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Influence of γ- radiation stress on scavenging enzyme activity and cell ultra structure in groundnut (*Arachis hypogaea* L.)

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ABSTRACT

The biological effect of gamma rays on plant cell is mainly due to interaction with water molecules producing free radicals, which can potentially damage important components of exposed cell. Consequently the balance between the production of Reactive oxygen species (ROS) and quenching activity of scavenging enzymes is upset resulting in oxidative damage. Among the major active oxygen species viz., superoxide radical (O^{-2}), hydrogen peroxide (H_2O_2), hydroxyl radical (OH) and singlet oxygen ($^1O_2$), the H_2O_2 and OH radicals are most reactive, toxic and destructive. The cell membrane and cellular organelles are the main targets for free radical attack. The aim of this study was to establish changes in the activity of the scavenging enzymes, plant pigments like chlorophyll which confer sensitivity to irradiation stress, and also to assess the damage caused by ionizing radiation on cell membrane and cellular organelles on exposure to gamma radiation. When the groundnut (Arachis hypogaea L.) cv. Narayani seedlings were subjected to gamma rays (0.00, 10, 20, 40, 50 and 100Gy) from a cobalt source (^{60}Co) at a dose rate of 3.06 kGy/hr, a dose dependent increase in the activity of peroxidase (POD) and superoxide dismutase (SOD) was observed in response to free radical generation. Further, gradual decline in leaf chlorophyll content was observed with increased dose and 100Gy exposure resulted in lowest leaf chlorophyll content (0.895mg/g FW) due to maximum pigment deterioration. The gamma ray induced ultra structural changes included distortion of nuclear membrane, chloroplast swelling, thylakoid dilation, rupture of chloroplast outer membrane and swollen endoplasmic reticulum. Damage to ultra structure, accumulated with exposure time and led to both vesiculation in the chloroplast stroma and endoplasmic reticulum of the cells after the exposure to gamma rays at a dose of 100Gy.

Key words: Groundnut; γ- Radiation; SOD; POD; Chlorophyll; TEM.

ABBREVIATIONS

µM	Micro Mole
60Co	Cobalt 60
BRNS	Board of Research in Nuclear Sciences
C *a* + *b*	Total Chlorophyll
C*a*	Chlorophyll a

$CaCO_3$	calcium carbonate
C*b*	Chlorophyll b
EDTA	Ethylene Diamine TetraAcetic acid
g Fw	Gram Fresh Weight
Gy	Gray
H_2O_2	Hydrogen peroxide
HO•	Hydroxyl free radical
LET	Linear Energy Transfer
mg	Milli Gram
Min	Minute
mM	Milli Mole
nm	Nanometer
O^{-2}	Superoxide radical
POD	Peroxidase
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
TBq	Tera Becquerel
TEM	Transmission Electron Microscopy
U	Unit
UV	Ultraviolet
VIS	Visible
w/v	Weight/ Volume
v/v	Volume/Volume
γ	Gamma

INTRODUCTION

Gamma rays belong to the ionizing electromagnetic group of radiations and are highly penetrating because of low Linear Energy Transfer (LET). Gamma irradiation causes oxidative stress and affects biomolecules by causing conformational changes, oxidation, rupture of covalent bonds and formation of free radicals (Cheftel *et al.*, 1985) [4]. The hydroxyl (HO•) and superoxide anion (O2•-) radicals generated by radiation stress could modify the molecular properties of the cell (Halliwell and Gutteridge 1986)[10].

Exposures of eukaryotic cells to ionizing radiation results in the immediate formation of free radicals lasting only for a matter of milliseconds and causes oxidative stress through radiolysis of body water which is often referred to as the indirect effect of radiation. This coupled with the 'oxygen effect' enhances tissue injury. Both these effects are more pronounced for low LET radiations, accounting for more than 70% of molecular damage. Consequently, low or high doses of ionizing radiation were used to stimulate or inhibit seed germination, plant growth and development in various plant and animal organisms (Korystov and Narimanovo 1997 [12]; Sagan 1987) [20].

For Several years, extensive research has been carried out on the effect of irradiation on plant cells. When ionizing radiation is absorbed in biological material, there is a possibility of acting directly on critical targets in the cell. Alternatively, the radiation may interact with other atoms or molecules in the cell, particularly with water, to produce free radicals which can diffuse far enough to reach and damage different important cell components. This indirect effect of irradiation is important in vegetative cells, the cytoplasm of which contains about 80% of water. Gamma radiation is very important in influencing biological systems, such as plant materials. Free radicals attack healthy cells of the plant causing them to lose their structure and function. Reactive oxygen species (ROS) are formed because of interactions between one electron carrier with two electron carriers in different ways as Super oxide is formed from one electron reduction of oxygen, Hydrogen peroxide is formed from a two electron reduction, Hydroxyl radical is formed via a three electron reduction of oxygen and is particularly reactive and destroys membranes by initiating oxidation of fatty acids in membrane lipids. There was compelling evidence which showed that the activities of enzymes involved in ROS scavenging were altered by several environmental stresses, including gamma irradiation. At present our knowledge concerning the role of the antioxidant systems in protecting plants under gamma irradiation stresses is limited and studies in this direction were attempted on very few plant species and rarely were reported so far for groundnut.

By determining the morphological changes in cellular organelles at the ultra structural level, we can gain insight into the damaging mode of ionizing radiation. Therefore, present study was planned to elucidate the irradiation induced changes in the activity of superoxide dismutase and peroxidase, the enzymes involved in oxidative stress defense and the detrimental influence on the leaf chlorophyll content was also assessed. An attempt had also been made to ascertain the effect of selected doses of gamma irradiation on leaf cells of groundnut at ultra structural level through Transmission Electron Microscopy.

MATERIALS AND METHODS

The seeds of Groundnut (*Arachis hypogaea* L.) cv. Narayani were collected from Regional Agricultural Research station, Tirupati, Andhra Pradesh, India. 50 seeds per each radiation dose were surface sterilized with double distilled water for 10 min. After successive washings, they were planted in earthen pots. The seedlings were raised for 15 days at room temperature and were subjected to gamma irradiation treatments at 10, 20, 40, 50, and 100Gy, as generated by a Gamma Chamber- 5000 (60Co, 444TBq capacity, BRNS) at Quality Control Laboratory, Acharya N. G. Ranga Agricultural University, Hyderabad, Andhra Pradesh, India. Following these treatments, growth of the seedlings was observed up to 7 days and leaves were collected for scavenging enzyme activity analysis and Transmission Electron Microscopic studies.

Enzyme extraction and assay

Enzyme extraction for Superoxide Dismutase and Peroxidase was carried out by following the method of Costa (Costa *et al.*, 2002) [5]. One gram of plant tissue collected and frozen in liquid nitrogen from both irradiated and control samples were homogenized with extraction buffer containing 50 mM phosphate buffer (pH 7.5) and 1 mM EDTA.

Superoxide Dismutase (SOD)

The assay is based on the formation of blue coloured formazone by interaction between nitro-blue tetrazolium and O2- radical, which is absorbed at 560 nm and the enzyme (SOD), decreases the absorbance due to reduction of O^{-2} radical by the enzyme. Fifty percent reduction in absorbance was considered as one unit of enzyme activity (Dhindsa *et al.*, 1981) [6]. The activity of SOD was expressed in unit per minute per gram of fresh weight.

Peroxidase (POD)

The assay is based on the absorbance due to the formation of tetra –guaiacol recorded at 470 nm (Castillo *et al.*, 1984) [2] and the enzyme activity was calculated as per extinction coefficient of its oxidation product, tetra-guaiacol. Enzyme activity was expressed as µM tetra- guaiacol formed per minute per gram of fresh weight.

Chlorophyll Content

Chlorophyll content of irradiated and non-irradiated plantlets was determined as per (Lichtenthaler 1987)[15]. Leaf tissue was added to a pre-chilled mortar in an ice bath and were ground with pestle in calcium carbonate (CaCO3) (Spectrum, CA) at a ratio of 1g of plantlets to 2g of CaCO3 together with 10ml of 80% (v/v) acetone. The sample extracts were filtered using Whatman no. 1 filter paper followed by washing with 80% (v/v) acetone. The extraction volume was made up to 50ml with 80% (v/v) acetone. Sample extracts were subjected to UV-VIS spectrophotometric determination (CECIL Aquarius CE 7200, Double Beam Spectrophotometer) of chlorophyll at 646nm and 663nm. The chlorophyll *a* (C*a*) and chlorophyll *b* (C*b*) content in milligram per litre was determined according to the formulae given below and further expressed in milligram per gram of fresh weight of plant material.

Chlorophyll *a*,

C*a* = 12.25(OD 663) – 2.79(OD 646)

Chlorophyll *b*,

C*b* = 21.50(OD 646) – 5.10(OD 663)

Total chlorophyll,

C *a* + *b* = 7.15(OD 663) + 18.71(OD 646)

Transmission Electron Microscopy (TEM)

Groundnut leaves of non-irradiated and irradiated seedlings (100Gy) were cut into 1- mm2 segments and placed immediately in freshly prepared mixture of 3% (w/v) glutaraldehyde in 0.05M phosphate buffer (pH-7.2) for 24 hrs at 4^{o}C and post fixed in 2% aqueous osmium tetroxide in the same buffer for 2 hrs. After post fixation samples were dehydrated in a graded series of Acetone / Alcohol, infiltrated and embedded in Araldite 6005 resin (Glauret *et al.*, 1958[9] & Mollenhauer *et al.*, 1959[17]) Semi-thin sections (300 – 500 nm) and ultra-thin sections (50 – 70 nm) were made with a glass knife on a leica ultra cut (UCT-GA-D/E-1/00) microtome. Semi thin sections were mounted on glass slide stained with toludine blue for light microscopy (Olympus AX-70) and ultra thin sections were mounted on grids and stained with saturated aqueous Uranyl Acetate and counter stained with 4% Leaf Citrate, for Transmission Electron Microscopy (Hitachi, H-7500) (John J. Bozzola and Lonnie D. Russell 1998)[11].

RESULTS AND DISCUSSION

Gamma irradiation was reported to induce oxidative stress with overproduction of reactive oxygen species (ROS) such as superoxide radicals, hydroxyl radicals and hydrogen peroxides, which react rapidly with almost all structural and functional organic molecules (Salter and Hewitt 1992)[21]. To avoid oxidative damage, plants have evolved various protective mechanisms to counteract the effects of ROS in cellular compartments (Foyer *et al.*, 1994)[7]. One of the protective mechanisms was the enzymatic system, which operates with the sequential and simultaneous actions of a number of enzymes including Peroxidase (Kovacs and Keresztes 2002)[13]. Exposure of plant cells to gamma radiation leads to the formation of ROS and therefore, SOD removes superoxide formed during radiation exposure and also inhibits formation of more reactive pro oxidants. Radiation is a well known factor that affects antioxidant status and increases free oxygen radical generation. In the present study, this was evident due to significant increase in the activity of SOD exposed to ionizing radiation. These findings indicated increased antioxidant activity. The observed increase of SOD of the antioxidant system indicated that increase in oxidative stress caused by radiation may be overwhelmed by this enzymatic system.

Table 1: The effect of selected doses of gamma radiation on SOD, POD and Chlorophyll content in leaves of Groundnut

Dose Gy)	SOD absorbance	POD absorbance	Chlorophyll - a (Ca) (mg/g FW)	Chlorophyll - b (Cb) (mg/g FW)	Total Chlorophyll (C a + b) (mg/g FW)
Control	0.078	0.600	1.388	0.374	1.762
10	0.087	0.437	1.272	0.358	1.630
20	0.049	0.636	1.220	0.269	1.489
40	0.045	0.761	1.169	0.232	1.401
50	0.033	0.798	1.098	0.232	1.330
100	0.028	0.950	0.775	0.120	0.895
Grand Mean	0.053	0.697	1.154	0.264	1.418
SED	0.003	0.087	0.031	0.022	0.052
CD>0.05	0.007	0.189	0.067	0.047	0.113
CD>0.01	0.009	0.265	0.094	0.066	0.159
CV%	6.970	15.260	3.270	10.050	4.480

The results presented in Figure 1 and 2, revealed a variable degree of stimulation in the activities of SOD and POD in leaves of 21 day old seedlings of groundnut (*Arachis hypogaea* L.) irradiated with selected doses of gamma rays. Enzyme induction was significantly correlated with the dose of irradiation. Results for groundnut (*Arachis hypogaea* L.) cv. Narayani seedlings irradiated with gamma radiation, indicated fluctuation in the activity of superoxide dismutase (SOD) correspondingly with the irradiation dose. Figure.1 explains the activity of superoxide dismutase in the irradiated groundnut (*Arachis hypogaea* L.) seedlings. For superoxide dismutase estimation the absorbance values of blue coloured formazone varied between 0.087 to 0.028 in comparison to 0.078 in non-irradiated control. SOD activity increased in the irradiated samples as the absorbance is inversely proportional to SOD activity, fact which concluded that irradiation had a stimulatory effect on the enzyme except at the lower dose 10Gy, 0.087. It is of great importance to analyze the changes in peroxidase activity after gamma irradiation because peroxidase is essential for a variety of cellular functions such as lignification, cell wall biosynthesis and plasticity, which all may be altered upon exposure to gamma irradiation.

Figure 1: Effect of Gamma Radiation on Superoxide dismutase activity in Groundnut

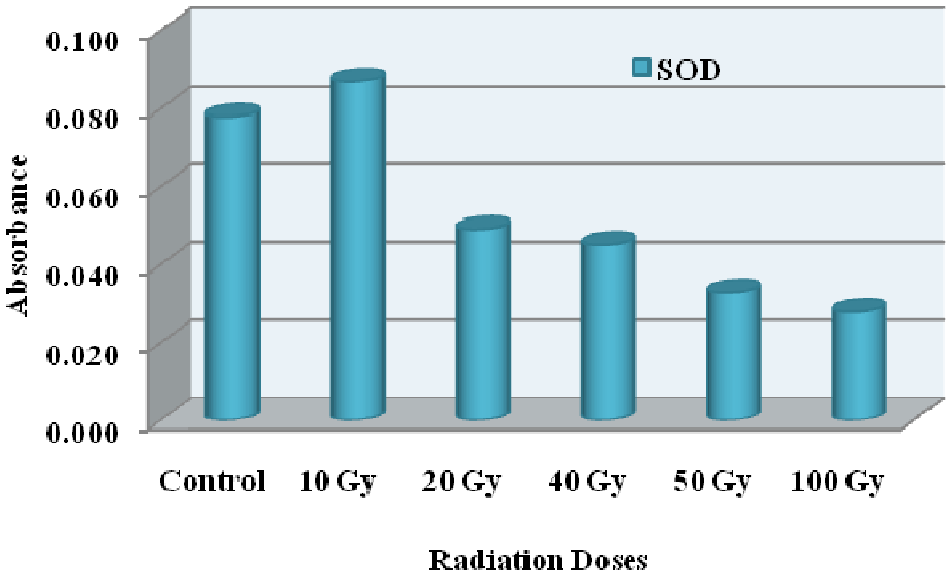
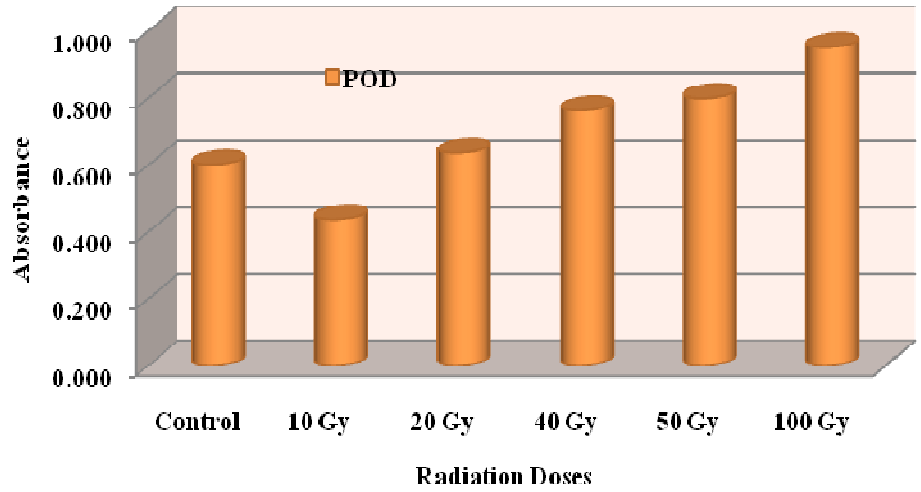


Figure 2: Effect of Gamma Radiation on Peroxidase activy in Groundnut



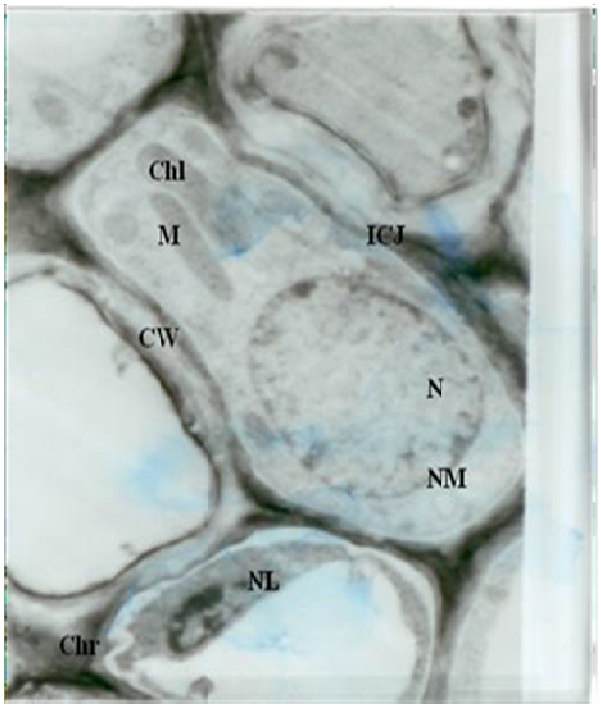


Figure 3

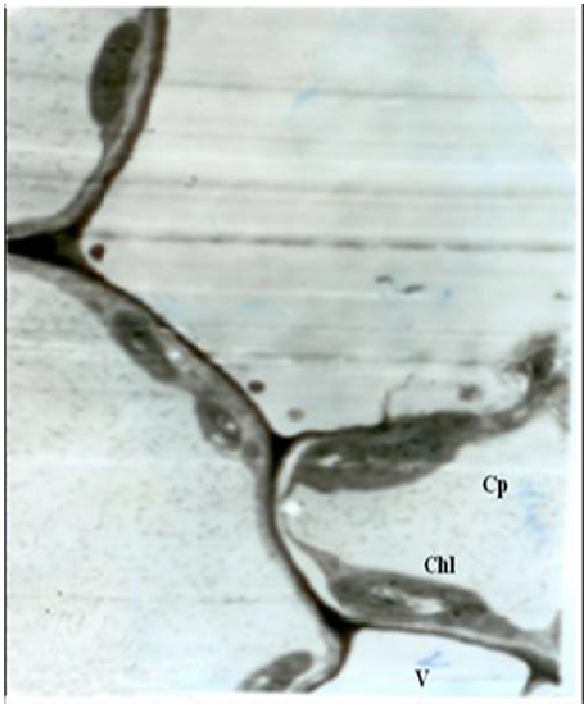


Figure 4

Figure. 3 & 4. Transmission Electron Microscopy of a Non-irradiated Groundnut leaf cell (Control)
Chloroplast (Chl), Mitochondria (M), Cell wall (CW), Chromatin (Chr), Nucleus (N), Nuclear membrane (NM), Nucleolus (NL), Inter Cellular Junction (ICJ), Cytoplasm (Cp) and Vacuole (V)

Considerable increase in peroxidase activity of 0.795 and 0.950 was recorded at 50 and 100Gy respectively (Figure.2). Plantlets irradiated at 20 and 40Gy, exhibited peroxidase activity of 0.667 and 0.764 respectively which were significantly different from non-irradiated sample (0.600). This indicated that plantlets irradiated at these doses recorded increase in peroxidase activity in excess of 111.1% and 127.3% respectively over the non-irradiated plantlets. However, irradiation at 10 Gy reduced the Peroxidase activity to the lowest level of 0.430 resulting in a decrement by 71.6%, as compared to the non-irradiated plantlets. Conversely, plantlets subjected to irradiation at higher dosages 50 and 100Gy displayed a remarkable enhancement of peroxidase activity than non-irradiated plantlets. However, doses 40 and 50Gy displayed peroxidase activities that were not significantly different from each other. The results of this study revealed significant increase in peroxidase activity of irradiated plants. Peroxidases located in the cytosol, vacuole, and cell walls as well as in extra-cellular spaces use guaiacol as electron donors and utilize hydrogen peroxide in the oxidation of various inorganic and organic substrates (Shah *et al.*, 2001)[22]. The expression patterns of peroxidase genes exhibited increased transcripts upon gamma irradiation of Groundnut (*Arachis hypogaea* L.) which accounted for the gradual increase in specific activity of peroxidase as the gamma dosage increased. Several reports with other crop species provided evidence of enhanced activity of Peroxidase upon gamma irradiation treatment. Increase in the activity of peroxidase with a corresponding decline in growth of *Triticum aestivum* plants under higher irradiation dosages (20, 40, 60, 80Gy) was also reported (Chaomei and Yanlin 1993)[3]. It has also been indicated that gamma irradiation enhanced peroxidase activity of two *Phaseolus vulgaris* cultivars (Plovdiv 10 and Plovdiv 11) (Stoeva 2002)[23]. In this regard it was earlier made clear that over expression probably occurs by an efficient regulatory mechanism, adjusting enzyme expression by positive regulation of the corresponding genes to provide cells with resistance to gamma rays (Zaka *et al.*, 2002)[27]. Photosynthesis is one of the most studied processes under the effects of gamma irradiation accompanied mainly by growth experiments. Despite the diversity of gamma ray targets in plants, it seems that the photosynthetic apparatus is among the main action sites of gamma rays (Kulandaivulu and Noorudeen 1983)[14]. Photosynthetic pigments can be destroyed by gamma irradiation, with concomitant loss of photosynthetic capacity (Strid *et al.*, 1990)[25].

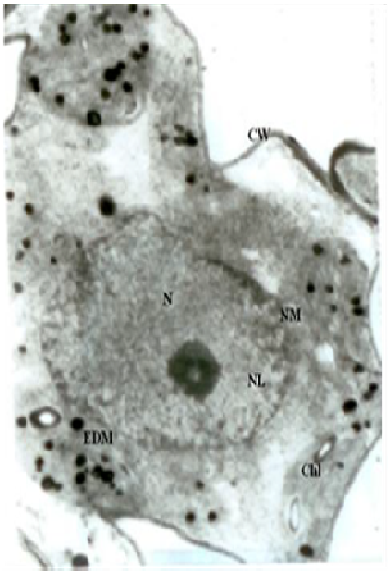


Figure 5

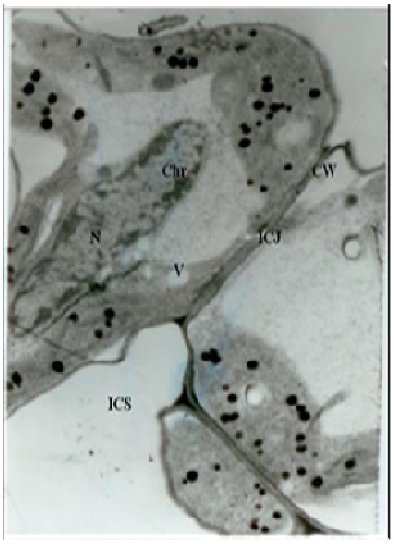


Figure 6

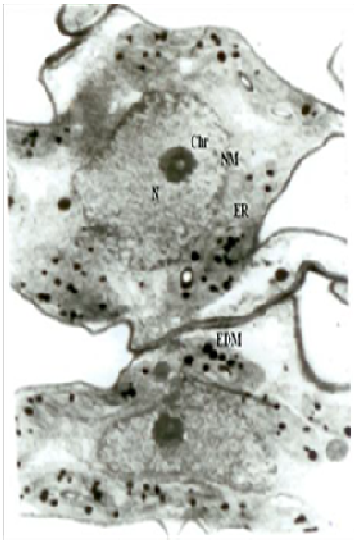


Figure 7

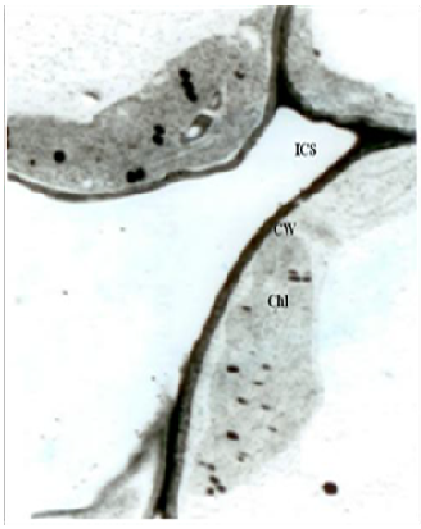


Figure 8

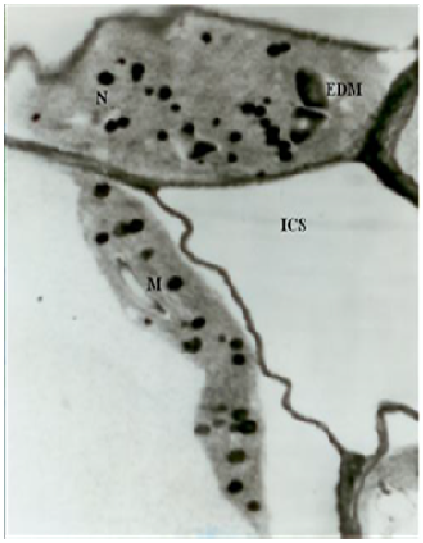


Figure 9

Figure. 5-9. Transmission Electron Microscopy of Groundnut leaf cell irradiated with 100Gy. Chloroplast (Chl), Mitochondria (M), Cell wall (CW), Chromatin (Chr), Nucleus (N), Nuclear membrane (NM), Nucleolus (NL), Inter Cellular Junction (ICJ), Cytoplasm (Cp) and Vacuole (V), Inter Cellular Space (ICS), Electron Dense Material (EDM)

The results of Chlorophyll concentrations of various treatments revealed that increased gamma radiation dosage caused reduction of chlorophyll *a* and *b* concentrations. All the irradiated plantlets exhibited lower amount of chlorophyll *a* and *b* as compared to the non irradiated plantlets. Plantlets irradiated at 10, 20 and 40Gy exhibited total chlorophyll concentration of 1.630mg/g FW, 1.489mg/g FW and 1.401mg/g FW respectively. There was a decrease of 7.5% of total chlorophyll content in plantlets at 10Gy as compared to the non irradiated plantlets (Table.1). Plantlets irradiated at 50 and 100Gy had 1.330mg/g FW and 0.895mg/g FW respectively and displayed a decrement of 24.5% and 49.2% in total chlorophyll as compared to the control. The total chlorophyll at 50 and

100Gy were significantly different from each other. A remarkable decline (49.2%) in total chlorophyll content was observed in plantlets irradiated at the highest dose of 100Gy. As illustrated in Table.1, the concentration of chlorophyll *a* was higher than chlorophyll *b* in both irradiated and non-irradiated plantlets. Concentration of chlorophyll *a* was 81.37% while, chlorophyll *b* was 18.63% on overall mean basis.

In the present study, the chlorophyll content of gamma irradiated groundnut (*Arachis hypogaea* L.) displayed a gradual decrease as the gamma dosage increased. In addition to that, it can be observed that the concentration of chlorophyll *a* was relatively higher than chlorophyll *b* in all the doses. Further, In previous studies, it was reported that gamma irradiation resulted in greater reduction in the amount of chlorophyll *b* as opposed to chlorophyll *a* (Strid *et al.,* 1990)[25]. This statement confirmed the results of this study. The reduction in chlorophyll *b* was 67.92% in comparison to control sample while, chlorophyll a registered only 44.17% reduction at the highest dose of 100Gy. This could be attributed to selective destruction of chlorophyll *b* biosynthesis or degradation of chlorophyll *b* precursors (Marwood and Greenberg 1996)[16]. The perusal of results clearly indicated the adverse effect of gamma irradiation at dose (100Gy) on the parameters assessed in this study. The data also revealed a clear cut association between vital physiological phenomena in groundnut viz., concomitant increase in levels of scavenging enzyme production in response to vigorous free radical generation as a result of radiation stress and the degradation of chlorophyll pigment. Both these phenomena can be visualized by deposition of free radicals and the physical damage caused by such deposition on the ultra structure of the cell.

Further, the lowest absorbance value of 0.028 due to radiation stress in this study was registered at 100Gy which indirectly indicated the highest activity of SOD. Similarly, at the same dose the POD was generated at maximum level (0.950) and arrested the irreversible damage caused by stress related free radicals to certain extent. Interestingly, the same terminal dose of 100Gy was critical in triggering maximum pigment deterioration in respect of chlorophyll. The impairment of photosynthetic apparatus in an environment dominated by the presence of deleterious free radicals would render the groundnut (*Arachis hypogaea* L.) plant to such a physiological state from where recovery to normalcy becomes very difficult and the field performance of such stressed out plants would be biologically, agronomically and genetically inferior. To further investigate the hazards of radiation stress on the morphology of cell and cell organelles at ultra structural level was attempted through Transmission Electron Microscopy (TEM) at the terminal dose of 100Gy.

As illustrated in Figure. 3 and 4, leaf material from control plant was characterized by well defined cell structure. All of the cellular components like cell wall (CW), cytoplasm (Cp), nucleus (N), nucleolus (NL), nuclear membrane (NM), chloroplast (Chl), endoplasmic reticulum (ER) and mitochondria(M) seen here were normal in appearance. The chloroplasts showed a typical structure, having an ellipsoidal shape, very well developed mitochondria, nucleus with nuclear membrane.

In contrast, the chloroplast structure in cells of the leaves at 100Gy irradiation was obviously altered. It is well established that a major site of damage by gamma rays is the chloroplast, leading to impairment of photosynthetic function (Bornman 1989)[1]. The damaged cell contained a chloroplast, which had begun to lose its integrity. The orderly pattern of grana and stroma thylakoids has been lost, and some of the thylakoids appeared slightly dilated.

Gamma radiation may cause more serious ultra structural alterations as were found in other plant species. In this study, the gamma ray induced ultra structural changes included distortion of nuclear membrane (NM), chloroplast swelling, thylakoid dilation, rupture of chloroplast outer membrane. Damage to ultra structure accumulated with exposure time and led to both vesiculations in the chloroplast stroma of the cells after the exposure to gamma rays (Figure 5). The power house of cell that is mitochondria (M) was gradually degenerated due to heavy stress and was comparatively more sensitive than other cell organelles. Mitochondria are crucial sites for energy generation and ATP synthesis by tetravalent reduction of oxygen by mitochondrial cytochrome oxidase. Radiation stress, besides affecting ATP synthesis, can compromise energy transduction by faultily synthesized proteins or lead to DNA fragmentation etc. (Yoshikawa *et al.,* 2000)[26]. The oxidative damage to mitochondria can also lead to membrane permeability transition, cytochrome c release and dysfunction of mitochondria associated with decrease in membrane potential (Yoshikawa *et al.*, 2000)[26].

The nucleus (N) was degenerated with loss of integrity; intercellular junction (ICJ) was thinner at higher dose of gamma irradiation (Figure 6). In the control the cell size, structure, surface areas were well organized. However, in seedlings exposed to 100Gy, the cell surface area was totally altered and cytoplasm was found with electron dense

material (EDM) deposition. The cell organelle like endoplasmic reticulum (ER) was lost with free radical deposition. Condensed chromatin (Chr) in the nucleolus, vacuole formation, scattered cytoplasm with heavy free radical deposition, degenerated nucleolus with thin nuclear membrane (NM), completely deformed cell were also encountered due to radiation damage (Figure 7).

Further it was evident that the nucleus (N) was condensed with migration of chromatin material, loss of integrity of nuclear membrane (NM), chloroplast (Chl) was detached with the cell membrane with vacuolation (V) and electron dense particles (Figure 7 & 8). These results are similar to those reported for plants exposed to environmental stresses, such as UV radiation, toxic metals, acidic rain, and high light intensities (Gabara *et al.*, 2003[8]; Molas 2002[18]; Quaggiotti *et al.*, 2004 [19] and Stoyanova and Tchakalova 1997)[24]. The TEM studies suggest that an increase in free radicals by high-dose gamma irradiation may have been involved in changing membrane structure and integrity. Heavy vacuolation was noticed in the mitochondria (M) of the leaves exposed to high- dose irradiation and their sizes were significantly increased with widely spread inter cellular spaces (ICS), (Figure 9).

CONCLUSION

It can be concluded that imposition of gamma radiation induced a concentration dependent oxidative stress as evidenced by biochemical changes, oxidative damage and scavenging enzyme activity. Ultra structural distortions suggested that the mechanism of gamma radiation might be characterized by oxidative stress.

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