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ORIGINAL RESEARCH ARTICLE

Risk assessment of a Disinfection By-Product (DBP) on mitotic chromosomes using *Allium* root-tip bioassay

Monika Yadav[1], Vivek Singh[1*] and Amit Kumar Singh[2]

[1]Department of Botany, Shri Jai Narain Mishra PG College, Lucknow 226001, (Uttar Pradesh), India
[2]Department of Botany, Lucknow University, Lucknow 226007, (Uttar Pradesh), India
[*]Corresponding author's E-mail: viveksingh_2@yahoo.com

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ABSTRACT

The process of disinfection of water, using chemicals like chlorine, bromine or iodine, leads to formation of certain chemicals called Disinfection By-Products (DBPs). These chemicals were once considered potentially harmless but as their levels are increasing in potable water, concerns over their toxic effects are growing worldwide. Chlorinated DBPs are of highest concern in case of swimming pool waters as they provide greatest exposure to skin, eyes and mouth of the swimmers. This study was therefore planned with an aim to evaluate the cytotoxic effects of DBPs on mitotic chromosomes of *Allium cepa* roots under controlled laboratory conditions. For this study, Trichloroacetic Acid (a common DBP) was dissolved in water in concentration levels that are common in swimming pool waters. This was used as a medium to grow roots in *A. cepa* bulbs. The cells of the roots grown in exposure of Trichloroacetic acid (TCAA) were then cytologically analysed. Results showed mitotic abnormalities like micronuclei, bridge formation, fragmentation, stickiness etc. with total abnormalities reaching up to 26.81%. Active Mitotic Index showed a dose dependent reduction with a mito-inhibition of 41.68% at highest treatment dose. Such an assessment of cytotoxic potential of TCAA on plant cell mitosis has rarely been attempted which makes the study novel. This bioassay showed that DPBs definitely have the mutagenic potential even under short term exposure and can become a bigger problem with successive bio-magnification. Hence, it proves the effectiveness of *Allium* mitosis bioassay in testing cytotoxicity of DBPs.

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INTRODUCTION

Chlorination has long been thought of as a panacea for water borne diseases, as it could reduce mortality rate without harmful side effects. In 1974, Rook (1974) identified Tri-Halomethane as the first Disinfection By-Product (DBP) in drinking water. Due to its very low concentration, it was supposed to be of no harm to plants or animals. But as the sewage and organic pollution increased in natural waters, higher amounts of chlorine had to be added to disinfect water. By the year 2006, more than 600 DBPs were known (Richardson *et al*., 2007). In last two decades, various studies started reporting substantial levels of DBPs in potable waters of different locations. Certain organic acids present in natural surface water bodies, viz., Humic and Fulvic Acid, react with Chlorine to form. DBPs can be of diverse chemical nature like Trihalomethanes, Haloacetic acid, Haloketones, Haloaldehydes, Haloamines, Halophenols and Haloamides. Their levels are higher in waters which receive frequent chlorination for disinfection like that of swimming pools.

A number of researchers suggested that presence of DBPs in swimming pool waters can cause oculus, nose and throat infections, skin problems and even carcinoma (Hinckley *et al*., 2005; Villanueva *et al*., 2007; Fantuzzi *et al*., 2010; Manasfi *et al*.,

2016). Plewa *et al.* (2012) reported that Trihalomethanes present in water can be carcinogenic. Kogevinas *et al.* (2010) also reported that regular swimmers in public pools can contract genotoxic diseases due to DBPs. Irrigation with such water on a regular basis disturbs the soil flora and cause reduction in plant growth. Also, Cirillo *et al.* (2016) reported that DBPs in Italian fish pond water have genotoxic effects on common Carp fish. The reviews of Carter *et al.* (2019) and Manasfi *et al.* (2016) provide a deep insight into the sources, types and health hazards of common DBPs in swimming. In another study, Daiber *et al.* (2016) compared the concentration of DBPs in swimming pools after a few cycles of disinfection, with the initially filled waters. He found a 610% and 900% appreciation in total DBP concentration. In a similar study the levels of five Haloacetic Acids in swimming pools of Tehran varied between 148 to 3488 mg/L, setting an average of 1045.26 mg/L (Dehghani *et al.*, 2018). A massive study by Carter *et al.* (2019) on 39 DBPs in 6 pools of Australia, revealed levels of Mono, Di and Tri-Chloroacetic Acid to be ranging between 200 and 479 µg/L. The studies by Shi *et al.* (2020) on Chinese swimming pools also reported high amounts of trihalomethanes (THMs) and haloacetic acids (HAAs). Majority of DBPs have been shown to be cytotoxic, clastogenic and even carcinogenic in nature (Richardson *et al.*, 2007). Generally toxic substances reach into the human system after a long time but as far as DBPs are concerned human beings seem to be the ones having primary exposure as they use disinfected water for drinking, bathing, washing, cooking and even for recreation.

It is evident with the above background, that DBPs are affecting the physiological as well as genetically aspects in all living systems that are exposed to them. It is therefore worthwhile to investigate various DBPs for their effects on chromosomes and genes. Plant systems have long been used as models for assessing the genotoxic effects of various chemicals on chromosomes. In the last few years, higher plants have gained importance in assessment of cytotoxic and mutagenic potential of various environmental pollutants (Lutterbeck *et al.*, 2015). However, there are very few studies on effects of DBPs on plant systems. In one such study, genotoxicity of DBPs was tested using *Vicia faba* root bioassay which showed mitotoxicity and genotoxicity of levels similar to common mutagens (Hu *et al.*, 2017). This gap in research on plant systems as models for testing toxicity of DBPs can be observed in previous literature. It was therefore planned to investigate the effects of a common DBP on mitotic chromosomes of *Allium cepa*. Since Halo Acetic Acids are one of the most important DBPs, we selected Tri Chloro Acetic Acid as our model DBP. The idea was to use a single DBP and use it to concentrations matching total DBPs in water as per previous studies mentioned above. In this way, a tentative chart of genotoxicity may be tabulated.

Thus, in the present study, Tricholoroacetic acid (TCAA) has been taken as a representative of all Disinfection By-Products (DBPs). TCAA is a derivative of acetic acid with replacement of three H atoms of the Methyl (CH_3) group by Chlorine atoms. Chemically, it is CCl_3COOH. It is formed by Chlorination of water using Cl_2 or hypochlorite. The WHO (2022) has established a provisional guideline of 100 µg/L for Trichloroacetic acid in drinking-water. Bureau of Indian Standards (BIS, 2012) has specified desirable limits for a few Triholomethanes like Chloroform, Bromoform etc. to 0.2 mg/L. However, in swimming pool waters, the levels of DBPs are 500 to 1000 times of the established WHO limits (Chowdhury *et al.*, 2014). This water comes in direct contact of humans through skin, eyes and buccal cavity through ingestion.

The concentrations of TCAA for treatment are been based on previously reported levels of DBPs found in swimming pool waters. *Allium cepa* was taken as the model as it is one of the best known plant systems where effects of any chemical on mitosis can be studied easily. Thus, this study envisages revealing the effects of high concentrations of DBPs on chromosomes using *Allium cepa* root mitosis bioassay.

MATERIALS AND METHODS

Based on previous studies, the concentrations of 25 mg/l, 50 mg/l, 75 mg/l and 100 mg/l of TCAA were taken for assessment of cytoxicity. Plane deionized water was used as blank control.

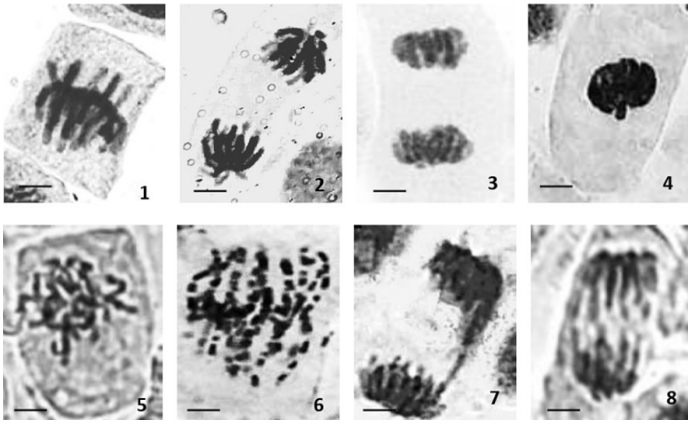
Standard solutions of TCAA were prepared according to IS-2316 (BIS, 1990). Tricholoroacetic Acid (AR Grade, Merck) was mixed with deionized water to prepare 25 mg/l, 50 mg/l, 75 mg/l and 100 mg/l solutions (w/v). These were filled in wide mouthed bottles and healthy onion bulbs were placed over it to cover the mouth. Suitable controls were maintained using distilled water. These were left in dark for 72 hours till the roots attain 2-3 cm size.

These roots were then plucked and fixed in Carnoy's fixative (3:1 Absolute Alcohol: Glacial Acetic Acid) for 24 hours. They were then stored in 70% Alcohol at 4°C for cytological analysis.

For analysis, squash preparations of root tips were done. Three slides per treatment dose were prepared and analyzed thereby observing around 1000 cells per treatment and control sets. The cytological analysis followed the standard techniques given by Darlington & La Cour (1976). The data was analyzed using standard statistical tools for central tendency and standard error of mean

RESULTS AND DISCUSSION

Control sets showed normal mitosis with 14 chromosomes at Metaphase and 7:7 separations at Anaphase (Figures 1-3). The Active Mitotic Index (AMI) was 14+1.12 and total abnormality percentage was recorded as 1.24+0.06%. However, various anomalies were encountered in TCAA treated sets which showed a dose dependent increase. AMI recorded gradual decrease with increasing concentrations of TCAA (Table 1). The common chromosomal aberrations encountered were un-orientation, fragmentation and stickiness at Metaphase, laggards, bridges, delayed separation etc. at Anaphase (Table 2 and Figures 4-8). In the treated cells, Metaphase recorded stickiness, clumping and scattering of chromosomes as the most



Figures 1-8. *Normal Mitosis in Onion root tip cells, 1- Normal Metaphase, 2- Normal Anaphase, 3- Normal Telophase, 4- Abnormalities induced by DBPs, 4- Clumping at Metaphase, 5-Unorientation at Metaphase, 6-Fragmentation, 7- Cytoplasmic Bridge at Anaphase, 8- Delayed separation at Anaphase (Scale Bar: 1cm=4μ).*

Table 1. Effects of DBPs on active mitosis and chromosomal anomalies in *A. cepa* root meristem.

Concentration (mg/L)	No. of dividing cells	Active mitotic index	Abnormality (%)
Control			
0	104 ± 2.65	10.4±0.09	0.86 ± 0.04
TCAA			
25	89 ± 1.18	8.9 ± 1.65	1.25 ± 0.08
50	75 ± 1.25	7.5 ± 0.89	6.05 ± 0.12
75	69 ± 2.05	6.9 ± 0.55	1.25 ± 0.05
100	54 ± 3.14	5.4 ± 0.03	6.81 ± 0.05

Table 2. Chromosomal abnormalities induced by TCAA in *A. cepa* root tip mitosis.

Treatment (%)	TAd	AMI (%)	MIn (%)	Metaphase abnormalities (%)					Anaphase abnormalities (%)			TAd (%)
				Un	Sc	Pm	Fr	St	Mn	Br	Cl	
Control	104 ± 2.65	9.26 ± 0.04	-					0.41			0.43	0.86 ± 0.04
TCAA (mg/l)												
25	89 ± 1.18	8.9 ± 1.65	3.88	1.86	0.38	1.55	1.62	0.93	2.05	1.62	1.24	11.25 ± 0.08
50	75 ± 1.25	7.5 ± 0.89	19.01	2.39	2.80	1.40	1.99	2.59	1.70	1.99	1.19	16.05 ± 0.12
75	69 ± 2.05	6.9 ± 0.55	25.48	2.20	4.41	0	3.81	1.40	2.61	3.41	3.41	21.25 ± 0.05
100	54 ± 3.14	5.4 ± 0.03	41.68	1.93	4.64	1.93	4.81	1.29	3.23	3.87	5.11	26.81 ± 0.05

TAd=Total number of Actively dividing cells; AMI=Active mitotic Index; MIn=Mitoinhibition; Tab=Total number of abnormal cells; Un=Unorientation of chromosomes; Sc=Scattering of chromosomes; Fr=Fragmentation of chromosomes; Pm=Precocious movement of chromosomes from the Metaphase plate; St=Stickiness of chromosomes; Cl=Clumping of chromosomes; Lg=Lagging chromosomes; Mn=Micronuclei; Br=Chromatin bridge between the poles; Tab (%)=Total percentage of abnormal cells.

common aberrations. Precocious movement and fragmentation of chromosomes were also observed but in lesser numbers. Fragmentation was more common at higher doses. Anaphase abnormalities were less common. However, chromatin bridge formations, unequal separation and disturbance in polarity were encountered in some cells. Total abnormality reached a high of 26.81% in 100 mg/L dose. Higher doses were able to induce definite mito-inhibition and thus may be expected to be growth retarding. Types and levels of abnormalities observed in the treated sets can be used as an insight into the possible effect of DBPs on cell division and growth in plant systems.

According to Istifli *et al.* (2019), a cytotoxic compound has the ability to alter the rate of cell proliferation. Although, the DBP in question produced alterations which might not seem as toxic as many other chemicals yet reduction in AMI, and development of various aberrations suggest its potential threat in future if the concentrations rise. Presence of chromosome bridges, stickiness, unorientations etc. are quite similar to those induced by heavy metals and pesticides. Dose based reduction in Active Mitotic Index is indicative of the fact that the DBPs or their derivatives are interfering in the normal process of mitosis thus stopping many cells from transitioning from Interphase to Prophase. One reason of this might be disturbance in DNA synthesis (Schneidernam *et al.*, 1971). Energy is needed for movement of chromosomes during division. It is likely that the DBPs might be interfering in the energy releasing pathways of respiration resulting in lower ATP production. This is corroborated by Jain & Sarbhoy (1988) who have reported that respiration inhibiting chemicals also inhibit cell division. Similarly, Epel (1963) also suggested that mitosis rate is directly proportional to levels of ATP. He added that cell division could be blocked by use of respiratory blockers at different stages.

A close observation of the types of aberrations encountered allows us to divide them into broad categories like agglutination, spindle dysfunction, breakage of chromatin and unusual associations of chromosomes. In our study, agglutination was most common aberration manifested in forms of Stickiness and Clumping of chromosomes. There are many hypotheses regarding the mechanism of agglutination but the precise process is yet to be understood. It may happen because of improper functioning of histone and non-histone proteins or due to change in electrical charge on the chromosome surface or due to dissolution of some nucleoproteins (Turkoglu *et al.*, 2007). Klasterska *et al.* (1976) blames the aberration on defective folding of chromatic fibers which might lead to subchromatid connections between them. Jayabalan & Rao (1987) view this as a result of changes in viscosity brought about by imbalance in biochemical reactions.

The other type of anomaly is spindle dysfunction which is related to improper functioning of microtubules due to certain chemicals. Majer *et al.* (2005) have called these types of substances as aneugenic as they cause damage to the microtubule

organization thus leading to abnormalities. Some of these chemicals might also act as clastogenic agents and produce breaks in the chromosome backbone. This is the cause of next type of anomaly related to breakage of chromosomes which can be seen in form of fragments, laggards etc. Some workers have attributed this abnormality to direct action of chemicals on DNA (Bignold, 2009). If such broken DNA strands survive or get left out during repair, they may form fragments. In such case, these chemicals are known as Clastogens. Some researchers have suggested that fragments result from the failure of broken chromosomes to recombine or due to misrepair of DNA (Evans, 1976). It was also suggested by some, that the upsetting of nucleic acid metabolism might lead to disturbed protein re-duplication thereby causing chromosomes to break at several loci (Sharma & Sharma, 1960). Chromosomes which are broken and connected to faulty spindle may form unusual associations, bridges, disturbed polarity etc.

At present a clear cut explanation of the pathway of action of TCAA on chromosomes is not known but there are reports by workers like Pals *et al.* (2013) that this may indirectly involve the oxidative damage caused by the DBP. In his studies, Wang *et al.* (2020) has demonstrated that most of the Halo Acetic Acids induce high levels of ROS production thereby making the antioxidant enzymes less active and creating an oxidative stress. This in turn will lead to significant loss of functionality of molecules like DNA, lipids and amino acids. Studies by Dad *et al.* (2013) and Ali *et al.* (2014) have also reported that higher levels of ROS can interfere in the actions of certain cellular components which are responsible for proper distribution of chromosomes during division. This may lead to various abnormalities. According to Bonassi *et al* (2021), chromosomal aberrations like micronuclei and fragmentation indicates non-repair of DNA damage and can lead to genetic problems in future generations. They further add that high chromosomal abnormality percentage may produce several health issues like infertility, hypoglycemia, heart problems and neuromuscular ailments in humans. These chromosomal abnormalities are biomarkers of carcinogenic risk also.

Pérez-Albaladejo *et al.* (2023) explored the cytotoxicity of three HAAs viz. chloroacetic acid, bromoacetic acid and iodoacetic acid using a bioassay of human lung and placental cells. They could observe EC_{50} values at concentrations 7.5 µg/l for Iodoacetic acid and 250 µg/l for monochloro acetic acid. These values were lower since the exposure was given directly to the human cells. In natural conditions cells are not directly exposed to the outer levels. Plant based systems are generally considered less sensitive but our study showed sensitivity even at lower concentrations. In our study, the EC_{50} value, where the actively dividing cell reduced to 50% of the control sets numbers, was attained at 100mg/l concentration. Concentrations of DBPs even higher than this have been reported by various workers in swimming pool waters. This is an alarming observation as halogen based disinfection of water in swimming pools is still being carried out unregulated, in countries like India.

Conclusion

In conclusion, the overall mito-inhibition and cytotoxicity was observed in the treated sets, under laboratory conditions, was specific for concentrations of TCAA based on secondary data. Typical clastogenic aberration even at low concentrations prove that this plant based bioassay is a reliable and rapid measure of cytotoxicity that can be used to screen out more dangerous DBPs and act towards their reduction. Chromosomal anomalies like micronuclei, fragmentation and clumping clearly point out towards non-repair of DNA damage which can be seen as a pointer towards mutagenic potential of DBPs. These results are not applicable for drinking water as they are based on high concentrations of TCAA found only in swimming pool waters. However, swimming pools provide exposure to humans, which is not less than that provided by drinking water. We have limited our study to just one DBP to evaluate its specific effect but under actual conditions there is a cocktail of various DBPs which can interact with each other and enhance overall toxicity. Future studies may focus on the combination of various DBPs to compare the effects. The study establishes that *Allium* bioassay is sensitive towards change in concentrations of DBPs in water can be effectively used for rapid and in vitro tests to reduce dependency on in vivo experiments.

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DECLARATIONS

Authors' contribution

Conceptualization, Methodology and Design: V.S. and A.K.S.; Investigation: M.Y. and V.S.; Data collection: M.Y.; Writing and draft preparation: M.Y. and V.S.; Review and editing: A.K.S.; Supervision: V.S. and A.K.S. All authors have read and agreed to the published version of the manuscript.

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