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EVALUATION OF GERMINATION, VEGETATIVE DEVELOPMENT AND GENOTOXICITY OF LETTUCE FROM IRRADIATED SEEDS

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ABSTRACT

Agriculture has benefited from the use of radiation techniques, which provides plant varieties with distinguish characteristics, such as higher productivity, precocity and greater resistance to disease, pests and harsh weather conditions. Therefore, this study aimed on the analysis of greenhouse morphological development of *Lactuca sativa* originated from irradiated seeds; as well as test their genotoxic effect. The seeds were irradiated at doses of 25, 50, 75, 150 and 300 Gy. In order to determine the germination index, the number of seedlings emerged from each well was counted. Biometric and weight measurements were taken during the development and post-harvest stages. Genotoxicity tests were performed based on the biological assay Allium cepa. The results demonstrated that the best vegetative development was observed for individuals originated from seeds irradiated with doses of 25 and 50 Gy when compared with the control, while this dose did not differ significantly from 75 Gy The calculated germination index remained constant at all dosages. Inhibition of vegetative growth was observed on 150 and 300 Gy dosed individuals. It was also observed that the increasing rate of irradiation is inversely proportional to the mitotic index. A relationship can be established between increased levels of irradiation with increasing percentage of aberrant cells.

1. INTRODUCTION

L. sativa stands out among the most consumed vegetables in Brazil. The lettuce-growing areas are located in the green belts of the great cities of São Paulo State and mountainous areas in the Southeast. There are large losses in vegetable crops in Brazil, regarding the depreciation of the products quality, mainly due to attack of pests and diseases in the production and post-harvest deterioration [1]. The problem of post-harvest losses of vegetables has been occurring throughout Brazil and has been analyzed at different stages of the supply chain and distribution channels [2]. One technique that aims to optimize the production process, as well as ease the post-harvest losses is the ionizing radiation process of the seeds [3].

Radiation techniques have already been proved to be helpful tools in the agronomic field [4]. Among them is the process of food irradiation, which is an efficient technique for conservation because it reduces the losses caused by natural physiological processes, and eliminates microorganisms, parasites and pests, conserving its physical-chemical sense and providing safer goods for the final consumer [5]. There are techniques also involving the improvement of existing crops varieties, throughout the irradiation of plant seeds, in order to aggregate more agronomic value [6]. The individuals generated by irradiated seeds germination are used as matrix plant to obtain new varieties of species of agronomic interest (Rashid et al., 2009). Within this context, the radiation becomes an important tool to induce mutations, a technique which targets the increase of genetic variability in plant breeding [8].

Despite the low doses used for seeds gamma radiation, it is necessary to test the application of cytotoxic and genotoxic to ensure the product quality and biosafety of the final consumer [9,10,11,12]. In this context, the Allium cepa test was identified as a fast and accurate assay for analyzing DNA damage such as chromosomal aberrations, disturbances in the mitotic cycle and formation of micronuclei [13], besides, it presents advantages over other rapid tests which require the preparation of solution in advance or require external metabolites system [14, 15].

This study aims to evaluate the potential germination of irradiated seeds and morphological development of the plants of L. sativa with different bands of radiation through the counting of germinated seeds and biometric measurements at different stages of development. It also aims, through the Allium cepa test, assess levels of cytotoxicity and genotoxicity of ethanol extracts of irradiated lettuce.

2. METHODOLOGY

Pure, dry and dormant seeds of variety Veronica *L. sativa L.* were obtained from the Center for Nuclear Energy in Agriculture (CENA), University of São Paulo, Piracicaba - SP, Laboratory of Radiobiology and Environmental Sciences. The seeds were irradiated with doses of 25, 50, 75, 150 and 300 Gy. For the seeds irradiation process, a cobalt 60 source (^{60}Co)-Gammacell 220 (0,409 KGy.h-1) was utilized. Also seeds without irradiation were used as control. The cultivation part of the project was developed in partnership with the Santo Antonio Ranch, located in the city of Palmital – SP (lat. 22° 47' 45.42"/ long. 50° 12' 38.77/ alt. 508 m).

The seeds were planted in a plastic tray with 200 cells (10x20) previously sterilized with inorganic contact fungicide type (Copper oxychloride - Cuprogarb ® 500). In order to promote aeration and rooting, a pine-bark based substrate containing vermiculite, charcoal, limestone and chemical fertilizers was used. 20 cells were used for each treatment, and in each compartment it was planted 3 seeds, summing 60 seeds per treatment. To break dormancy, the tray was kept in the dark at room temperature for 3 days and then it was taken to a greenhouse. No pesticide or fertilizer was applied during the crop development.

On the fourth day after seeding, the germination index analysis was carried out, when it was counted the numbers of germinated seedlings in each compartment and compared to the original number of seeds. The seedlings were transferred to the greenhouse fields through random distribution and the standard agronomic practices and plant protection measures were adopted.

The plants were periodically observed until the development of three primordial leaves, then a selection was made using as criteria the size of each seedling. Biometric measurements from samples in each dosage and control were performed with the aid of calipers and ruler. All seedlings had their aerial parts measured, the largest leaf of the set was considered for the dimension measurement. Also the post-cropped weigh was measured before and after drying the seedlings on a vacuum induced dryer oven, using an analytical weigh balance (Model AR 2140). The measurements were listed in tables with the computer program Microsoft Excel (Windows Office Pack ® 2007) to calculate the mean, standard deviation, ANOVA and Tukey factors.

For the preparation of the ethanol extract, the fragmented fresh leaves were extracted in a food processor, using ethanol PA at a ratio of 1:10 (w / v) at room temperature until the obtain of a homogeneous extract. Later, the extract was filtered under reduced pressure on a filter paper. The extract was dried at a temperature of 333.15K using a Rota evaporator. After the extract was lyophilized using a bench Lyophilizer followed by the maceration of the solid and measurement of each extract weight. It was prepared, using the powder extract, dilutions in distilled water of 50, 100 and 200 mg of extract / ml of water.

The test was conducted with Allium cepa onion bulbs (*Allium cepa L.*, 2n = 16) obtained commercially in the city of Assis- SP. They were cleaned, leaving an intact ring containing the primary roots. For growth of the roots it was used a solution of cultivation -Hoagland's solution [16]. The bulbs were kept suspended in 100 ml beakers leaving the ring of roots in contact with the growing solution, which was changed every 24 hours for a period of 72 hours, kept at a controlled photoperiod (18h/6h light / dark) and temperature (295 K) greenhouse BOD. Bulbs with roots measuring above 0.2 m were used in the experiment.
To evaluate the mitotic index and the induction of chromosomal aberrations (aberrant anaphase and telophase), six onion bulbs were suspended for each concentration of the aqueous extract (50, 100 and 200 mg/mL) and ethanol extract (50, 100 and 200 mg/mL) of the treated *L.sativa* and untreated as control. For the negative control it was used the untreated plant extract and for the positive control solution of Methylmethanesulfonate (MMS, Sigma-Aldrich ®) 10 mg / L.
At the end of 48 hours exposure and 24 hours recovery in culture solution, the roots of the treated solution bulbs and controls were cut and fixed in a solution of ethanol: acetic acid (3:1, v / v). These are hydrolyzed in 1N HCl at 333.15 K for 360 s, and washed with distilled water. The roots were stained with Acetic Carmine for 10 minutes, with the edges carefully removed and squeezed between the slide and sealing coverslip. Five slides were prepared for each treatment and for the control groups. 5000 cells were counted per treatment; the mitotic index was calculated as the number of dividing cells per 5000 cells observed. The ratio of aberrant cells was calculated based on the number of aberrant cells (anaphases and telophases) analyzed for total cells for each treatment and control [17].

3. RESULTS AND DISCUSSION

For the germination index, 60 seedlings were counted in each treatment, thereby presenting a germination rate of 100%.
During the vegetative growth of lettuce, biometrics measures and weight were obtained from the seedlings. The medium growth (X) and weight (Y) in cm and its respective standard deviation (SD) are shown in Table 1.

Table 1- Average measurements of plant growth (X) in centimeters, average humid and dry plant weight (Y) in grams and its standard deviation (SD) obtained from biometrics measurements from each dose of radiation and positive control (NC).

Doses (Gy)	Growth -cm (X±SD)	Humid Weight - g (Y1 ± SD)	Dry weight - g (Y2 ± SD)
NC	4.33 ± 0.89a	638.91 ± 1.80a	143.84 ± 0.92a
25	4.46 ± 0.93b	825.34± 0,47b	137.53 ±0.74a
50	4.67 ± 0.68b	857.26 ± 0.96b	260.60 ± 0.59b
75	4.32 ± 0.48a	741.20 ± 1.20c	226.7 0± 0.57b
150	3.77 ± 0.84c	707.13 ± 1.50c	153.62 ± 0.81a
300	1.78 ± 0,98d	515.78 ± 0.76d	140.21 ± 0.98a

Medium ± Standard deviation. Same letters in column do not differ statistically, medium evaluated with krustal-wallis ($p<0.05$) statistical test.

According to the results it was found that the average growth of the plants from the negative control group was not significantly different when compared to the treatment of 75 Gy. Since treatment with doses of 25 and 50 Gy showed no significant difference between them, resulting in higher average growth compared to the control group. The treatments with doses of 150 and 300 Gy had the lowest average growth between samples. From the humid weight results it was observed that treatments with 25 and 50 Gy presented the higher average weight with no statistical differences from each other; while doses of 75 and 150 Gy did not differ significantly from each other but still had higher average weight when compared to the negative control group and 300 Gy dosed group which presented the lowest average humid weight among the samples. Average dry weights measurements results has shown that treatments exposed to 50 and 75 Gy did not differ significantly from each

other and presented the higher measurements; while the other treatments revealed lower average dry weight and did not differ from each other.

It is known that water radiolysis is the main effect of ionizing radiation in live organisms, leading to Reactive Oxygen Species (ROS) formation, which can affect structural and functional organic molecules [18]. However, doses of 25 and 50 Gy induced a positive vegetative growth; this may be associated with activation of stress-induced antioxidant enzyme systems that reduced the mitotic cycle, resulting in an increased meristem cell division and stimulation of plant development under this specific stress condition [19]. In the case of doses 150 and 300 Gy, there was inhibition of vegetative growth of lettuce, since the high doses may have generated large amount of biological damage, which caused a disturbance in mitotic cycle, leading to retardation of the meristematic growth rate.

From the counting of 5000 cells for each treated dilution, it was possible to generate mitotic index data and the number of aberrant cell based on phonotypical visual analysis, and its standard deviation (SD). Results are shown in Table 2; Table 3 and Table 4.

Table 2 - Chromosomal aberrations and mitotic index for the ethanol extract of Lactuca sativa irradiated at doses of 25, 50, 75, 150 and 300 Gy treated at concentration 50µg/mL. Negative control (NC) treated with mineral water and a positive control (PC) treated with Methylmethanesulfonate (MMS) to 10 mg / L.

Doses	% Mitotic Index (MI±SD)	Aberration		Total Aberrant Cells	Aberrant Cells Ratio (R± SD)
		Anaphases	Telophases		
50µg/mL					
NC	21.6±0.98a	15	10	25	0.005±0.0001a
25Gy	25.1±1.23a	20	10	30	0.008±0.0002b
50Gy	19.3±0.78a	00	00	00	0.000±0.0000c
75Gy	21.3±1.45a	15	00	15	0.003±0.0001a
150Gy	28.3±1.78a	40	15	11	0.011±0.0003d
300Gy	15.9±0.34b	30	15	45	0.009±0.0002d
PC	10.2±0.59b	30	25	55	0.011±0.0004d

5000 cells evaluated per treatment. Medium ± Standard deviation. Same letters in column do not differ statistically, medium evaluated with krustal-wallis ($p<0.05$) statistical test.

Table 3 - Chromosomal aberrations and mitotic index for the ethanol extract of Lactuca sativa irradiated at doses of 25, 50, 75, 150 and 300 Gy treated at concentration 100 µg/mL. Negative control (NC) treated with mineral water and a positive control (PC) treated with Methylmethanesulfonate (MMS) to 10 mg / L.

Doses	% Mitotic Index (MI±SD)	Aberration		Total Aberrant Cells	Aberrant Cells Ratio (R± SD)
		Anaphases	Telophases		
100 µg/mL					
NC	24.6±1.89a	05	00	05	0.001±0.0001a
25 Gy	22.8±2.23a	35	15	50	0.010±0.0005b
50 Gy	23.5±1.98a	10	0	10	0.002±0.0001a
75 Gy	20.1±1.56a	30	05	35	0.007±0.0003b
150 Gy	18.1±0.78a	20	10	30	0.006±0.0001b
300 Gy	13.1±1.99b	30	25	55	0.011±0.0003b
PC	11.6±1.89b	20	40	60	0.012±0.0007b

5000 cells evaluated per treatment. Medium ± Standard deviation. Same letters in column do not differ statistically, medium evaluated with krustal-wallis ($p<0.05$) statistical test.

Table 4 - Chromosomal aberrations and mitotic index for the ethanol extract of Lactuca sativa irradiated at doses of 25, 50, 75, 150 and 300 Gy treated at concentration 100 µg/mL. Negative control (NC) treated with mineral water and a positive control (PC) treated with Methylmethanesulfonate (MMS) to 10 mg / L.

Doses	% Mitotic Index (MI±SD)	Aberration		Total Aberrant Cells	Aberrant Cells Ratio (R± SD)
		Anaphases	Telophases		
200µg/mL					
NC	28.4±1.23a	40	00	40	0.008±0.0002a
25 Gy	31.2±2.78a	30	05	35	0.007±0.0001a
50 Gy	22.3±2.34a	05	05	10	0.002±0.0001b

75 Gy	16.1±2.11b	10	10	20	0.004±0.0001b
150 Gy	16.6±1.99b	10	35	45	0.017±0.0003c
300 Gy	20.5±2.01a	65	35	100	0.020±0.0004c
PC	10.7±1.89b	25	45	70	0.014±0.0002c

5000 cells evaluated per treatment. Medium ± Standard deviation. Same letters in column do not differ statistically, medium evaluated with krustal-wallis ($p<0.05$) statistical test.

The rates observed on the mitotic index results corroborates the relationship between increasing dosages of radiation and retardation of mitotic cycle [20], however no relationship could be established based on the extract concentration. The expected increase of mitotic index on the 25 and 50 Gy was not observed.
The aberrant cells ratio results confirm that the increasing radiation dosage exposure lead to higher number of aberrant cell formation. As observed for the higher exposed treatment 300 Gy, a direct correlation with the increasing extract concentration can be assumed; while at the higher concentration of 200µg/mL the aberrant cell ratio from 300 Gy is 42% higher than the positive control group ratio. The aberrant cells increased index can be explained by the negative effects via oxidative stress DNA lesions; these direct damages such as strand breaks, deletions and indirect damages – free-radical products from water radiolysis reacting in the nucleotides vicinity- may lead to dividing aberrant cells [21].

4. CONCLUSIONS

From the analysis of the germination ratio, there was no significant difference in the rate of germination between the treatments. It follows therefore that irradiation did not affect the rate of germination of the lettuce crop in this experiment. Through the biometric and weight measures data, it was concluded that the vegetative growth of lettuce plants could be affected by ionizing radiation technique.
Regarding the cytotoxicity test, it is conclusive that high doses of radiation may be associated with increased mitotic disturbances and growing number of chromosomal aberrations. It is also known that the extent that increases the rate of irradiation, there is a decrease in the mitotic index, which can be related to low a vegetative growth when exposed to higher doses.

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