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Somatic embryogenesis and Plantlet Regeneration under Salinity Stress Conditions in Mature Caryopsis Culture of Indian Red Rice (*Oryza sativa* L.) and Assessment of Genetic Fidelity in Regenerants

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The main objective of this study was to establish the stable and efficient protocol for somatic embryogenesis and plantlet regeneration under NaCl-salt stress conditions in traditional Indian red rice (cv. Poongar) using mature caryopsis as explant and also to assess the genetic fidelity in regenerated plantlets. High frequency (98.88±1.33%) of callogenesis was recorded in MS (Murashige and Skoog, 1962) medium fortified with 10µM of 2,4-D (2,4-Dichlorophenoxyacetic acid) while maximum percentage (95.0±3.9%) of somatic embryogenesis and also maximum number (47.3±3.9) of somatic embryo per callus was recorded in presence of 5µM of 2,4-D alone. Furthermore, maximum frequency (85.33±2.02%) of germination of somatic embryos into plantlets was found in MS medium supplemented with 2.0mg/L of IAA (Indole-3-acetic acid), 3.0mg/L of BAP (6-Benzylaminopurine) and 1.0mg/L of Kn (Kinetin). During the salinity stress treatments, various concentrations (10mM, 25mM, 50mM and 100mM) of NaCl were used in this study in order to induce somatic embryogenesis and plantlets regeneration. Significantly, 100mM of NaCl shows the strong inhibitions for callus induction (26.67±0.63%) and somatic embryogenesis (31.3±1.8%), moreover, weight of the callus was also found to get decreased (94±0.5mg) than control (156±4.3mg) treatment. Interestingly, the highest concentration 150mM of NaCl was proved to be completely inhibitory and <1% of callogenesis was recorded. Moreover, due to the inhibitory response of NaCl, number of somatic embryos per callus was also decreased to 12.7±0.9 whereas the low concentration (10mM) of NaCl proves to be the little inhibitory and thus maximum frequency (91.1±2.33% and 67.5±2.33%) for both callogenesis and embryogenesis respectively. To produce salinity tolerant plantlet, the somatic embryos were transferred to regeneration medium supplemented with respective concentrations of NaCl. The high concentration (100mM) of NaCl causes very low percentage (20.7±0.6%) of regeneration of somatic embryo whereas the very low concentration of NaCl (10mM) supports the high frequency (74.52±1.2%) of plantlet regeneration. These salinity tolerant regenerated plantlets were later aacclimatized into autoclaved soil mixed with vermiculite at the ratio of 3:1. Furthermore, RAPD / ISSR-PCR profiles were analyzed to confirm genetic homogeneity between *in vitro* regenerated control plantlet and salinity tolerant plantlet. Among the four primers tested, all the primers could show clear polymorphic DNA bands. Hence, the stress tolerant plant and control plantlets are probably not homogenic in nature.

Key words: Genetic fidelity, Mature Caryopsis, Red rice, Salinity, Somatic embryogenesis

Rice is a staple and major source of food throughout the world which provides 50-80% of calories of their daily calories (Khush, 2005). Poongar rice is also called as red rice or The Women Rice. Comparing to white rice, red rice is rich in vitamins, minerals, amino acids, proteins, lipids and phenolics which consumed as whole grain (Min *et al*., 2011; Santos *et al*., 2021; Peng *et al*., 2021). Red rice prevents cancer, diabetic and improves anti-inflammation properties through antioxidant compounds (ferulic acid, coumaric acid and anthocyanins) and phenolic compounds which are present in it (Xu *et al.,* 2001; Rohrer and Siebenmorgen, 2004). Significantly, consuming red rice reduces the risk of chronic diseases and cancer (Mbanjo *et al.,* 2020) and also improves the level of haemoglobin.

During the process of soaking, the nutrient from the husk gets absorbed by rice grain which increases the nutrient value and anti-oxidant property of rice (Komatsuzaki *et al.,* 2007; Rahman, 2019). Some of the *indica* and *japonica* varieties have been considered as recalcitrant rice crops in terms of callogenesis (Sahoo *et al.,* 2011). Large number of plantlets could be produced by using PGRs through callogenesis and plantlet regeneration. These PGRs play vital role in cell division and differentiation during somatic embryogenesis (Kumar, 2016; Mostafiz *et al.,* 2018).

Previous studies also reveal on *in vitro* regeneration in different pigmented rice using mature caryopsis culture (Diawuoh *et al.,* 2016, Thi Linh *et al.,* 2017, Sedeek *et al.,* 2024), anther culture of Indonesian *indica* black rice (Maharani *et al.,* 2020), mature embryo culture (Artadana *et al.,* 2017).

Due to biotic and abiotic stresses, the productivity of rice gets decreased which causes the significant deficit in required supply (Sankar *et al.,* 2011). Salinity is one of the major factors which affects the productivity of rice in saline prone areas throughout the world (Lee *et al.,* 2003). In order to increase the yield of rice crops around saline prone areas especially coastal areas, *in vitro* regenerated stress tolerant crops have been produced. Many literatures are available on production of salinity tolerant plant through tissue culture techniques on white rice cultivar (Binh *et al.,* 1992, Htwe *et al.,* 2011; Siddique *et al.,* 2014; Taratima *et al.,* 2022).

Since literature on establishment of somatic embryogenesis and plant regeneration under salinity stress conditions in Indian red rice is lacking completely, so present study was aimed to optimize an efficient and stable protocols for the induction of somatic embryo and plantlet regeneration in Indian red rice (Poongar) using mature caryopsis explant tissues under salinity stress conditions.

MATERIALS AND METHODS

Source of Explant and Sterilization

Dry and healthy mature caryopses of Poongar, Indian red rice were collected from Perunthalaivar Kamaraj Krishi Vigyan Kendra (PKKVK), Puducherry (India). These seeds of red rice were de-husked manually after drying and washed to remove dust from the de-husked seeds. Washed seeds were further treated with tween 20 (teepol) for 10 mins followed by proper wash under running tap water to remove the detergent traces. Seeds were later surface sterilized with 70% ethyl alcohol (V/V) for 30 sec under laminar air flow to remove the microbes from the caryopsis explant tissues.

Finally, the seeds were treated with 0.1% of $HgCl_2$ (W/V) about 10mins to remove endophytes from the seeds followed by washing with sterilized distilled water for 2 times under aseptic conditions (under laminar air flow chamber). The sterilized seeds were finally dried on sterile filter paper to remove the excess water on the surface of the seeds to avoid water born contaminations.

Callus Induction Medium

For induction of callus, the sterilized mature caryopsis was inoculated on the MS Medium (Murashige and Skoog, 1962) supplemented with 30g/L of sucrose (W/V), 8g/L of agar as gelling agent in presence of various concentrations of auxin 2,4 D (5 µM, 10 µM, 20 µM, 30 µM and 40 µM). Finally, the pH of the nutrient media was adjusted to 5.5-5.8 and were autoclaved at 121°C at 15 psi for 20 min.

After sterilization of nutrient medium, the sterilized explants were transferred into the fresh medium under laminar air flow. The inoculated seeds were incubated

for 21-days at 28±2°C under dark condition for induction of callus from the explant.

Determination of Fresh Callus Weight

After 21-days of culture initiation, incubated calli from the callogenic media were used to determine the fresh weight of the calli and 10 identical calli from different treatments were used to calculate the mean fresh weight of the callus.

Embryogenic Induction Medium

The callus from the explant was removed and inoculated into the MS medium which contains 30g/L of sucrose as carbon sucrose and 0.8% of agar included with various concentrations of PGRs (2.5 µM and 5µM of 2,4-D alone, 5µM of 2,4-D with 1.0µM of Kn and 10µM of 2,4-D with 2.0µM of Kn). Furthermore, sub-cultured calli were incubated under dark conditions for 14 days at 28±2°C for proliferation of embryogenic callus.

Plantlet Regeneration Medium

Healthy embryogenic calli were transferred into regeneration medium which contains MS-salts, 30gm/L of sucrose, 8.0gm/L of agar and various combinations of plant growth regulators (PRM1-PRM6) Further, cultures were incubated at 28±2°C and light intensity of 1500-1600 lux 16 hours light for 30-days.

Salinity Stress Treatment

To understand the effects of salinity stress on callogenesis, the sterilized explant was inoculated into the nutrient medium with optimal concentrations of nutrient medium with sucrose, agar and 2,4-D along with different concentrations of sodium chloride (10mM, 25mM, 50mM, and 100mM) and without NaCl as control which were inoculated under dark conditions for 21-days.

The calli from the explants were transferred into the optimal concentration of embryogenesis induction medium and regeneration medium with corresponding stress conditions of NaCl for embryogenesis and regeneration of plantlets from the embryogenic callus.

Acclimatization

In vitro plantlets were removed from the medium and washed thoroughly using sterilized water to remove the adhering medium on the root which were transferred into the pot which contains autoclaved soil and vermiculite at the ratio of 3:1 and covered with polyethylene bags followed by irrigation with the same salt solutions regularly.

Isolation of DNA

The genomic DNA of *in vitro* regenerated plantlets of Poongar (Red Rice) was isolated from the leaf tissues which were grown with and without NaCl stress treatments by using CTAB method (Doyle and Doyle, 1990). Moreover, agarose (0.8%) gel electrophoresis was later used to identify the quality of isolated DNA.

RAPD, ISSR- PCR Analysis

To identify the homogeneity of stress tolerant plant and *in vitro* regenerated control plantlets, RAPD (OPA 1, OPA 4 and OPA 5) and ISSR (UBC 823) primers were employed for PCR amplification that were carried out in PCR Thermal Cycler (GeneAmp PCR).

Gel loading dye (6X) added to the PCR products were analyzed by 1.2% agarose gel electrophoresis in 0.5 X TBE buffer which contains ethidium bromide (0.5µg/ml). The PCR products were loaded into the well and the electrophoresis was completed at 75V for 1 hour. Moreover, 2-log DNA ladder (NEB) was used as molecular standard. Electrophorized gel was visualized under UV transilluminator (Genei) and the image was captured under UV light using gel documentation system (Bio-Rad).

Statistical Analysis

The experiments were repeated for three times each with 10 replicates. During the entire experiments, various traits in terms of percentage of explants showing callogenesis, percentage of callus shows somatic embryogenesis, frequency of plantlets regeneration, mean number of plantlets per callus and callus morphology with browning percentage were calculated.

Statistical data were performed by IBM SPSS Statistics 22, Duncan's Multiple Range Test (DMRT) and ANOVA were performed at 5% level of significance.

RESULTS

Induction of Callus

In the present study, among all the concentrations of 2,4-D, the maximum amount of callus was found with the lower concentration of 2,4-D (10µM), which shows the highest percentage of callogenesis (98.88±1.33%)

exhibited creamy, white and compact callus formation **(Fig. 1B & Table 1)**. Significantly, the maximum callus weight (156±4.3mg) was also recorded with the same treatment containing lower concentration of 2,4-D (10µM) whereas with the control experiment in basal medium, mature caryopsis was failed to show callus induction instead simple germination of mature caryopsis **(Fig. 1A)** was observed and thus, nutrient medium without 2,4-D was found to be non-responsive in terms of callogenesis even after 21-days of culture initiation.

However, MS-medium supplemented with the increased concentration of 2,4-D (20µM), the percentage of explants showing callus induction was decreased to 70.0±1.88% and exhibited friable and white callus in appearance **(Fig. 1C)** and also, the fresh weight of the callus was recorded to be declined as 120±2.6mg. Moreover, with the further increase in the concentrations of 2,4-D (30µM and 40µM) **(Fig. 1D & E)** respectively**,** the percentage of callogenesis was obtained as (46.67±1.96% and 23.3±1.01%) respectively **(Table 1)**.

Similarly, fresh weight of the calli were also decreased (103±1.7mg and 93±0.4mg) respectively with the increase in 2,4-D concentrations in the nutrient media. The nature of the callus was also observed to be affected and turns to pale yellow after 21-days of incubation.

Induction of Somatic embryogenesis

Healthy calli were sub-cultured into the embryogenic induction medium, the maximum percentage (95.0±3.9%) of somatic embryogenesis was found with 5µM of 2,4-D alone **(Fig. 2 B)** and the mean no. (47.3±3.9%) of somatic embryos per callus **(Table 3)** while it was followed by 10µM of 2,4-D in combination with 1.0µM of Kn **(Fig. 2C)** in terms of percentage of somatic embryogenesis (51.33±3.1%) and mean number of somatic embryos (13.7±0.6) per callus **(Table 3)** after 14-days of incubation. Moreover, reduction in frequency of somatic embryogenesis was obtained with the increase in Kinetin (2.0µM) concentration with 2,4-D (10µM) and therefore, 31.67±2.9% of somatic embryogenesis was recorded at 10µM of 2,4-D with 2.0µM of Kn and number of somatic embryos per callus (11.2±0.51) was also reduced **(Fig. 2D)**.

In contrast, basal medium shows the minimum percentage of somatic embryogenesis and no. of somatic embryos per callus (27.9±2.3% and 7.3±0.31%) **(Fig. 2A & Table 3)** respectively.

Effects of Salt Stress on Callogenesis and Embryogenesis

There is significant difference between control and salt treated callus. At lower concentration of NaCl (10mM), induced callus was white and compact. A significant reduction in percentage of callogenesis (91.1±2.33%) **(Fig.1F & Table 2)** and mean callus weight (145±0.8mg) was recorded after 21-days of incubation. The salt -treated calli were embryogenic and shows small, white nodular structure and maximum percentage of embryogenesis was recoded as 67.5±2.33% **(Fig. 2E & Table 4)** whereas control shows somatic embryogenesis (95.0±3.9%) after 15-days of sub-culture.

However, the higher concentration (100mM) of NaCl is lethal and shows least percentage of callogenesis (26.67±0.63%) **(Fig. 1I & Table 2)** and somatic embryogenesis (31.3±1.8%) **(Fig. 2H &Table 4)**. Significantly, further increase in concentration of NaCl (150mM) was proved to be completely lethal and the calli were necrosed, whereas lower concentrations (25mM and 50mM) of NaCl could show relatively high (76.67±3.36% and 62.23±1.09%) percentage of callogenesis **(Fig. 1G & H)** respectively **(Table 2)** and also for somatic embryogenesis (52.9±1.8% and 45.6±2.3%) respectively after 15-days of sub-culture **(Fig. 2F & G)** respectively **(Table 4)**.

There is significant difference between control and salt-stress treated cultures at 5% level of significance (p=0.05). the mean percentage of callogenesis, callus weight and somatic embryogenesis was affected to (91.1±2.33%, 145±0.8mg and 67.5±2.33) in control treatments (98.88±1.33%, 156±4.3mg and 95.0±3.9) respectively **(Tables 2 & 4)**.

Plantlet Regeneration

Two weeks old embryogenic calli were sub-cultured into the various combinations of auxin and cytokinins,

among all the combinations tested, nutrient medium (PRM6) contained with IAA (2.0mg/L) + BAP (3.0mg/L) + Kn (1.0mg/L) shows maximum percentage of plantlet regeneration (85.33±2.03%) **(Fig. 3B & Table 5)**. Moreover, percentage of callus necrosis was also exhibited to be low (10%) when compared to other combinations of PGR, followed by the medium containing 2.0mg/L of IAA +2.0mg/L of BAP + 0.5mg/L of Kn (PRM5) shows 63.3±1.76% **(Fig. 3A &Table 5)** of plantlet regeneration.

There is significant difference between these two different combinations of nutrient media in terms of frequency of plantlet regeneration and percentage of callus necrosis. 57.0±1.67% of plantlet regeneration was recorded with PRM4 and mean number of regenerated plantlets (12.3±0.3) per embryogenic callus **(Table 5)** was recorded.

Significantly, with the medium containing 2.0mg/L of IAA, 3.0mg/L of BAP, and 1.0mg/L of Kn (PRM6), mean number of plantlets (34.3±1.3) per callus was obtained after 4-weeks of incubation **(Fig. 3B)**.

Effect of Salt Stress on Plantlet Regeneration

Embryogenic calli were sub-cultured into the respective (PRM6) plantlet regeneration medium (2.0mg/L of IAA, 3.0mg/L of BAP, and 1.0mg/L of Kn) with and without various concentrations of NaCl. Lower concentration of NaCl (10mM) shows 74.52±1.2% of plantlet regeneration and 23.3±1.8 tolerant plantlets per embryogenic callus **(Fig. 3C & Table 6).** It was followed by 25mM and 50mM of NaCl (65.5±1.5 & 16.67±1.18) and (49.3±0.97% & 12.33±0.67) respectively **(Fig. 3D & Table 6)**.

Moreover, in contrast, 85.33±2.02% of plantlet regeneration and 34.3±1.3 plantlet per callus was obtained in control experiment. Lowest Percentage of 20.7±0.6% was observed at higher concentration 100mM of NaCl **(Fig. 3E &Table 6)** after 30-days of sub-culture.

Genetic Fidelity analysis using ISSR and RAPD markers

The RAPD primers OPA-1, OPA-4, and OPA-5) and ISSR primer (UBC 823) each was seen to amplify in forms of several bands in NaCl (100mM) treated samples and in control regenerants as well **(Table 7).** Every RAPD amplified band was evident in between the ranges from 100bp-3Kb while ISSR bands were visible in range of <3Kb and also exhibit polymorphisms leading to genetic dissimilarities **(Fig. 4A-D**). The RAPD primer OPA-1 could amplify overall 10 scorable bands. Significantly, 7 among 10 were observed to be polymorphic and shows 70% of polymorphism rate **(Fig. 4A)**. The maximum rate of polymorphism (86%) was exhibited by the primer OPA 4 in which the numbers of amplicons were 7 and 6 showing the genetic heterogeneity **(Fig. 4B)**.

Similarly, the OPA 5 band produces around 6 amplicons among 5 are polymorphic bands and rate of genetic dissimilarities was observed to be 83% **(Fig. 4C & Table 7).** Moreover, the ISSR Primer UBC 823 shows 4 polymorphic band among 5 shows 80% genetic dissimilarity **(Fig. 4D & Table 7)** Hence, the salinity tolerant plantlet was treated as genetically altered and exhibits the polymorphism in order to overcome the salinity stress condition compared with control plantlet. Moreover, probably in order to adapt the NaCl stress the regenerated plantlet exhibits genetic differences and shows polygenic in nature.

Table 1: Poongar- Red rice (*Oryza sativa* L.); effects of various concentrations of auxin 2,4-D on callus induction in mature caryopsis culture after 21-days of culture initiation. Significant at 5% level of significance. Table indicates the same letters in a column, don't differ significantly as per Duncan Multiple Range Test (DMRT) calculation.

Concentration of 2,4-D (µM)	Callogenesis (% Mean±SE)	Callus Weight (mg) (Mean±SE)	Callus Morphology
0	0	0	Seedling germination
5.0	9.13±0.86 [a]	20±0.19[a]	Seed germination with little callus
10	98.88±1.33[e]	156±4.3[e]	Compact, Creamy, White
20	70.0±1.88[d]	120±2.6[d]	Friable, Creamy, White
30	46.67±1.96[c]	103±1.7[c]	Pale Yellow
40	23.3±1.01[b]	93±0.4[b]	Pale Yellow

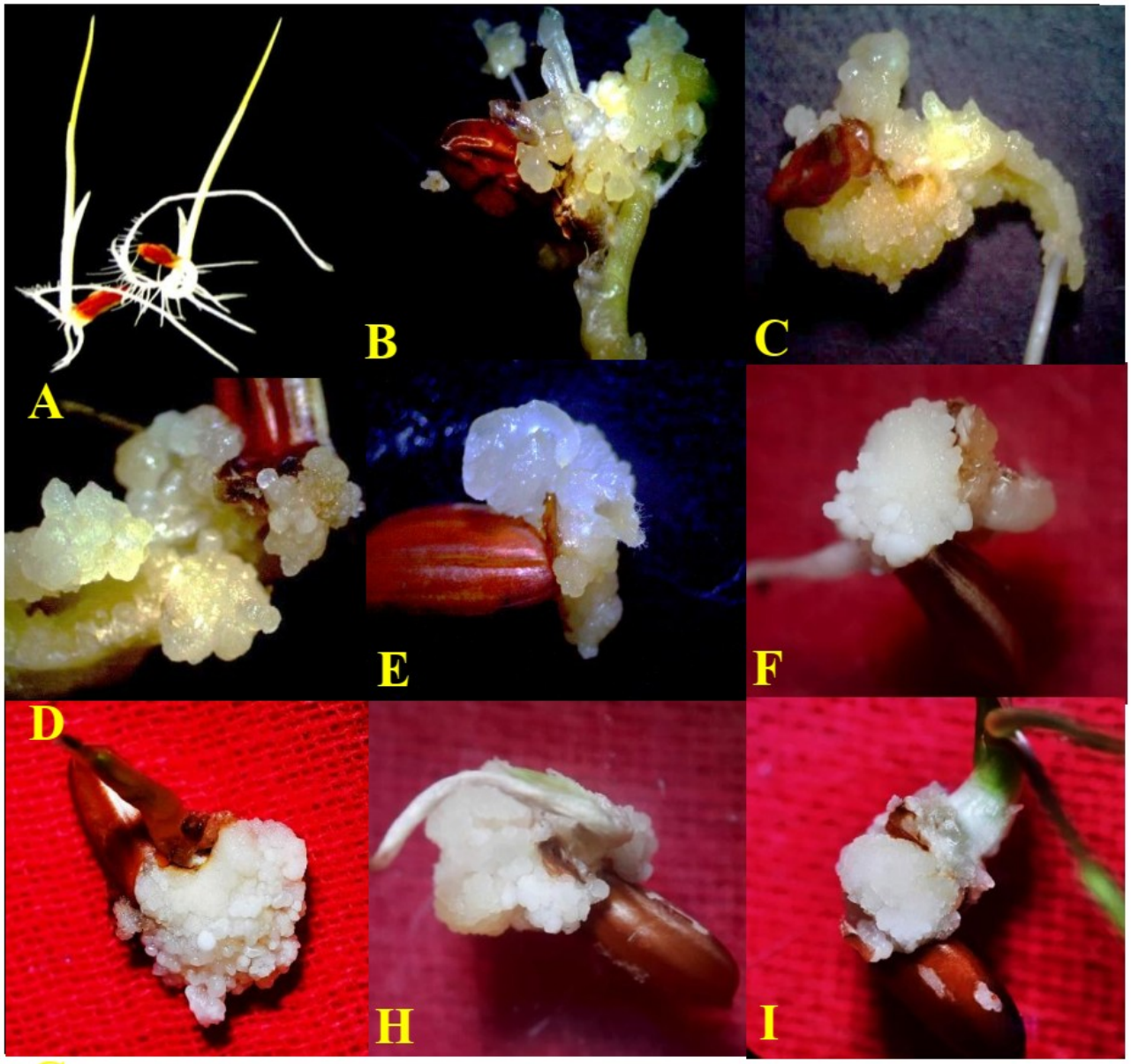

Figure 1. Poongar-Red rice (*Oryza sativa* L.)**;** Mature caryopsis culture showing effects of NaCl (salinity stress) on callogenesis; **(A)** Control **(B)** 2,4-D (10µM) **(C)** 2,4-D (20µM) **(D**) 2,4-D (30µM) **(E**) 2,4-D (40µM) **(F**) 2,4-D (10µM) + 10mM of NaCl **(G)** 2,4-D (10µM) + 25mM of NaCl **(H)** 2,4-D (10µM) + 50mM of NaCl **(I)** 2,4-D (10µM) + 100mM of NaCl salt treatments (after 21-days of culture initiation).

Table-2: Poongar- Red rice (*Oryza sativa* L.), effects of salinity stress (NaCl) on frequency of callogenesis and mean weight of the callus in mature caryopsis culture after 21-days of culture initiation. Significant at 5% level of significance. Table indicates the same letters in a column, don't differ significantly as per Duncan Multiple Range Test (DMRT) calculation.

Concentration of NaCl (mM)	Concentration of 2,4-D	Callogenesis (%Mean±SE)	Callus Weight (mg) (Mean±SE)
0	10µM	98.88±1.33 ^{e}	156±4.3 ^{e}
10		91.1±2.33^{d}	145±0.8 ^{d}
25		76.67±3.36^{c}	120±0.1 ^{c}
50		62.23±1.09^{b}	118±0.8 ^{b}
100		26.67±0.63^{a}	94±0.5 ^{a}

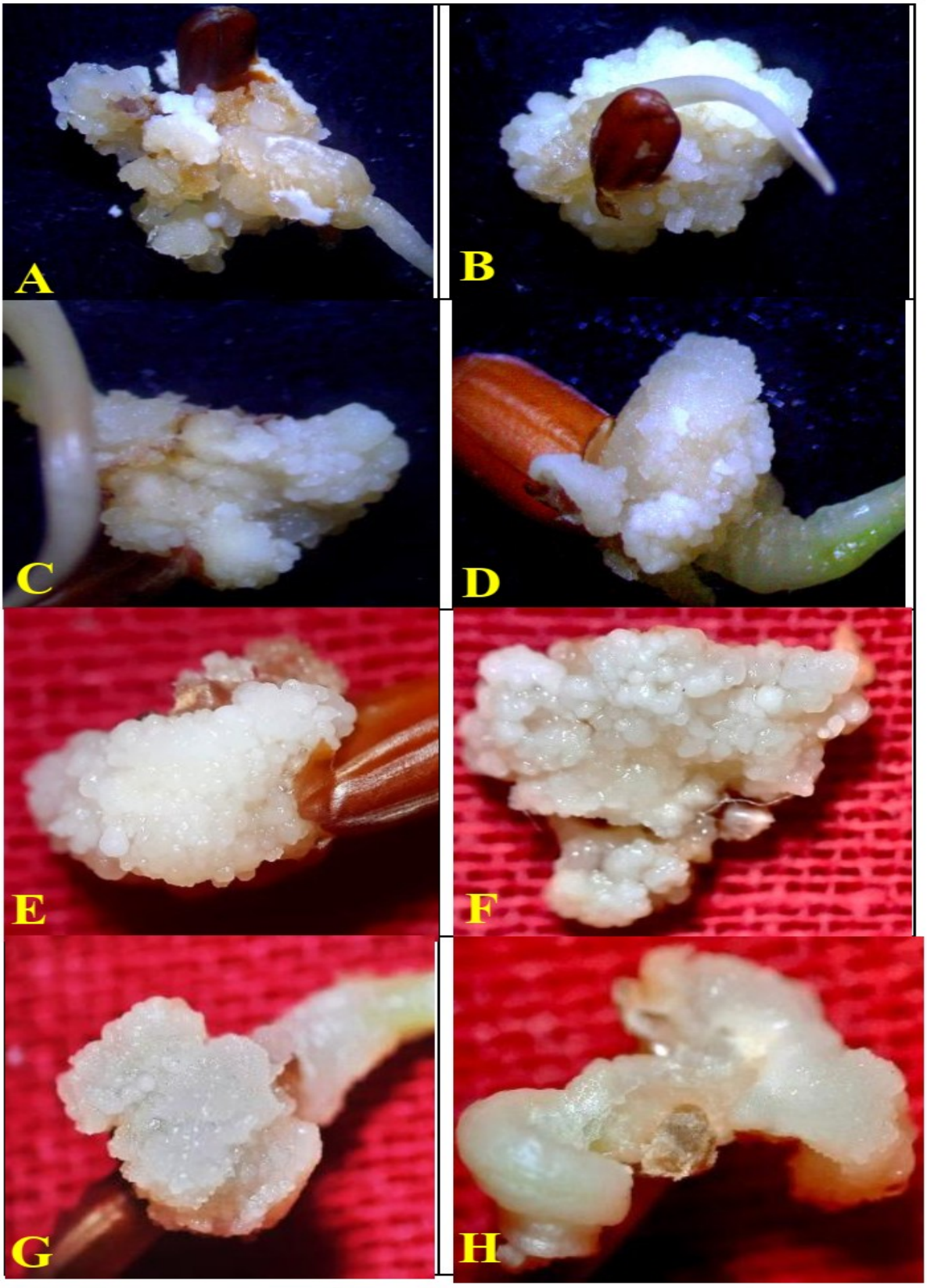

Figure 2. Poongar-Red rice (*Oryza sativa* L.)**;** Mature caryopsis culture showing effects of NaCl (salinity stress) on callogenesis; **(A)** Control **(B)** 2,4-D (10µM) **(C)** 2,4-D (20µM) **(D)** 2,4-D (30µM) **(E)** 2,4-D (40µM) **(F)** 2,4-D (10µM) + 10mM of NaCl **(G)** 2,4-D (10µM) + 25mM of NaCl **(H)** 2,4-D (10µM) + 50mM of NaCl **(I)** 2,4-D (10µM) + 100mM of NaCl salt treatments (after 21-days of culture initiation).

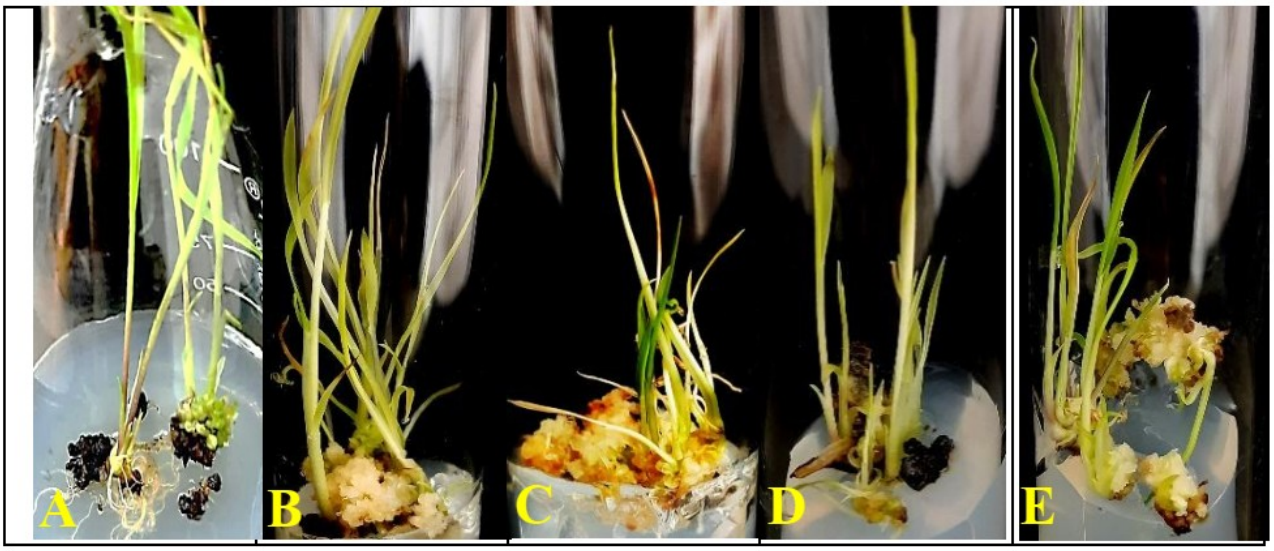

Figure 3. Poongar-Red rice (*Oryza sativa* L.)**;** Mature caryopsis culture showing effects of NaCl (salinity stress) on plantlet regeneration; **(A)** PRM5 **(B)** PRM6 **(C**) PRM6 +10mM of NaCl **(D**) PRM6 + 50mM of NaCl **(E**) PRM6 + 100mM of NaCl salt treatments (after 30-days of culture initiation).

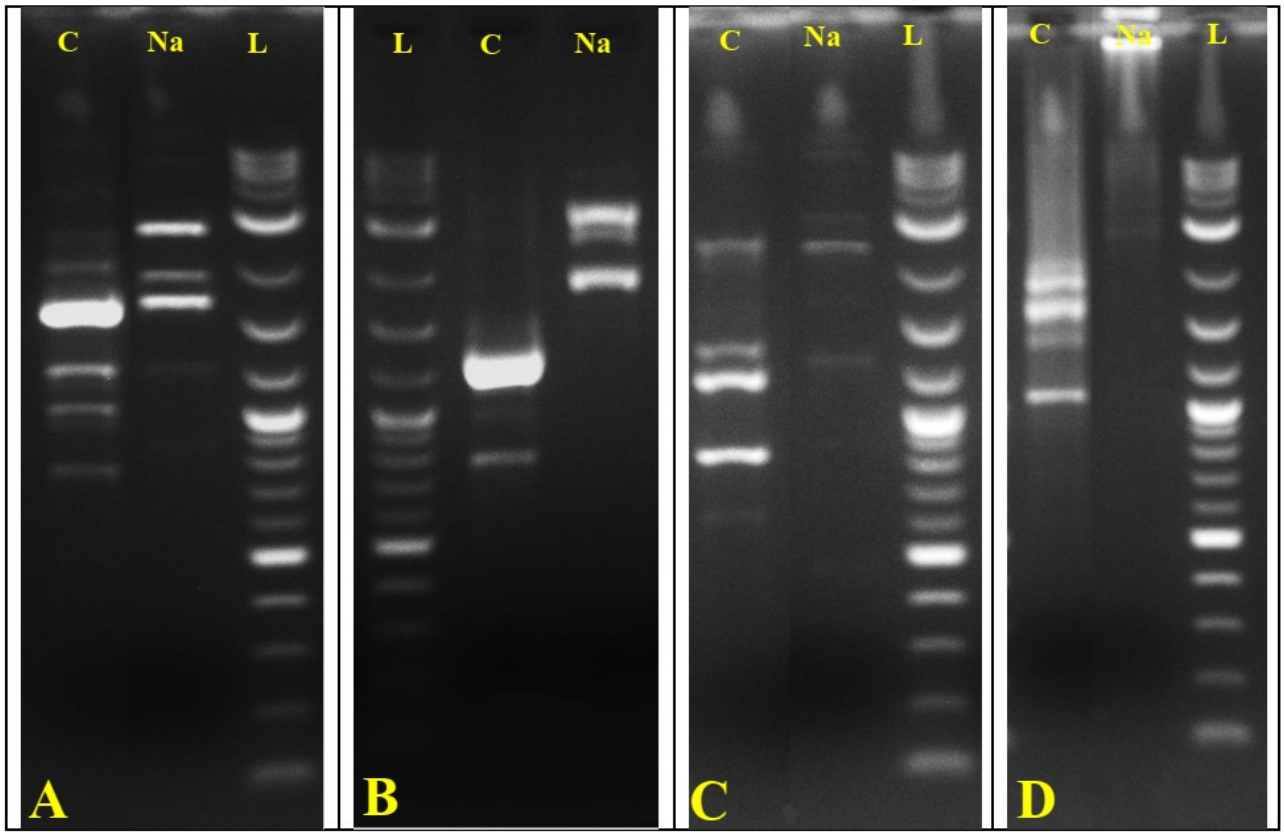

Figure 4. Poongar-Red rice (*Oryza sativa* L.)**;** RAPD and ISSR amplification profile of *in vitro* regenerated plantlets; Control (C), NaCl (Na), Ladder (L) using four different primers; **(A)** OPA-1 **(B)** OPA-4 **(C)** OPA-5 **(D)** UBC-823.

Table-3: Poongar- Red rice (*Oryza sativa* L.), effects of various concentrations of auxin (2,4-D) with various concentrations of cytokinin (Kn) on somatic embryogenesis after 14-days of sub-culture. Significant at 5% level of significance. Table indicates the same letters in a column, don't differ significantly as per Duncan Multiple Range Test (DMRT) calculation.

Combination of Auxin with Cytokinin		Somatic Embryogenesis (%±SE)	No. of Somatic Embryos/Callus (Mean±SE)
2,4-D (µM)	**Kn (µM)**		
0	0	27.9±2.3[a]	7.3±0.31[a]
2.5	0	40.21±3.5[c]	11.9±1.9[c]
5.0	0	95.0±3.9[e]	47.3±3.9[e]
10	1.0	51.33±3.1[d]	13.7±0.6[d]
10	2.0	31.67±2.9[b]	11.2±0.51[b]

Table-4: Poongar- Red rice (*Oryza sativa* L.), Exhibiting the impacts of salinity stress (NaCl) on frequency of somatic embryogenesis and number of somatic embryos per callus in mature caryopsis culture after 14-days sub-culture of embryogenic callus. Significant at 5% level of significance. Table indicates the same letters in a column, don't differ significantly as per Duncan Multiple Range Test (DMRT) calculation.

Concentration of NaCl (mM)	Concentration of 2,4-D	Somatic Embryogenesis (%Mean ± SE)	No. of Somatic Embryos/ Callus (Mean ± SE)
0	5µM	95.0±3.9[e]	47.3±3.9[e]
10		67.5±2.33[d]	31.2±2.5[d]
25		52.9±1.8[c]	22.9±1.9[c]
50		45.6±2.3[b]	18.6±1.5[b]
100		31.3±1.8[a]	12.7±0.9[a]

Table-5: Poongar- Red rice (*Oryza sativa* L.), effects of various concentrations of auxin (IAA) with various concentrations of cytokinins (BAP and Kn) on germination of somatic embryo and plantlet regeneration in mature caryopsis culture after 30-days sub-culture of embryogenic callus. Significant at 5% level of significance. Table indicates the same letters in a column don't differ significantly as per Duncan Multiple Range Test (DMRT) calculation.

	Combination of Auxin with Cytokinins (µM)			Plantlet Regeneration (%Mean±SE)	No. of Regenerated Plantlets/Callus (Mean±SE)	Necrosed Callus (%)
	IAA	**BAP**	**Kinetin**			
PRM1	0	2.0	0.5	10.7±1.2[a]	4±0.6[a]	70
PRM2	0	3.0	1.0	45.0±2.87[b]	7.3±0.3[b]	44
PRM3	1.0	2.0	0.5	52.0±2.30[c]	10±0.6[bc]	45
PRM4	1.0	3.0	1.0	57.0±1.67[d]	12.3±0.3[c]	39
PRM5	2.0	2.0	0.5	63.3±1.76[e]	27.7±0.3[d]	35
PRM6	2.0	3.0	1.0	85.33±2.03[f]	34.3±1.3[e]	10

Table-6: Poongar- Red rice (*Oryza sativa* L.), Exhibiting the impact of salinity stress (NaCl) on percentage of callus showing regeneration of plantlets, number of plantlets per embryogenic callus and frequency of necrosed callus after 30-days sub-culture of embryogenic callus. Significant at 5% level of significance. Table indicates the same letters in a column don't differ significantly as per Duncan Multiple Range Test (DMRT) calculation.

Concentration of NaCl (mM) + PRM6	Plantlet Regeneration (%Mean ± SE)	No. of Regenerated Plantlets/ Callus (%Mean ± SE)	Necrosed Callus (%)
0	85.33±2.02[e]	34.3±1.3[e]	10
10	74.52±1.2[d]	23.3±1.8[d]	19
25	65.5±1.5[c]	16.67±1.18[c]	23
50	49.3±0.97[b]	12.33±0.67[b]	45
100	20.7±0.6[a]	8.2±0.89[a]	64

Table-7: Poongar- Red rice (*Oryza sativa* L.), effects of RAPD and ISSR primers used for amplification for genetic fidelity assessment in control and salinity (100mM NaCl) tolerant plantlets.

Primers	**OPA 1**	**OPA 4**	**OPA 5**	**UBC 823**
Primer Sequence (5′-3′)	AATCGGGCTG	AATCGGGCTG	AGGGGTCTTG	AGGGGTCTTG
Total No. of Amplicons	10	7	6	5
Band length of Amplicons (bp)	100bp-3KB	100bp-3KB	100bp-3KB	<3KB
Monomorphic Bands	3	1	1	1
Polymorphic bands	7	6	5	4
Frequency of Polymorphism (%)	70%	86%	83%	80%

DISCUSSION

Approximate duration of Poongar red rice crop is 70-90 days suitable to grow in all seasons but most common period for cultivation is December to March. An average height of the crop is 129.9cm and produces 80 grains per ear head. Average yields of this crop are 600Kgs/acre grows in alluvial and clay soil (Karpagalakshmi *et al.,* 2021) mostly cultivated in southern part of India.

Effects of NaCl-Salt on Callogenesis

Callus is considered as an unorganized and undifferentiated mass of parenchymatous cells (Bhatia, 2015). Most commonly used synthetic hormone for callus induction is 2,4-D alone (Castillo *et al.,* 1998; Shahsavari, 2010), however, some of the other studies reveal that the combination of 2,4-D (3.0mg/L) with NAA (2.0mg/L) causes high percentage (up to 90%) of callogenesis (Trejo-Tapia *et al.,* 2002; Ali *et al.,* 2004; Din *et al.,* 2016). Since the variation in genotypes between different variety of rice is common, therefore, some of the hormonal combinations have been proved to be not suitable.

Significantly, in this study 2,4-D alone (10µM) is turned out to be more effective and thus, shows maximum mean frequency of callogenesis (98.88%) and fresh weight of the induced callus (156mg). In parallel, there is decrease in frequency of callus induction if the concentration of 2,4-D gets increased (Ramesh *et al.,* 2009). Depends on explants used, the nature of the callus differs and callus induction can also be affected due to pH, PGRs' light intensity etc. (Barman *et al.,* 2016),

The most commonly used medium for induction of callus and regeneration of callus are MS, LS, and N_6 (Pandey *et al.,* 1994) but MS medium shows the maximum frequency of callus induction and regeneration of embryogenic callus (Khanna and Raina, 1998). Simultaneously, mature de-husked seed has been emerged as a good source of explant for callogenesis (Ge *et al.,* 2006; Khaleda and Al-Forkan 2006). Callus from scutellum of the mature caryopsis is also proved as a suitable source of explant for *in vitro* regeneration (Dina *et al.,* 2016). Significantly, due to the availability of mature caryopsis throughout the year, mature caryopsis was used during present study in red rice.

In previously available report based on mature caryopsis culture, the mean weight of the callus was recorded to be lower (84.0mg) during 100mM of NaCl treatment in the cultivar BRRI38 whereas the control treatment shows relatively higher mean callus weight (97.0mg) while with further increase in concentration of NaCl (150mM), the weight of the callus was reduced to half of the weight (34.0mg) of callus that induced with 100mM of NaCl-treatment (Zinnah, 2013). With the increase in concentration of NaCl in nutrient medium, fresh weight of the callus decreases which indicates the negative effect of salinity stress (Shankhdhar *et al.,* 2000; Priya *et al.,* 2011).

Similarly, in case of the cv. Chinikanai, the weight of

the callus without salt stress was reported as 240mg which was later decreased to 129mg during the treatment with 100mM of NaCl stress. Significantly, in terms of callogenesis, the rice cv. Chinikanai tolerates up to 200mM of NaCl and produces the least callus weight (35.0mg) after one month of incubation (Zinnah *et al.,* 2013).

In presence of medium level of salt-stress, the yield gets decreased up to 60% (Zeng *et al.,* 2002). Furthermore, previous studies also indicate that addition of salt into the medium decreases the frequency of somatic embryogenesis and plantlet regeneration (Ping *et al.,* 2006, Tariq *et al.,* 2008). By ionic and osmotic mechanisms, the plant cell gets damaged through the ions Na^+ and Cl^- which are present in NaCl (Chinnusamy *et al.,* 2005).

Morphology of the calli at nutrient medium without stress were globular, creamy and friable which were getting worst when the concentration of NaCl getting increased (Rattana and Bunnag, 2015; Sidek *et al.,* 2024). Effects of salinity on callus induction were observed in terms of decreased size of callus, callus necrosis, and presence of large vacuoles cause unorganized meristematic zone (Atabaki *et al.,* 2018). In the Malaysian variety MARDI Siraj 297, the viability of callus was decreased and led to necrosis of callus at the concentration of 150mM. Hence, 100mM of NaCl was considered to be threshold level for callogenesis (Sidek *et al.,* 2024).

Additionally, previous studies reveal that 11.7g/L of NaCl shows reduced frequency of cell viability (45.33%) where control shows 85.33% in the cultivar BRRI dhan47 while the frequency get less viability (10.67%) in the BR10 cultivar. Moreover, BRRI dhan32 was completely affected and shows 0% of viable calli after 4-weeks of incubation (Siddique *et al.,* 2014).

Moreover, during this study in red rice cv. Poongar, lower concentration of 2,4-D (10µM) alone proves to be enough for induction of callus from mature caryopsis culture and shows 98.88± 0.86 percentage of callogenesis, while even concentration of NaCl (50mM) in presence of 2,4-D (10µM) was found to be inhibitory and shows significantly reduced percentage of callogenesis (62.23±1.09%). Interestingly, increase in further higher concentration 100mM of NaCl, frequency of callogenesis (26.67±0.63%) was sharply declined and the callus appeared to be pale yellow even after 2-days of incubation.

Effects of NaCl- Salt on Somatic Embryogenesis

Several reports are available indicating the high concentration of NaCl strongly affects both the embryogenesis and regeneration in rice (Subhashini and Reddy,1989; Vajrabhaya *et al.,* 1989). Studies reported that most of the embryogenic calli were yellow and show similar morphological structures (Sahoo *et al.,* 2011). Due to genotypic variation between the rice, composition of medium, and plant growth regulator used, considerable differences in embryogenesis could be observed in the induced callus like size, colour, number and morphology (Lee *et al.,* 2003).

In another study, embryogenic callus was distinguished into 4 types Type-I (white or cream colour and compact organized callus), Type-II (yellow and organized callus), Type-III (yellow or brown unorganized callus), and Type-IV (Rhizogenic callus) (Visarada *et al.,* 2002). Furthermore, during previous study to increase the embryogenesis and regeneration of calli, small amount of Kn could be used among the indica rice varieties (Nhut *et al.,* 2000; Afrasiab and Jafar, 2011) while this study in Poongar rice, low concentration of 2,4-D (5µM) alone was proved to be effective to induce higher rate of embryogenesis.

Among all the different concentrations, very low concentration of 2,4-D (5µM) alone without addition of NaCl salt shows highest percentage of somatic embryogenesis (95.0±3.9%) and also the maximum number (47.3±3.9) of somatic embryos per callus. Significantly, among all the concentrations of NaCl treated callus, 100mM of NaCl in presence of 2,4-D (5µM) treatment shows the lowest frequency of embryogenesis (31.67±2.9%) and also the minimum number (11.2±0.51) of somatic embryos per callus. In contrast, the lower concentration of NaCl (10mM) in presence of 2,4-D (5µM) shows the highest (67.5±2.33) percentage of somatic embryogenesis after 14-days of salinity treatment.

Effects of NaCl-Salt on Plant Regeneration

Factors like explants source, genotype, culture

conditions and combinations of plant growth regulators, osmotic pressure, and partial desiccation affect the frequency of shooting (Vennapusa *et al.,* 2015). Reports are available on addition of proline into the culture medium gives positive impact on regeneration of rice varieties (Ge *et al.,* 2006; Shahsavari, 2011).

Addition of Kn into the regeneration medium increases the frequency of regeneration of plantlet from the somatic embryos (Nhut *et al.,* 2000; Afrasiab and Jafar, 2011). Some reports suggest that Kn is more effective compared to BAP in terms of regeneration (Lee *et al.,* 2003, Barman *et al.,* 2016).

Addition of auxin plays vital role during plantlets regeneration (Prodhan *et al.,* 2001; Mostafiz *et al.,* 2018). Many reports suggest that composition of medium, source of explant and culture environment can affect the potential of regeneration (Khatun *et al.,* 2003; Hoque and Mansfield 2004). Moreover, appearance of green spots on the embryogenic callus indicates the initiation of regeneration and these green spots later form adventitious shoots (Artadana *et al.,* 2017).

Earlier studies reveal that the concentration of BAP in regeneration medium influences the regeneration of plantlet (Shaheenuzzaman *et al.,* 2001 and Malek *et al.,* 2007). However, MS-medium alone is enough for formation of root from the shoot after 2-weeks of incubation (Kumar *et al.,* 2016).

Although, there is regeneration in NaCl-treated calli but there will be physiological and molecular changes (ionic, osmotic, and tissue tolerance) due to the stress conditions (Roy *et al*., 2014; Bhatia, 2015; Isayenkov *et al.,* 2019). Highest frequency of regeneration in terms of rooting *japonica* is better than *indica* (Mikami and Kinoshita, 1988). Addition of NaCl to the medium helps to study the effect of salinity stress on different stages of regeneration (Priya *et al.,* 2011).

Significantly, high level of NaCl affects the frequency of regeneration in general and rice in particular. Moreover, in previous study, 128mM of NaCl was found to be inhibitory in terms of regeneration (Basu *et al.,* 1997). NaCl inhibits *in vitro* plant regeneration more than other salt stress like Na_2So_4 which indicates that Cl^- containing salts are more inhibitory for *in vitro* plant regeneration (Arefin, 2018).

Previous study reports that the percentage of regeneration without stress was recorded as 80% but higher concentration of NaCl 100mM shows the maximum inhibitory response and thus, minimum percentage of regeneration (20%) was recorded. With further increase in concentration of NaCl (150mM), there was no regeneration of plantlet from the embryogenic calli in the cultivar cv. BRRI 38 (Zinnah *et al.,* 2013).

In contrast, cv. Chinikanai shows 20% of plantlet regeneration with the concentration of 150mM of NaCl into the regeneration medium, which was further decreased to 0% when increases the concentration of NaCl to 200mM in comparison to control treatment where the frequency of regeneration was obtained 60% (Zinnah *et al.,* 2013).

During present study, the lowest concentration of NaCl (10mM) treatment could show the maximum frequency (74.52±1.2%) of plantlet regeneration and also number of regenerated plantlets (23.3±1.8) per callus while in contrast, the highest concentration of NaCl (100mM) resulted the minimum frequency (20.7±0.6%) of plantlet regeneration and also the minimum number (8.2±0.89) of regenerants per callus regeneration were obtained in comparison to the control (PRM6) treatment where 85.33±2.02% of plantlets regeneration and 34.5±1.3 plantlet per callus were noted.

Assessment of Genetic Fidelity analysis in Regenerants

Among the *in vitro* regenerated plantlets, the genetic variability or dissimilarity can be assessed through different ISSR, and RAPD primers which are DNA based molecular markers (Rawat *et al.,* 2013). It is the technique used to find the homogeneity or to find the difference among *in vitro* regenerated plantlets and *in vitro* stress tolerant plantlets. Four RAPD and ISSR primers (OPA1, OPA4, OPA5 and UBC 823) were used in present study and all the primers could show dissimilarities in terms of generation of polymorphic bands. Among all the four primers tested, RAPD primer (OPA 4) shows the maximum frequency of polymorphism (86%) and the polymorphic bands were positioned in between 100bp-3Kb.

CONCLUSION

In this study, an efficient and stable protocol for high frequency of somatic embryogenesis and plantlet regeneration from mature caryopsis in red rice (Poongar) could be established. The induction of callus and differentiation of somatic embryos along with the production of salinity (NaCl) tolerant plants could be also successfully achieved in red rice cv. Poongar. Further, genetic homogeneity of *in vitro* regenerated control plantlets and *in vitro* regenerated stress tolerant plantlets were assessed by using RAPD and ISSR markers resulted in generation of polymorphic DNA bands.

CONFLICTS OF INTEREST

The authors declare that they have no potential conflicts of interest.

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