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Cytogenetic effects of Fulvic acid on *Allium cepa* L. root tip meristem cells

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Abstract. Fulvic acid is a class of compounds of humic substances and is found in a significant proportion of the substances in the environment. It has been used for many years in industry, agriculture, and complementary medicine. In this study, cytogenetic effects of fulvic acid purified from Muğla Milas Hüsamlar leonardite (TURKEY) on *Allium cepa* root tip meristem cells were investigated using the Allium test. For this purpose, 10 mg/ml stock solution of fulvic acid was prepared by dissolving in citric acid and it was diluted with distilled water to 10, 20, 40, 80 and 100 µg/mL concentrations. Onion bulbs were exposed to these concentrations of the fulvic acid for macroscopic and microscopic analysis. Tap water was used as a negative control, 40 µg/mL citric acid was used as solvent control (fulvic acid solvent), and 0.02M Ethyl methane sulfonate (EMS) (a mutagenic, teratogenic, and possibly carcinogenic organic compound) was used as a positive control. There has been statistically significant stimulation of root growth depending on fulvic acid concentration in comparison with the control groups ($p<0.05$). Furthermore, in fulvic acid treatment groups, breaks, stickiness and polar deviations appeared at very low rates, and total chromosome aberration ratios were insignificant compared to the control groups. These results suggest that fulvic acid does not have cytotoxic and genotoxic effects on *A. cepa*.

Keywords. Allium test, Chromosome aberrations, Fulvic acid, Mitotic index.

1. INTRODUCTION

Fulvic acid is a class of compounds of humic substances, and it is a mixture of polyphenolic compounds formed through the degradation of organic substances such as plants, microbes and animals by chemical and biological processes (Motojima et al. 2011). It is a type of humic acid. Compared to other humic acid types, the fulvic acid is soluble in both acid and alkaline solutions, is lower in molecular weight, and has a greater biological activity (Stevenson, 1994; Bai et al. 2013; Yong 2001; Zhang et al. 2011). Piccolo (2002) redefined fulvic acid as associations of small hydrophilic molecules in which there are enough acid functional groups to keep the fulvic clusters dispersed in solution at any pH. Because, while humic acids precipitate when the pH is adjusted to 1-2, fulvic acids remain in solution after the alkaline extracts are acidified (Canellas et al. 2015).

Recently, it has been reported that fulvic acid has nutraceutical, neuroprotective (Cornejo et al. 2011; Guzmán-Martinez et al. 2013), antimicrobial, antioxidant, and anti-inflammatory properties (Van Rensburg et al. 2001; Yamada et al. 2007; Sherry et al. 2013). Fulvic acid has also been used as a medicine by people in China, Mexico, India, South America and Russia for centuries. Fulvic acid has a large capacity to retain transition metals, forming metalorganic complexes, which cause these metals to be more or less available for plants which include them into the food chain. The food industry also uses it as an ion exchanger, because it holds heavy metals very well (Pena-Mendez et al. 2005).

Over these last decades, more than 200 short-term bioassay utilizing plants, microorganisms, and insects have been developed and used to evaluate the environmental risks (Marcato-Romain et al. 2009). Plant assays are highly sensitive, easy to use in an experiment, inexpensive, and good predictors of genotoxicity and carcinogenicity (Ennever et al. 1988). The Allium test has been used by many researchers as a bioindicator of environmental pollution (Bagatini et al. 2009; Leme and Marin-Morales 2009) and genotoxicity of various agents (Aşkın Çelik and Aslantürk 2007, 2009, 2010) for a long time. With this test, mutagenic effects of substances may be analyzed by monitoring macroscopic parameters, like the appearance and growth of the roots or by genotoxic parameters, like type and frequency of chromosome aberrations, and abnormal cell division. Another advantage of this test is the presence of an oxidase enzyme system, which is essential for promutagen evaluations (Fiskesjö, 1985; Nielsen and Rank,1994). The Allium test is important, since it is an excellent model *in vivo*, where roots grow in direct contact with the test substance enabling possible damage to DNA of eukaryotes to be predicted. Therefore, results from this test can be extrapolated for all animal and plant biodiversity (Tedesco and Laughinghouse IV 2012).

Although fulvic acid is found in a significant proportion of the substances in the environment, and has been used for many years in industry, agriculture and complementary medicine, there is still minimal scientific evidence of its biological properties. In this study, cytogenetic effects of fulvic acid on *Allium cepa* root tip meristem cells were investigated using the Allium test.

2. MATERIALS AND METHODS

2.1. Supply of Fulvic acid

Fulvic acid purified from Muğla Milas Hüsamlar leonardite (TURKEY) in Chemical Engineering Laboratory of Gazi University in Ankara (TURKEY) was used in this research (Sönmez 2011). This study was conducted between March and December 2017.

2.2. Preparation of the Fulvic acid solution

The 10 mg/ml stock solution of fulvic acid was prepared by dissolving in citric acid, as it has a structure, which is soluble in weak acid. Stock fulvic acid solution was diluted with distilled water to 10, 20, 40, 80 and 100 µg/mL concentrations. Fresh solution was prepared just before the experiment.

2.3. Allium Test

Small bulbs (1.5–2.0 cm in diameter) of the common onion, *A. cepa*, (2n = 16) were purchased at a local supermarket in Aydın, Turkey. Prior to initiating the test, the outer scales of the bulbs and the dry bottom plate were removed without damaging the root primordia.

For each treatment, seven onion bulbs were placed on top of test tubes filled with tap water (pH 7.3) for 48 h. The test tubes were kept in an incubator at 22±1°C. After 48 h, two unhealthy onions with the most poorly growing roots were removed and the other healthy onion bulbs in water were treated with 10, 20, 40, 80 and 100 µg/mL fulvic acid for 24 hours. 0,02M Ethyl methane sulfonate was used as positive control for 3 h, 40 µg/mL citric acid was used as solvent control, and tap water was used as negative control.

Citric acid is a weak organic acid that has the chemical formula $C_6H_8O_7$. It occurs naturally in citrus fruits. In biochemistry, it is an intermediate product in the citric acid cycle, which occurs in the metabolism of all aerobic organisms (Berovic and Legisa, 2007). Ethyl methanesulfonate (EMS) used as positive control in experiment is a mutagenic, teratogenic, and possibly carcinogenic organic compound and its chemical formula $C_3H_8SO_3$. EMS is often used in genetics as a mutagen. Mutations induced by EMS can then be studied in genetic screens or other assays (Merck Index, 1989).

After the completion of treatment the roots were counted and their lengths were measured for each onion. To determine mean root length in a root bundle for each bulb, root lengths of experimental and control bulbs were measured with ruler at the end of treatment time. After then root tips were removed from the bulbs, fixed in 3:1 (v/v) ethanol:glacial acetic acid and stored overnight at 4°C. The next day they were placed in 70% (v/v) aqueous alcohol and refrigerated until use. An average of five slides was made for each bulb using

five root tips which hydrolyzed in 1N hydrochloric acid (HCl) for 3 min, and microscope slides were prepared by squashing the stained root tips in 2% (w/v) acetic orcein. Each slide was examined using Olympus BX51 at a total magnification of 40×10. Chromosomal aberrations were determined by scoring cells with bridges, fragments, sticky chromosomes, and polar deviations in 1000 cells per slide. Also micronucleus formation was determined in 1000 cells per slide. 5000 cells scored in total for each bulb (Fiskesjö 1993, 1997; Pavlica et al. 2000).

2.4. Statistical Analysis

Statistical analyses were performed using the SPSS 20.0 software package program. Data on physicochemical parameters, root length, root number, and mitotic index and chromosomal aberrations were compared using analysis of variance (One Way ANOVA) to confirm the variability of the data and validity of results. Post-hoc test was used to describe the magnitude of variability. Differences between corresponding controls and exposure treatments were considered statistically significant at p <0 .05.

3. RESULTS

3.1. Morphological Analysis

The results of the morphological analysis (root number and root length) are presented in Table 1. These results show that all tested concentrations of fulvic acid caused increase in the root growth, and average root number in comparison to negative control, positive control, and solvent control. The measured average root length is 2.26±1.07 cm in negative control, 1.40±0.40 cm in positive control, and 1.36 cm in solvent control. The average root length after 20 and 40 µg/ml fulvic acid treatment is found very high (4.10±0.41 and 4.22±0.69 cm, respectively) compared to controls (Table 1). The number of roots also increased in fulvic acid treatment groups compared to control groups. The highest root number is found in group treated with 80 µg/ml fulvic acid (Table 1). The root morphology in fulvic acid treated groups was thinner and more fragile compared to the negative control group.

Table 1. The average root numbers and root lengths in control and treatment groups after 24h treatment (Analysis were carried by One Way ANOVA).

Concentrations	Average root number ± SD	Average root lengths (cm ± SD)
Negative control	19.2 ± 8.07	2.26 ± 1.07
Solvent control	20.2 ± 8.87	1.36 ± 0.24
EMS (positive control)	21.0 ± 7.81	1.40 ± 0.40
FA10	37.2 ± 4.43*	3.36 ± 0.54
FA20	31.8 ± 4.56	4.10 ± 0.41*
FA40	37.6 ± 5.92*	4.22 ± 0.69*
FA80	39.2 ± 5.17*	3.98 ± 1.29*
FA100	34.8 ± 6.14*	3.68 ± 0.68

One Way ANOVA Analysis *p<0.05 is significant (EMS: 0.02M Ethyl methane sulfonate; Solvent control: 40 µg/ml citric acid; FA10: 10 µg/ml fulvic acid; FA20: 20 µg/ml fulvic acid; FA40: 40 µg/ml fulvic acid; FA80: 80 µg/ml fulvic acid; FA100: 100 µg/ml fulvic acid).

3.2. Cytogenetic Analysis

With the objective of investigating the possible mechanism involved in root growth stimulation, cytogenetic analysis was performed. Fulvic acid was found to stimulate mitotic index. A statistically significant difference in the mitotic index of root meristems was found in negative, positive and solvent control. The increase in the mitotic index was found to be positively correlated with the increase in concentration of the fulvic acid (Table 2). In the positive and solvent control groups, the mitotic index decreased significantly compared to the control group, and the mitotic index value approached zero in the solvent control group (Table 2).

Cytogenetic alterations were investigated, and the results are described in Table 2 and Figure 1. Table 2 presents the percentage of the aberrant cells in dividing cells. Very few cells with polar deviation were observed in the negative control group. No chromosome aberration was observed except for polar deviation. In this group, total chromosome aberration was found very low (0.07%). The chromosome aberration rate in the positive control group was found to be significantly higher than the control group (36.09%). Especially the anaphase bridge and stickiness have been observed to appear at a very high rate ($p<0.05$). In addition, breaks and polar deviations were observed in the positive control group. No chromosome aberration was observed in the solvent control group (citric acid), because there were only two divided cells in total and the mitotic index value was near zero in this group. The cell membrane and nucleus were deformed (Fig. 1a).

In the groups treated with fulvic acid, breaks, stickiness and polar deviations appeared at very low rates. Total chromosome aberration percentages in these groups were insignificant compared to control and solvent control groups. The highest total chromosome aberration percentage in fulvic acid treated groups was 20

Table 2. Mitotic index values, percentage of chromosomal aberrations and thousandths of micronuclei in control and treatment groups after 24h treatment.

Concentrations	Total cells	Total dividing cells	Mitotic index (MI ± SD)	Breaks (%± SD)	Anaphase bridge (%± SD)	Stickiness (%± SD)	Polar deviation (%± SD)	Total aberrant cells (%± SD)	Micronuclei (‰ ± SD)
Negative control	25000	1241	4.96 ± 0.31	0 ± 0	0 ± 0	0 ± 0	0.07 ± 0.03	0.07 ± 0.03	0 ± 0
Solvent control	25000	2	0.01 ± 0.17*	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
EMS (positive control)	25000	209	0.83 ± 0.11*	1.17± 0.26	10.47 ± 9.59*	20.05 ± 6.90*	4.39 ± 6.03	36.09 ± 5.48*	0 ± 0
FA10	25000	2893	11.57 ± 1.93*	0.04 ± 0.01	0 ± 0	0± 0	0.51 ± 0.61	0.52 ± 0.60	0.04 ± 0.01
FA20	25000	3111	12.44 ±0.51*	0 ± 0	0 ± 0	0.03 ± 0.07	2.87 ± 0.53	2.90 ± 0.52	0 ± 0
FA40	25000	3559	14.23± 0.41*	0 ± 0	0 ± 0	0 ± 0	2.90 ± 0.42	2.90 ± 0.42	0 ± 0
FA80	25000	3566	14.26 ± 0.35*	0.03 ± 0.06	0 ± 0	0.03 ± 0.06	2.68 ± 0.51	2.73 ± 0.61	0 ± 0
FA100	25000	2988	11.95 ± 0.54*	0.03 ± 0.08	0 ± 0	0 ± 0	2.38 ± 0.17	2.41 ± 0.21	0 ± 0

One Way ANOVA Analysis *p<0.05 is significant (EMS: 0.02M Ethyl methane sulfonate; Solvent control: 40 µg/ml citric acid; FA10: 10 µg/ml fulvic acid; FA20: 20 µg/ml fulvic acid; FA40: 40 µg/ml fulvic acid; FA80: 80 µg/ml fulvic acid; FA100: 100 µg/ml fulvic acid. 25000 cells/group were evaluated for MI and CA)

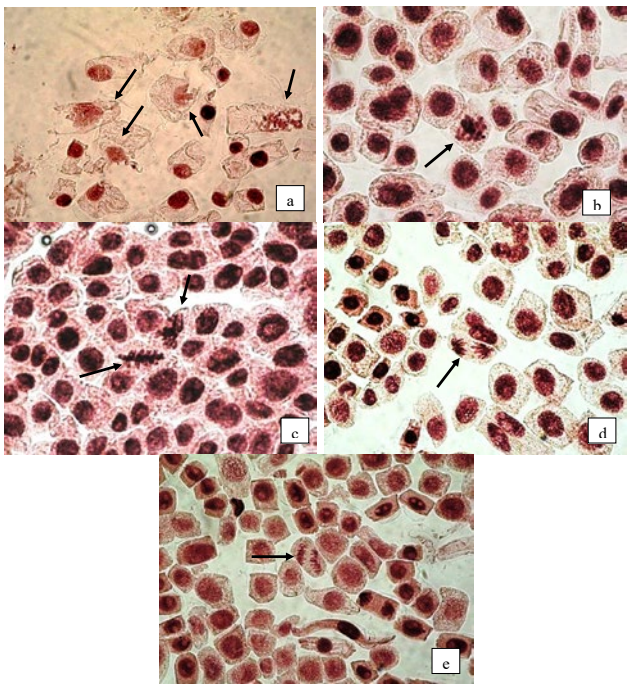

Fig. 1. a: membrane and nucleus deformation in solvent control (citric acid) group; b: stickiness; c: stickiness and polar deviation in positive control group; d: polar deviation in 20 µg/ml fulvic acid treatment group; e: polar deviation 80 µg/ml fulvic acid treatment group.

and 40 µg/ml, respectively (Fig. 1d, e). This percentage is statistically insignificant when compared to the control and solvent control group. In addition, this percentage is very low compared to the chromosome aberration value obtained from the positive control group (EMS), and the difference is statistically significant ($p < 0.05$). In the positive control group (EMS), chromosome aberration percentage has been found high, especially stickiness and anaphase bridge.

Micronucleus formation results are also present in Table 2. Micronucleus formation was found at a very low level of 0.04 ‰ in 10 µg/ml fulvic acid treated group only which was statistically not significant. No micronucleus formation was found in other experimental groups (including negative, positive and solvent controls).

As a result of this study, root length and mitotic index results show that fulvic acid promotes root growth by inducing division in *Allium cepa* root meristem cells. Chromosome aberration and micronucleus results also indicate that fulvic acid does not induce cytotoxic and genotoxic effects in the root meristem cells.

4. DISCUSSION

In this study, cytogenetic effects of fulvic acid were evaluated by analyzing root growth and root morphology. Fulvic acid caused an increase in root growth and number, and there was a statistically significant difference between fulvic acid and control groups (negative, positive and solvent controls). Cyto- and genotoxicity were estimated by observing cytological parameters, such as the mitotic index and number of chromosome abnormalities, including chromosome breaks, stickiness, and polar deviations. The mitotic index (MI) of *A. cepa* meristem cells treated with the EMS and citric acid (solvent control) was significantly decreased (0.83% and 0.01%, respectively) in comparison to negative control. Although EMS and citric acid significantly decreased the

mitotic index, fulvic acid treatment increased the mitotic index in *A. cepa* meristem cells at all concentrations significantly (Table 2). The mitotic index is measure of the mitotic activity of a cell population. It measures the proportion of cells in the M-phase of the cell cycle (Rojas et al. 1993). Therefore, the increase of MI in groups treated with fulvic acid, in comparison to negative control, suggests that fulvic acid could have proliferative effect on the meristem cells of *A. cepa*. The increased mitotic index in *A. cepa* root tip cells treated with fulvic acid is probably due to induction of DNA synthesis and promotion of cell cycle. The MI results of fulvic acid treatment groups are consistent with literature data. Previous studies suggest that humic substances including fulvic acid enhanced stimulation of seedling germination and growth of plants (Kulikova et al. 2002; Pena-Méndez et al. 2005; Van Rensburg 2015). Humic substances affect the development of organisms. Being utilized as a substrate (a source of organic carbon) or nutrient source (N, P, trace elements and vitamins), humic substances can serve as a moiety of the biosynthesis chains. On the other hand, beneficial effects of humic substances on the plants are often attributed to hormone-like activity of these substances (Nardi, 1994; Nardi et al. 2002; Piccolo et al. 1992; Kulikova et al. 2002). Since humic substances originate from the chemical and biological decomposition of plant and animal residues, and from metabolic activities of microorganisms, they might have characteristics of hormones. It was shown that humic substances enhanced plant growth by exhibiting auxin-like activity (Kulikova et al. 2002). Some researchers reported that humic and, in particular, fulvic acids showed some auxin, gibberellin or cytokinin-like activity (Phuong and Tichy 1973; Nardi, 1994; Kulikova et al. 2002). Furthermore, fulvic acid, as a plant growth regulator, is involved in plant response to several environmental stress factors, and is reported to affect growth and development of plants (Heil 2005; Shahid et al. 2012).

There are also studies of growth promoting effects of fulvic acid on plants as well as growth enhancing effects on animals (Nardi et al. 2002; Heil 2005; Bai et al. 2013), because of its antioxidant, antimicrobial and anti-inflammatory properties (Yamada et al. 2007; Van Rensburg et al. 2001; Sherry et al. 2013).

Gao et al. (2017) have shown that when fulvic acid is used as food supplements for 60 days, it increases growth performance of *Paramisgurnus dabryanus* (Sauvage) and improves its intestinal health conditions (Gao et al., 2017). Also, Bai et al. (2013) reported that supplementation of diets with fulvic acid is an effective way to increase growth performance, reduce backfat thickness, and improve meat quality in growing-finishing pig (Bai et al. 2013).

Chromosome aberration and micronucleus results of this study show that fulvic acid does not induce genotoxic effects in the root meristem cells in comparison with control groups. Although in the positive control group (EMS), chromosome aberration rate (especially stickiness and anaphase bridges) has been found high, but in the fulvic acid treatment groups, breaks, stickiness and polar deviations appeared at very low rates. The total chromosome aberration percentages in fulvic acid treatment groups were found insignificant compared to control and solvent control groups (Table 3).

As a result of literature screening, different data on cytotoxic, genotoxic and mutagenic effects of fulvic acid have been reached. Qui et al. (2007) reported that fulvic acid has protective effect against copper toxicity to the polychaete *Hydroidas elegans* larvae, and such an effect is caused by the reduction in labile copper due to Cu-FA (copper-fulvic acid) complexation (Qui et al. 2007). Also, it has been suggested that humic and fulvic acids have desmutagenic effect (inactivation of mutagens outside the cell) on *Vicia faba* root tip cells treated with maleic hydrazide, whereas they have no antimutagenic effect (Ferrara et al. 2000). Ferrara et al. (2004) reported anticlastogenic, antitoxic and sorption effects of humic substances (soil humic acid, peat humic acid and peat fulvic acid) on the maleic hydrazide tested in leguminous plants, *Vicia faba* and *Pisum sativum* L (Ferrara et al. 2004). However, no data has been found on the direct cytogenetic effects of fulvic acid in the literature. Therefore, the data obtained in this study is important in terms of its contribution to the scientific literature in this regard. Furthermore, fulvic acid is currently being used in planting and growing plants, especially in agriculture, and as complementary in treatment of human and animal health. Its use is becoming increasingly widespread. Therefore, it is important to determine whether this substance is safe for the environment, and animal and human health. The results of this study suggest that fulvic acid stimulate the root growth in *A. cepa*, and it does not have cytotoxic and genotoxic effects on *A. cepa* root meristem cells. These results are important, because it is a preliminary study on the safety of using of fulvic acid. However, in order to be able to say that the use of fulvic acid is safe, more detailed studies have to be carried out using different test systems.

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DISCLOSURE STATEMENT

No potential conflict of interest was reported by the author.

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