Physiological and biochemical responses of Makhana (*Euryale ferox*) to gamma irradiation

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Abstract

The impact of gamma irradiation on growth and physiology of *Euryale ferox* was described in the present investigation. *E. ferox* is an underutilized aquatic food crop that grows in shallow-water bodies in lower Assam regions and north Bihar of India. The seeds of *E. ferox* were irradiated with different doses of gamma irradiation ranging from 0 to 500 Gy. It was observed that the germination and survival percentage was inhibited by increasing the irradiation dose. However, plants developed from seed exposed to an irradiation dose beyond 100 Gy did not survive more than 1 month. Further growth parameters (leaf size and number, number of thorns, root number and length, and number of flower and seeds) were also compared with respect to non-irradiated plants. Physiological parameters, viz. chlorophyll *a*, chlorophyll *b*, total chlorophyll, photosynthetic rate, transpiration rate, stomatal conductance, and intracellular CO₂ content was higher in the irradiation population of *E. ferox*. Superoxide dismutase (SOD) and ascorbate peroxidase (APX) activities were observed low in irradiated population of *E. ferox*. The proline and glycine betaine content was enhanced with increasing the irradiation dose. The present investigation explores the potential use of gamma rays in genetic improvement of *E. ferox* and improves understanding of the physiological responses inflicted by gamma irradiation.

Keywords *Euryale ferox* · Gamma irradiation · Germination, growth · Physiology

1 Introduction

Euryale ferox Salisbury is a leading fresh water aquatic cash crop, but has remained ignored, probably due to the absence of suitable improved varieties. *E. ferox* has a wide ranging distribution in East Asia. In India, it is mainly distributed in Bihar, Manipur, West Bengal, and Assam. *E. ferox* cultivation provides livelihood to thousands of poor farmers, mainly in Bihar and Manipur states of India. Its seeds are edible after being popped [1, 2]. It has very high nutritional as well as medicinal

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properties as it has a high ratio of lysine+ arginine/proline (4.74–7.6) and amino acid index (89–93%) [3]. As compared to staple food, it has high caloric value (3.62 kcal/g) [4]. In India, it has a prominent place in the nutritional chart with medicinal values for reproductive, respiratory, circulatory renal, and digestive diseases [5]. In Japan, it is categorized as ‘vulnerable’, and listed in the Red List of threatened plants [6]. In China, it is mostly cultivated for food and medicine. The major drawback of *E. ferox* cultivation is that the interlacing ribs of leaves and petioles are prickly. The mature fruits are borne on long pedicels and are difficult to harvest due to the stout prickles on the outer surface. Farmers are not able to do fish cultivation simultaneously with *E. ferox* cultivation due to the presence of thorns on whole surface of *E. ferox* [7].

E. ferox is an exclusively self-pollinated plant and the fertilization in flowers takes place at a very early (hermetically sealed) stage under the surface water. It is a monotypic genus and the available genetic variability is very limited [8]. Although it is an important aquatic crop, work on its improvement was not initiated earlier using the conventional breeding and induced mutagenesis. Because it is a monotypic genus, induced mutagenesis is the best available method for its improvement. Induced mutagenesis through gamma rays has proved to be more effective as compared to other mutagenic agents because of their penetration power. Ionizing radiation affects cellular components; thus, potentially inducing morphological changes in plants. Evaluation of these morphological changes is very important, as they occur through such physicochemical changes. Gamma radiation has been used successfully to induce useful mutations for genetic improvement of several crop plant [8–13]. Considering the importance of gamma irradiation on genetic improvement of crop plants, the present investigation was carried out to demonstrate the effects of gamma irradiation on growth and physiology of *E. ferox*.

2 Materials and methods

2.1 Plant material

Fresh seeds of the Swarna Vaidehi variety of *E. ferox* were obtained from the ICAR-RCER-Research Centre Makhana, Darbhanga, Bihar, India for the present study.

2.2 Gamma irradiation treatment

Seeds were irradiated with 0, 50, 100, 200, 300, 400, and 500 Gy of gamma rays in ambient condition at Food Technology Division, Bhabha Atomic Research Centre, Mumbai.

2.3 Determination of seed germination and survival percentage

Irradiated and non-irradiated (control) seeds were sown in a glass jar for determination of germination percentage and survival percentage under natural condition. Seeds were counted as germinated when more than 2 mm of radical extension was observed after 6 weeks. Germination percentage of seeds were calculated by using the equation:

$$\text{Germination percentage} = \text{Number of germinating seeds} / \text{Total number of seeds} \times 100$$

Germinated seeds are considered alive when leaf development occurs on seedlings. Counts of survived germinated seedlings were made after 10 weeks. Survival percentage of seeds was calculated by using the equation:

$$\text{Survival percentage} = \text{Number of live germinated seeds} / \text{Total number of germinated seeds} \times 100$$

Seed germination was not observed when seed was exposed more than 100 Gy of irradiation, hence 50 Gy and 100 Gy irradiation dose was applied to seed in the present investigation. Irradiated and control seeds were sown in a pond to determine the most suitable dose of gamma rays for large-scale cultivation.

2.4 Growth measurements

Morphological traits such as shoot length, number of leaves, leaf size, thorn number, root length, root number, number of flowers and number of seeds per flower were measured in blooming phase, i.e., during start of flowering.

2.5 Extraction of photosynthetic pigment

Chlorophyll *a*, *b*, total chlorophyll from the leaf tissues of control and gamma-irradiated plants were extracted using dimethyl sulphoxide (DMSO) [14]. Fifty milligrams of fine pieces of fresh leaf was suspended in a test tube containing 10 ml of dimethyl sulphoxide (DMSO) and incubated at 65 °C for 4 h. The supernatant was transferred and a further 10 ml of DMSO was added to the residue and incubated at 65 °C for 4 h. The supernatants were pooled and the final volume was adjusted to 20 ml by adding DMSO and the absorbance was taken at 663 and 645 nm using a spectrophotometer (Perkin Elmer, USA). Concentrations of chlorophyll *a*, *b*, chlorophyll *a/b* ratio, and total chlorophyll were calculated with the following equation [15].

$$\text{Chlorophyll a (mg/g)} : 12.7(A_{663}) - 2.69(A_{645})$$

$$\text{Chlorophyll b (mg/g)} : 22.9(A_{645}) - 4.68(A_{663})$$

$$\text{Total Chlorophyll (mg/g)} : 20.2(A_{645}) + 8.02(A_{663})$$

2.6 Measurements of photosynthesis rate, transpiration rate, stomatal conductance, and internal CO₂

Photosynthesis rate, transpiration rate, stomatal conductance, and internal CO₂ were measured in blooming phase, i.e., during start of flowering with an LI 6400 portable infrared gas analyzer (IRGA, Li-Cor®, Inc., Lincoln, NE, USA). Measurements were carried out under atmospheric CO₂ and full sunlight in the field.

2.7 Total protein and enzyme extraction and assays

Leaf (500 mg) was homogenized at 4 °C in 2 ml of extraction buffer [(200 mM Tris-HCl, 10 mM ethylene diamine tetra acetic acid (EDTA), 0.5 M sucrose, 2 mM phenyl methyl

sulfonyl fluoride (PMSF), and 1% insoluble polyvinyl pyrrolidone (PVP) (pH 8.5)]. The homogenate was centrifuged at 10000 rpm for 30 min and the supernatant was used for calculation of protein content [16] and enzyme activity.

Superoxide dismutase (SOD; E.C. 1.15.1.1) enzyme activity was estimated according to the method of Beauchamp & Fridovich (1971) [17]. One hundred microliters of enzyme extract mix with 3-ml reaction mixture (13 mM of methionine, 75 μ M of NBT, 2 μ M of riboflavin, 50 mM of phosphate buffer (pH 7.8), 0.1 mM of EDTA), was illuminated using fluorescent lamps for 15 min. The absorbance of reaction mixture was read at 560 nm using a spectrophotometer. A non-illuminated reaction mixture served as the control. 50% of NBT reduction as a unit of SOD activity intended SOD activity was obtained by the following formula:

$$SOD\ activity = \frac{Control - Sample}{Control / 2}$$

The activity of ascorbate peroxidase (APX; E.C. 1.11.1.11) was calculated according to the method of Nakano and Asada (1981) [18]. Fifty milliliters of enzyme extract was mixed with 2-ml reaction mixture (0.1 mM H_2O_2 , 1 mM EDTA, 0.5 mM ascorbic acid, 50 mM potassium phosphate buffer (pH 7.0), and was incubated at 25 °C for 1 min and measure the change in absorbance at 290 nm. The decrease in concentration of ascorbate was followed by a decrease in the OD at 290 nm, and activity was calculated using the extinction coefficient for ascorbate.

2.8 Extraction and estimation of proline and glycine betaine

According to Bates et al. (1973) [19], free proline content was calculated; 500 mg of fresh leaves was homogenized in 3 ml of 3% aqueous sulfosalicylic acid and centrifuged at 10000 rpm for 10 min. Two milliliters of supernatant was mixed with 2 ml acid ninhydrin (625 mg ninhydrin dissolved in 10 ml 6 M orthophosphoric acid and 15 ml glacial acetic acid), and 2 ml glacial acetic acid mix in a test tube and boiled at 100 °C for 30 min. The reaction was stopped by cooling the tubes in an ice bath. The chromophore formed was extracted with 6 ml of toluene and the absorbance of the resulting organic layer was measured at 520 nm. The proline concentration was calculated by referring to L-proline standard curve.

According to Grieve and Grattan (1983) [20], glycine betaine was calculated; 500 mg of fine powdered leaf tissue was mixed with 20 ml of de-ionized water for 24 h at 25 °C. The samples were filtered and diluted as 1:1 with 2 N sulfuric acid and cooled in ice for 1 h. Two hundred microliters of chilled potassium iodide iodine reagent was added to diluted sample and after mixing centrifuged at 10000 rpm for 15 min at 8 °C. After 3 h, the periodite crystals were dissolved in 9 ml 1, 2-dichloro ethane and absorbance was measured at 365 nm.

2.9 Determination of mineral content

For estimation of K^+ and Na^+ , 100 mg leaf tissue were dried at 80 °C in oven and further digested with a nitric-perchloric acid mixture. The concentration of K^+ and Na^+ of the extract were calculated using a flame photometer.

3 Results

3.1 Seed germination and survival percentage

As compared to irradiated seed, a higher germination percentage was observed in control seed. As illustrated in Fig. 1, the germination percentage was inhibited with increasing irradiation dose. Maximum germination percentage (94%) was observed in control whereas, only 10% germination was observed in 500 Gy treatment of gamma irradiation (Figs. 1 and 2).

There was a reduction in survival percentage observed with increasing dose of gamma irradiation. Maximum survival percentage (92%) was observed in the control, whereas only 34% germination was observed in 100-Gy treatment of gamma irradiation. No survival of the plants was observed beyond 100-Gy treatments (Figs. 1 and 2).

3.2 Growth measurements

A gradual reduction in plant height was observed after 50-Gy and 100-Gy irradiation dose. Number of leaves increased in the 50-Gy treatments as compared to the control, but again decreased at 100-Gy gamma irradiation treatments. A greater reduction in leaf size was observed in the 50-Gy treatments of gamma irradiation. The number of thorns either on the upper or lower surface of mature leaf as well as on plant surface increased in the 50-Gy treatment of gamma irradiation. However, it was decreased in treatment with 100-Gy irradiation dose (Table 1). Lower root length and root number was observed in gamma-irradiated population as compared to the control. Root length and root number decreased at 50 Gy but again increased at 100-Gy gamma irradiation treatments. It was observed that the gamma-irradiated population required more time for full flower blooming as compared to the control. There was no observable differences was observed with respect to number of flowers per plant, and number of seed per fruit in control and irradiated population (Table 1).

Fig. 1 Effect of gamma irradiation on seed germination and survival percentage of *Euryale ferox*. Mean $\pm$ SE, $n = 3$

Fig. 2 Effect of gamma irradiation on seed germination and survival of *Euryale ferox*. **a** In vitro condition **b** In field

3.3 Photosynthetic pigment content

We recorded 28% high chlorophyll *a*, and 20% high total chlorophyll content in plants treated with 100-Gy irradiation, whereas there were no remarkable differences observed in content of chlorophyll *b* in plants exposed to either 50-Gy or 100-Gy gamma irradiation. Maximum content of chlorophyll *a* (110 $\mu\text{g/g}$ FW) was observed in 100-Gy gamma-irradiated population, whereas only 84 $\mu\text{g/g}$ FW chlorophyll *a* content was observed in 50-Gy treatment of gamma irradiation. Maximum content of total chlorophyll (240 $\mu\text{g/g}$ FW) was observed in 100-Gy gamma-irradiated population, whereas only 200 $\mu\text{g/g}$ FW chlorophyll *a* content was observed in 50-Gy treatment of gamma irradiation (Fig. 3).

3.4 Measurements of photosynthesis rate, transpiration rate, stomatal conductance, and internal CO_2

High photosynthetic rate was observed in irradiated population as compared to control plants; a 42% higher photosynthetic rate was in the irradiated population. It was increased from

Table 1 Effect of different doses of gamma irradiation on different parameters of growth and development of *Euryale ferox*

Growth parameters	Control	50 Gy	100 Gy
Plant height (cm)	67 $\pm$ 1.5	64 $\pm$ 1.7	62 $\pm$ 1.5
No. of leaves per plant	6.66 $\pm$ 0.33	8.33 $\pm$ 0.33	6 $\pm$ 0.57
Leaf size (cm^2)	1820 $\pm$ 51	1260 $\pm$ 40	1686 $\pm$ 70
No. of thorns on upper surface of the leaf (number of thorns/ cm^2)	13 $\pm$ 1.15	19 $\pm$ 1.5	12 $\pm$ 1.8
No. of thorns on lower surface of the leaf (number of thorns/ cm^2)	26 $\pm$ 1.5	39 $\pm$ 5.7	21 $\pm$ 2.1
No. of thorns on plant surface (number of thorns/ cm^2)	39 $\pm$ 2	59 $\pm$ 5.5	34 $\pm$ 3.5
Root length (cm)	59 $\pm$ 3.1	37 $\pm$ 2.5	58 $\pm$ 4.4
Total number of roots	66 $\pm$ 8.8	50 $\pm$ 5.7	56 $\pm$ 4.4
Number of days of full flower bloom	172 $\pm$ 4	189 $\pm$ 6.4	189 $\pm$ 6.6
Number of flowers/plant	5 $\pm$ 0.57	4 $\pm$ 0.33	4 $\pm$ 0.33
Number of seeds /fruit	34 $\pm$ 2.7	31 $\pm$ 2.2	37 $\pm$ 3.5

Fig. 3 Effect of gamma irradiation doses on chlorophyll *a*, chlorophyll *b*, total chlorophyll, and total chlorophyll content ($\mu\text{g/g FM}$) of *Euryale ferox*. mean $\pm$ SE, $n = 3$

7 mmol CO_2 / m^2/s (control) to 12 mmol CO_2 / m^2/s (irradiated population). No remarkable difference was observed in photosynthetic rate of 50-Gy and 100-Gy irradiated population (Fig. 4a). A low transpiration rate was observed in the irradiated population as compared to control plants. An 18 and 13% transpiration rate was decreased at 50-Gy and 100-Gy irradiated population, respectively, as compared to the control (Fig. 4b). Low stomatal conductance was observed in the irradiated population as compared to control plants; 16 and 8.3% stomatal conductance was decreased at 50-Gy and 100-Gy irradiated population, respectively, as compared to control (Fig. 4c); 61% less intracellular CO_2 was observed in the 50-Gy irradiated population as compared to the control but no remarkable difference was observed between the 100-Gy treated population and the control (Fig. 4d).

3.5 Total protein and enzyme extraction and assays

Total soluble protein content was 20% decreased at 100-Gy treatment of gamma irradiation as compared to the control, whereas there were no remarkable differences observed between the 50-Gy and 100-Gy irradiated population and control population.

A 50 and 75% decrease in the activity of SOD was observed as a function of 50-Gy and 100-Gy gamma irradiation, respectively, as compared to the control (Fig. 5a). Similarly, APX activity was 50 and 89% decreased at 50-Gy and 100-Gy treatment of gamma irradiation, respectively, as compared to the control (Fig. 5b).

3.6 Extraction and estimation of proline and glycine betaine

High proline and glycine betaine content was observed in the gamma-irradiated population as compared to the control. Gradual enhancement of proline and glycine betaine content was recorded with increasing irradiation dose. Only proline content at 100 Gy was decreased as compared to the control (Fig. 6a).

Fig. 4 Photosynthetic characteristics. **a** Photosynthetic rate, **b** transpiration rate, **c** stomatal conductance, and (intracellular CO₂ concentration) of leaf of *Euryale ferox* plants as affected by different doses of gamma irradiation. Mean $\pm$ SE, $n = 3$

3.7 Determination of mineral content

Na⁺ accumulation reduced with increasing treatment of gamma irradiation. It was observed that Na⁺ content decreased 46 and 72% at 50-Gy and 100-Gy irradiated population, respectively, as compared to the control. K⁺ content 42% declined at 50-Gy treatments but there was no remarkable difference in K⁺ content observed between 100-Gy irradiated population and the control population. Similarly, Na⁺/K⁺ ratio is high at 100-Gy treatment and low at 50-Gy treatments as compared to the control (Fig. 6b).

4 Discussion

Mutation through gamma irradiation is one of the reliable, well-known, and safe approaches for genetic improvement of crop plants. It is well reported that gamma rays directly or indirectly control cell division and elongation and thus the phenotypical traits and subsequently the associated physiological characteristics changes [21–23]. In the present investigation, it

Fig. 5 Effect of different gamma irradiation on the total soluble protein concentration and on the **a** superoxide dismutase (SOD) and **b** ascorbate peroxidase (APX) activities of the leaves of *Euryale ferox*. Mean $\pm$ SE, $n = 3$

was observed that gamma irradiation had a significant effect on seed germination and survival percentage of *E. ferox*. Inhibition of seed germination and survival percentage was observed with increasing dose of gamma irradiation and after 100-Gy irradiation treatments, seedlings did not survive. Seed germination was also delayed with increasing dose of gamma irradiation [8, 24, 25]. Reduction in survival was recorded in all the treatments and the complete germinated seeds died in the 100-Gy treatments. A gradual reduction in plant height was recorded after treatments of 50 and 100 Gy as compared to control. The number of leaves was higher in the 50-Gy irradiation as compared to 100-Gy irradiated and control population, whereas a reduction in leaf size was observed at 50-Gy gamma treatments, which may be due

Fig. 6 Effect of different gamma irradiation treatment on **a** glycine betaine and proline content, and **b** content of Na and K of *Euryale ferox*. Mean $\pm$ SE, $n = 3$

to the increased number of leaves at 50 Gy. The number of thorns on the upper surface of the mature leaf as well as on plant surface is reduced at 100-Gy treated population, which may be of help in the co-cultivation of fish. The number of leaves as well as leaf size was reduced due to which pond water is exposed more to light intensity which induced BOD of pond which is essential for cultivation of fish in pond. There were no differences observed in the number of flowers per plant, but the number of seeds per flower was affected after gamma irradiation. The number of seeds per flower was also high at 100-Gy treatments of gamma rays. Verma et al. also observed a similar result [8]. Radiation is an important environmental stress that affects the morphological and physiological structure of all living organisms. It was also observed that gamma irradiation was found to be effective in regulating chlorophyll content. High Chl *a* and total chlorophyll content was found in the 100-Gy-treated population as compared to the 50-Gy and control [25, 26]. A higher photosynthetic rate was observed at the 100-Gy-irradiated population. A high amount of chlorophyll content, photosynthetic rate, and leaf area in 100-Gy-irradiated plants hints at the good efficiency of irradiated plants for the development of both reproductive and vegetative mass [27]. Transpiration rate, stomatal conductance, and intracellular CO₂ concentration was inhibited in irradiated population as compared to the control, which may be due to the varied number and size of leaves, and content of chlorophyll [27]. SOD and APX activity gradually decreased with increasing dose of gamma irradiation [26]. Proline and glycine betaine act as osmoregulators by contributing to hydrophobicity, active oxygen scavenging, maintaining cell pH, stress tolerance, and protection [28]. Remarkable differences in proline and glycine betaine accumulation due to gamma irradiation treatment observed in the present study are in agreement with previous reports [29]. It was also observed that Na⁺/K⁺ ratio decreased as a function of gamma irradiation dose. A low Na⁺/K⁺ ratio could be essential for many plant species than solely continuing small a concentration of Na⁺ [30]. Na⁺ and K⁺ takes part in many enzymatic activities in plant cells and maintaining the cytosolic Na⁺/K⁺ ratio is a key requirement for growth under any stress conditions [31]. Different characters of *E. ferox* were found to be sensitive to gamma rays and 100-Gy radiation was found to be an effective dose for variation. Variants from the present experiment are being maintained to study their performance in subsequent generations. Thus, the present study clearly indicates that gamma irradiation can develop new varieties of this crop with improved commercial traits.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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