposed to clodinafop-propargyl at different concentrations (30 to 480 ppm) using alkaline SCGE [12]. Hence, the genotoxicity of clodinafop-propargyl appears to depend upon the model system used as well as the genetic endpoint evaluated. To our knowledge, no data are available about the genotoxicity of clodinafop-propargyl and cloquintocet-mexyl combination, so it is important to supply more reports about its adverse effects.

Test systems to determine the genotoxicity or mutagenicity can be divided into groups based on the biological system employed and their genetic endpoint detected. Bioassays with prokaryotes enable the detection of agents that induce gene mutation and primary DNA damages. On the other hand, analyses with eukaryotes enable the detection of a greater damage extent, varying from gene mutations to chromosome damages and aneuploidy [13, 14] Using both pro- and eukaryotic test system strengthen and correlate the results to make sure if the chemical(s) really has/have any badly effects on the genes [15]. Higher plants have been proposed as test organisms for the detection of genotoxic substances in the environment. Several plant test systems are already in use and are found to be as sensitive and reliable as other short-term tests [16, 17]

Allium test is one of the best-established test systems in order to determine the toxicity in the laboratories [18, 19, 20, 21, 22, 23] because onions are easy to store and to handle, and also macroscopic and microscopic parameters can be observed easily. Moreover, this system is well correlated with the data obtained from eukaryotic and prokaryotic systems [24]

The objective of this study was to investigate the cytotoxic and genotoxic potency of a commercial formulation of clodinafop-propargyl by *Allium cepa* test. To our knowledge, this was the first report describing the genotoxicity assessment of clodinafop-propargyl and cloquintocet-mexyl combination using *Allium cepa* test. Throughout the document, the name of the commercial formulation will be used: Topik.

2. Materials and methods

2.1. Chemicals

A commercial formulation of clodinafop-propargyl [2-propynyl-(R)-2-[4-(5-chloro-3-fluoro-2-pyridinyloxy)-phenoxy]propionate] was kindly provided by the institute of technology of cultures, department of Guelma, Algeria as Topik 80 EC. Other chemicals used for staining and fixation were purchased from Sigma (USA), Glaxo (Bombay, India) and Biochem Chemopharma (Montreal, Quebec).

2.2. Test organism and growth conditions

Equal-sized bulbs of common onion (*A. cepa* L. 2n = 16), untreated with plant growth regulators, were obtained from local farms. The *Allium* test was performed according to [18, 25]. The yellow-brownish outer scales were removed carefully, leaving the ring of root primordia intact. The bulbs were grown and observed in the laboratory. The test procedure was performed in room temperature (at 20 ± 2°C) and protected from direct sun light. All experiments were set up in completely randomized design.

2.3. Root growth inhibition test

The toxicity assay is performed as 96 h semi-static exposure test according to Rank [25] method, and ten concentrations of the test chemical were used. First, the bulbs were placed onto test tubes filled with tap water of good quality, which was renewed every 24 h. The experiments started when newly emerging roots had reached 15–20 mm in length, using a series of six bulbs for each concentration and control group. The test solutions of topik (10, 50, 100, 150, 200, 250, 300, 350, 400 and 450 ppm of clodinafop-propargyl) were selected on the basis of preliminary studies and prepared in tap water (pH = 6.5). Every 24h, the test solutions were replaced by fresh solutions. After 96 h exposure, the length of the whole root bundle from control and experimental sets was measured as described by Fiskesjö [18]. The concentrations of the chemical were plotted against root length as a percentage of the control to estimate EC50 value. Other signs of toxicity such as changes in roots consistency and color, and the presence of tumors, hook and twisted roots were also examined.

2.4. Genotoxicity assay

The genotoxicity assay is carried out with five sample concentrations based on EC50 value. Tap water was used as negative control. Six onions are exposed to each concentration under the same laboratory conditions described above. For the first 24 h, the onions are grown in tap water, where after they are exposed to topik for 48 h, which is close to two cell cycles. The test solutions are changed after 24 h and at the end of the exposure, the onions are prepared for microscopy. The root tips from each test group were immediately placed in a chilled Carnoy's fixative for 24 h at 4°C, than they are conserved in 70% ethanol at 4°C until use. [26, 27, 28]. For the microscopic observation, six slides were prepared for each test group and randomly coded and scored blindly. The root tips were hydrolyzed during 8 min in 1N HCl at 60°C and stained by the Feulgen reaction, than the apical 2mm were squashed in a drop of 45% acetic acid on slides and cover slips were lowered carefully, to exclude air bubbles. The cover slips were sealed to the slides with clear fingernail polish [19, 29].

2.5. Mitotic index (MI), mitotic phase (MP) and chromosome aberrations (CA) analysis

For MI and MP, the different stages of mitosis were counted in 6000-7000 cells per concentration and expressed as a percentage as described by Kwankua et al [30]. CAs (bridges, breaks, stickiness, C-mitosis and laggards) were analyzed in 600 dividing cells for each test group.

2.5. Statistical analysis

Statistical analysis of variance (ANOVA) with Tukey test and non linear regression for the determination of the 50% effective concentration (EC50 value) were performed using XLSTAT for windows. The level of statistical significance was $p \leq 0.05$.

3. Results and discussion

3.1 Root growth inhibition

The *Allium cepa* assay is an efficient test for chemical screening and *in situ* monitoring for genotoxicity of environmental contaminants. The test has been widely used to study genotoxicity of many pesticides that can be found as residues in food [31] and in most environmental compartments [32]. These compounds can induce chromosomal aberrations in root meristems of *A. cepa* [15, 21, 33]. The effect of Topik on root elongation is summarized in Table 1. A significant inhibition of root growth was observed after topik treatment with an estimated effective concentration (EC50) of 126.51 ppm. After 96 h, the root bundles in the control group had an average length of 9.3 ± 1.2 cm. No macroscopic changes were observed until 250- 450 ppm concentrations where the root tips become brown to dark.

From the point of toxicity, it would suggest that these concentrations are toxic for *A. cepa* [18, 34, 35, 36]. The inhibition of root growth is generally related to apical meristematic activity [37] and to cell elongation during differentiation [35]. Over 150 ppm concentration of topik, slightly brown and dark coloration in roots was observed. These roots may indicate the retardation of growth and cytotoxicity [23].

Table 1. Results of Allium root growth inhibition test

Concentration (ppm)	Average length (%) \pm SD	Decrease in growth (%)
Negative control-96h	100.77 \pm 7.51a	0
10	89.21 \pm 7.89b	11.55
50	67.79 \pm 8.37c	32.97
100	53.66 \pm 4.31d	47.11
150	41.15 \pm 4.91 ^e	59.61
200	39.72 \pm 3.35 ^e	61.04
250	28.66 \pm 2.76f	72.10
300	25.69 \pm 2.49fg	79.18
350	21.58 \pm 5.80fg	75.07
400	17.71 \pm 0.48g	83.05
450	16.18 \pm 0.87g	84.58

3.2. Genotoxicity assay

3.2.1. Mitotic index and mitotic phase

Topik clearly decreased the MI with all tested concentrations. The inhibitory effects were dose-dependent and significant from the lowest concentration tested (Table 2). Decreasing levels of MI were accompanied by increasing levels of phase index (PI) of interphase cells (PI-I) but lower levels of mitotic phase index (Prophase, Metaphase, Anaphase) when compared with the control set. However, the percentage of telophase stage increased at concentrations <150 ppm but it decreased at the two highest concentrations (150 and 250) when compared with the control group.

Table 2. The effect of topik on mitotic index and mitotic phase of *Allium cepa* root meristem cells

Concentration (ppm)	Mean of MI (%) \pm SD	Mitotic phase (%) \pm SD*				
		Interphase	Prophase	Metaphase	Anaphase	Telophase
Negative control-48h	100.34 \pm 2.52a	18.71 \pm 2.24f	60.26 \pm 3.73a	3.73 \pm 0.65a	3.57 \pm 0.32a	13.70 \pm 4.58b
30	91.07 \pm 0.75b	26.23 \pm 0.67e	41.47 \pm 3.13b	4.38 \pm 0.94a	3.52 \pm 0.79a	24.38 \pm 3.24a
60	71.61 \pm 2.56c	41.99 \pm 2.28d	30.84 \pm 7.75cd	2.44 \pm 0.97b	1.83 \pm 0.90b	22.87 \pm 6.14a
120	65.05 \pm 2.83d	47.31 \pm 2.52c	29.75 \pm 3.44d	1.08 \pm 0.28c	1.44 \pm 0.64b	20.39 \pm 1.92a
150	53.50 \pm 3.90e	56.66 \pm 3.47b	38.61 \pm 2.96bc	0.34 \pm 0.18c	1.36 \pm 0.56b	3.01 \pm 0.78c
250	43.99 \pm 1.91f	64.36 \pm 1.7a	28.89 \pm 3.70d	0.63 \pm 0.26c	1.61 \pm 0.67b	4.49 \pm 3.09c

SD Standard Deviation

*Means with the same letters do not significantly differ at 0.05 level.

Mitotic index is a reliable parameter to identify cytotoxicity [18]. The cytotoxicity levels of an agent can be determined by the increase or decrease in the MI [38]. MIs lower than the negative control may indicate that the growth and development of exposed organisms have been affected by test compounds. On the other hand, increasing MIs are result of the induction of cell division, and may be characterized as detrimental event to cells by leading to both uncontrolled proliferation and tumor formation [39]. According to Mesi and Koplikua [40], the decrease below 50% inducts sub-lethal effect on the test organism while below 22% causes lethal effect [41, 42]. In our study, topik samples significantly decreased the mitotic index in onion root tip cells showing sub-lethal effect at 250 ppm concentration. The decrease of mitotic index indicates that topik can

arrest cell growth and it may interfere in the normal cell cycle resulting in decreased number of dividing cells [43]. According to Badr and Ibrahim [45], decrease of mitotic index indicates mitodepressive effect resulting in the inhibition of the cell access to mitosis [45] or the inhibition of certain cell cycle specific proteins as a possible pesticide target site which inhibit DNA polymerase and other enzymes [34] or a blocking in the G2 phase of the cell cycle [43]. This is explained by anti-mitotic effect [34]. Hence, it can be due to the decreased ATP level or suppression of engine of energy production [46]. Several pesticides have been reported to inhibit mitosis [15, 47, 48]

The suppression of mitotic activity is often used to assess cytotoxicity [49] Mitodepressive action on the cell division may be the reason of mitotic index reduction in *A. cepa* roots [40]. When the mitotic phase frequencies at different topik concentrations were compared to the control group, there have also been statistically meaningful results. Increase of (PI) of interphase cells (PI-I) but lower levels of mitotic phase index (Prophase, Metaphase, Anaphase and telophase). The mitotic depression may be because inhibition of DNA synthesis and microtubule formation or arrest of the 24-h cycle of *A. cepa* in G1 and G2 phases, impaired nucleoprotein synthesis and reduced level of ATP to provide energy to spindle elongation, microtubule dynamics and chromosomes movement [50]. The potential target enzymes which mediate these biological processes include DNA polymerase, DNA gyrase, RNA polymerase and kinases [51]. In general, the frequency of prophase and metaphase stages was decreased on the expense of the other mitotic stages in *A. cepa* for topik treatment. This may indicate that topik influence the sequence of mitotic division. Topik can be accepted as pre-metaphase inhibitor. Similar results have been reported after treatment of *A. cepa* root tip cells with various other pesticides. [15, 48, 52]

3.2.2. Chromosome and mitotic aberrations

The results regarding the type and frequency of abnormalities in root meristematic cells of *Allium cepa* following treatment with different concentrations of topik for 48 h are given in table 3. Topik treatments increased significantly the frequencies of aberrations with all tested concentrations when compared with control set. This increase was dose-dependent at concentrations <150 ppm but the frequencies of aberrations decreased with the two highest concentrations (150 and 250 ppm) when compared with the other concentrations and this decrease was accompanied by a decrease in cell counting number (CCN). The observed abnormalities types were breaks (which seem to be the most frequent type), bridges, C-mitosis, laggards and stickiness.

Table 3. Chromosome and mitotic aberrations in root meristematic cells of *Allium cepa* following treatment with different concentrations of Topik for 48 h

Concentration (ppm)	Aberration (%) (Mean ± SD)*						
	CCN	Breaks	Bridges	C-mitosis	Laggard	Stickiness	TA% ± SD
Negative control-48h	600	0.39 ± 0.15c	0.67 ± 0.14d	0.59 ± 0.22b	0.49 ± 0.31b	0.36 ± 0.15c	2.52 ± 0.68e
30	600	6.19 ± b1.31	2.09 ± 0.86cd	3.51 ± 1.84b	1.88 ± 0.98b	3.31 ± 1.41bc	17.00 ± 2.22d
60	600	7.45 ± 1.33b	5.61 ± 1.50b	2.38 ± 1.28b	2.82 ± 1.12b	6.52 ± 3.49b	24.81 ± 6.39c
120	575	7.88 ± 2.02b	4.94 ± 1.69bc	9.99 ± 2.77a	7.40 ± 2.11a	11.25 ± 2.10a	41.48 ± 2.88a
150	467	19.33 ± 8.71a	2.00 ± 2.70 cd	8.60 ± 3.78a	2.27 ± 2.53b	2.23 ± 3.00c	34.45 ± 6.65b
250	397	5.46 ± 1.33b	12.07 ± 4.76a	2.27 ± 1.86b	3.33 ± 1.61b	2.08 ± 1.76c	25.22 ± 3.69c

SD Standard Deviation

*Means with the same letters do not significantly differ at 0.05 level

The total aberrations percentage significantly increased in all topik concentrations when compared to the negative group. A relative decrease of TA% at 150 and 250 ppm can be explained by the cytotoxic effect which was stronger and could masquerade the genotoxic effect. As discussed elsewhere [40, 50] bridges, stickiness and breaks are due to chromatin dysfunction. Bridges result from chromosome and/or chromatid

breakage and fusion, they could happen during the translocation of an unequal chromatids exchange or due to dicentric chromosome presence. These bridges cause structural chromosome mutations [53]. On the other hand, Stickiness is considered to be a chromatid type aberration and attributed to the effect environmental pollutants on degradation or depolymerization of chromosomal DNA [54], on DNA condensation [55] and on entanglement of interactions between chromosomes [56, 57]. They are a common sign of highly toxic effects on chromosomes and irreversible type that probably leading to cell death [18, 53].

Stickiness can be caused by sub-chromatid linkage between chromosomes [59] or chromosomes lose their movement abilities and get stuck in anywhere and cannot go to final destination [58]. This can also be explained as physical adhesion of the chromosome proteins [57]. C-mitosis and laggard chromosome are due to spindle failure [40]. In *A. cepa*, c-mitosis indicates that spindle formation was adversely affected [40, 53], that results in c-mitosis aneuploidy [24]. Laggard chromosomes are also due to spindle failure [40] and they increase the risk for aneuploidy [60, 61].

Conclusion

The results presented in this study indicate that topik have cytotoxic and genotoxic effects when tested on *Allium cepa*. It decreased mitotic index which was always accompanied by higher levels of PI-I and lower level of mitotic phase-index. This finding implied that the reduction of MI might be a result of inhibition of the cell cycle, thus slowing down the progression through mitosis. More cells were arrested at the interphase stage which consequently led to the reduction in rates of cell division. Genotoxicity was evident in the forms of chromosome and mitotic aberration in the plant root tip cells. The results obtained in previous genotoxicity studies on the safener of topik revealed negative results [7, 8], but previous genotoxicity studies on the active ingredient of topik, clodinafop-propargyl, were contradictory. Negative effect was reported by [5] and positive response was revealed by Kashanian *et al* [11]. Hence, the genotoxicity of clodinafop-propargyl appears to depend upon the model system used as well as the genetic endpoint evaluated. These findings support the idea that *Allium cepa* assay serves as an excellent monitoring system to detect substances that may pose genetic hazard. However, full reports must be supplied about adverse human effect of topik during manufacture or formulation and arising from use, misuse and abuse.

Conflicts of interest: There are no conflicts of interest to declare.

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