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Induced mutation and *in vitro* techniques as a method to induce salt tolerance in Basmati rice (*Oryza sativa* L.)

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Abstract

Embryogenic callus of indica rice (*Oryza sativa* L.) cv. Basmati 370 induced on MS medium containing 9.05 µM2, 4-D was irradiated at 50 Gy of gamma rays of ^{60}Co for creating genetic variability against salinity. Irradiated and non-irradiated calluses were screened *in vitro* through three consecutive proliferation phases at 4.0, 6.0, 8.0 and 10.0 d/Sm electrical conductivity of NaCl. Growth value and number of adapted mutagenized callus was more than that of non-mutagenized callus. Salinity levels beyond 6 d/Sm and 8 d/Sm were lethal to growth and adaptation of non-irradiated and irradiated callus respectively. NaCl adapted irradiated callus showed 2.0%-4.75% regeneration frequency on MS regeneration medium containing 5.37 M NAA and 9.29 µM Kinetin. Non-mutagenized salt adapted callus did not show any regeneration. From gamma ray mutagenized cultures, 2 putative lines (M_2 generation) with moderate salt tolerance were obtained at seedling stage. The results suggest that in vitro technique in connection with gamma rays may be used as a versatile approach to improve the level of salt tolerance in Basmati rice for saline environment.

Key words: *Rice, induced mutation, NaCl stress, callus, regeneration*

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Introduction

It is notable that the grain yield especially of rice has not been harvested in commensuration to its existing genetic potential in almost all rice-growing ecosystems. One of the major reasons behind this failure is the sensitivity of this crop to abiotic stresses particularly salinity (Grover, *et al.*, 2000). Tolerance and yield stability are complex genetic traits that are difficult to establish in crops since salt stress may occur as a catastrophic episode, be imposed continuously or intermittently become gradually more severe at any stage during development (Yokoi, *et al.*, 2002). Basmati rices are highly sensitive to saline environments; current guidelines indicate that salinity affects the yield at or above 3.0 d/Sm (Stephen, *et al.*, 2002). The typical mechanism of salinity tolerance in rice is the exclusion or reduction of Na uptake and increased absorption of K to maintain a good balance of Na-K ratio in the shoot.

Breeding for salinity tolerance in rice requires reliable screening techniques. These techniques must be rapid to keep pace with the large amount of breeding materials generated. Screening under field conditions is difficult due to stress heterogeneity, presence of other soil related stresses, and the significant influences of temperature, relative humidity and solar radiations. Melchers and Bergman (1959) reported the use of cell culture for mutant selection as the easiest way of large scale screening of cell populations. The high efficiency of in vitro selection system is based on the fact that it is possible to grow millions of cells in petri dish or in a flask and achieve a rapid multiplication of cell populations on defined media. Additional genetic variations against stresses can be induced by physical and chemical mutagens (Ahloowalia, 1997).

Following mutation induction, the regenerated plants can be pre-selected *in vitro* by exposing regenerative cells or tissues to stress conditions and pre-selected genotypes can then be selected for the desired traits in the field. Success for obtaining abiotic stress mutants by the application of mutation coupled with *in vitro* systems have been attained in some cop plants including rice (Ahloowalia, 1990; Das, *et al.*, 2000; Pathirana, *et al.*, 2002; Lee, *et al.*, 2003). The identification of the salt tolerant lines obtained from *in vitro* mutagenesis was carried out at germination and seedling stage on the solution by other authors (Miah, *et al.*, 1996; Sathish, *et al.*, 1997). The solution based screening is rapid and widely acceptable under controlled conditions instead

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of field screening (Gregorio, *et al*., 1997).

Water logging and salinity are common in irrigated agriculture in Pakistan. Out of 14.8 x 10^6 hectares irrigated land; 6 million hectares are salt affected causing an economic loss of 3.0 million US $ annually on account of decrease in agriculture production (Anonymous, 2003). Huge amount of capital is required to reclaim salt affected soils, which seems impractical. Unlike in South and South Asian countries, rice mostly of Basmati type is not considered as subsistence crop in Pakistan. It is cash crop grown for export and contributes about 4478.5 thousand tones of rice with worth of 559 million US $ (Anonymous, 2003). Development of salt resistant or tolerant varieties is one of the most effective approaches to coexist with saline soils. The objective of the present study was therefore, the isolatiton of gamma-induced rice mutants better adapted to saline environments using integrated technique of tissue culture and mutagenesis. This reaserch was conducted at Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan during 2000-2004.

Materials and Methods

Manually dehusked seeds of Basmati rice (*Oryza sativa* L. cv. Basmati 370) were surface sterilized by immersion in 50% v/v commercial bleach containing 5.25% sodium hypochlorite (NaOCl) for 30 minutes followed by three washings with sterilized distilled water. The seeds were placed on CI medium (modified MS medium containing MS salts and vitamins as described by Murashige and Skoog, 1962) supplemented with 2, 4-dichlorophenoxyacetic acid (2, 4-D) 9.05 µM, sucrose 30 g/land bacto agar (Difco) 10 g/l. The pH of the medium was adjusted to 5.8. The cultures were placed in dark at a temperature of 25±1 °C for callus induction for four weeks. Observations regarding the morphology of the callus were noted. Non-Embryogenic callus was discarded. Embryogenic callus was excised aseptically and divided into two portions. Half portion was kept as non-irradiated (control) while remaining half was irradiated at 50 Gy of gamma rays of ^{60}Co. Both non-irradiated and irradiated calluses were divided into about 1 mm. in diameter and proliferated onto non-saline and saline CI medium for three consecutive phases with interval of 45 days. Four electrical conductivity (EC) levels of NaCl (4.0, 6.0, 8.0 and 10.0 d/Sm) were established in CI medium with the same amount of 2, 4-D, sucrose, bacto agar and with similar culture conditions. CI medium was replaced with fresh medium at each proliferation phase. Growth of tissue was expressed by value determined by the ratio of final weight to the initial weight. Calluses with maximum growth value were used as source of inocula in the next phase. Thus adapted callus lines at different EC of NaCl were obtained. Finally all adapted calli were transferred to MSR medium (modified MS medium containing MS salts and vitamins supplemented with kinetin, 9.2 µM; naphthalene acetic acid, 5.37 µM; sucrose 30 g/l; agar 10 g/l; pH 5.8) for regeneration. All the cultures were placed in a growth room under 16/8 h. light / dark period at 25±1 °C. Cultures were transferred to fresh MSR medium after three weeks to regenerate plants (M_1 generation). Emergence of green shoots above 3.0 cm. was considered to calculate regeneration frequency (RF) as under:

RF = Number of embryo derived calli showing green shoots/Total number of calli transferred x 100

The regenerated mutants were transferred to hormone free MS medium for vigorous root development and then to hydroponic solution (Yoshida, *et al*., 1976) for root hardening. Each regenerated plant was assigned a plant line number and grown to maturity in NaCl-free field conditions. Agronomic data of interest was recorded and seed was collected at maturity.

Screening for salt tolerance in M2 generation

A rapid screening was done following method of Gregorio, *et al*., (1997). Ten seed from each M_2 plant line and parent cultivar Basmati 370 were surface sterilized with fungicide vitavax. These were placed in petri dishes with moistened filter paper and incubated at 30 °C- 48 °C to germinate. Two germinated seeds were sown per hole of a Styrofoam float placed on rectangular tray filled with distilled water. Total number of rows on float was 10 in fashion of 10 holes per row. Thus 8 M_2 lines were compared with 2 lines of control. When seedlings were established within 3-4 days, the distilled water was replaced by nutrient solution (Yoshida *et al*., 1976). Nutrient solution was initially salinized at 2 d/Sm EC of NaCl. Later on salinity was increased to 4 and 6 d/Sm with gradual interval of three days by adding NaCl while stirring up to desired level of EC. The pH of the solution was adjusted to 5.0 and checked twice a day i.e., at 0900 and 1500 hour. Adding distilled water compensated the loss of solution due to evaporation and transpiration. Old nutrient solution was replaced with fresh solution

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after every 8-day and pH was maintained at 5.0 daily. Each M_2 line was rated at 10 and 16 day after initial salinizaiton. Shoot and root length of seedlings of M_2 line and Basmati 370 was recorded to note tolerance level. Modified standard evaluation score (Gregorio, *et al*., 1997) of visual salt injury at seedling stage were employed for rating M_2 line along with original variety using score of 1 (highly tolerant), 3 (tolerant), 5 (moderately tolerant), 7 (susceptible) and 9 (highly susceptible).

Results

Callus was obtained after four weeks of seed inoculation onto CI medium. The embryogenic callus was essentially nodular and pale yellow. Pieces of callus of 1 mm. diameter were cultured on the medium with 4, 6 and 8 and 10 d/Sm electrical conductively of NaCl respectively. Study on growht value of non-irradiated (control) and irradiated calluses under saline conditons revealed that growth value of irradiated calluses were higher than that of non-irradiated calluses (Figure 1).

Table 1: Effect of NaCl on calluses under control and saline conditions

EC (NaCl) d/Sm	Irradiated adapted calluses (%)	Non-irradiated adapted calluses (%)
4	67	40
6	50	23
8	23	-
10	-	-

Similarly, the number of irradiated adapted calluses was more (23%-67%) than that of non-irradiated calluses (23%-40%) as shown in Table 1.

Irradiated and non-irradiated calluses cultured at 10 d/Sm and 8-10 d/Sm of NaCl respectively could not tolerate the high levels of NaCl stress and became necrotic, hence these were discarded.

Plant regeneration response is indicated in Table 2. The application of gamma rays was effective between 4 and 6 d/Sm with regeneration frequency of 4.75% and 2.0%. Total number of green plants obtained were 27 (19 and 8 at 4 and 6 d/Sm respectively) as compared to zero regeneration response from non-irradiated ones The differentiation of green spots was higher (5.25%-9.5%) with lower (3%-6%) albino plant formation.

Morphological characterization of M_1 mutant plants

Considerable variations for heading date (100-120 days), plant height (60-100 cm), fertility (60%-80%), yield per plant (14-20 g), seed size and color of the grains were noticed in 27 M_1 plants.

Screening for salt tolerance in M_2 generation

Screening for evaluation of salt was conducted by taking a sample of 270 M_2 seedlings, (10 seedlings for each M_2 line) and 80 seedlings of variety Basmati 370 as under:

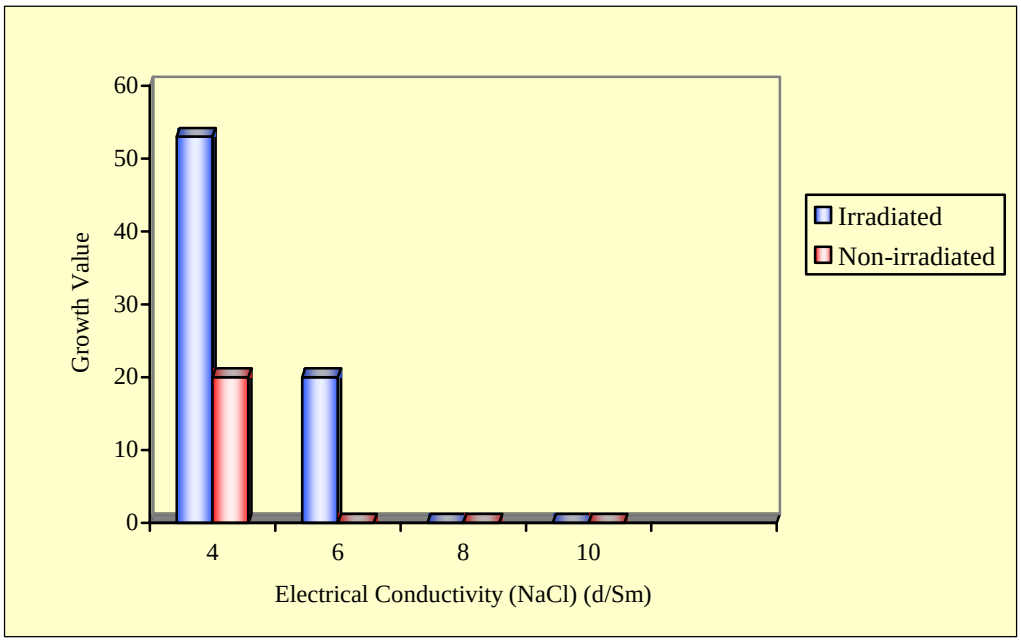

Figure 1: Comparative analysis for growth of irradiated and non-irradiated callus

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Table 2: Regeneration frequency of irradiated and non-irradiated adapted callus

Dose (Gy)	EC (NaCl) d/Sm	No. of calli inoculated	No. of green spots	Regeneration frequency	No. of albino
50	4	400	38 (9.5%)	19 (4.75%)	24 (6%)
50	6	400	21 (5.25%)	8 (2%)	12 (3%)
50	8	400	6 (1.5%)	-	-
50	10	-	-	-	-
0	4	400	15 (3.75%)	-	-
0	6	400	4 (1%)	-	-

a) Screening of M_2 lines obtained at 4 EC of NaCl in M_1 generation

At 10 day of salinization, 2 lines out of 19 M_2 lines were assigned score 5 showing moderate tolerance. Growth was severely retorted, most of the leaves rolled and only few were elongating. However at 16 days one of them lost the tolerance and became susceptible scoring 7. At this point there was complete cessation of growth; most of the leaves became dry leading to death. One M_2 line remained at moderate level of tolerance with score 5. Six plants were exhibiting tolerance while other four died in this line. The root and shoot length was increased considerably. The original control variety Basmati 370 was highly susceptible even at 10 day of the salt stress and there was complete death of its all seedlings.

b) Screening of M_2 lines obtained at 6 EC of NaCl in M_1 generation

One M_2 line found to be moderately tolerant at 10 and 16 day of salinization with score of 7. Half of the pants in each line were susceptible. The shoot and root lengths of this M_2 line were more increased than those of control. Seedlings of the rest of lines and Basmati 370 registered susceptible to high susceptible response at 10 day of salinization.

Discussion and Conclusion

Decrease in growth value and percentage of adapted calluses through consecutive phases, is the result of stress injuries, which has been accounted for increased level of NaCl and the ionic osmoticum nature of NaCl (Basu, *et al.*, 1996 and Basu, *et al.*, 1997). Moreover high salinity causes hyperosmotic stress and ion-disequilibrium that produces secondary effects or pathologies (Hasegawa, *et al.*, 2000 and Zhu, 2001) on growth of calluses. The combined use of mutagenic treatment with *in vitro* culture was applied to focus on heritable genetic variations. This was depicted from higher growth value and green plant regeneration frequency of irradiated NaCl adapted calluses as compared to that of non-mutagenized control (Table 2). Similar results were disclosed by other authors (Bhagwat and Duncan, 1998; Cheema, *et al.*, 2002; Lee, *et al.*, 2003). It was believed that minimal stress or proper stress on the callus by irradiation may not induce irreversible genotypic changes but can stimulate callus formation and plant regeneration. The emergence of considerable morphological variations between M_1 mutant lines provided additional genetic variability for the selection of other trait (s) of interest in succeeding generations. The usefulness of such variations have been documented in various papers aiming to develop or improve cultivars in almost all major crops (Ahloowalia and Maluszynski, 2001; Ahloowalia, *et al.*, 2004).

Keeping in view the highly sensitive nature of Basmati 370, the levels of salinity were gradually increased from 2 d/Sm to 6 d/Sm to assure salt tolerance selection through the screening which was done on the most sensitive seedling stage. Out of 27 M_2 lines, only 2 lines were scored at 16 day of salinization with moderate tolerance at 6 d/Sm of NaCl while all other lines became salt susceptible. In many cases plant regenerated from salt tolerant or resistant cell lines have lost the selected traits in the following generations due to instability of cellular modifications (Zhu, *et al.*, 2000). It was clear from our results that spectrum of genetic variability for salt tolerance with in and between the M_2 lines was variable however, none of the M_2 lines could proved to be salt tolerant. From the studies it can be concluded that the technique of tissue culture in connection with induced mutation is effective to improve salt tolerance. It has screened two M_2 lines on seedling stage as moderate tolerant to salinity. Field trials need to be conducted in succeeding generations on these putative M_2 lines to prove genetic stability of salt tolerance.

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