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Evaluation of Tobacco Industrial Wastewater for Genotoxic Characteristics on *Allium cepa* L. Root Cell Mitosis

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Authors' contributions

This work was carried out jointly by all the authors. Authors AAA, CCN and KOO designed the study. Author AAA performed the statistical analysis, wrote the protocol and the first draft of the manuscript. Authors CCN and KOO did the analyses of the study while Author AAA conducted the literature search. All authors read and approved the final manuscript.

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ABSTRACT

Due to increased environmental and toxicological problems originating from the release of toxic contaminants in the environment, it has become obvious that cheaper and reliable methods of evaluating genotoxicity of these contaminants are needed. Hence, the effect of industrial effluent from tobacco on *Allium cepa* L. root mitosis was investigated with the view to ascertaining its genotoxic effects using the biological test. Healthy sprouted onion roots were treated with concentrations of 20%, 50% and 100% of Tobacco wastewater for 6 h, 12 h and 18 h time duration respectively, while distilled water served as control. Healthy roots were harvested after

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the treatment periods and fixed in 1:3 acetic alcohol for 24h. After this, roots were hydrolysed in 1N HCl before squashing, staining with FLP-Orcein and observed on the microscope. There was significant difference in the mitotic indices between the treatment concentrations (27.71; 32.32; 27.09; 33.85) and (21.98; 26.89; 16.17; 34.04), and the control (20% 50%; 100%; control), at 6 h and 12 h respectively. However, exposures of root cells to even the least concentration beyond 12 h to 18 h produced a toxic effect on the cells and a complete inhibition of mitosis of the root cells. Similarly, the treatment concentrations induced various mitotic chromosomal aberrations such as Stickiness, C-metaphase, bridges, unequal distribution of chromosomes, breaks, laggards, vagrant and ring chromosomes at concentrations of 20%, 50% and 100%, and between 6 and 12 h exposure. These observations confirm the assumption that wastewater of tobacco has a genotoxic potential and capable of affecting DNA biosynthesis, especially when high concentrations are absorbed for a prolonged period of time.

Keywords: Mitosis; chromosomal aberration; Tobacco wastewater; Mitotic Index; DNA biosynthesis.

1. INTRODUCTION

One of the most powerful and largest firms in the world is the tobacco industry whose cigarette production economic value is said to be bigger than thousands of billion dollars per year [1]. The transnational companies have been looking harder towards developing countries as revenue declined in developed countries during the last decade [2]. Tobacco farmers can also receive practical as well as financial assistance from the tobacco industries for the cultivation of tobacco as an attractive substitute crop to food, at the same time, as a source of income although this is not necessarily the case [3-5]. At the government level, the cultivation of tobacco can also be regarded as highly encouraging, generating financial reimbursement through trading and taxation, at least for a short period [6]. From the point of view of the tobacco industries, the cost of production in the developing world is lower and the market is less regulated [7], this makes it more conducive as an operating environment. Since the late 1970s, concerns have been registered by a number of environmental agencies about the impact of tobacco production [8].

The processes involved in the manufacturing of tobacco products leave behind various industrial by-products and wastes, which include ammonia, nicotine, hydrochloric acid, ethylphenol, toluene among others [9]. These wastes and by-products are toxic and capable of inducing various health hazards dangerous to the environment, when not properly managed or discharged accidentally into the environment untreated.

The proper discarding of these chemicals and also the biological treatment of wastewater is rigorously kept in place in developed countries; the reverse might be the case in developing countries where the production of tobacco is becoming more intense [8]. Nigeria, one of the world's developing countries where tobacco is manufactured and consumed widely has a poor, inadequate infrastructure for the management and regulation of tobacco use and its wastes according to report by the Global Youth Tobacco Survey Collaborative Group [10]. Similarly, the impact of industrial wastewater effluent in the environment is to a large extent ignored by the local government. The awareness of genotoxicity of tobacco wastewater and its effects on the environment or in countering the transnational tobacco companies is minute to non-existent by the citizens and the local government [2].

Therefore, it is highly crucial to determine wastewater genotoxicity to ultimately regulate the exposure of the population to such potential chemical hazards. The screening of water contamination for potentially carcinogenic compounds consequently presents a major concern for humans, animals and microorganisms in the environment. It is tremendously difficult to quantify the risk factors associated with these effluents as they usually occur in concentrations too low for analytical determination, and putative mutagens, with few exceptions, yet undetected [11]. Besides, the effects of mixtures cannot be measured through analytical methods, since these effects are often expressed on biological species. Hence, both bacterial genotoxicity and biological tests are often employed to determine toxicity of effluents in which a prior knowledge of toxicants identity and physiochemical properties is not necessary.

Due to increased environmental and toxicological problems originating from the release of toxic contaminants in the environment, it has become

necessary that rapid methods of evaluating genotoxicity of these contaminants are needed. A living organism does not respond to a single chemical, and usually the final response is the result of cumulative effects, therefore, it is highly important to know if the substances found in wastewater are genotoxic.

For many years, *in vitro* tests for genotoxicity have been developed and standardised [12]. They are widely employed in different sectors, and play vital regulatory role in risk assessments, such as in detecting potential mutagenic and carcinogenic agents which can result in various health hazards. The *in vitro* genotoxicity tests for chemicals and pharmaceuticals industries were recommended by authoritative international guidelines. Among the diverse tests are:

1. The Ames test to determine mutagenicity using *Salmonella typhimurium* strains with or without S9 metabolic activation,
2. The SOS-chromotest using *Escherichia coli* strains to test for genotoxicity, the lymphocyte chromosomal aberration test for determining the cytotoxic effects of the wastewater and
3. The *Allium cepa* test to determine genotoxic effects on plants [13].

The *Allium cepa* test will be explored in evaluating the genotoxic effects of tobacco wastewater effluent because of its high degree of sensitivity and economic cost. *Allium cepa* is a vegetable bulb producing plant belonging to the family of Amaryllidaceae. It has been used by researchers over time to provide useful information on the genotoxicity of most industrial products and chemical waste [12].

Hence, the aim of this study is to investigate the genotoxicity of wastewater from a tobacco company in Nigeria, by examining their effects on the mitotic division of growing roots of *Allium cepa*.

2. MATERIALS AND METHODS

2.1 Area of Study

This research work was conducted in the laboratory of the Department of Biosciences and Biotechnology, Babcock University, Ilishan-Remo, Ogun State, Nigeria. The study area was around a Tobacco Company in Ibadan industrial area which is one of the most rapidly developing and heavily polluted industrial belts of Ibadan. The industrial area is spread over 863.18 hectares of land consisting of about 20 large and medium scale industries like engineering units, steel processing industries, chemical units, paints, pharmaceutical units, textile industries etc. The study area lies between latitude 7º23'47"N longitudes 3º55'0"E. The main water source for the industrial consumption is bore holes. The industrial area utilizes a lot of fresh water per day. However, specific amount of water used was not documented. The effluent discharge, treated and untreated is released into neighbouring environment. This has created health hazards not only for local population but also resulted in disturbances of aquatic life of the Odo-Ona River, flowing near the industrial area.

2.2 Sampling of Industrial Waste Water Effluent and Sample Preparation

The industrial wastewater samples (number of samples collected, n = 4) were collected randomly from all major discharge points of the tobacco company (name withheld for confidentiality) in the year 2012. Polythene bottles of 2.5 L and 2.0 L were used to collect the grab water samples. The bottles were thoroughly cleaned with hydrochloric acid, washed with tap water to render free of acid, washed with distilled water twice, again rinsed with the water sample to be collected and then filled up with the sample leaving only a small air gap at the top. The sample bottles were stoppered and sealed with paraffin wax. The samples were refrigerated at 4ºC throughout the period of the study.

Onions (*Allium cepa* L., Family Amaryllidaceae), were obtained commercially at the Ilishan-Remo Market, Ogun State, Nigeria, and sun-dried for 2 weeks. Thereafter, the healthy dry bulbs were used for the test.

2.3 Protocol

Thirty six (36) onion bulbs were used for this study: 12 bulbs were used for each treatment factor. Three onion bulbs were used in each of the three concentrations (20%, 50%, 100% and control) and for each of the three durations (6h, 12h and 18h).The onion bulbs were dressed at the base removing old roots and placed on 30ml beakers filled with distilled water with the base of the onion touching the water surface to induce rooting. The rooted bulbs were later placed on beakers that contain the different treatment concentrations of tobacco wastewater with the roots dipped in the solutions for 6 h, 12 h and 18 h as shown in Figs. 1 and 2. The roots that

emerged from the bulbs that were placed on the beakers with distilled water were the control following the method of Nwangburuka and Oyelana [13]. Sixty (60) healthy roots were randomly chosen from the three bulbs for the treatment and analysis of the mitotic activities at the end of treatment.

2.4 Mitotic Studies and Analysis

At the end of each treatment period, harvested roots were fixed in 1:3 acetic alcohol for 24h at room temperature and later transferred into 70% alcohol before preservation in the refrigerator. Slides were prepared for Mitotic studies by treating the roots in a solution of 1NHCl for 5 min before squashing the root tips on glass slide and stained with FLP-Orcein as described by Okoli [14] and modified by Nwangburuka et al. [15]. Photomicrographs were taken at x1000 magnification under oil immersion while the slides were counted at x400 using National Digital Microscope 163 phase contrast with motic image 2000, 2.0 version computer programme. The number of dividing cells was scored and the mitotic indices calculated for the treatments and the control. The mitotic index (MI) was calculated by dividing the number of cells undergoing mitosis with the total number of cells examined for each treatment [16]. Thus, the data acquired were analysed to ascertain the effects of the treatments (concentration and duration of treatment) on mitotic activities of *A. cepa* cells.

The significance between treatments on the mitotic index was determined with the use of a t-test.

2.5 Statistical Analysis

Data was analysed using SPSS software program version 16.0 (Chicago: SPSS Inc.). One-way analysis of variance (ANOVA) and Duncan's mean range test (DMRT) were used to separate the means.

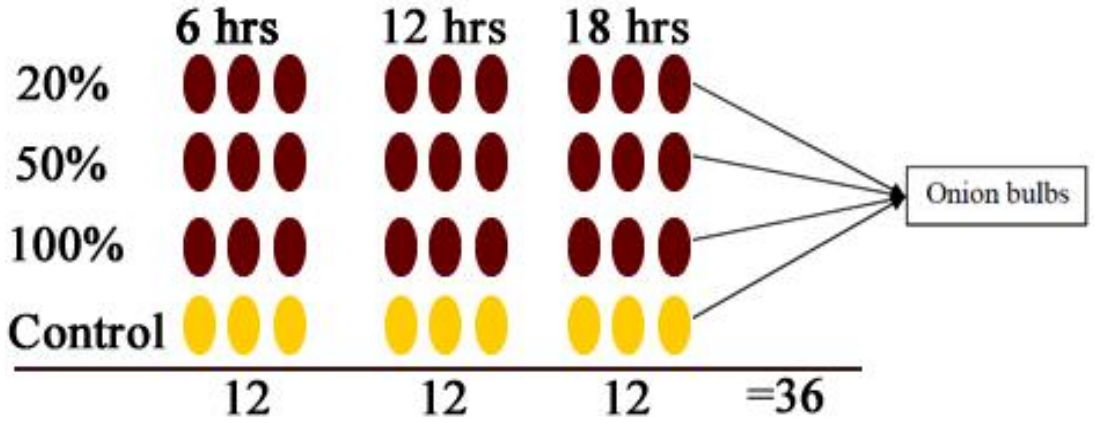

Fig. 1. Experimental layout

Fig. 2. Photograph of experimental layout

3. RESULTS

The study showed that all concentrations (25%, 50% and 100%) of the unadulterated wastewater reduced the mitotic division rates in the root meristem and this led to the various chromosomal anomalies observed. The effluent significantly decreased MI in the treatment groups compared with the control at all concentrations and treatment periods. It is worthy to note that the decrease of the MI was dose-dependent (Table 1). Furthermore, the reduction in the mitotic activity was significantly increased when exposure time increased from 6 to 18 h. All concentrations (25%, 50% and 100%) of the wastewater were toxic at the 18-h treatment period, and no dividing cells were obtained (Table 1). There were significant differences in MI between treated groups and control groups (Table 1). The data obtained show highly significant differences in MI between 50% and 100% for the 6-h, 12-h and 18-h treatment periods (Table 1).

Fig. 3 shows samples of different types of aberration induced by tobacco wastewater in *Allium cepa* root tips. They include irregular chromosome splits, lagging chromosomes, Polar chromosomes, C-mitosis, vagrant chromosomes, sticky chromosomes, unequal distribution of chromosomes, ring chromosome, Spindle breakdown, bridge and micronucleus. The percentages of these abnormalities are given in Table 1.

4. DISCUSSION

Several chemicals and food preservatives have been reported to inhibit mitosis [17-19] in *Allium cepa* and animal cells. Inhibition of DNA synthesis or a blocking in the G2 -phase of the cell cycle, preventing the cell from entering mitosis, are reasonable explanations for the reduction in the mitotic activity [20]. Nwangburuka and Oyelana [13] likewise observed low MI and inhibited mitosis of *Allium cepa* root in high concentration of Chloroquine solution treatment. Similarly, exposure of root tips of *A. Cepa* and *Viciafaba* to high concentrations of the herbicide atrazine also led to inhibition of DNA synthesis [21]. This suggests that the wastewater may cause inhibition of DNA synthesis due to pesticides and other chemicals used in production and flavouring of the tobacco. The effluent also caused a change in the frequencies of different cell stages by increasing the percentage metaphases after the 6-h treatment period while decreasing the percentage of metaphase and anaphase–telophase stages after 12-h and 18-h treatment period. Results identical to these observations have been reported after treatment of *A. cepa* root-tip cells with various other chemicals and food additives [13,17,18,22].

The effluent treatment induced various mitotic abnormalities when compared to the control in the root tips of *A. cepa*. Their percentages increased as the concentration of the applied wastewater and the duration of treatment increased when compared with the control groups. There were statistically significant differences between control and treated groups in chromosome abnormalities. It should also be noted that for all treatment times, there were significant differences between the 100% dose and all other treatment doses.

In the *A. cepa* root tips, various types of abnormalities were recorded at different mitotic stages in all treatments: stickiness, c-mitosis, laggards, bridges, micronuclei, ring chromosome, fragmented and unequal chromosome distribution. The appearance of C-mitosis indicated that the wastewater caused inhibition of spindle formation similar to the effect of colchicine [23]. There are suggestions that induction of C-mitosis is commonly associated with spindle poisons or as a result of lipophilic chains of spindle proteins that cause the bending of polypeptides [21]. Such C-mitosis cells were also reported to be induced by treatments with various other chemicals [17,18,22,13]. The heavy metals in the wastewater were suspected to have induced bridges in treatments in the 6-h period and in 50% of the 12-h treatment period. The bridges noticed in the cells are probably formed by breakage and fusion of chromosomes and chromatids. Such chromosome bridges were also reported to be induced by other chemicals [22]. Chromosome bridges may also be due to chromosomal stickiness and/or unsuccessful separation of chromosomes at anaphase, otherwise, it may be attributed to unequal translocation or inversion of chromosome segments [18].

Laggards were also observed after treatments with the wastewater. This was due to the inability of the chromosomes to move to either of the poles [18]. Another chromosome aberration induced by the effluent was the micronucleus,

Table 1. Cytogenetic analysis of *A. cepa*root tips exposed to different concentrations of tobacco wastewater for different periods

Duration of treatment (h)	Concentrations (%)	total cells examined	Total mitosis	% Prophase	% Metaphase	% Anaphase	mitotic index (mean±S.E)	A-B	C-M	L	S	Total abnormalities[a]
	Control	1025	347	48.55	20.87	30.58	33.85±0.02^{ad}	-	-	-	-	0.00^{a}
	20	1007	279	52.93	15.04	32.03	27.71±1.05^{be}	0.85	3.58	-	1.56	5.46^{bd}
6	50	1021	330	54.80	33.80	11.40	32.32±2.27^{a}	1.18	3.64	0.50	0.50	5.49^{b}
	100	1015	275	43.44	38.55	18.01	27.09±1.96^{b}	0.68	3.22	0.56	-	14. 02^{c}
	Control	1028	350	44.52	27.09	28.39	34.04±1.02^{d}	-	-	-	-	0.00^{a}
	25	1028	226	54.00	37.02	8.98	21.98±0.44^{f}	-	7.84	-	-	7.84^{f}
12	50	1019	274	36.54	58.68	4.78	26.89±0.87^{e}	0.90	1.68	-	1.68	4.35^{d}
	100	1014	164	42.75	34.03	23.22	16.17±0.28^{g}	-	17.97	-	-	17.97^{h}
	Control	1035	287	52.65	34.08	18.57	27.73±0.98^{b}	-	-	-	-	0.00^{a}
	25	1007	116	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic
18	50	1018	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic
	100	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic

A-B: anaphase-telophase bridges; C-M: C-mitosis; L: laggards; S: stickiness. [a]Means with the same letters do not significantly differ at 0.05 level (DMR Test)

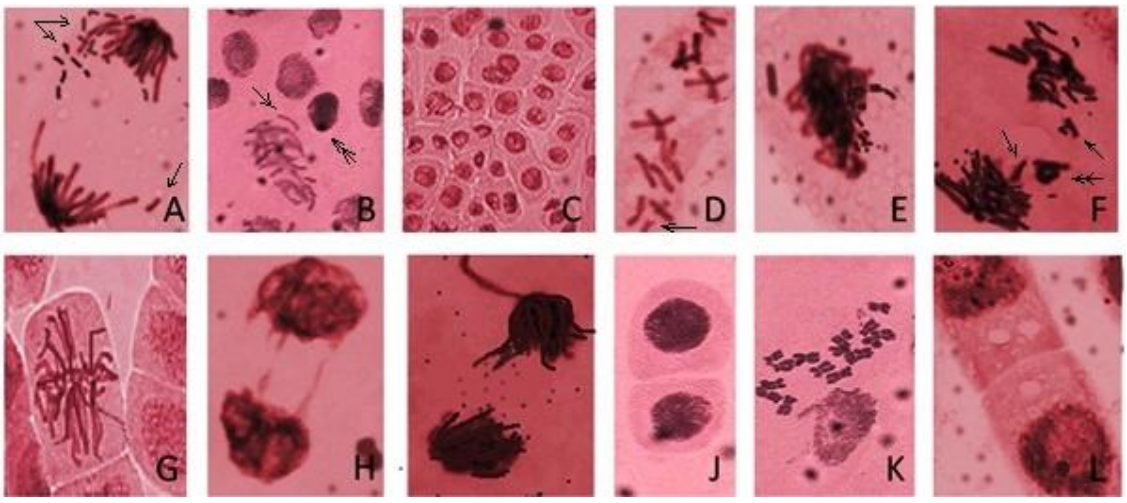

Fig. 3. Samples of different types of aberration induced by tobacco wastewater in *Allium cepa* root tips: (A) irregular chromosome splits and lagging fragments (arrowed), (B) Polarity at metaphase and vagrant chromosomes (arrowed), micronucleus at interphase (doubled arrow head), (C) non-dividing cells (D) C-mitosis and chromosome break (arrowed), 2n=16 (E) sticky metaphase (F) unequal distribution of chromosomes, laggingchromosomes (arrowed), ring chromosome (double arrow head),(G) disorientatedchromosomes,spindle breakdown (H)telophase bridge (I) sticky anaphase with lagging chromosome, (J) Normal telophase (K) Regular *Allium cepa* chromosome (control), 2n=16, and (L) micronucleus

Which occurred at very substantial percentages after treatment of *A. cepa* for 12-h period. Micronucleus formation indicated loss of genetic material. Micronuclei are the consequences of acentric fragments or lagging chromosomes that could not integrate successfully into either of the daughter nuclei during telophase of the mitotic cells [23,24]. This implies that the effluent contained clastogens that induced chromosome breaks and/or aneugens that induced lagging chromosomes. Similarly, micronuclei were recorded by many researchers following treatments with different chemicals [18,25,26].

In this study, abnormalities such as chromosome stickiness, breaks and unequal chromosome distribution were also detected. Chromosome stickiness is classified as a physiological effect exerted by the wastewater in *A. cepa*, which alter the proteins of the chromosomes (Table 1; Fig. 3E). Chromosome stickiness can also lead to apoptosis due to toxic effects of the wastewater. Again, these results are in line with other studies on the effects of different chemicals on plants and human cells [13,17,18,27]. Chromosome breaks induced by clastogens in tobacco wastewater involved an action on the DNA [18]. More studies involving the effects of the tobacco effluent on DNA and RNA are required. Unequal distribution of chromosomes and breaks have been observed in plant and animal systems as a result of treatment with toxic chemicals [18,28] Ring chromosome may result from the union of broken ends of segments of chromosomes without centromere, preventing them from moving to the poles.

It may be envisaged that similar genotoxic effects may occur in other plant and animal systems exposed to effluent of tobacco companies. However, further studies will be required to confirm such effects especially in human cell lines.

4. CONCLUSION

Tobacco wastewater was genotoxic, as shown by its inhibitory effects on onion root cells and depression of MI. It also induced an array of structural chromosomal aberrations and abnormal chromosomal behaviour ranging from C-mitosis, vagrant and lagging of chromosomes, breaks, stickiness, irregular distribution of chromosomes and micronuclei among others. It further revealed that tobacco wastewater could be used in plant mutagenic studies since it has the tendency of interfering with DNA biosynthesis. The simplicity and economical cost of the procedure make *A. cepa* genotoxicity bioassay desirable for environmental monitoring and risk assessment and a requisite tool in the

evaluation of wastewater toxicity before its discharge into the environment.

Finally, the study revealed the effectiveness of cytogenetic methods in determining the toxicity of chemical pollutants and their influence on living organisms. Further cytogenetic studies dealing with clastogenicity and genotoxicity of industrial effluents are desirable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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