Cellular Molecular Changes in *Nerium oleander* (L.) Cell Culture Under Gamma Radiation Stress

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**ABSTRACT**

This study was done to analyze the relationship between the various effects of five different doses of gamma ray treatments (control, 0, 100, 200, 300 and 400 rad) on cell suspension culture of *Nerium oleander* belonging to the family Apocynaceae, Plant samples were collected from Egyptian flora. The five treatments of the plants were characterized by analyzing variability in frozen biomass cell suspension culture of *N. oleander* through SDS PAGE and peroxidase isozymes. The electrophorogram showed a total of 36 bands of proteins with molecular weight ranging from 10 to 225 KDa. The protein diversity analysis was done based on the presence or the absence of bands thus interpreting their relevance. The histogram analysis clearly showed a high degree of diversity along these five treatments of the plant.

The results of electrophoretic patterns of peroxidase isozymes that was extracted from frozen biomass cell suspension cultures after receiving the different gamma doses revealed remarkable molecular changes in all treatments. These changes in peroxidase isozymes and protein bands indicate the effect of the different irradiation treatments on the gene expiration.

*Keyword: Nerium Oleander, Gamma Radiation, SDS Page Protein, Peroxidase Isozymes.*

**INTRODUCTION**

*N. oleander* L., which belongs to the Apocynaceae family, is an evergreen shrub, that is localized in northern Africa and the Mediterranean regions. It is widely planted as an ornament in warm temperate and subtropical regions due to its abundance and long-lasting flowering period and its tolerance against heat, salinity, and drought. Many cultivars have been selected due to their colors or doubled flowers (white and pink), and also the complete green and variegated cultivars produced. (1)

*N. oleander* L. has been considered historically as a poisonous plant because some of its compounds may exhibit toxic, especially to animals, when consumed in large amounts. Among these compounds are oleandrin and oleandrigenin, known as cardiac glycosides, which are known to have a narrow therapeutic index and can be toxic when ingested. Toxicity studies that have been conducted on dogs and rodents administered oleander extracts by intramuscular (IM) injection indicated that on an equivalent weight basis, doses of an oleander extract with glycosides ten times in excess of those likely to be administered therapeutically to humans are still safe and without any serve toxicity observed. (2)

Symptoms can include drowsiness, tremors or shaking of the muscles, seizures, collapse and even coma that can lead to death. Oleander sap can cause skin irritations, severe eye inflammation and irritation, and allergic reactions characterized by dermatitis. (2)

*N. oleander*, a member of the Apocynaceae family, is an ornamental plant and widely exists in the tropic and sub-tropic regions including Mediterranean region. It is the only species currently classified in the genus *Nerium*. *N. oleander* has a prolonged flowering period during the dry season (3).
N. oleander has included cardiac glycosides (4) and the aqueous extract contains polysaccharides, some cardinals and triterpenoids (5). Although the plant has toxic agents (6) it is used as an anticancer drug (7) antidepressant (8) and cardiotonic (9) purposes. In addition, chemical extract of N. oleander has antimicrobial and antifungal effects (10) compared to the distillate of N. oleander (11).

In this study, it was aimed to verify the changes at the molecular level as a result of exposure to gamma ray for a different period of time in order to produce lines of cells of economical value, and to extract high pharmaceutical compounds such as vincristine and vinblastine that can be used in the treatment of many types of cancer through biotechnological and genetic Engineering applications.

**MATERIALS AND METHODS**

**Plant material and cultural conditions**

Explants used in the regeneration experiments were taken from young (seedlings and seedling-derived cultures from N. oleander (L.) and from adult plants of the local cultivar with pink rose flowers. This cultivar grows in Egypt. To obtain young plant material, random bulk seeds were collected from oleander plants growing in their natural habitat (Egypt).

Seeds were sterilized by immersion in a 25% solution of commercial bleach for 15 min (50 g/L of active chloride) followed by three rinses with distilled sterile water. Seeds were then germinated in test tubes containing 20 ml of basal medium (BM).

Basal medium (BM) consisting of Murashige and Skoog (MS) major and minor salts (12), B5 vitamins (13), and 3.0% sucrose. The pH was adjusted to 5.8 and the medium was solidified with 0.7% agar before being autoclaved for 20 min. at 120°C.

Seedling-derived cultures were maintained by transferring shoot apices to glass vessels containing 40 ml BM with the ammonium nitrate salt reduced to half of its original concentration (BMN/2) and 4.4 mM 6-benzylaminopurine (BA). Tips of leaves from these proliferating cultures were used as explants. The cultured jars were incubated in a growth chamber at 26 ± 2°C at day and 22°C at night applying a photoperiod of 16 h/day of 2000 LUX intensity.

**Callus induction**

Apical shoot tips (1 to 2 mm in length) from seedlings (15-day-old) and previously established shoot cultures of seedlings were individually transferred to test tubes containing 20 ml of proliferating media. Four proliferation media containing the MS major salts (BM), MS major salts with ammonium concentration reduced to a the half (BMN/2), (14) major salts (SHM) were tested. Media also that contained MS minor salts, B5 vitamins, 3% sucrose, and 0.7% agar and were supplemented with 0, 4.4, or 8.8 mM of BA. The cultured jars were incubated in a growth chamber at (26 ± 2°C) at day and 22°C at night applying a photoperiod of 16 h/day of 2000 LUX intensity.

**Cell suspension culture**

Calluses were divided and transferred to vessels containing 40 ml above of the prepared media, which was used in callus induction, without agar, then the vessels were placed on gyratory shakers (130) rpm while incubated under the same conditions in callus induction. After two weeks cell suspensions were irradiated at doses of 100, 200, 300 and 400 rad.

**Cesium irradiation source**

C. roseus. Cell suspension cultures were exposed to gamma rays. The source used to be 137Cs with dose rate 0.48 Gy/min. located at National Center for Radiation Research and Technology, Nasr City, Cairo, Egypt. The doses were 0, 100, 200, 300 and 400 rad.

**Biochemical assays:**

**1- SDS-Page protein electrophoresis**

Five samples of the crude extracted were prepared by homogenizing 100g of frozen biomass from each sample in a blender pre-cooled by liquid nitrogen. Sodium dodecyl sulphate polyacrylamid gel-electrophoresis (SDS PAGE) was performed according to the method described by (15) and modified by (16).
2- Peroxidase isozymes

Native – polyacrylamide gel-electrophoresis (Native – PAGE) was used to identify the effect of various treatments on the enzyme (peroxidase) finger prints. Their isozymes finger prints Peroxidase isozymes were determined, according the procedures described in previous studies (17).

I- Extraction of isozymes

Five samples were extracted from crude N. oleander. (L.). Cell suspension was prepared by homogenizing 10 g of frozen biomass from each sample in a blender pre cooled by liquid nitrogen cell culture was soaked in distilled water for 24 hours to allow maximum imbibitions only, by using 4 g were extracted with 1 ml extraction buffer (pH 7.5). Each sample was vortexed for 15 seconds by electric vortex and centrifuged for 10 min at 10,000 rpm at 5°C. The supernatant was transferred to new Eppendorf tube and kept in a deep-freezer until used for electrophoresis analysis.

II- Enzymes assay

The staining solutions were prepared as follows; [1 M sodium Acetate, pH 4.7, 50 ml & Methanol 50 m l & 3,3 , 5,5-tetramethylbenzidine 50 ml & TMBZ 2 ml & 30 % H2O2] and enzyme assays were carried out according to the method described in earilier studies (18).

RESULTS AND DISCUSSIONS

Irradiation of N. oleander cell suspensions, with a dose level higher than 400 rad of gamma rays resulted in a significant mortality of these cells ranged from 60 to 90 % accordingly, no data were obtained after exposure of cell suspension to a dose level exceeding 400 rad.

SDS – Page electrophoresis

The results of electrophoretic patterns of protein extracted from the frozen biomass Cell suspension culture of N. oleander bush different doses of γ-rays (control, 100, 200, 300 and 400 rad) in the taxa, are shown in table (1) and figure (1). The Figure showed a maximum number of ten bands in taxa with lane 4 corresponding to 300 rad, while the minimum showed that five bands in the control (0) and 100 rad were observed.

The proteins Zymograms isolated from cell suspension culture of the four irradiated doses of N. oleander were subjected to SDS-PAGE analysis and observed a total of 36 bands (Table 1). The band no. one and six corresponds to 225 and 50 KDa, respectively, were present in all samples treated and control. The band no. 2 corresponding to 150 KDa was present in 200 and 300 rad. This band was absent for treatments with other doses. The dose of 100 rad exhibited 5 bands with lower intensity than other treated samples. Such result justifies a radiation effect on gene expression. The third band corresponding to 100 KDa was absent in 400 rad, but was present in all treatments, the forth bands corresponding to 85 KDa appeared in 300 and 400 rad samples but was absent in other treatments. Bands 5 corresponding with 75 KDa was present in all treated samples, but was absent in the control (0) rad. Therefore, irradiation modified genes expression in these treatments. Band no. 7 corresponding to 35 KDa appeared in control and 400 rad, but absent in other treatments, So we could consider this fragment as a molecular marker for 400 rad.

Band no. 8 corresponding to 30 KDa was present in all treatments, but was absent in control (0) rad and 100 rad treatments. Band no. 9 corresponding to 25 KDa was present in all treatments, but disappear in 200 rad treatments. The tenth band corresponding to 15 KDa was present in 200, 300 and 400 k rad, but was absent in control (0) and 100 rad. Finally, the eleventh band corresponding to 10 KDa was absent in all treatments but appeared in 300 and 400 rad.

Our results agree with (19) who studied the relationship among five different varieties of N. oleander L. through SDS PAGE. The electrophorogram showed a total of 45 bands of proteins with molecular weight ranging from 4.29 to 160.94 KDa. The protein diversity analysis was done based on the presence and absence of bands thereby interpreting their relevance.
Other researchers\(^{(20)}\) reported that the difference between populations of two species from Apocynaceae family, *Razya stricta* Decasine and *N. oleander* L. seed samples taken from the southeast of Iran in SDS-PAGE analysis of their proteins. The results showed 35-88% and 40-80% similarities between populations of the two species and *Catharanthus roseus* L. another species of the Apocynaceae family, 47-55% similarity was observed.

**Peroxidase isozyme**

Peroxidase are heme-containing enzymes ubiquitous in the plant kingdom. The term peroxidase means an enzyme catalyzing oxidation-reduction between hydrogen peroxide and reductants (from \(\text{H}_2\text{O}_2 + \text{AH}_2\) to \(\text{H}_2\text{O} + \text{A}\)). Peroxidases belong to a super family, which comprises Class I enzymes from mitochondria, chloroplasts, and bacteria; Class II from fungi; and Class III from higher plants based on their structural and catalytic properties\(^{(21)}\).

The results of electrophoretic patterns of peroxidase isozyme extracted from the frozen biomass cell suspension culture of the different treated samples (0, 100, 200, 300 and 400 rad in the *N. oleander* taxa are diagrammatically illustrated in Fig. (2). The figure showed a maximum number of six bands.
In control (0) rad with lanes 1 showed five bands, in the 100 rad sample treatment exhibited five bands of the same intensity within the treated samples. So the radiation exposure induced an effect on gene expression that was negative in the 100 rad dose.

In 200 rad treatment, lane 3 showed that band 6 was absent in control as well as other treatments while present in 200 rad dose, so that should be considered as a conferring modification of gene expression caused by irradiation.

The pattern of 300 rad lane 4 exhibited six bands, band 2 was present in the 200 rad, but was absent in the control and other treatments, indicating the effect of 200 rad irradiation treatment and the remarkable modification of gene expression. Therefore, irradiation treatments affected on modification of gene expression in Sour Orange.

The peroxidase zymogram of 400 rad lane 5 showed six bands, band 1 was very faint compared to that of the control treatment and other treatments, so the 400 rad dose irradiation treatments effected at gene expression in N. oleander cell suspension culture.

Unfortunately, studies on the effect of ionizing radiation, such as (gamma, beta and alpha ray) in N. oleander plant as a biotech electors were infrequent and far fetched. However, there was some studies on the effect of the gamma in N. oleander cells related to terpenoid indole alkaloid biosynthesis.

Our results agree with the (22) who studied the purification and characterization of enzyme peroxidase from leaves of C. roseus, which belong to the Apocynaceae family, in order to characterize of peroxidase from C. roseus, kinetic properties viz. Km value with respect to substrate H2O2 as well as the optimum pH and optimum temperature, etc. It was found that the molecular weight of POD was estimated to be around 46-48 kDa by SDS-PAGE which is in agreement with that of peroxidases studied from other sources. Detection of two bands bearing POD activity on native gel might signify the existence of two distinct proteins with associated POD activity.

Other researchers (23) found that the total peroxidase activity and the activities of peroxidase isoforms in leaves of red oak Quercus rubra seedlings exposed to wounding and plant hormones in the greenhouse. Activity of specific peroxidase isoforms was induced differentiation by gypsy moth.
wounding, mechanical wounding, and the wound-associated plant hormone jasmonic acid. The activity of one isoform was enhanced modestly by treatment with salicylate. A study of peroxidase activity in naturally occurring galls elicited on red oak leaves by 12 hymenopteran and dipteran insect species lead to the detection of 16 peroxidase isoforms, 11 of which were differently induced or suppressed in galls compared with leaves. In both studies, total peroxidase activity that was measured spectrophotometrically was not clearly related to the activity of these isoforms. These results indicated that red oak seedlings and trees may respond specifically to wounding, particular insects, and plant signals induced by changes in the activities of individual isozymes.

A reported change in protein and peroxidase isozymes was caused by changes in pH of medium cell culture as well as, a reported change in permeability of the cell membrane due to the consequent change in RNA in cytosol.

These results were demonstrated by (24) while studying other species of the same family. This studied species was C. rouses. They found that pH values of C. roseus cell suspension, increased after irradiation with UV rays for 5 min. then, the value decreased after that. They reported that on increasing pH values the charge and polarity of the cell surfaces were directly affected and late activate MPK enzyme. This enzyme could be inducing an increase in indole alkaloids through RNA transcription within the cell nucleus.

CONCLUSION

Results clearly indicate different effects of Gamma radiation on the medium and protein bands including peroxidase isozymes. The medium alkalization works on changing the permeability of charge active membrane so that ions flux namely Ca$^{2+}$ and influx H$^+$, K$^+$ and Cl$^-$ efflux. Among these processes, Ca$^{2+}$ play an important role in transduction of the signal elicitor, induced accumulation of secondary products inside the cell.

REFERENCES