Determination of genotoxic damages of picloram and dicamba with comet assay in Allium cepa rooted in tissue culture and distilled water

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Abstract

Background:

Many genotoxicity tests allow us to understand the mechanism of damages on genetic material occurring in living organisms against various physical and chemical agents. One of them is the Comet test. The current study aimed to evaluate genotoxic damages by picloram and dicamba to root meristems of Allium cepa utilizing comet assay.

Methods and Results:

Two different protocols were used for rooting and auxin/pesticide application. (i) A. cepa bulbs were rooted in MS medium and then treated with MS medium (control) and 0.67, 1.34, 2.01, 2.68, 3.35, 4.02, and 8.04 mg/L of Picloram and Dicamba using aseptic tissue culture techniques. (ii) A. cepa bulbs were then rooted in bidistilled water and treated with 0 (control), 0.67, 1.34, 2.01, 2.68, 3.35, 4.02, and 8.04 mg/L of Picloram and Dicamba in distilled water. The A. cepa root tip cells in both treatment groups were examined using comet test to find the possible DNA damaging effects of Picloram and Dicamba. The results obtained at all the concentrations were statistically compared with their control groups. Almost at all the concentrations of Picloram and Dicamba increased comet tail intensity (%) and tail moment in roots treated in MS medium. Two highest concentrations revealed toxic effect. On the other hand, DNA damaging effect of both auxins was only noted on the highest concentrations (>4.02 mg/L) in roots treated in distilled water.

Conclusions:

This study approve and confirm genotoxic effects of tow growth regulators on plants.

1. Introduction

Due to the rapid increase of the world population, a large part of the existing agricultural lands was occupied by industrial and residential areas. Both conventional farming practices and plant biotechnology are used to obtain more yield from the available land per unit area. In both productions, chemicals called plant growth regulators (PGR) are used to influence plant growth and development such as repressing shoot growth, increasing branching, increasing blooming, detaching excess fruit, or modifying fruit maturity [1]. Auxins are among the most widely used groups of PGRs. They are used in plant tissue culture, especially for callus induction and tissue and organ differentiation. They are available in both natural and synthetic forms. Dicamba, 2,4-D, and Picloram are two classes of synthetic auxins. They tend to exhibit higher activity compared to other auxins like NAA (1-Naphthaleneacetic acid), IAA (3-Indoleacetic acid), and IBA (Indole-3-butyric acid), promoting somatic embryo development in tissue culture [1]. In the last decade, loss of the 30% of the agricultural production in the world has resulted from agricultural pests; about 13% of these are weeds [2]. Picloram and Dicamba are commonly

used auxin herbicides in combating weeds that cause high crop loss each year, competing with cultivated crops in terms of nutrients, water, light, and location they take from the soil [3].

Picloram or 4-amino-3,5,6-trichloropicolinic acid is also included in the class of picloram picolinic acid herbicides or pyridine herbicides due to its herbicidal action [3]. Picloram usage comes after glyphosate, throughout the world [4]. This auxin herbicide is commonly used to kill dicotyledonous weeds in crop fields and pastures [5]. According to the data described in the EPA (1995) report, Picloram does not cause chromosomal damage; declared non-toxic at low doses to birds, mammals, and aquatic species. In high concentrations, it has been reported to cause vascular tissue damage and inhibit cell division [6].

Dicamba or 3,6-dichloro-2-methoxybenzoic acid, on the other hand, is a systemic broad-spectrum herbicide from benzoic acid derivative synthetic auxins and is mostly used in agricultural areas to control single and perennial weeds. Besides, there are many commercial and industrial applications such as the maintenance of pasture and forest areas, cleaning of golf courses, and parks-gardens in residential areas from weeds [7]. With the development of Monsanto's dicamba-resistant GM soybeans and cotton, dependence on dicamba has increased in recent years [8], which means that concentrations on non-target crops and in the environment will increase steadily [9]. While the use of auxin herbicides provides a great advantage in killing harmful plants, they are common causes of soil and water pollution [10]. Furthermore, many macro and microorganisms with their residues in the soil, they can be washed from the soil and directly reach groundwater and surface water such as rivers, lakes, and sea [11], or accumulate in organisms living there and negatively affect the food chain [12, 13]. Samanic et al. [14] noted that farmers in agricultural areas, where dicamba was applied, were more frequently caught in lung and colon cancer than other individuals, according to the agricultural health study data. Similarly, Lerro et al. [15] revealed a relationship between the usage of dicamba and many types of cancer based on the data of agricultural health studies conducted in the last twelve years. These results reveal that exposure of living organisms to chemical agents such as herbicides can cause damage to their genetic material.

Many genotoxicity tests allow us to understand the mechanism of damages on genetic material occurring in living organisms against various physical and chemical agents. One of them is the Comet test or SCGE-Single Cell Gel Electrophoresis that was firstly used in 1984 by Östling and Johanson to evaluate DNA damage. Comet technique is a fast, reliable, and cost-effective method for observing DNA damage [16]. It is currently used to directly determine DNA damages and repair in both animal and plant materials [17–22]. There are several model species like *Arabidopsis thaliana, Vicia faba, Nicotiana tabacum* or *Allium cepa* to perform Comet test [20, 23]. *Allium cepa* is the most widely preferred plant species and has been used in genotoxicity studies related to many chemical agents [19, 21, 24, 25]. When the literature was searched, it was seen that there were only a few studies conducted in recent years on the DNA damaging effect of picloram in plants. In one of these studies, Taspinar et al. [26] have revealed changes in DNA methylation using CREDRA (Coupled Restriction Enzyme Digestion Random Amplification) techniques in bean (*Phaseolus vulgaris*) and DNA damage using RAPD (Random Amplified Polymorphic DNA) techniques. Mohammed and Ma [27] have demonstrated the genotoxic effect of picloram using the micronucleus test on Tradescantia.

RAPD and CREDRA methods were applied to test the effects of picloram and dicamba, respectively, on genetic and epigenetic changes in mature embryo culture of barley (*Hordeum vulgare* L.). While authors have observed DNA hypomethylation following dicamba treatment, hypermethylation of DNA were confirmed utilizing higher doses of picloram [28]. However, as far as we know, no study has been found investigating the genotoxic potential of picloram using the comet test.

A few studies were conducted using different genotoxicity tests to check the effects of Dicamba on nontarget plants. For example, Abass et al. [29] have examined the genotoxic effects of Dicamba on the Hillawii variety of palm (*Phoenix dactylifera* L.) plant in tissue culture using the RAPD technique, and no significant result has been found compared to the control. Using RAPD and CREDRA techniques, the genotoxic effect of dicamba has been examined in wheat (*Triticum aestivum* L.) [30]. Authors have reported DNA alterations in this species. Studies on the genotoxic effect of dicamba by the comet test are extremely limited. In one of these studies, Reynoso et al. [31] have investigated the genotoxic activity of dicamba-atrazine mixture by comet assay on *Zea mays* L. and have confirmed significant induction of genetic damages in corn plants at all the concentrations used over the negative control. The genotoxic potential of Dicamba and 2.4 D have been investigated using RAPD and Comet assays and found that both herbicides generated similar damages in the genomic DNA of beans [32]. Çakmak et al. [23] examined the DNA damaging potential of dicamba in callus obtained from anther culture of sunflower (*Helianthus annuus* L.). They have revealed the genotoxic effect of dicamba by the comet test.

Due to there is no available literature on *A. cepa* evaluating the genotoxic potential of Picloram and Dicamba under *in vitro* conditions, this experiment aimed to determine the genotoxic effect of different concentrations of Dicamba and Picloram used in tissue culture and germinated in distilled water on *A. cepa* using the Comet test. It is understood that the results would give important insights into the use of dicamba and picloram in both tissue culture and agricultural practices.

2. Material And Methods

2.1. Herbicides

Picloram's chemical name is 4-Amino-3,5,6-trichloropyridine-2-carboxylicacid. Empirical Formula: $C_6H_3Cl_3N_2O_2$, Mol. Weight: 241.46 g/mol, and Catalog No. 1918-02-1 [33]. Dicamba's chemical name is 3,6-dichloro-2-methoxybenzoic acid. Empirical Formula: $C_{l2}C_6H_2$ (OCH_3) CO_2H, Mol. Weight: 221.04 g/mol, and Catalog No. 1918-00-9 [33].

2.2. Rooting procedures and treatments

For rooting and treatment of commercially obtained *A. cepa* L. bulbs with a diameter of 1.5-2 cm and a weight of 2–4 g, two different protocols were established. 1) The bulbs were rooted in MS culture medium and then treated with 7 different concentrations of both Picloram and Dicamba prepared in MS medium. Here, MS culture medium served as the negative control. 2) The bulbs were rooted in bidistilled

water and then treated with the same concentrations of both Picloram and Dicamba, as in 1, prepared in distilled water dd H_2O was utilized as negative control in the experiment.

Sterilization and rooting in tissue culture

Before rooting, *A. cepa* bulbs were surface sterilized with 25% commercial bleach (5% NaOCl) for 10 minutes [21] and then rinsed in sterilized dd H_2O for 3 times × 5 min. The bulbs were left in a nutrient medium containing 1×MS medium [34] (Murashige and Skoog 1962) having 30 g L^{-1} sucrose and 7 g L^{-1} agar under aseptic conditions for regeneration and growth of roots up to 1.5-2 cm length for 5–6 days.

The pH of culture treatments used in this study was set to 5.7 ± 0.1 with either 1M HCl or 1M KOH before the addition of agar. All culture treatments were subjected to autoclave at 104.5 kPa atmospheric pressure at the temperature of 121°C for a period of 20 min. These cultures were regenerated at 24 ± 2°C in a laboratory growth chamber using 16 h light (42 µMol photons m^{-2} s^{-1}) and an 8 h dark photoperiod.

*Treatment with Picloram and Dicamba: S*tock solutions of both auxins were prepared as per instruction of the supplier by dissolving 10 mg of the chemical in 0.25 mL ethyl alcohol (absolute alcohol) and subsequently completed to 10 mL with ddH_2O water. The Sigmaaldrich [33] suggests 1–10 mg/L picloram for use in plant tissue culture studies. To meet this range, previously applied concentrations by Ozel et al. (2018) (0.67, 1.34, 2.01, 2.68, 3.35, 4.02 mg / L) were choosen. Besides, in this study, one more concentration (8.04 mg / L), which is just under 10 mg/L, was added.

There is no available literature about comet test for auxin Dicamba. Again; Sigmaaldrich [33] recommend 1–10 mg/L Dicamba for in vitro studies, therefore, the doses used for picloram were considered appropriate for this auxin as well. The *A. cepa* bulbs rooted for 5 days were treated with the above mentioned respective concentrations of picloram and dicamba for 24 h. A total of 12 bulblets were used for each experimental treatment. These were equally divided into six replications of two bulblets each.

2.3. Application of the Comet (SCGE) Assay

The method used by Juchimiuk et al. [35] in the comet assay was applied with some modifications. All comet work was done under dimmed red light. Root tips were cut and kept on ice in a Petri dish by spreading it with 400 µL cold 0.4 M Tris-HCl buffer (pH 7.5). They were chopped finely with a razor blade. The suspension of nuclei (90 µL) was mixed with 90 µL of 0.75% LMA (Low melting agarose) (prepared in Tris-HCl) and evenly spread on the slide precoated with 1% NMA (Normal melting agarose). The covered slides were kept on ice for 5 minutes. After removing the coverslips, the slides were placed in an electrophoresis tank having cold electrophoresis-buffer (1 mM EDTA, 300 mM NaOH, pH > 13) for 20 min to facilitate DNA unwinding. Electrophoresis was run at 27 V, 300 mA for 20 min at 4°C. Then the slides were rinsed three times with neutralization buffer (0.4 M Tris, pH 7.5), stained with 80 µL of EtBr. Tail intensity (%) and moment of the randomly selected 25 comets for each concentration, were evaluated under the fluorescence microscope (Olympus BX51, with an excitation filter of 515–560 nm and a barrier filter of 590 nm) using "Comet Assay IV" (Perceptive Instruments Ltd., UK).

2.3. Statistical analysis: The results were given as mean ± standard error. Every set of experimental data between the control and treated groups were compared statistically using Student's t-test.

3. Results

The DNA damage at the root tips of *A. cepa* germinated in tissue culture with Picloram and Dicamba was given in Table 1 and Figs. 1 and 2. All the concentrations of Picloram significantly increased the comet tail intensity and tail moment (except 3.35 mg/L). Furthermore, the two highest concentrations showed toxic effects and no enough cells could be observed. Similarly, all the concentrations of Dicamba significantly increased comet tail intensity and comet tail moment compared to the control group (Table 1, Figs. 1 and 2). These results show that Picloram and Dicamba auxins have DNA damaging effect on *A. cepa* root tips germinated in tissue culture.

Table 1
DNA damage in *A. cepa* root tips germinated in tissue culture with Picloram and Dicamba

Concentrations (mg/L)	Tail Intensity ± SE	Tail Moment ± SE
Picloram		
Control	32.38±2.50	29.67±2,72
0.67	55.99±4.74*	58.06±6,13*
1.34	47.61±5.47*	68.97±12,64*
2.01	68.94±5.17*	122.91±17,83*
2.68	76.16±2.52*	116.13±7,39*
3.35	43.68±3.57*	32.71±3,14
4.02	Toxic	Toxic
8.04	Toxic	Toxic
Dicamba		
Control	37.86 ± 6.75	31.38 ± 8.30
0.67	32.68 ± 3.86	21.94 ± 3.51
1.34	61.80 ± 5.63*	77.04 ± 11.24*
2.01	68.57 ± 5.78*	90.33 ± 10.09*
2.68	83.61 ± 3.49*	117.86 ± 7.86*
3.35	82.38 ± 4.45*	98.37 ± 10.00*
4.02	80.43 ± 3.18*	125.28 ± 11.74*
8.04	63.60 ± 5.14*	67.26 ± 7.71*

SE= Standart Eror

* * Significantly different from the control $p < 0.05$ (t-test)

Table 2 contains data on DNA damage occurring in *A. cepa* root tips germinated in water with Picloram and Dicamba. While most of the Picloram treatment increased both the comet tail intensity and tail moment, increasing was significant at three concentrations, 1.34, 4.02, and 8.04 mg/L, compared to the negative control. Most of the applications of Dicamba have also elevated the DNA damage determined

by comet assay, however, this increase was significant at the two highest concentrations (4.02 and 8.04 mg/L) for tail intensity and the 4.02 for the tail moment, compared to the control (Table 2 and Fig. 1, 2). These observations reveal that especially high concentrations of both auxins can cause DNA damage in *A. cepa* root tips germinated in water.

Table 2
DNA damage in *A. cepa* root tips germinated in water with Picloram and Dicamba

Concentrations (mg/L)	**Tail Intensity ± SE**	**Tail Moment ± SE**
Picloram	38.68 ± 8.09	51.10 ± 12.39
Control		
0.67	44.31 ± 7.33	63.11 ± 11.67
1.34	74.64 ± 5.93*	119.06 ± 11.39*
2.01	39.73 ± 5.61	41.51 ± 6.84
2.68	47.83 ± 7.32	56.11 ± 9.03
3.35	36.97 ± 7.07	40.28 ± 10.03
4.02	77.87 ± 6.20*	119.57 ± 13.41*
8.04	80.50 ± 3.29*	136.66 ± 11.80*
Dicamba		
Control	43.09 ± 8.14	62.28 ± 13.20
0.67	51.99 ± 5.13	82.84 ± 9.04
1.34	58.91 ± 4.14	77.57 ± 6.31
2.01	55.95 ± 2.06	66.22 ± 3.80
2.68	57.52 ± 3.36	74.81 ± 6.14
3.35	47.18 ± 2.35	47.27 ± 3.28
4.02	78.06 ± 1.96*	132.13 ± 6.92*
8.04	64.76 ± 3.86*	95.58 ± 8.41
SE = Standart Error		
* Significantly different from the control $p < 0.05$ (t-test)		

4. Discussion

Synthetic auxins are widely used around the world involving their exogenous applications in plant regeneration under *in vitro* conditions and their use as herbicides to kill weeds in agriculture. Continuous accumulation and misuse of these auxin-herbicides to soil are causing serious threat to the environment

and ecosystem. Plants are constantly exposed to greater amounts of pollutants than any other organism [25]. Constant accumalation of auxin-herbicides can induce stress on plants cells and tissues after passing threshold limit. This stress ends up with generation of reactive oxygen species (ROS) [36, 37]. If the formation of ROS is not stopped immediately and promptly, it can destroy cell membranes and nuclear membrane along with increased permeability ending up with lipid peroxidation, increased conductivity, and significant electrolyte and small organic molecules leakage [38].

It is possible to identify damages using model plant species (*Allium cepa*, *Hordeum vulgare*, and *Vicia faba* etc.) [16, 18, 20] in genotoxicity tests against physical and chemical agents. These tests easily check genomic instabilities and damages to the genetic material in the living organisms [25]. Comet assay is one of the genotoxicity tests commonly used to determine the genotoxic potential of various agents. In recent years, specifically, the Allium-Comet test has been used as a sensitive and reliable method. The comet test used in the current study exhibited a precise measurement of the potential of Picloram and Dicamba auxin herbicides to induce damage on *A. cepa* root tip cells under both tissue culture conditions and in water. Optimization is important in plant regeneration studies during plant tissue culture. Success of tissue culture depends on many variables like dose of disinfectant used for sterilization, duration of application period, sucrose, growth regulators and pH. These auxins were studied under *ex vitro* (in water) conditions as a control treatment to understand the effects of auxin independent of these variables. The results exhibited that both of the auxins induced more stress and more pronounced DNA damage in *Allium cep*a root tip cells using > 4.02 mg/l under tissue culture conditions compared to treatments in water.

Taspinar et al. [26] also revealed similar observations. They found that both dicamba (0.2, 0.4, and 0.6 ppm) and picloram (5, 10, 20, and 40 mg/L) could induce profile changes in *Phaseolus vulgaris* seedlings, along with template instability and induced DNA hypomethylation and hypermethylations. Reynoso et al. [31] used comet assay to determine the effects of dicamba-atrazine (1000–2000 and 2000–4000 ppm) mixture and found significant increase in DNA damage at all doses they used. Another investigation by Özel et al. [24] have determined that *A. cepa* root meristems exposed to picloram for 24 and 48 hours showed decreased mitotic index without any abnormalities. In another study, the sunflower anthers were pre-treated with cold or hot stress, then they were grown in tissue culture containing different concentrations and combinations of 6-benzylaminopurine, 2,4-dichlorophenoxyacetic acid, α-naphthalene acetic acid, and indole-3-acetic acid. Oxidative stress factors and DNA damage levels (with comet assay) were evaluated in calli tissues. The results revealed that the mentioned culture factors induced DNA damage and oxidative stress [23].

Mohammed and Ma [27] noted that picloram increased the frequency of MN in the *Tradescantia*-micronucleus test in dose dependent manner. Similarly, Cenkci et al. [32] demonstrated that in the roots of *Phaseolus vulgaris* L. treated with 2,4-D and Dicamba for 96 h, DNA fragmentation was observed by comet assay. Aydin et al. [28] reported genetic and epigenetic changes after treatments with increasing concentrations of (2–10 mg/L) 2,4-D, picloram, and dicamba for 21 days. Decreasing in the genomic

stability was also revealed in *Hordeum vulgare.* Arslan [30] has also noted a positive correlation between the doses of Dicamba and the degree of genetic damage.

All of these results make it important to identify the potential genotoxic effects of these chemicals on different organisms [39–41]. These findings also support the ecotoxicity concerns using picloram and dicamba as suggested by Office of Pollution Prevention and Toxics of the U.S. EPA [42, 43]. The results of the present study are also consistent with the findings of other researchers on different organisms. This outcome is contrary to the findings of Miller et al. [9] who reported that dicamba (0.22 or 2.2 µg/L for 48 h) was non-toxic, did not induce oxidative stress, and alter the DNA methylation profile in *Salvelinus confluentus* and *Oncorhynchus mykiss* hepatocytes. Larvae and adult of *Drosophilla melanogaster* were treated with Pesticide Palace (2,4-D and picloram mixture) and noted that the toxin had ecotoxicological adverse effects on *D. melanogaster* [44].

The toxic effects of plant growth regulators have also been demonstrated in urine and blood samples of humanbeings, particularly in agricultural workers engaged with these chemicals [10]. They found a significant difference in nuclear division index (NDI) value between workers and control group healthy indviduals [45]. Weichenthal et al. [12] have reported an increased association of cancer with dicamba.

Another important finding on auxins was that these herbicides contaminated groundwater. In the compilation study reporting this situation, the absorption and degradation rates of some auxins in the soil were compared. Researchers reported that mecoprop-P and dichlorprop-P had a greater leach capacity into the groundwater [13]. Additionally, a study conducted in the United States with glyphosate and dicamba-resistant soybeans has shown that increased dependence on these herbicides would also increase concentrations in other crops [8]. These invetsigations reveal the potential damage that plant growth regulators may cause to the environment.

All of these studies demonstrate that the accumulation of both auxins in the environment could have long-term adverse effects on the agroecosystems. These results are in agreement with European Union are major cause of the decline in dynamics of humans and plant [46, 47] and animal populations.

Conclusions

These auxin herbicides are major environmental pollutants. Their unstopped use and accumulation in the environment could have unprecedented negative impacts, especially on the genetic material of living organisms. Furthermore, these findings give an evidence of DNA damage in *A. cepa*. Therefore, to conserve ecosystem and to pose minimum threat to life, both picloram and dicamba should only be used in appropriate and recommended concentrations in agriculture.

Declarations

Authors' contributions: C.A. Ozel and D. Yuzbasioglu designed the research and carried out the experimental stages, collected the experimental data and wrote the article. F. Unal contributed the comet

assay and the statistical analyses and write up of the article. E. Avuloglu-Yilmaz and E. Erikel contributed to all the experimental stages including writing of the article. S. Mirici contributed to tissue culture stages. All authors have read and approved the manuscript.

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Availability of data and materials: All data generated or analyzed during this study is included in this article.

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Figures

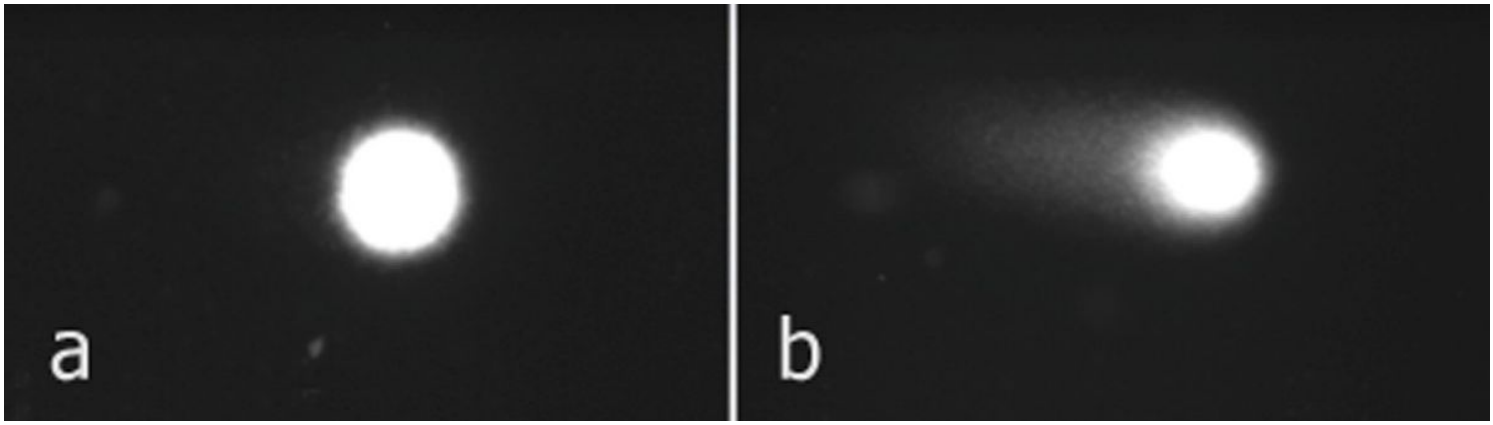

Figure 1

Comet assay representative images of *A. cepa* root tips treated with Picloram (a) Control group (b) Damaged DNA

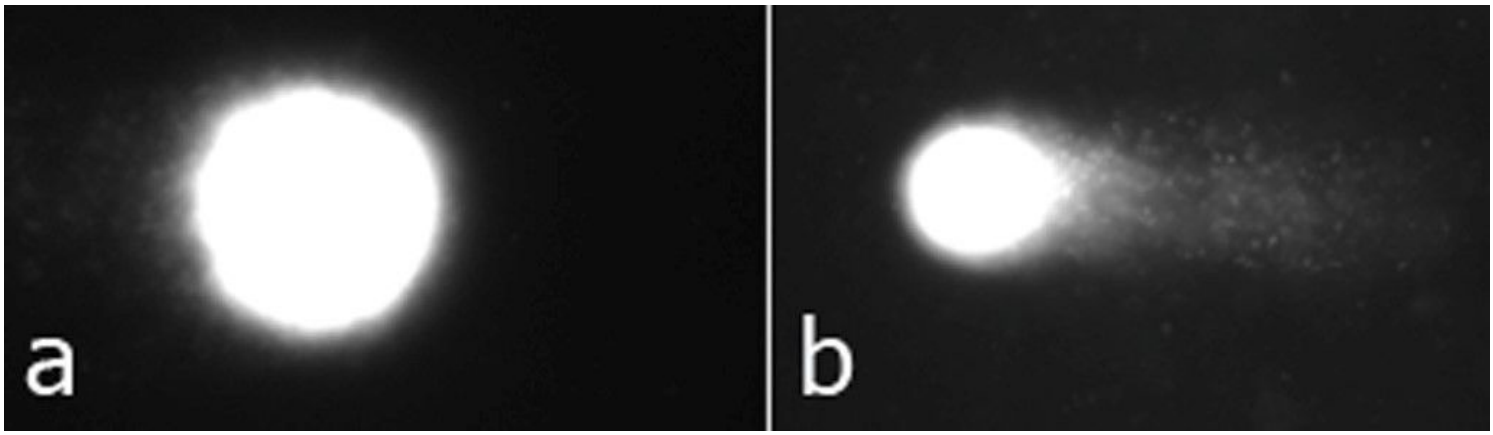

Figure 2

Comet assay representative images of *A. cepa* root tips treated with Dicamba (a) Control group (b) Damaged DNA