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Gamma Irradiation and NaCl Treatment on the Embryonic Callus of Rice (*Oryza sativa* L.) Cultivar Kuliyadichan and CO 43 for Salt Tolerance

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A B S T R A C T

The present study investigated the effect of gamma irradiation and NaCl treatment on the embryonic calli of *indica* rice variety Kuliyadichan and CO43. Embryonic calli obtained from both the varieties were exposed to gamma irradiation (0, 20, 30, 40 and 50 Gy) and lethal dose LD_{50} based on calli survival and regeneration capacity was determined. The LD_{50} based on calli survival was deduced as 34.31 Gy and 31.01 Gy for Kuliyadichan and CO43 respectively. Similarly the LD_{50} based on regeneration capacity was deduced as 27.09 Gy and 24.01 Gy for variety Kuliyadichan and CO43. Furthermore, embryonic calli from both varieties were exposed to four different NaCl concentrations with electrical conductivity of 4, 8, 10 and 12 dS/m and the lethal concentration LC_{50} based on calli survival and regeneration capacity was determined. The lethal concentration LC_{50} based on calli survival was fixed as 11.01 dS/m for Kuliyadichan and 10.24 dS/m for CO43. Similarly, the LC_{50} based on regeneration was fixed as 10.6 dS/m for variety Kuliyadichan and 9.19 dS/m for variety CO43. Based on overall consideration, the variety CO43 showed more sensitivity to gamma irradiation and NaCl stress than the variety Kuliyadichan.

Introduction

Rice (*Oryza sativa* L.) is an important staple food for more than half of the world's population. It serves 20% of the calories to the human diet. World human population is increasing day by day and it is expected to be around 9-10 billion by 2050.Global consumption of rice has increased from 437.12 metric tons in 2008 to 495.5 metric tons in 2019 (Statista, 2020) and was projected to reach 852 metric tons by the year 2035 (Khush and Gurdev, 2013). But the agricultural production is dwindling year by year due to climate change, rainfall variability, and other abiotic stresses. Among the abiotic factors, salinity is a major threat that reduces the growth and development of plants. It is estimated that 32% of the irrigated lands and 20% of the total cultivated agricultural lands are affected by high salinity. Irrigation with saline water, high

surface evaporation and poor cultural practices increase the rate of salinization by 10% annually and estimated to be increased up to 50% by 2050 (Jamil *et al.,* 2011).

There are two ways to manage the salinity stress: 1) to change the environment that adopts for normal growth of plants and 2) to change the genetic architecture of the crop that adopts to salinity tolerance. The first method is a tedious and time-consuming process and requires a lot of soil amelioration process. The second approach *i.e.,* breeding crops with in-built salinity tolerance is the most promising, socially accepted approach and requires less resource consumption. In light of the situation, crop genetic improvement in the agronomically important crops that are tolerant or resistant to salinity is important (Reddy *et al.,* 2017).

Induced mutation is one of the ways to create genetic variability in plants. There are three ways to induce mutation viz., T-DNA insertion, using physical mutagens such as gamma rays, fast neutrons and electron beam and chemical mutagens such as MNU, EMS, and NaN_2 (Serrat *et al*., 2014). Among these, physical and chemical mutagens are most commonly used (Penna *et al.,* 2012). Once the plants have been treated with mutagens, the mutants are to be screened for any improvement in traits. Screening through conventional breeding requires more time and a large population to identify a desired mutant (Schaart *et al.,* 2015). In such scenario, tissue culture techniques could be more advantageous than conventional breeding. The culture media could be modified by adding selection agents and the desirable mutants could be screened in a short period and in controlled conditions (Bado *et al.,* 2015). The combined use of *in vitro* culture methods such as anther culture, somatic embryogenesis, protoplast fusion, and induced mutations can help to overcome some limitations in both seeds and vegetatively propagated crops (Maluszynki *et al.,* 1995).

In vitro mutagenesis and somoclonal variations can help to improve agronomic traits like salinity and drought tolerance in different crop plants (Arzani and Ahmad, 2008). The present experiment was conducted to study the effect of gamma rays and NaCl salt on rice callus.

Materials and Methods

Seeds of rice cultivars *viz.,* Kuliyadichan and CO 43 were selected because of the high callus induction frequency and regeneration frequency (Amaravel, 2019). Embryogenic calli generated from these seeds were used as experimental materials.

Callus induction

Selected healthy, fully matured seeds from each variety were dehusked by removing the lemma and pale a using sand paper and taken in a sterile centrifuge tube. The seeds were surface sterilized by immersing in 70%ethanol for 1 min followed by50% commercial bleach (~5% NaOCl) for 10 min with shaking at 120 rpm. The seeds were rinsed with sterile deionised water (3 to 5 times) and dried for 5 min by placing on a sterilized tissue paper. Sterilized seeds were inoculated in callus induction medium (CIM) comprising of MS media (Murashige and Skoog, 1962) supplemented with 2.0mg/l of 2, 4-dichlorophenoxyacetic acid (2, 4 –D) 0.5mg/l of kinetin and 30 g/l of sucrose as described by Amaravel (2019). The pH of the media was adjusted to 5.6-5.8 before adding Clerigel (Himedia, India) 3g/l as a gelling agent. The medium was sterilized by autoclaving at 15 psi for 15 min. The cultured plates were placed in the dark at a temperature of 27 ± 2° C for three weeks for callus induction. The callus induction

percentage of each variety was observed at the time of initiation

$$\text{Callus induction percentage} = \frac{\text{Number of seeds that produced callus}}{\text{Total seeds cultured}} \times 100$$

After three weeks, induced calli from seeds were irradiated with gamma rays and were used to establish tolerance to salinity.

Mutagenesis of callus by gamma irradiation

Determination of sensitivity and growth response of irradiated callus

Healthy embryonic calli of the three rice cultivars were irradiated with gamma rays (Gamma chamber 5000, Indian Institute of Horticultural Research, Bangalore) with three replications and 12 calli per replication. Post irradiation, the calli were divided into 3-4 mm diameter tissues using a sterile scalpel. Up to 150 calli were transferred into freshly prepared CIM (Murashige and Skoog, 1962) and subcultured two times for every 13 days to eliminate the radiolysis hazards. The proliferating calli were observed by the cream color and the increase in mass and any browning due to phenolic exudation were considered irresponsive. After one month, the survival and weight of the callus were observed and recorded.

$$\text{Relative differentiation rate (RDR \%)} = \frac{\text{No of survived calli in treatment}}{\text{No of survived calli in control}} \times 100$$

Relative growth percentage = $(W_n\text{-}W_0 / W_0) \times 100$,

where W_n is the weight of the callus at the end of the experiment

W_0 is the weight of the callus at the start of the experiment.

Effect of gamma radiation on regeneration percentage

After two subcultures, some of the survived irradiated calli were transferred to regeneration media comprising of MS media supplemented with BAP 3 mg/l, NAA 1.0 mg/l, maltose 30g/l and clerigel 3g/l (Amaravel, 2019). The cultures were maintained at27 ± 2°C with a 16/8 hr light and dark period for 3-4 weeks. The regeneration response of each variety at different doses was recorded.

The regeneration frequency was calculated based on the formula,

$$\text{Regeneration percentage} = \frac{\text{Number of calli that producedgreen shoot}}{\text{Total number of calli transferred to regeneration media}} \times 100$$

Determination of optimal dose of selection factor for callus to salt tolerance and growth response

Non irradiated calli were grown in CIM with electrical conductivity of 4, 8, 10, and 12dS/m salt stress to study the effect of salinity on callus formation and to determine the optimal dose for selection factor. The cultures were maintained at 27 ± 2°C in dark for a period of 3-4 weeks. The treatments consisted of three replicates and each replicate consisting of 12 calli per rice variety. After three weeks, survival of calli and relative growth rate were observed.

Effect of NaCl treatment on regeneration percentage

After 4 weeks, some of the NaCl stress survived calli were transferred to regeneration media comprising of MS media supplemented with BAP 3 mg/l, NAA 1.0 mg/l, maltose 30g/l and clerigel 3g/l (Amaravel, 2019) with

electrical conductivity of 4, 8, 10 and 12 dS/m. The cultures were maintained at 27 ± 2°C with a 16/8 hr light and dark period for 3-4 weeks. The regeneration response of each variety at different doses was recorded.

Statistical analysis

Radio sensitivity assay of irradiated callus and salt sensitivity of non-irradiated callus for each variety based on relative differentiation rate was analysed by linear regression in Completely Randomized Design (CRD). The LD_{50} and LC_{50} was fixed by using the Curve Expert1.4 program. The *in vitro* growth response data were analysed by analysis of variance (ANOVA) and mean comparison by Tukey HSD at 0.05% significance by SPSS software version 16.0.

Results and Discussion

Callus induction percentage

In rice tissue culture, callus induction depends upon number of factors such as, genotype of the mother plant, composition of the basal salts and organic compounds, type of explants, and plant growth regulators used in callus induction (Khaleda and Al-Forkan, 2006).Even within the *indica* rice varieties, significant variations in *in vitro* culture of different genotypes were observed. The callus induction media for the rice cultivars used in this study was standardized previously (Amaravel, 2019).

The best combination for obtaining maximum callus was observed as MS medium, (Murashige and Skoog, 1962) supplemented with 2, 4 –D 2.0mg/l, kinetin 0.5mg/l and sucrose 30 g/l. Among the varieties tested, maximum callus induction was observed in Kuliyadichan (85.6%) followed by CO 43 (82%).

Mutagenesis of rice calli by gamma irradiation

Determination of radiation sensitivity of the irradiated callus

Fresh actively proliferating embryonic calli showed sensitivity to gamma irradiation. The survival rate of the embryonic calli decreased with the increase in irradiation dose and the different rice cultivars showed different percentage of survival rate (Fig. 1A-D). The relative differentiation rate of irradiated callus based on survival of calli and the ability to produce regeneration was calculated (Table 1 and 2). This suggests that the genotype of the cultivar is important in determining the radiation stress survival rate. Furthermore, it emphasizes the need for fixation of optimal radiation dose for every cultivar to get maximum heritable mutations with limited lethality. The irradiation causes the production of free radicles such as H^+ and OH^-due to the interaction with the water molecules present in the treated cells which leads to plant cell death (Taheri *et al.,* 2014) Also a reduction of survival percent was reported in rice (Abdelnour *et al.,* 2020) (Yunita *et al.,* 2020), in sugarcane (Nikam *et al.,* 2015) (Patade *et al., 2008*) and in *Citrus recticulata* cv. Limau Madu (Agisimanto *et al.,* 2016).

In any mutation-induced program, the determination of the radiosensitivity is essential for identifying the suitable dose for inducing mutation in particular cultivars. Radiosensitivity of irradiated embryonic callus (LD_{50}) can be found from the relative differentiation rate based on callus survival percentage and the regeneration rate.

The linear regression equation for two different varieties based on the percentage of survival and regeneration is shown in Figure 2. The lethal doses (LD_{50}) based on callus

survival percentage of the two cultivars viz., Kuliyadichan, and CO 43 was fixed as 34.81 Gy, and 31.01 Gy respectively. Similarly, the lethal dose (LD_{50}) based on regeneration percentage was fixed as 27.09 Gy for variety Kuliyadichan and 24.21 Gy for variety CO 43.

The LD_{50} of the irradiated embryonic callus of upland rice variety Situpatenggang and Batutegi was 24.68 Gy and 22.15 Gy (Yunita *et al.*, 2020). The lethal dose of irradiated embryonic calli was 200Gy and 60 Gy based on survival percentage and plant regeneration percentage respectively in rice variety CR 5272 (Abdelnour *et al.*, 2020). Agisimanto *et al.*, (2016) determined that the mean lethal irradiation dose (LD_{50}) of *Citrus reticulate cv.* Limau Madu was 30Gy for the callus induction. Saif *et al.*, (2001) reported that the $LD_{50}$for the sugarcane variety CP-43/33 and potato cv Cardinal was 20Gy. Thereby each crop variety with varying response to gamma radiation has been documented.

Table.1 Effect of gamma irradiation on relative differentiation rate of embryonic calli of Kuliyadichan and CO 43

S.No	Gamma radiation dose (Gy)	Kuliyadichan		CO 43	
		No.of survived calli	RDR (%)	No. of survived calli	RDR (%)
1	0 (control)	143	100	145	100
2	20	118	82.51	120	82.75
3	30	63	44.05	65	44.82
4	40	60	41.25	45	31.03
5	50	46	32.16	26	17.93

Table.2 Effect of gamma irradiation on the regeneration of rice varieties Kuliyadichan and CO 43

Variety	Dosage of gamma irradiation (Gy)	No. of calli inoculated	No. of calli that turned green	No. of calli regenerated	Regeneration percentage (%)	Relative differentiation rate (%)	No. of albino plants
Kuliyadichan	0	35	30	24	68.57%	100	_
	20	35	30	16	45.7%	66.66	_
	30	35	21	10	28.57%	41.66	1
	40	35	10	5	14.2%	20.83	1
	50	35	8	3	8.5%	12.5	1
CO 43	0	35	28	20	57.14%	100	-
	20	35	18	13	37.14%	65	-
	30	35	12	7	20.1%	35	1
	40	35	3	2	5.7%	10	2
	50	35	0	0	0	0	-

Table.3 Relative growth rate of rice varieties Kuliyadichan, and CO 43 subject to gamma irradiation

Gamma irradiation (Gy)	Fresh weight of callus (g)	
	Kuliyadichan	CO 43
0	$2.08_{a} \pm 0.03$	$1.71_{a} \pm 0.03$
20	$1.87_{a} \pm 0.09$	$1.30_{b} \pm 0.09$
30	$1.28_{b} \pm 0.04$	$0.81_{c} \pm 0.10$
40	$0.94_{c} \pm 0.07$	$0.60_{cd} \pm 0.06$
50	$0.70_{d} \pm 0.07$	$0.27_{d} \pm 0.04$
CD at 0.05%	0.217	0.253
CD at 0.01%	0.308	0.361

Data represent the mean of the replicates ± S.E values. Mean followed by the same letter in the same column does not differ according to post hoc test Tukey HSD analysis at the level of 5% significance

Table.4 Effect of different salt stress on relative differentiation rate of embryonic calli of Kuliyadichan and CO 43

S.No	NaCl Stress media (dS/m)	Kuliyadichan		CO 43	
		No. of survived calli	RDR (%)	No. of survived calli	RDR (%)
1	4 (control)	34	100	35	100
2	8	28	82.35	29	82.85
3	10	21	61.76	18	51.42
4	12	12	35.2	10	28.57

Table.5 Effect of NaCl on the regeneration of rice varieties Kuliyadichan and CO 43

Variety	NaCl Stress media (dS/m)	No.ofcalli Inoculated	No. of calli regenerated	Regeneration percentage (%)	Relative differentiation rate (%)
Kuliyadichan	4 (control)	10	8	80	100
	8	10	7	70	87.5
	10	10	5	50	62.5
	12	10	2	20	25
CO 43	4 (control)	10	6	60	100
	8	10	5	50	83.33
	10	10	3	30	50
	12	10	0	0	0

Table.6 Effect of NaCl on the relative growth rate of calli in rice varieties Kuliyadichan and CO 43

S.No	NaCl stress media (dS/m)	Relative growth rate (RGR)	
		Kuliyadichan	CO 43
1	4 (control)	$2.01_{a} \pm 0.03$	$1.70_{a} \pm 0.07$
2	8	$1.63_{b} \pm 0.03$	$1.45_{a} \pm 0.08$
3	10	$1.28_{c} \pm 0.05$	$0.81_{b} \pm 0.06$
4	12	$0.76_{d} \pm 0.15$	$0.50_{b} \pm 0.11$
	C D at 0.05%	0.120	0.132
	C D at 0.01%	0.175	0.201

Data represent the mean of the replicates± S.E values. Mean followed by the same letter in the same column does not differ according to post hoc test Tukey HSD analysis at the level of 5% significance

Fig.1 Effect of gamma irradiation on the embryonic calli of rice. (I)Embryonic calli formation at 10th day after inoculation of seed (A) Kuliyadichan and (B) CO 43.(II)30Gy gamma irradiated callus after 26 days (C) Kuliyadichan and (D) CO 43.(III)Regeneration of 30 Gy gamma irradiated callus after 26 days (E) Kuliyadichan and (F) CO 43

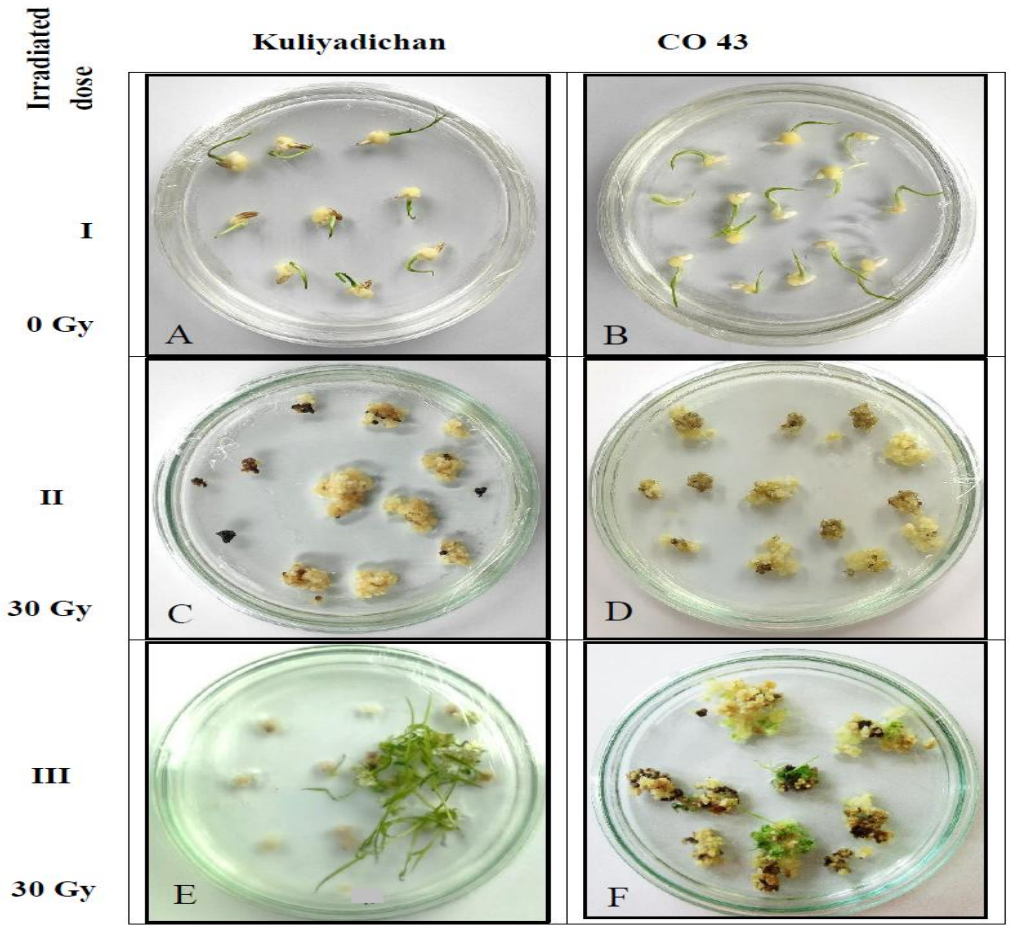


Fig.2 Radiosensitivity assay of irradiated embryonic calli based on relative differentiation rate from survival percentage of callus (A) Kuliyadichan and (B) CO 43, and from regeneration percentage (C) Kuliyadichan and (D) CO 43

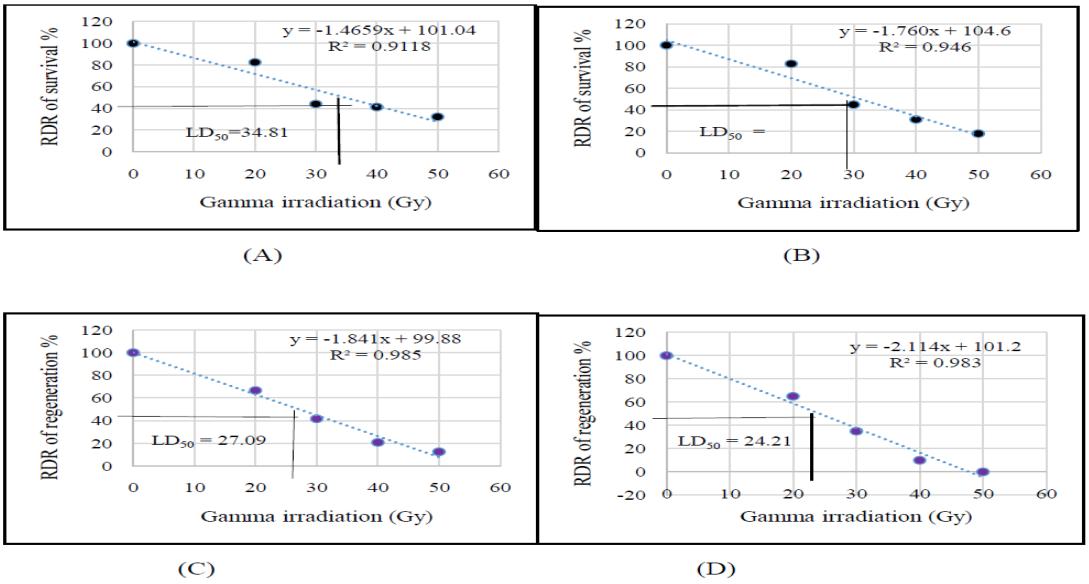


Fig.3 Effect of NaCl on the embryonic calli of rice.(I)Morphology of embryonic calli after 3 weeks in non-stress (control) medium. (A) Kuliyadichan and (B) CO 43 and(II) in 10 dS/m Nacl stress medium (C) Kuliyadichan and (D) CO 43.(III) Regeneration of embryonic calli in 10 dS/m NaCl stress media after 3 weeks (E) Kuliyadichan and (F) CO 43

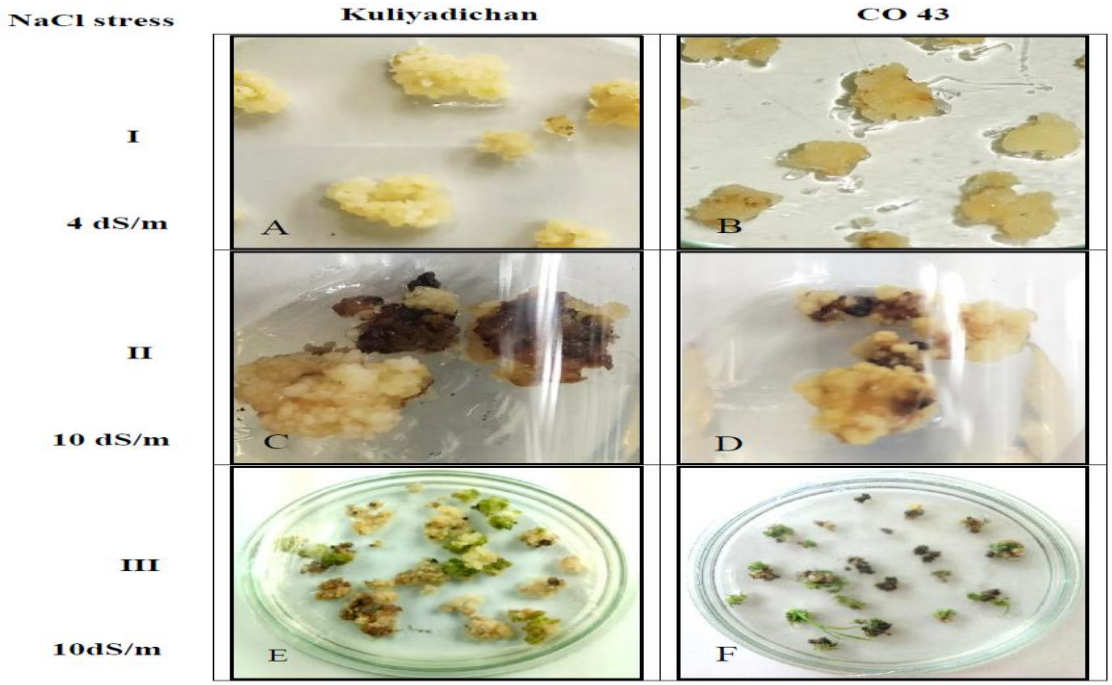
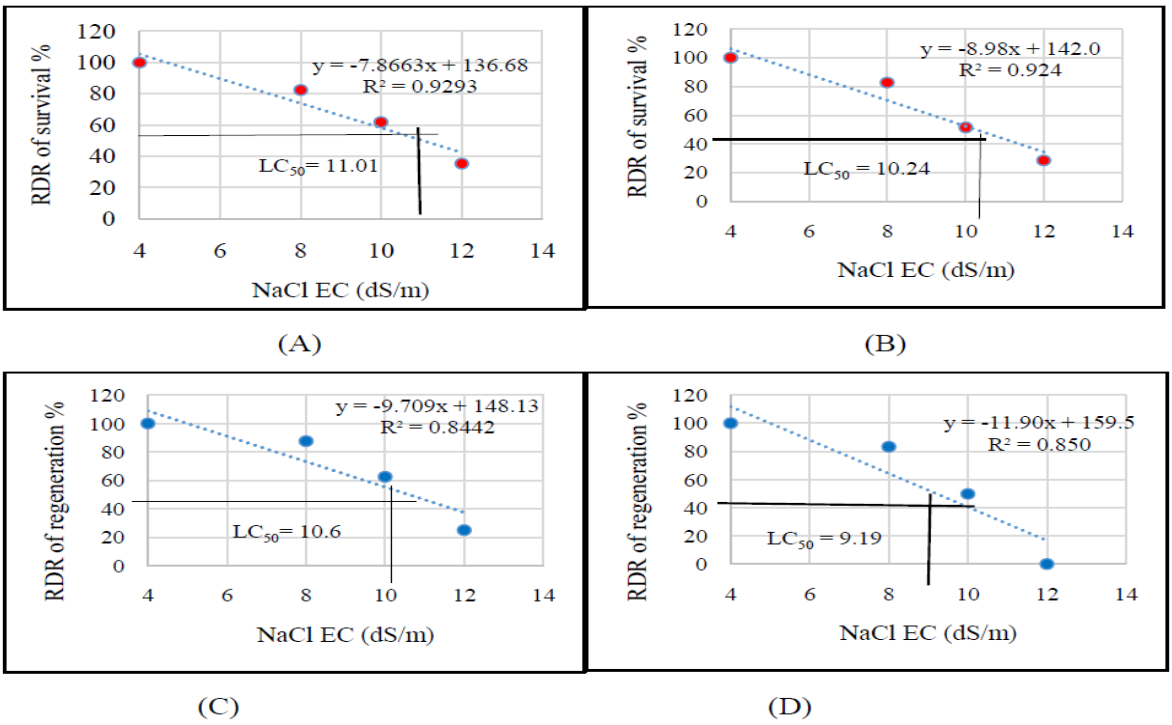


Fig.4 Salt sensitivity assay for embryonic calli based on relative differentiation rate from survival percentage of callus (A) Kuliyadichan and (B) CO 43, and from regeneration percentage (C) Kuliyadichan and (D) CO 43



***Invitro* growth response of irradiated callus**

A gradual decrease in the fresh weight of callus was observed in the irradiated calli when compared to control. In this study, the relative growth rate of callus showed a decreasing trend as the irradiation dose increased from 0 to 50Gy, and a significant difference was observed among each genotypes. In the two rice cultivars, the RGR was higher in control and lower in calli irradiated with 50 Gy dose (Table 3).

In this case, the radiation has a strong influence on callus growth and development. Physiologically gamma rays affect the cell wall and cell membrane and may cause changes in callus tissues. The callus growth inhibition at higher doses may be attributed to the cell cycle arrest at the G2/M phase during the somatic cell division or due to damage in the entire genome (Preuss and Britt, 2003)

The callus proliferation was reported to be affected in sugarcane (Nikam *et al.*, 2015) (Patade *et al.*, 2008), Gerbera (Hasbullah *et al.*, 2012) and *Phaseolus vulgaris* (Bajaj *et al.*, 1970). According to (Nikam *et al.*, 2015) a 50% reduction of callus growth was observed when exposed to low dose gamma irradiation of 10 and 20 Gy (Khalil *et al.*, 2015) observed a significant reduction of callus proliferation in the irradiated callus of *Stevia rebaudiana* when compared to the untreated callus culture.

Regeneration response of irradiated calli

The regeneration percentage was calculated based on the emergence of green shoots with shoot length more than 3cm. The regeneration percentage of the plants decreased with the increasing radiation dose. Among the two varieties, the highest regeneration percentage of 68.57 % was observed in Kuliyadichan

followed by CO 43 with 57.14 %in calli without gamma irradiation. Similar to the callus survival percentage, increasing the gamma irradiation dosage resulted in reduction of regeneration percentage. About 50% reduction of regeneration percentage was observed based on RDR at a dosages between 20-30Gy in the two experimentally taken cultivars (Fig. 1E-F).

Moustafa *et al.,* (1989) observed decreased plant regeneration capacity with the increasing levels of radiation dosage and 50% inhibition was found at 25Gy. (Patade *et al.,* 2008) also reported better regeneration potentials of non-irradiated embryonic callus of sugarcane than the irradiated callus. Saif *et al.,* (2001) obtained only 4-5% regeneration frequency when sugarcane and potato calli were irradiated with 60Gy dose.

In addition, abnormality in plant shoot growth like weak culm and albino were observed in the calli irradiated above 30Gy radiation. (Hasbullah *et al.,* 2012) observed in the callus tissues, the loss of capacity to differentiate and form shoots when irradiated with a higher dose of gamma irradiation.

The *invitro* regenerated shoots were transferred to half MS media for the development of roots. The irradiated calli took 30-40 days for sufficient root growth before the hardening process. Some of the plants of around 1-2% showed poor or no root formation. After the complete plant establishment, they were maintained in liquid half MS medium without hormones for one week and then transferred to the greenhouse for hardening

Determination of optimal dose of selection factor for callus to salt tolerance

Increasing the salt stress in media decreases the survival and relative growth rate of embryonic calli. The relative differentiation rate of embryonic calli based on survival percentage of callus and regeneration capacity in different salt stress media is shown in (Table 4 and 5). Two rice cultivars Kuliyadichan and CO 43 showed different sensitivity to NaCl. The color of the calli turned to brown or black when exposed to increasing concentration of NaCl in the culture media (Fig. 3A-D).

The linear regression equation for two different varieties based on the relative differentiation rate of survived calli and regeneration is shown in the Figure 4. The optimal dose of selection factor (LC_{50}) for the variety Kuliyadichan and CO 43 was fixed as 11.01 EC and 10.24 EC respectively. Similarly, the LC_{50} based on regeneration percentage was fixed as 10.6 EC for variety Kuliyadichan and 9.19 EC for variety CO 43.

Increasing the NaCl level in the medium and the consequent nature of ionic osmoticum accounts for the decrease in the percentage of survival of callus. Callus browning and necrosis increased when NaCl concentration was elevated.

This could be due to the genotype effect and low osmotic potential of cells that create differences in the survival percentage within the genotypes (Haque *et al.,* 2017). Rattana *et al.,* (2015) reported that the calli showed a decrease in the survival percentage when exposed to increasing NaCl concentration in culture media.

The callus browning is the indicator of tissue damage (Wu *et al.,* 2005) and the abiotic and biotic stress induce the synthesis of phenolic compounds that may result in callus browning (Koc *et al.,* 2009). The influence of salinity on callus viability is greatly influenced by genotype (Haque *et al.,* 2017).

In vitro callus growth response under NaCl stress

The addition of NaCl in the culture media caused a decrease in callus growth in the two genotypes and a significant difference was noticed in variety Kuliyadichan. Table 6 shows the effect of increasing salt stress on the relative growth rate of callus. In each variety, the relative growth rate of control was higher than the calli exposed to increasing concentrations of NaCl stress with electrical conductivity of 4 (control),8, 10 and 12dS/m.

In the present study, the RGR was decreased with the increasing NaCl concentration. (Haque *et al.,* 2017) reported this phenomena of reduced relative growth rate in NaCl stress might happen due to the reduction of water availability and loss of turgor pressure in the cells. A similar result was reported by (Sankepally *et al.,* 2016) and (Lutts *et al.,* 1998) in rice and (Patade *et al.,* 2008) in sugarcane.(Sankepally *et al.,* 2016) observed reduction of callus growth in six cultivars of rice when exposed to different NaCl concentrations of 50, 100, 150, 200, 225, and 250 mM in culture media. Reddy and Vaidyanath (1986) observed decline in the fresh weight of callus from 212mg in control condition to 120mg in 2% NaCl stress condition in culture media. Shankhdhar *et al.,* (2000) observed decrease in the fresh weight of callus with increasing salt concentration and reported that the reduction of water availability might be a reason for the decrease in fresh weight with an increase in salt concentration.

Invitro regeneration response under NaCl stress

The inherent capacity of the rice calli to regenerate in salt-stress conditioned tissue culture medium was evaluated on the basis of regeneration potential. The regeneration percentage of calli was decreased in linear fashion with increase in salt stress (Table 5). Normal plant regeneration was observed in media without NaCl (control), but increased NaCl concentration in the medium decreased the regeneration capacity in rice varieties Kuliyadichan and CO43 (Fig.3E-F). Regeneration percentage of rice variety Kuliyadichan was 80% at4 dS/m (control) but further decreased to 50% in 10dS/m of NaCl and to 20% in 12dS/m of NaCl. In the rice variety CO43, the plant regeneration on 4 dS/m (control) was 60% which further decreased to 30% on 10 dS/m of NaCl. No regeneration was found in medium with 12 dS/m NaCl stress. Similar result of decreased regeneration capacity of rice callus when exposed to increasing concentration of NaCl stress was reported elsewhere (Yunita *et al.,* 2014) and (Shanthi *et al.,* 2010). (Aditya and Backer, 2006) reported that the reduction of differentiation capability was due to presence of NaCl in the regeneration medium.

In conclusion the overall study on the effect of gamma irradiation and NaCl treatment on the embryonic calli survival, callus growth and regeneration of two rice varieties, Kuliyadichan and CO43 concluded that the variety CO43 was more radiosensitive and salt sensitive than the variety Kuliyadichan. The callus survival, callus growth and regeneration were profoundly affected by gamma irradiation and NaCl in both varieties with linear fashion of increasing dose and NaCl stress.

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