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Genetic Consequences of Fluted Pumpkin (*Telfairia Occidentalis* Hook F.) Seed Exposure to Different X-Rays Doses

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***Abstract* - This study was carried out to investigate the genetic consequences of exposing the seeds of fluted pumpkin (*Telfairia occidentalis* Hook F.) to x-ray doses. The seeds were exposed to varying doses of x-ray irradiation before planting and experiment was set up in a randomized complete block design (RCBD) with five replications. M_1 and M_2 generations were raised, fresh fluted pumpkin leaves of both generations were subjected to molecular assessment. Data collated were further examined using phylogenetic analysis. Several kinds of SNP mutation in the form of indels and nucleotide substitutions were observed. A total of 17 indels were recorded and at different positions. One substitution was recorded. Both transversion and transition occurred in this study and were in the ratio 4:1 except for G: T transversion and A: G transition at positions 495 and 87 respectively which both had a ratio of 1:1. A total of 7 transversions and 4 transitions were recorded. The highest number of SNP's mutation frequency was recorded on the M_1 and M_2 generation seeds exposed to 14.08mGy (4 and 8). This was followed with the M_1 and M_2 generation seeds exposed to 6.75mGy and 18.75mGy. The least number of point mutations (frequency) was recorded in the unexposed seeds in both generations followed by 10.08mGy exposures of both generations. The phylogenetic analysis revealed the formation of two major Claude from the phylogenetic clusters. These alterations in the DNA arrangement of the fluted pumpkin seeds indicate the effectiveness of x-ray as a mutagenic substance.**

Keywords: Mutation, *Telfairia occidentalis,* X-ray, SNP, genetic consequence, molecular analysis.

I. INTRODUCTION

It is obvious that x-rays has the potency to induce changes in cells, tissues and organs of living organism. These changes could be very harmful as it can cause cell mutation, cell killing (necrosis and apoptosis), skin burns, hair loss, birth defects, illness, cancer, and death (WHO 2016). However, in the last 2 decades, there were increasing evidence reporting that low dose ionizing radiations also have beneficial effects on living organisms, including immune enhancement (Farooque et.al 2011), anti-inflammatory (Dhawan et.al 2020; Royo et.al 2020) radiation hormesis (Feinendegen 2005) cell growth stimulation (Yonezawa et.al 1996), lower mortality rate and cancer frequency (Cohen 1993; Kendal et.al 1992) and all these are as a result of DNA rearrangement.

Mutagens are agents of artificial mutation. They are categorized into alchemic (chemical) and palpable (physical) mutagens. Ionizing radiation and chemical mutagens, as well as physical mutagens such as x-rays, thermal neutrons, fast neutrons, gamma rays, ultraviolet, and beta radiation, have all been used to produce favorable mutations at high frequency (Ahloowalia and Maluszynski, 2001; Yakoob and Rashid, 2001). Aside from physical mutagens, chemical mutagens such as Ethylene Imine (EI), Ethyl Nitroso Urea (ENU), N-nitroso-N- Methyl urea (NMU), Ethyl Methane Sulphonate (EMS), Methyl Nitroso Urea (MNU), and sodium azide (SA) are also frequently used to induce mutagenesis in crop plants. Physical mutagens stimulate great lesion, with regards to chromosomal rearrangement or abbreviations while chemical mutagens also stimulates point mutations (Kharkwal and Shu, 2009). Dhanavel and Girija (2009) has shown that gamma ray and Ethyl Methane Sulphonate combination treatment in cowpea is effective and efficient. In addition, when compared to gamma ray and combination treatments, EMS is considered to be more effective and efficient in triggering mutations. According to Mba (2013) the frequency and forms of mutations are directly an outcome of the dosage exposed to the mutagen rather than its type. Therefore, choice of any particular mutagen is based on researcher's circumstance which includes the safety precaution applied in usage, effectiveness of use, mutagen availability, cost involvement and available basic infrastructure with other important key factors.

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In this research, the different seed sets of fluted pumpkin were exposed to different doses of x-ray irradiation to determine the frequency and forms of mutation that can be induced by a particular x-ray dosage.

II. MATERIAL AND METHOD

2.1 Source of research material

The pods of the fluted pumpkin were sourced from the National Agricultural Seed Council of National Root Crop Research Institute Umudike after which they were cut open and the seeds extracted.

2.2 Treatment/exposure of material

The exposure of the different sets of the fluted pumpkin seeds to different doses of x-ray irradiation took place at Mecure Diagnostic Center of Abia State Specialist Hospital, Umuahia. Five samples of twenty seeds each were treated with different doses of X-ray irradiation (0.0 mGy, 6.75 mGy, 10.8 mGy, 14.8 mGy and 18.75 mGy) respectively using a therapeutic medical X-rays device (Clinac 23EX Linear Accelerator, Varian Medical Systems, USA).

2.3 Land preparation and planting dates

The field was properly cleared, ploughed and harrowed to fine tilt, beds were made and planting was done on March 20th 2018 to raise the M1 generation and March 1st 2019 to raise the M2 generation.

2.4 Experimental design

The experiment was designed in a randomized complete block with five replications. The plot size was 2m x 2 m with 0.5 m apart. The planting distance was 120 cm x 60 cm with 2 seeds sown per hole and later thinned to one. Cultural practices including staking/weeding and fertilizer application were carried out fourteen days and twenty eight days after planting respectively. Twenty one days after germination, fresh leaves of plants were harvested and evaluated individually based on different treatments. At maturity, seeds of harvested M1 generation were re-planted to raise the second generation.

2.5 Molecular assessment

This was carried out at the Bioinformatics Service Center, Queen Elizabeth Road Ibadan. Samples of fresh leaves/seedlings of both M1 and M2 generations were harvested and subjected to molecular assessment to investigate the mutagenic effects (DNA Polymorphism) as induced by the different treatments using the Single Nucleotide Polymorphisms (SNP's) marker.

1. DNA Extraction:

This was carried out in accordance with the method of Dellaporta *et al.* (1983). Fresh leaves were harvested into sample bottles and were preserved using silica gel. Approximately 200 mg (0.2 g) of leave samples were ground into fine powder and was transferred to a well labeled microfuge tube. Addition of 700 µl of pre -heated (65°C) extraction buffer was made into the grounded leaves. It was then inverted for 6-7 time to mix the sample with buffer. The samples were incubated at 65°C for 20 minutes with occasional mixing to homogenize the sample. The tube was allowed to cool at temperature for 2 minutes and 500 µl of 5M ice-cold potassium acetate was added and mixed and then incubate on ice. The sample was spun at 12000 rpm for 10 minutes. The supernatant was transferred into new tubes. Thereafter, 700 µl of chloroform: isoamyl alcohol (24:1) were added and spun at 10000 rpm for 10 minutes. The supernatant was transferred to new tubes and 700 µl of ice-cold isopropanol was added and mixed by gently inverting the tubes for 6-10 times and was stored in a freezer (-20oC) for 1 hour. Samples were spun at 12000 rpm for 10 minutes and the supernatant was discarded. The pellet was washed twice in 300 µl of cold 70 % ethanol for 20 minutes and air dried. After drying, 100 µl of 1x TE and 2 µl of 10ng/ml RNase were added to remove RNA. Incubation was done for 40 minutes at 37°C with gently mix at 10 minutes interval.

2. Extraction of buffer components

10 ml of 1M Tris
10 ml of 0.5 EDTA
10 ml of Nacl
2 g of SDS
2 g of PVP

This was made up to 100 ml with water.

3. DNA Quantification

Approximately 1g of agarose was measured and mixed. Agarose powder with 100 mL 1xTAE was mixed in a microwavable flask. It was microwaved for 1-3 minutes until the agarose was completely dissolved. The solution was allowed to cool at about 50 °C. Then, 10 µL EZ vision DNA stain was added. The agarose was poured into a gel tray with the well comb in place. The newly poured gel was placed at 4 °C for 10-15 minutes.

4. Loading Samples and Running Agarose Gel

Loading buffer was added to each of the DNA samples and PCR products. The samples were allowed to solidify. The agarose gel was then placed into the gel box. Thereafter, the gel box was filled with 1xTAE (or TBE) until the gel was

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covered. A molecular weight ladder was carefully loaded into the first lane of the gel. The samples were carefully loaded into the additional wells created. The gel was run at 80-150 V for about 1-1.5 hour. The power source was turn off and the electrodes were disconnected from the power source, and the gel then carefully removed from the gel box. DNA fragments were finally visualized under UV Tran illuminator.

5. SNP-PCR Gene Amplification

i. PCR Mix component

The PCR mix was made up of 12.5 µL of Taq 2X Master Mix from New England Biolabs (M0270); 1 µL each of 10 µM forward and reverse primer; 2 µL of DNA template and then made up with 8.5 µL Nuclease free water.

ii. Primer Sequences

H1f F: CCACAAACAGAGACTAAAGC

Fofana R: GTAAAATCAAGTCCACCGCG

iii. Cycling Conditions

Initial denaturation at 94˚C for 5 minutes, followed by 40 cycles of denaturation at 94˚C for 30 second annealing at 55˚C for 30 second and elongation at 72˚C for 45 seconds. This was followed by a final elongation step at 72˚C for 7 minutes and hold temperature at 10 ˚C forever.

iv. Electrophoresis for PCR

Agarose powder was mixed with 100 mL 1xTAE in a microwavable flask. It was microwave for 1-3 minutes until the agarose was completely dissolved. The agarose solution was allowed to cool down to about 50 °C about 5 minutes. 10 µL EZ vision DNA stain was added. EZ vision binds to the DNA and allows you to visualize the DNA under ultraviolet (UV) light. The agarose was poured into a gel tray with the well comb inside and newly poured gel was placed at 4 °C for 10-15 minutes.

Samples were loaded and run by adding loading buffer to each PCR products. After solidification, the agarose gel was placed into the gel box (electrophoresis unit) and filled with 1xTAE (or TBE) until the gel was covered. A molecular weight ladder was carefully loaded into the first lane of the gel. Then the samples were loaded carefully into the additional wells of the gel. The gel was run at 80-150 V for about 1-1.5 hours. PCR product was visualized under UV Trans-illuminator.

v. Tracing of DNA polymorphism along sequenced regions.

DNA polymorphism along the ribulose bisphosphate carboxylase (RBCL) sequence regions were traced with the help of single nucleotide polymorphisms (SNP'S) markers and there exact positions and type of mutation were noted.

III. RESULTS

After the DNA extraction and amplification, fragments were visualized under a UV Trans illuminator and results were as shown in figure 1 below.

It was observed that the different treatment samples have DNA bands of varying base pairs along the DNA ladder. The sizes of this DNA bands ranged from 600-750BP and this purely indicate different type of mutation at different positions along the sequenced region.

With the aid of the single nucleotide polymorphism (SNP) markers, the different positions of the mutation were able to be traced as shown in fig 2 below.

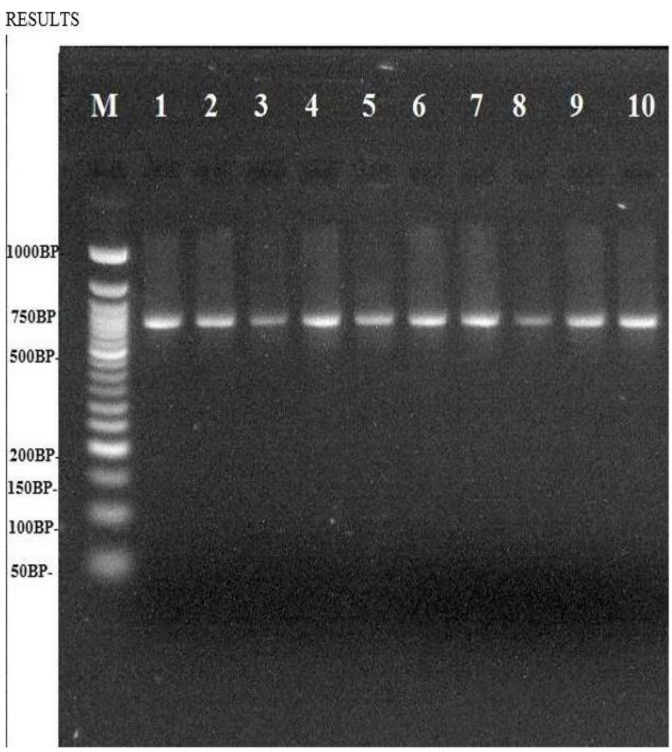

Figure 1: DNA amplification result

Legend (Treatment codes)

1= 0.00mGy (M_1), 2= 6.75mGy (M_1), 3= 10.08mGy (M_1), 4= 14.08mGy (M_1), 5= 18.75mGy (M_1)

10= 0.00mGy (M_2), 6= 6.75mGy (M_2), 7= 10.08mGy (M_2), 8= 14.08mGy (M_2), 9= 18.75mGy (M_2)

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3.1 Sequence alignment of the treated samples

The aligned sequence of the treated and untreated sequences reveals all kinds of SNP mutation in the form of indels and nucleotide substitutions however; the indels were the most occurred. A total of 17 indels were recorded with 5 of cytidine and 4 thymidine, adenosine and guanosine respectively. Cytidine, which had the highest indel occurrence were seen at positions 385, 460, 506, 545 and 551. Thymidine indel which had a total of 4 occurrence were at positions 46, 393, 417 and 472 while 4 guanosine indel were seen at positions 360, 339, 453 and 522 respectively. Adenosine indel were seen at positions 371, 423, 529, and 540.

Both the transversion and transition mutation occurred in this study and all were in the ratio 4:1 except for G: T transversion mutation and A: G transition mutation at positions 495 and 87 respectively which both had a ratio of 1:1. A total of 7 transversion mutation were recorded among which were the A:C at position 411, A:T at positions 88, 124, and 352, G:C at position 349 and G:T at positions 474 and 495. While only 4 transition mutations were recorded which were T:C at location 402 and A:G in positions 87, 435 and 530.

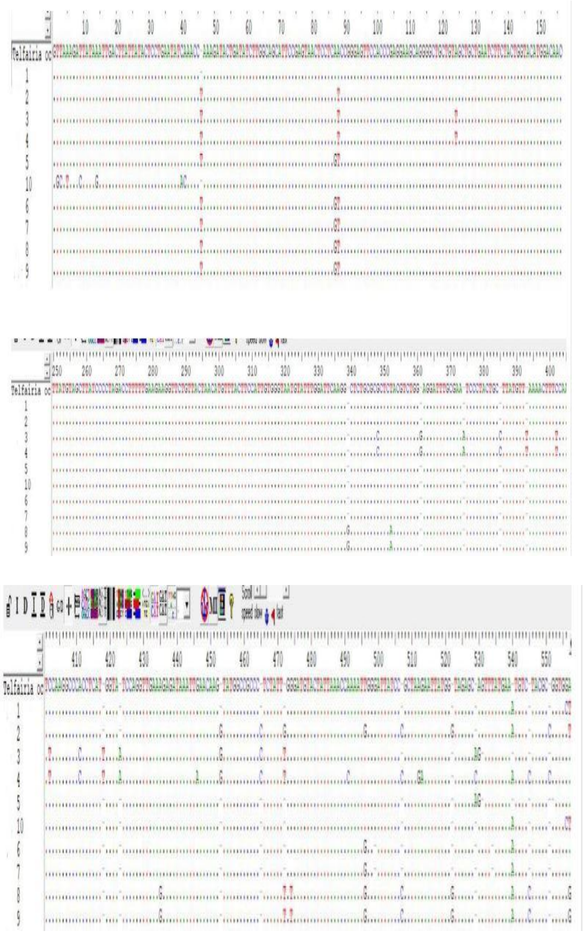

Figure 2: Sequence alignment of treated samples

3.2 The positions of Single Nucleotide Polymorphisms (SNP's) mutations along the Ribulose Bisphosphate Carboxylase (rbcl) sequence regions

Table 1 shows the positions of Single Nucleotide Polymorphism mutations along the RBCL sequence regions. The result revealed that four mutation types were identified. The indels occurred in CTGA at various locations. Nucleotide substitution occurred in A:C at position 411 whereas transition occurred in A:G at position 87, 435 and 530 respectively. Transversion occurred in all the nucleotide sequence at various positions.

Table 1: The positions of Single Nucleotide Polymorphisms (SNP's) mutations along the Ribulose Bisphosphate Carboxylase (rbcl) sequence regions

Mutation type	Description	Location
Indels/ Deletion	C	385, 460, 506, 545 and 551
	T	46, 393, 417 and 472
	G	360, 339, 453 and 522
	A	371, 423, 529 and 540
Substitution	A:C	411
Transversion	A:T	88,124,352
	G:C	349
	G:T	474, 495
	T:C	402
Transition	A:G	87, 435, 530

3.3 The number of Single Nucleotide Polymorphisms (SNP's) mutation according to the different levels of x-ray exposures

The genetic consequences of the different levels of x-ray exposure on fluted pumpkin seeds were also investigated to ascertain the total number of SNP's mutation induced and result is as tabulated in table 2 below.

The table shows that the highest number of SNP's mutation was recorded on the M_1 and M_2 generation seeds exposed to 14.08mGy (4 and 8) which had twenty five (25) and eleven (11) different points of SNP mutation respectively along the Ribulose Bisphosphate Carboxylase (rbcl) sequence regions. This was followed with the M_1 and M_2 generation

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seeds exposed to 6.75mGy and 18.75mGy. The least number of point mutations was recorded in the unexposed seeds in both generations followed by 10.08mGy exposures of both generations.

Table 2: Number of SNP's mutation influenced by the different levels/doses of x-ray exposures

Treatment code	Treatment/ exposure doses	Number of SNP mutation point
1	0.00mGy	0
2	6.75mGy	10
3	10.08mGy	9
4	14.08mGy	25
5	18.75mGy	3
10	0.00mGy	0
6	6.75mGy	4
7	10.08mGy	4
8	14.08mGy	11
9	18.75mGy	11

Legend (Treatment codes)

1= 0.00mGy (M_1), 2= 6.75mGy (M_1), 3= 10.08mGy (M_1), 4= 14.08mGy (M_1), 5= 18.75mGy (M_1)

10= 0.00mGy (M_2), 6= 6.75mGy (M_2), 7= 10.08mGy (M_2), 8= 14.08mGy (M_2), 9= 18.75mGy (M_2)

3.4 Influence of mutation along the amplified sequence

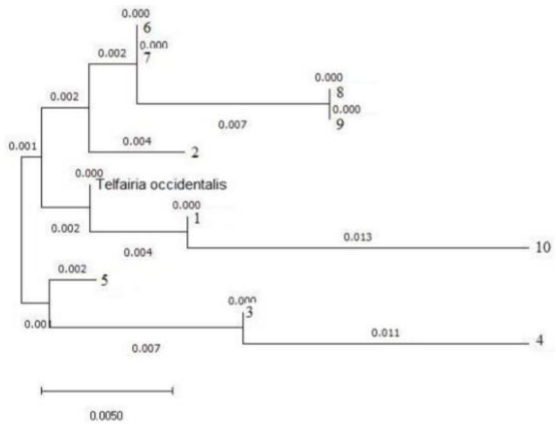
The influence of the mutations along the amplified sequence was investigated in the treated samples and examined with a phylogenetic analysis as shown in Table 3 below. The phylogenetic cluster formed 2 major Claude. The untreated 1 and 10 formed a close cluster with a genetic distance of 0.013 and this formed a close cluster with the reference *Telfaria occidentalis*. This cluster was closely related to another cluster formed by a close relationship between 6 and 7 (which were genetically similar) and also 8 and 9 (which were genetically similar).

The second Claude is a much smaller branch consisting of a close cluster between 3 and 4 with a genetic distance of 0.011 and this cluster formed a close relationship with 5 with a genetic distance of 0.021.

Table 3: Influence of the mutations along the amplified sequence as investigated in the treated samples and examined with a phylogenetic analysis

	T. occidentalis	1	2	3	4	5	10	6	7	8
T. occidentalis										
1	0.004									
2	0.006	0.006								
3	0.011	0.015	0.015							
4	0.017	0.021	0.020	0.011						
5	0.006	0.009	0.007	0.009	0.021					
10	0.017	0.013	0.019	0.028	0.034	0.023				
6	0.006	0.009	0.004	0.013	0.019	0.004	0.023			
7	0.006	0.009	0.004	0.013	0.019	0.004	0.023	0.000		
8	0.013	0.015	0.011	0.021	0.026	0.011	0.028	0.007	0.007	
9	0.013	0.015	0.011	0.021	0.026	0.011	0.028	0.007	0.007	0.000

Figure 3: A dendogram representing the morphological variation among the different treatments



IV. DISCUSSION

The application of mutation breeding in modern day agricultural and biotechnological activities has done honorably in closing out the gap created on reliance on evolutionary trend of different crop varieties. Physical mutagens are known to induce mutation in crops to create a desired variety (IAEA 1998). Different doses of ionization radiation exposures constitutes various effects on living cells. Higher doses of ionization radiation has been recorded to cause negative effects on physiological and biochemical traits in plants (Bernstein, H. et.al 1985; Esnault, M. A. et.al 2010; Evseeva, T. 2009). However, radio sensitivity has reveal different levels of susceptibility depending on the plant variety due to DNA-content-, repair-process-, antioxidant-reaction-, and cell cycle kinetics-related differences (Evseeva, T. I. et.al 2011; Fedak, K. M. et.al 2015; Fedotov I. S., et.al 2006; Foyer C. H and Noctor, G. 2016). In order to increase the mutation breeding efficiency, the selection of an appropriate radiation dose that could increase the mutation frequency without significantly affecting the plant's survival and reproduction is important.

In this research, the effects of different doses of x-ray irradiation on the DNA sequence of fluted pumpkin seed was examined and the result revealed that the different treatment

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samples has DNA bands of varying base pairs along the DNA ladder. The sizes of this DNA bands ranged from 600-750BP and this purely indicates different type of mutation at different positions along the sequenced region which was caused by the exposure to x-ray doses. This agrees with the report of Sharifi-sirchi et.al (2012) where the chromosome number of plants exposed to high dose (700-1200Gy) of x-ray was altered leading to a breakage. Also, Ballarini and Ottolenghi (2003), Ballarini et.al (2002) has similar reports. All these and more underscores the fact that ionization radiation alters the genetic materials in reproductive cells in the form of lesions in DNA including single and double strand breaks, DNA protein cross links, oxidized bases and abasic sites(Cadet et.al 1999) giving rise to mutant materials that can be transfer from one generation to another.

V. CONCLUSION AND RECOMMENDATION

Following the investigation carried out and results generated, it is pertinent to note that the impact of x-ray exposures on plants varies and also depends on plant type, variety and developmental stage. Ionizing radiations such as x-rays has proved to be effective in inducing mutations in the DNA arrangement of plants as revealed in this present study. Several point mutations in form of indels, substitutions, transversions and transitions which were recorded in the study are evidence of genetic mutations as induced by x-ray exposure. Highest number of SNP's mutation frequency which was recorded on seeds exposed to 14.08mGy indicates an effective x-ray dose to induce mutation in fluted pumpkin seeds.

It is therefore recommended that for an effective mutation to be induced in fluted pumpkin (*Telfairia occidentalis*) 14.08mGy dose of X-ray can be used.

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