Cytotoxic and Genotoxic Effects of Clopyralid Herbicide on Allium Cepa Roots

Eslem Amaç
Usak University Faculty of Arts and Sciences: Usak Universitesi Fen Edebiyat Fakultesi
Recep Liman (rliman@hotmail.com)
Usak University https://orcid.org/0000-0002-7944-4952

Research Article

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Abstract

Clopyralid is a one of the synthetic pyridine-carboxylate auxin herbicides and used to control perennial and annual broadleaf weeds in wheat, sugar beets and canola etc. In this study, dose dependent cytotoxicity and genotoxicity of clopyralid at different concentrations (25, 50, and 100 µg/mL) on the *Allium cepa* roots were evaluated at macroscopic (root growth) and microscopic levels (Mitotic index (MI), chromosome aberrations (CAs) in ana-telophase cells and DNA damage) using root growth inhibition, Allium ana-telophase and comet tests. The percentage root growth inhibition and concentration reducing root growth by 50% (EC_{50}) of clopyralid in relation to the negative control were determined by using various concentrations of clopyralid (6.25–1000 µg/L). The 96 h EC_{50} of clopyralid was recorded as 50 µg/L. The gradual decrease in root growth and the MI reveals the cytotoxic effects of clopyralid. All the tested concentrations of clopyralid induced total CAs (polyploidy, stickiness, anaphase bridges, chromosome laggards, and disturbed ana-telophase) and DNA damage dose and time dependently. This study confirmed cytotoxic and genotoxic effects of clopyralid on non-target organism.

1. Introduction

Clopyralid (3,6-dichloro-2-pyridine-carboxylic acid) is a one of the synthetic pyridine-carboxylate auxin herbicides (also known as the picolinic acid herbicides) like picloram and aminopyralid. It is a chlorinated pyridine selective herbicide used to control perennial and annual broadleaf weeds such as *Bifora radianis, Cirsium arvense, Matricaria spp., Lactuca serriola, Centaurea cyanus, Sonchus oleraceus, Senecio vulgaris, Chrysanthemum segetum, Vicia spp., Taraxacum officinale* and *Veronica hederifolia* in wheat, sugar beets and canola etc. It also has potential efficacy against woody species on pastures and rangeland. Clopyralid has been found very common in environment and even in drinking ground water due to its higher water affinity (around 1000 mg/L), nonadsorption to soil particles and its resistance to biodegradation (Tu et al., 2001; Donald et al., 2007; Westphal et al., 2013; Berberidou et al., 2016). Although, the mechanism of action of clopyralid is not well studied, it affects the plants in a dose-dependent manner. A low concentration of clopyralid enhanced the growth by inducing RNA, DNA, and protein synthesis leading to the unregulated cell division and disordered growth, and eventually, degradation of vascular tissue. However, at higher concentrations, it decreased cell division and growth leading to plant death by inducing by inducing ethylene biosynthesis through the synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC) and abscisic acid (ABA, Cremlyn 1991; Tu et al., 2001; Grossmann, 2010; Hura 2019). Some information regarding chemical properties of clopyralid are presented in Table 1.

Allium test, one of the most used plant based genotoxicity tests, is widely used for studying the cytotoxic and genotoxic functions of environmental pollutants like herbicides. It has been recognized as a good bio-indicator test system due to its simplicity, cheap, easy to perform and shows good correlation with other test systems, by the International Programme on Plant Bioassay, US Environmental Protection Agency (EPA), World Health Organization (WHO) International Programme on Chemical Safety and United Nations Environmental Programme.. Different parameters such as root growth, membrane integrity,

antioxidant enzyme activities, MI and CAs can be evaluated by Allium test due to its fast growing roots and its large and reduced chromosomes (2*n* = 16), respectively (Bianchi et al., 2016: Rosculete et al., 2019; Liman, 2020; Macar, 2020; Maity et al., 2020; Srivastava and Singh, 2020: Liman et al., 2021). In addition, the single-cell gel electrophoresis or comet assay using Allium roots is a sensitive tool for assessing dose dependent genotoxicity of by herbicides due to its versatility, sensitivity, low cost, ease of implementation, and the short time (a few days) required to complete an investigation (Karaismailoglu, 2015; Liman et al., 2015; Özkul et al., 2016; Silveira et al., 2017; Aydın and Liman, 2020; Sivaram et al., 2020; Taşdemir et al., 2020).

This study was planned to investigate dose dependent cytotoxicity and genotoxicity of clopyralid on bioindicator test plant roots (*A. cepa*) at macroscopic (root growth) and microscopic levels (DNA damage, MI, and CAs such as stickiness, anaphase bridges, chromosome laggards etc.) using root growth inhibition, comet and Allium ana-telophase assays.

2. Materials And Methods

Chemicals

Clopyralid (CAS Number 1702-17-6), sodium hydroxide, ethidium bromide, di-sodium salt of ethylene diamine tetra acetic acid (EDTA), trizma base, sodium chloride, hydrochloric acid, disodium hydrogen phosphate, MMS (CAS Number 67-27-3), low melting point agarose (LMPA), potassium phosphate monobasic, magnesium chloride hexahydrate, potassium chloride, triton X-100, normal melting point agarose (NMPA), basic fuchsin, and trizma hydrochloride were purchased from Sigma-Aldrich.

Allium root growth inhibition test

Similar-sized (20-30 mm in a diameter) and healthy *A. cepa* bulbs obtained commercially in Uşak, Turkey. EC_{50} of clopyralid was determined according to Aydın and Liman (2020) with some modifications. All the dry scales and roots of the bulbs were removed carefully with a sharp knife without destroying the roots primordia. The onions were treated with distilled water (negative control) and different concentrations of clopyralid (6.25, 12.5, 25, 50, 100, 250, 500 and 1000 µg/L) for 96 h at room temperature in the dark. After the exposure time, average root length of negative control and exploratory sets were measured (in cm) with a ruler by taking 50 roots from five best-developed bulbs to identify the percentage root growth inhibition and EC_{50} of clopyralid (The effective concentration reducing root growth by 50%) in relation to the negative control.

Allium ana-telophase test

Rank and Nielsen (1997) protocol were used for cytogenetic analysis with minor modifications. After germination with distilled water for 2 days, when the roots reached around 2-3 cm in length, three bulbs

for each concentration of clopyralid (25, 50 and 100 µg/L), distilled water (negative control), methyl methane sulfonate (10000 µg/L, positive control) were incubated for 24 h to 96 h at room temperature. Roots tips were fixed ethanol:acetic acid (3:1, v/v) solution for 24 h at 4 °C. Following fixation, the root tips were washed with distilled water and stored in 70% ethanol before the cytological studies in the refrigerator. 7-8 root tips for each application were hydrolyzed in 1 N HCl in a water bath (60 ° C) for 8-10 min and then rinsed with distilled water. Staining of root tips were performed with Schiff's reagent at room temperature for 25-30 m, and then slides were three times passed through the distilled water. The semi-permanent slides were prepared with darkly stained apical tips and 45% acetic acid for microscopic analysis. The slides were examined and photographed according to Saxena et al. (2005) using a trinocular Nikon light microscope (Eclipse Ci-L, Japan) coupled to Argenit Kameram 5 CMOS digital camera (Turkey). Randomly 5000-5220 cells and 500 ana-telophase cells from five slides of each treatment were analyzed for MI and CAs (stickiness, anaphase bridge, polyploidy, chromosome laggards, and disturbed ana-telophase), respectively by using following formulas.

$$MI = \frac{Number\ of\ cells\ in\ division}{Number\ of\ total\ cells} x100$$

$$Phase\ index\ = \frac{Particular\ phase}{Number\ of\ cells\ in\ division} x100$$

$$CA\ = \frac{Total\ ana-telophase\ anomalies}{100\ ana-telophase\ cells} x100$$

Comet assay on *A. cepa* root tips

The modified method of Tice et al. (2000) was applied for the determination of DNA damage induced by clopyralid using comet assay as described earlier by Aydın and Liman (2020). Three bulbs were used for each treatment and control groups under the same conditions as described Allium ana-telophase test. 600 µL ice-cold nuclear isolation buffer (pH 7.5, 4 mM $MgCl_2$-$6H_2O$, 0.5% w/v Triton X-100, 200 mM Tris) in a petri plate over an ice were used for isolation for nuclei by slicing the onion roots tips gently with a fresh razor blade. After centrifugation (1200 rpm for 7 min at 4 °C), 1:1 mixture of nuclear suspension and 1.5% LMPA in phosphate buffer saline (pH 7.4) were pipetted on the pre-coated slides with 1% NMPA at 37 °C with cover slips. The gel was solidified for 5-6 min on ice. After removing the cover slips, the slides were submerged in a fresh and chilled electrophoresis buffer (pH > 13, 1 mM EDTA and 0.3 M

NaOH) for 20 min prior to electrophoresis. The electrophoresis was carried out at 0.3 A and 25 V for 20 min. Following the neutralizing of the slides in Tris buffer (pH 7.5, 400 mM Trizma base), staining of the slides were performed with 20 µg/mL ethidium bromide at 4 °C for 5 min in dark. Coverslips were placed on the slides after removing the excess stain with cold distilled water. Randomly 150 comets for each sample (three slides per sample) were visually analyzed with a BAB TAM-F fluorescence microscope (Turkey) attached with BAB TC-5 CCD camera (Turkey) at ×400 magnification. The comets were classified into five damage classes according to head integrity and tail length ranging from 0 to 4 according to Collins (2004) as shown Fig 4. Total DNA damage per each sample expressed as arbitrary units was calculated using the following formula

$$Arbitrary\ Unit = \sum_{i=0}^{4} Ni \times i$$

Ni =Number of cells in *i* degree; *i* = degree of damage (0, 1, 2, 3, 4).

3. Data Analysis

SPSS version 23 was used for the statistical analysis of the results represented as mean ± standard deviation. One-way ANOVA along with Duncan test and Pearson correlation test were performed for comparisons and bivariate correlations of groups, respectively at $p \leq 0.05$ and $p=0.01$.

4. Results And Discussion

Findings of Allium root growth inhibition test is shown in Fig. 1. A significant growth reduction (r =-.991) was observed in clopyralid exposed onion bulbs in a dose dependent manner from 5.69% at 6.25 µg/L (4.31 ± 0.09 cm) to 93.44% at 1000 µg/L (0.3 ± 0.08 cm) compared to control group (4.57 ± 0.11 cm) indicating cytotoxic effects of the clopyralid. EC_{50} value of clopyralid was calculated as 50 µg/L (2.29 ± 0.1 cm) after 96 h by root growth inhibition test. The root and shoot lengths of *Zea mays* were reduced by clopyralid (Vettakkorumakankav et al., 2002). Root growth inhibition was also observed by other pyridine-carboxylate auxin herbicides such as triclopyr in *Populus trichocarpa* (Eslamiamirabadi et al. 2020), fluroxypyr in *Oryza sativa* (Wu et al., 2010) and other auxin herbicides such as 2,4-dichlorophenoxyacetic acid (2,4-D) and 2-methyl-4-chlorophenoxyacetic acid (MCPA) in *A. cepa* (Fiskesjö et al., 1981; Ateeq et al., 2002). Unlike our result, clopyralid did not show inhibitory effect on *Dunaliella primolecta* growth (Santin-Montanya et al., 2007).

The percentages of phase and mitotic index in the meristematic root cells exposed to Clopyralid are given in Table 2. Significant reduction of MI between 62.82 ± 0.81 to 55.71 ± 0.52 was observed at clopyralid exposed onion bulbs compared to negative and positive (except 24 and 96 h) control groups. The gradual decrease in the MI in both dose (r=-.957 for 24 h, r=-.919 for 48 h and 96 h, r=-.901 for 72 h) and time

(r=-.928 for 25 µg/L, r=-.866 for 50 µg/L, and r=-.878 for 100 µg/L) dependent manner reveals the cytotoxic effects of clopyralid. Clopyralid significantly decreased the percentage of metaphase and anaphase (except 24 h at 25 µg/L) but increased telophase indices compared to negative control group. The other synthetic auxin herbicides like picloram at the highest four concentrations (2.01, 2.68, 3.35, 4.02 mg/L, Özel at al., 2018), triclopyr (El-Khodary et al., 1989), three higher concentrations (2.68, 3.35 and 4.02 mg/L) of 2,4-D at 24 h and 4.02 mg/L of 2,4-D at 48 h (Özkul et al., 2016), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), MCPA and 2,4-D (Fiskesjö et al., 1981) was also reported to inhibit MI in *A. cepa* root tips. Significant decreases in MI were also detected with 1-Naphthaleneacetamide in human peripheral blood lymphocytes (Kocaman and Güven, 2015), MCPA in *Brassica napus* L. (Polit, et al., 2014), diclofop in *Triticum aestivum* (Morrison et al., 1981).

Types and percentages of mitotic abnormalities in clopyralid exposed *A. cepa* ana-telophase cells are presented in Fig. 2 and Fig. 3, respectively. All the tested concentrations of clopyralid induced CAs with increase in dosage concentration (r = .967 for 24 h, r = .939 for 48 h, r = .937 for 72 h, and r = .913 for 96 h) and time (r = .931 for 25 µg/L, r = .842 for 50 µg/L, and r = .731 for 100 µg/L) duration. No statistical significant difference was observed between the positive control group and the 100 µg/L clopyralid (except at 24 h) concentration. The gradual increase in the CAs reveals the genotoxic effects of clopyralid. Disturbed ana-telophase (Fig. 2a) and chromosome laggards (Fig. 2b) probably occur due to disruptions in the spindle formation and failures in chromosome movement towards poles (Tkalec et al. 2009: Khanna and Sharma, 2013). Stickiness (Fig. 2c) probably occur due to DNA depolymerization, contraction or condensation of chromosomes and partial dissolution of nucleoproteins (Sabeen et al., 2019). The chromosome breakage or fusion, formation of dicentric chromosomes, cross-links between proteins and chromosomes, unequal chromatid exchange or stickiness could be caused anaphase bridge (Fig. 2d, Feretti, et al., 2007: Dutta et al. 2018). Atypical scattering of chromosomes during replication or chromosome segregation may resulted polyploidy (Fig. 2e, Nefic et al. 2013; Palsikowski et al. 2018).Unlike our result, clopyralid did not induce CAs in Chinese hamster lung cells (Wang et al., 2012), in mammalian bone morrow cells (Ilyushina et al., 2019). The other synthetic auxin herbicides like triclopyr (El-Khodary et al., 1989), 2,4-D (Ateeq et al., 2002; Özkul et al., 2016), MCPA, 2,4-D and 2,4,5-T (Fiskesjö et al., 1981) were also reported to induce CAs in *A. cepa* root tips. The results of mitotic anomalies of the current study are in agreement with the work on plants reported previously such as such as with 2,4-D on *Raphanus sativus* L. and *Phaseolus vulgaris* L. (Truta et al., 2011), 2,4-D and dicamba on *Arabidopsis thaliana* (Filkowski et al., 2003).

The results of DNA damage in *A.* cepa root tips treated with clopyralid is summarized in Fig. 4. Clopyralid exposed root tips showed significantly higher DNA damage (between 124 ± 4.58 to 166.67 ± 2.52 Arbitrary Unit) than the negative control group (between 11.67 ± 1.53 to 13.67 ± 0.58 Arbitrary Unit). Induction of DNA damage was found to be dose (r = .972 for 24 h and 48 h, and r = .981 for 72 and 96 h) and time (r = .954 for 25 µg/L, r = .933 for 50 µg/L, and r = .915 for 100 µg/L) dependent. Clopyralid did not induce genotoxicity on *Salmonella typhimurium* and mice CD-1 strains (Ilyushina et al., 2019). DNA damage by using comet assay was also observed by other synthetic auxin herbicides like 2,4-D and dicamba on *Phaseolus vulgaris* root tips (Cenkci et al., 2010), triclopyr on *Anguilla anguilla* L. (Guilherme et al., 2015),

2,4-D on human Caco-2 cells (Syberg et al., 2015), Chinese hamster ovary cells (González et al., 2005, Laborde et al., 2020), on *Clarias batrachus* (Ateeq et al., 2005),on *Cnesterodon decemmaculatus (*de Arcaute et al., 2016), and on Syrian hamster embryo cells at 11.5 µM and 23 µM of 2,4-D (Maire et al., 2007), MCPA and 2,4-D on epithelioma papillosum cyprini cell line (Bokán et al., 2013), MCPA on mussel gill (Emmanouil et al., 2008). But negative results by using comet assay was reported with other synthetic auxin herbicides such as 2,4-D on *A. cepa* (Özkul et al., 2016), dichlorpop and mecoprop on epithelioma papillosum cyprini cell line (Bokán et al., 2013).

The cytotoxicity and genotoxicity of mechanism of clopyralid has not yet been fully elucidated. The suppression of root growth and reduction of MI associated with enhanced genotoxicity by clopyralid could be due to inhibition of cell division in root tips by inducing ethylene biosynthesis through the synthesis of ACC and ABA leading to ROS overproduction (Cremlyn 1991; Tu et al., 2001; Grossmann, 2003, 2010; Sunohara and Matsumoto, 2008; Christoffoleti et al., 2015; Hura 2019, Abou-Zeid et al., 2020). But high levels of ROS can also stimulate the ethylene and ABA biosynthesis (Xing et al., 2004; Kim et al., 2008, McCarthy-Suárez, 2017). Overproduction of ROS by auxin herbicides can damage the membranes, nucleic acids, proteins and enzyme activities (Bradberry et al., 2004; González et al., 2005; Rodríguez-Serrano et al., 2014).

Clopyralid induced cytotoxicity and genotoxicity on the *A. cepa* roots by a dose dependent decline in root growth and MI, and by a dose and time dependent increase in CAs and DNA damage. Clopyralid should be used carefully at appropriate doses. The cytotoxic and genotoxic mechanisms of clopyralid should be investigated with using both plant and animal models.

Declarations

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Author contributions

E.A. and R.L. contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript.

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to participate

Not applicable.

Consent to publish

Not applicable.

Data availability

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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Tables

Due to technical limitations, tables are only available as a download in the Supplemental Files section.

Figures

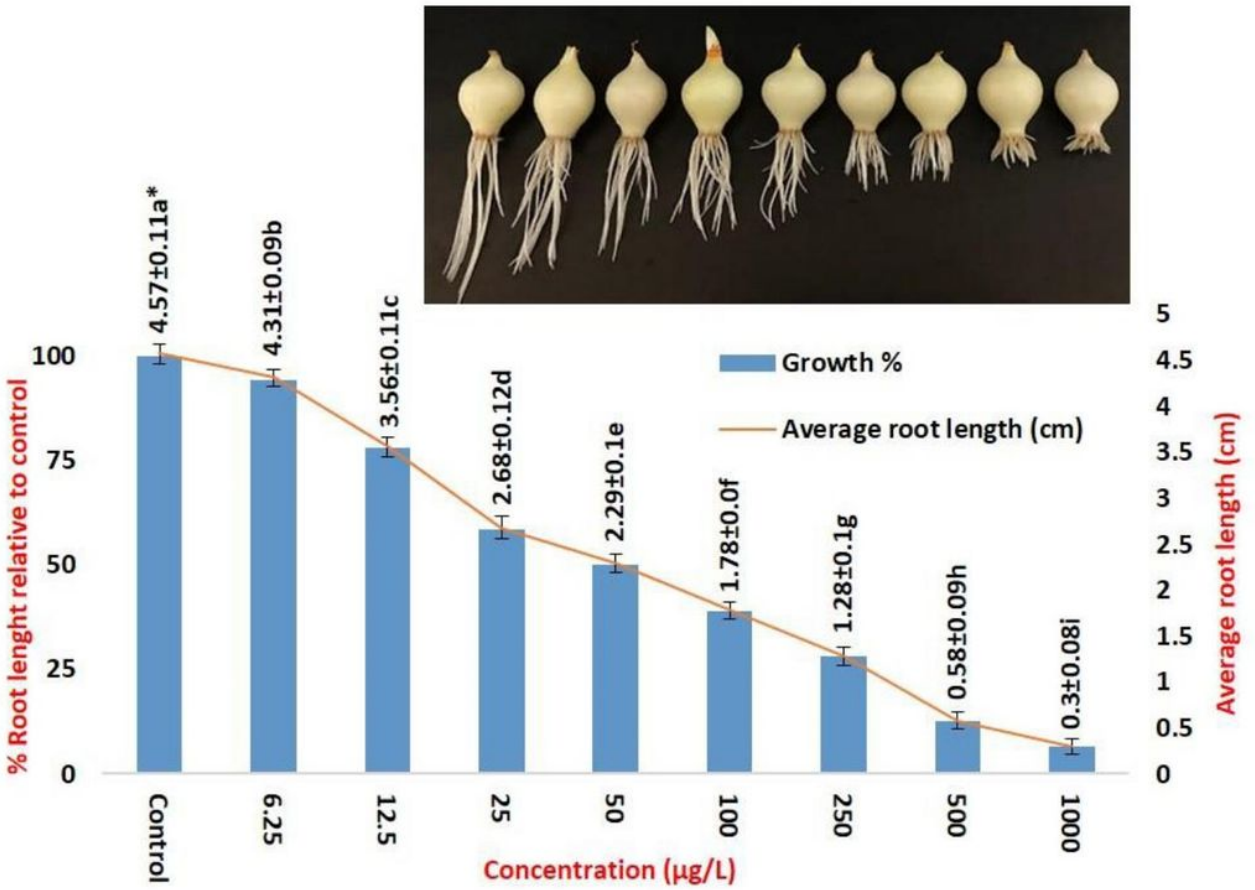

Figure 1

Inhibitory effect of clopyralid on A. cepa meristematic root growth. *Different letters are significantly different at $p \leq 0.05$.

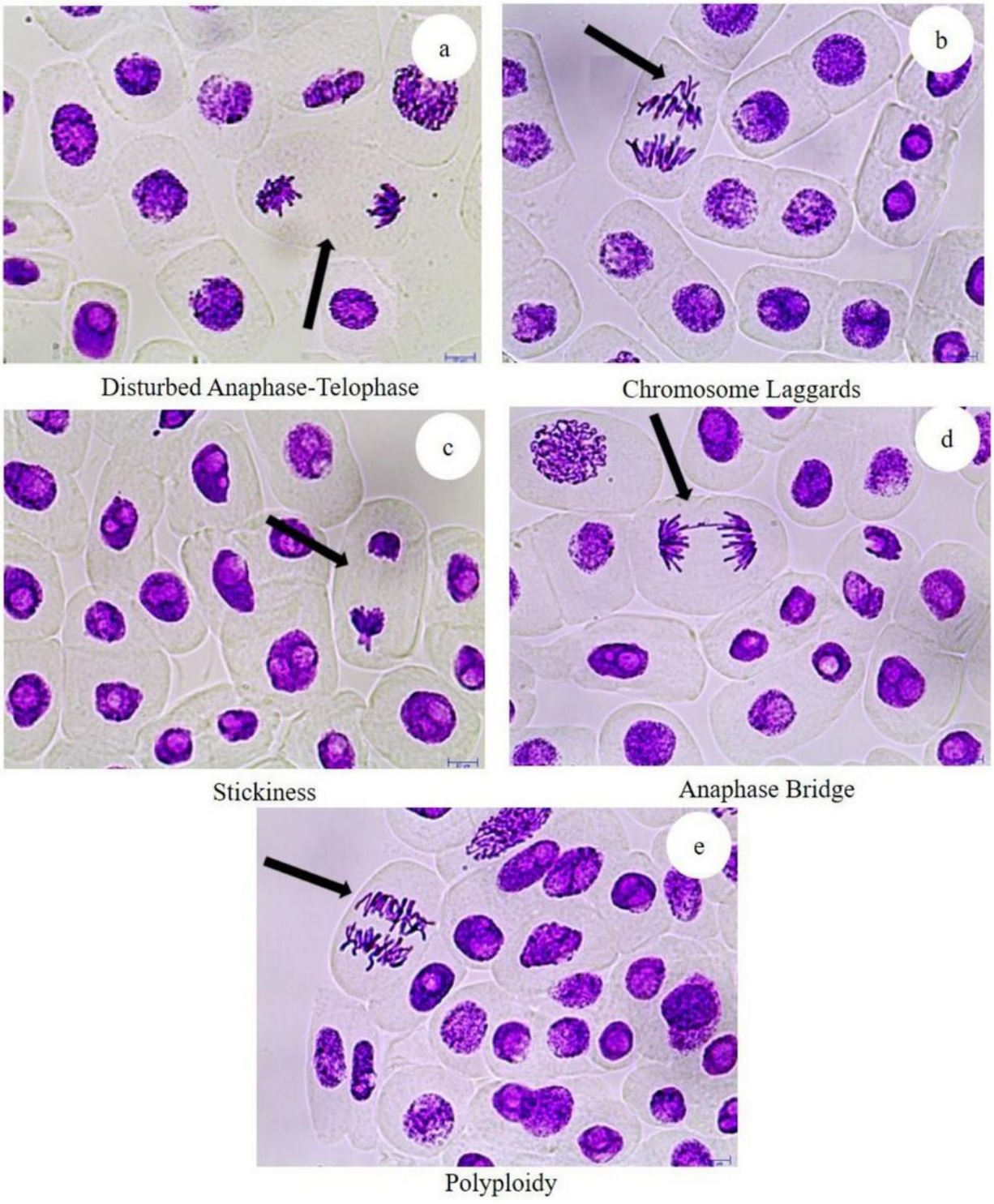

Figure 2

Clopyralid induced different ana-telophase anomalies in A. cepa roots. Scale bars 10 µm

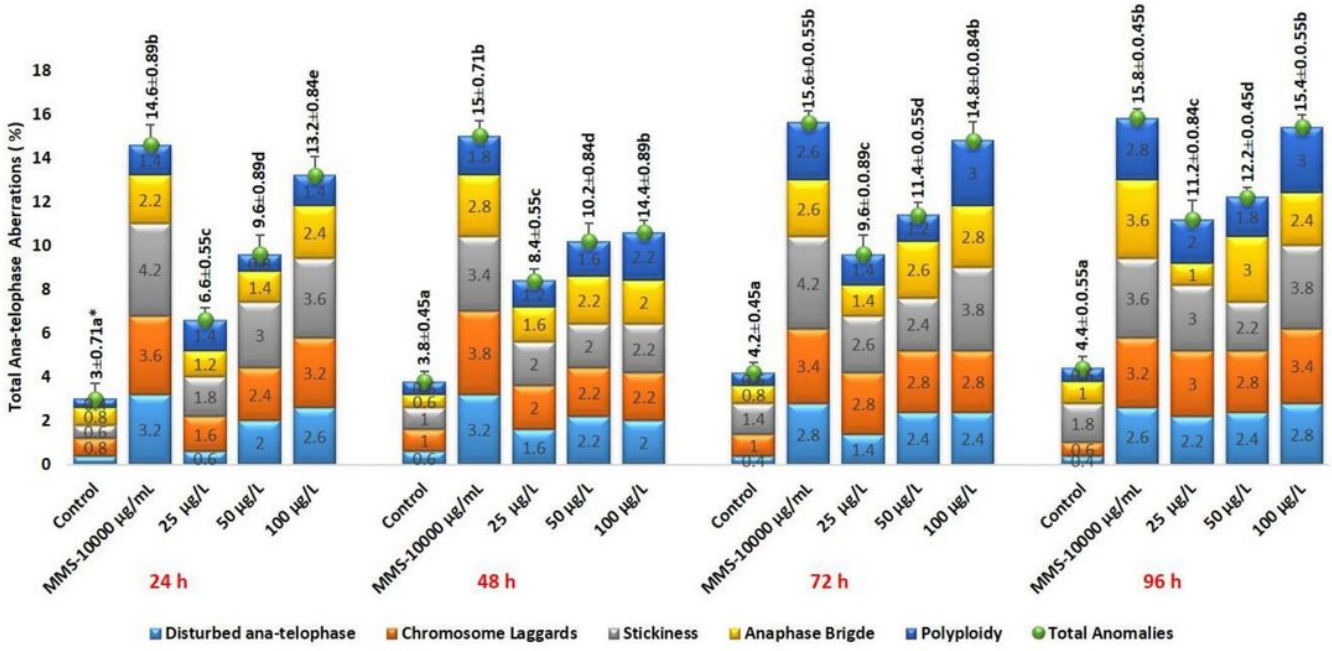

Figure 3

The effect of clopyralid on ana-telophase anomalies in A. cepa root tips. * Different letters for each treatment time are statistically significant ($p \leq 0.05$).

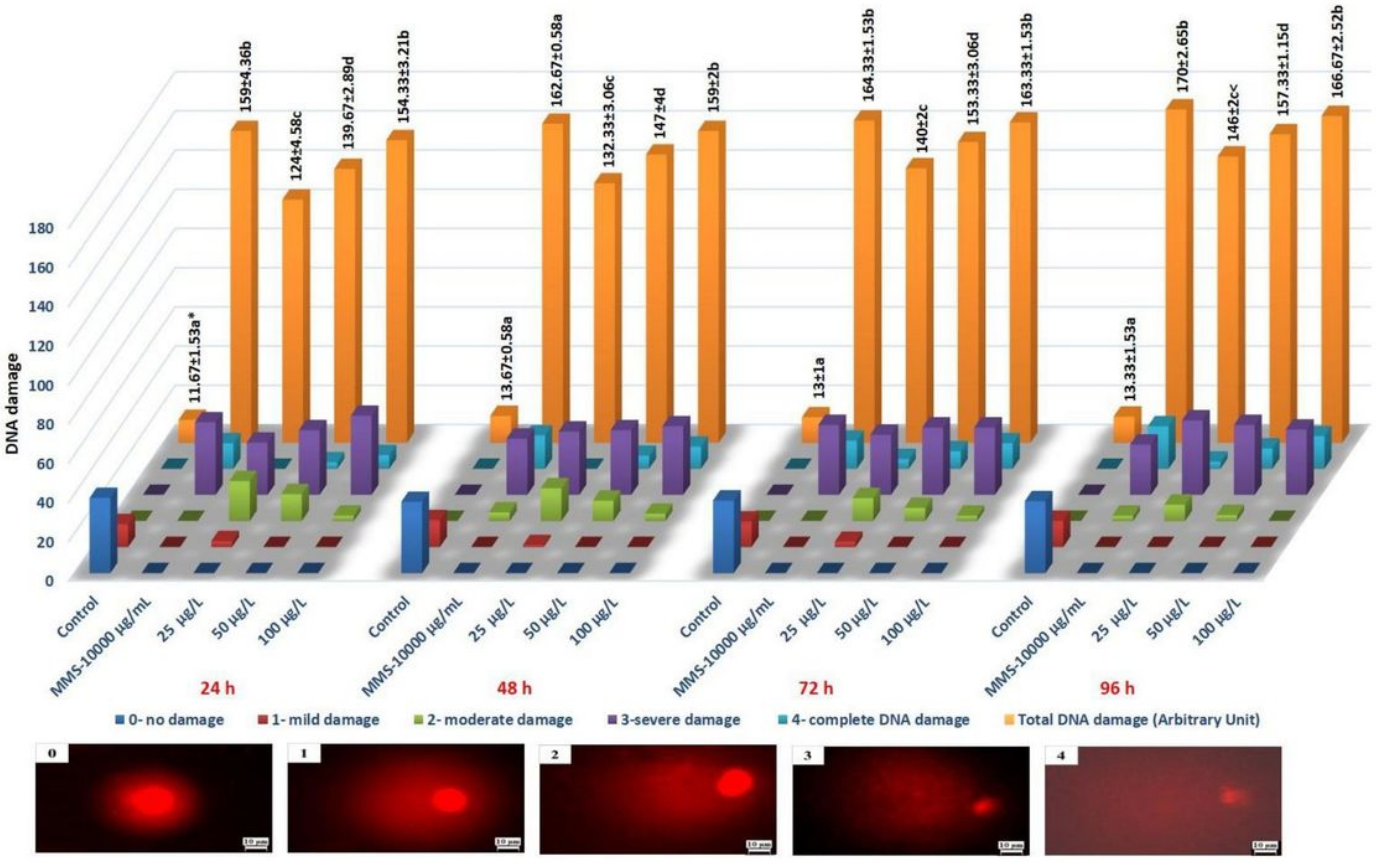

Figure 4

The effect of clopyralid on DNA damage in A. cepa root tips. * Different letters for each treatment time are statistically significant ($p \leq 0.05$).

Supplementary Files

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