Biologia Futura
https://doi.org/10.1007/s42977-024-00237-z

ORIGINAL PAPER

Effect of salt stress on plant regeneration efficiency in primed and non-primed seed's calli of rice (*Oryza sativa* L.) variety Swarna

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Received: 10 November 2023 / Accepted: 22 July 2024

Abstract
This study leads with the primed seeds of rice (var. Swarna) with distilled water (D.W.) and various concentrations of $Mg(NO_3)_2$ (0–8 mM)/Kinetin (0–5 ppm) alone or in combination with screen out the regeneration medium induced tolerance level of NaCl. To fulfill the objective, the primed and non-primed rice seeds were inoculated in MS medium supplemented with 30 gL^{-1} maltose + 1 gL^{-1} casein hydrolysate and 2 mgL^{-1} of 2,4-D for callus induction and cultured up to 45 days in two sets: one set for regeneration purpose in NaCl-induced regeneration medium and another set was used to study the physiological potentiality of the callus. The 45-day-old calli were transferred into regeneration medium MSR (MS medium for regeneration) (BAP: NAA: Kinetin = 4:1:1) containing NaCl with a concentration range of 0 to 300 mM. The number of regenerating calli and shoot regeneration percentage, number of plantlets obtained from one callus, recovery of plantlets from each concentration of NaCl and proline estimation from the leaf of the regenerated plantlets were determined from one set obtained after 45 days. The calli obtained from another set after 45 days, the frequencies of total and embryogenic calli induction percentage, fresh and dry weights, proline content, nitrate reductase and superoxide dismutase activities were measured. The calli obtained from 2.5 ppm kinetin + 4 mM $Mg(NO_3)_2$ primed seeds were showed best result as compared to the other treatments for the above-mentioned parameters in different concentrations of NaCl-induced medium and survive up to 200 mM concentrations of NaCl.

Keywords Somatic embryogenesis · NaCl stress tolerance · Priming · Nitrate reductase · Superoxide dismutase · Regeneration percentage

Introduction

Rice is an integral part of human history and major food crop in Asia and rest of the world (Mondal et al. 2013). The rice variety Swarna (MTU 7029) is a popular high yielding cultivar of India.

According to Assaha et al. (2017), as a result of climate change, agricultural fields will be converted into barren land due to improper drainage system, and 50% of all arable land is predicted to be impacted by salinity by 2050. More than 8×10^8 hm^2 land throughout the world is affected by salinity (FAO, 2008). Salinity is considered one of the major abiotic stressors which limits the growth and productivity of agricultural crops and having detrimental effects on germination, vigor index of plants and crop yield (Wang et al. 2019). The salinity causing toxic ions Na^+ and Cl^- derived from NaCl can damage the plant cells through the both osmotic and ionic mechanisms. According to Reddy et al. (2017), salinity stress affects seed germination, seedling growth, leaf size, overall shoot growth, number of tillers per plant, flowering stage, spikelet number, percent of sterile florets and productivity. However, in the recent years, researchers aimed to develop salt-tolerant rice cultivars through classic genetic and modern biotechnological approaches by using tissue culture techniques (Khattak et al. 2017).

Plant cells consist a unique property of dedifferentiation and the capacity to return to its meristematic state. Whereas, their developmental fates can be induced by endogenous or exogenous stimuli like the expression of different pluripotency factors or influenced by various phytohormones and stresses (Su et al. 2021). Somatic embryogenesis is a method

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Published online: 31 July 2024

of clonal propagation and a powerful tool for dedifferentiation and subsequent regeneration of plants (Mendez-Hernandez et al. 2019). Literatures suggest that several attempts already taken to improve rice callus regeneration in the presence and absence of salt stress but there are many successful stories and vice versa also. Khattak et al. (2017) tested 5 rice cultivars under one-step and multiple-step salinity in somatic embryogenesis study and observed that plant regeneration was reduced with the increased NaCl concentrations. Whereas, the intensity of reduction in plant regeneration rate under one-step salinity imposition was more than the multiple-step procedure. Similarly, Taratima et al. (2022) depicted that MS medium supplemented with 0.5 mg^{-1} IBA with 1 mg^{-1} 2,4-D was suitable for new plantlets regeneration from the surface of embryogenic calli of Luem Pua rice seeds. The calli were affected strongly in the presence of NaCl. Seedling growth and other physiological parameters under salinity treatment decreased when the concentration of NaCl increased.

On the other hand, it is already known that seed priming has gained ample popularity as it is a booming low-cost eco-friendly technology done by various ranges of inorganic and organic compounds which improves the quality of seeds by promoting germination and early seedling establishments and enzymatic activities and leads to improve stress tolerance by inducing stress imprint/memory mediated by proteins, transcription factors and epigenetic changes (Mondal et al. 2014; Mondal and Bose 2019). However, very few literatures are available where primed seeds were used in somatic embryogenesis for high-frequency plantlets regeneration. Recently, Mondal and Bose (2013) and Mondal et al. (2014) used the magnesium nitrate and kinetin primed seeds of rice variety Swarna, respectively, to observe the physiological potentiality of the callus as well as the regeneration capacity with better rooting ability of the plantlets via somatic embryogenesis. Therefore, this type of experiments elucidates a path where primed seeds can be used for the purposes of somatic embryogenesis to fulfill the goal of better regeneration of the embryogenic calli. This technology reopens the window toward the plant scientists to develop environmental stress resistance varieties by applying somatic embryogenesis technology for better understanding of the cellular mechanisms as well as to avoid the unnecessary interrupting factors. With these points kept in mind, the present experiment was designed in such a way where the induced salinity stress resistance capacity of the plantlets was tested by putting the embryogenic calli, obtained from various concentrations of magnesium nitrate/kinetin alone or in combination primed and non-primed seeds by using the somatic embryogenesis technique. Different concentrations of NaCl (0–300 mM concentrations) containing standardized culture medium for regeneration were used for this experiment. The aim of the study was to regenerate salt resistant plantlets from NaCl-induced regeneration medium by using primed and non-primed seed's embryogenic calli.

Materials and methods

Plant materials

The present work was carried out in Seed Physiology Laboratory of Department of Plant Physiology and Tissue Culture Laboratory, Department of Genetics and Plant Breeding, I.Ag.Sc., BHU, India. Dehusked seeds of mature grain of the rice (*Oryza sativa* L.) variety Swarna (MTU 7029) were used, procured from the Genetics and Plant Breeding Department of the same institute.

Seed priming

Healthy and bold seeds were surface sterilized by keeping them in 0.1% $HgCl_2$ (Mercuric chloride) solution for 2 min and then thoroughly washed with distilled water for 5–6 times. These sterilized seeds were used for experimental purpose. For priming, the sterilized seeds were soaked in distilled water (T_2), different concentrations of kinetin [2.5 and 5 ppm (T_3 and T_4)], magnesium nitrate [2, 4, 6 and 8 mM (T_5, T_6, T_7 and T_8)] and the combination of both kinetin and magnesium nitrate [2.5 ppm + 4 mM (T_9)] for 20 h. After 20 h these seeds were gently washed with distilled water and then dried back to its original weight at the room temperature by placing them under fan. After that these seeds were packed in separate paper bags for each treatment and used as per the requirement of the experiment. The seeds without any treatment referred as control (T_1: Non-primed seed). For the purpose of somatic embryogenesis experiment before the inoculation, dehusked primed and non-primed seeds were surface sterilized with 70% ethanol for 30 s and rinsed with autoclaved double distilled water for 3 times, after that the seeds were soaked in 0.1% mercuric chloride ($HgCl_2$) for 5 min with intermittent shaking followed by 5–6 rinses in sterile water (Raveendar et al. 2008). After surface sterilization, the dehusked seeds were kept on autoclaved filter paper in a Petri dish to remove the excess water.

Callus induction

Seeds were inoculated into culture tubes containing 30 gL^{-1} maltose + 1 gL^{-1} casein hydrolysate (M + CH) in MS basal media (10 mL media was poured in each culture tube) which was supplemented with 2 mgL^{-1} 2,4-D (9.04 µM) (2,4-dichlorophenoxyacetic acid) for callus induction in two series: one for regeneration of plantlets in NaCl-induced medium and another for the study of physiological potentiality of the callus. Each series containing 5 replications and

each replication consisting of 10 tubes and the experiment was repeated 3 times. The pH of the media was adjusted to 5.8 with 1N NaOH and 1N HCl using electronic pH indicator; maltose and agar concentrations in medium were 3% (w/v) and 0.8% (w/v), respectively. The media was autoclaved at a temperature of 121 °C and pressure of 15 psi for 20 min. Inoculation was carried out under a sterilized environment in a laminar air flow cabinet, and one seed was inoculated in each culture tube. After inoculation, culture tubes were incubated in dark at 25 ± 2 °C for 45 days. After 1 month, callogenesis rate, i.e., the percent of embryos having produced a callus, was determined. After 45 days, they were classified either as embryogenic or non-embryogenic by visual observation according to Nabors et al. (1983) and Peterson and Smith (1991): Embryogenic rice callus is compact, white in color, smooth with knobby appearance, while necrotic and non-embryogenic callus (yellow to translucent, wet and rough to crystalline in appearance) was discarded; callus presenting a heterogeneous appearance was considered as embryogenic.

After 45 days with the second series of calli, fresh and dry weights, proline content, nitrate reductase and superoxide dismutase activities were measured. Fresh and dry weights of the calli were determined by taking 3 calli for one replication and 5 replications were taken by using the electronic balance (Model No. ADAIR DUTT INSTRUMENT PVT. LTD.). Dry weight of the calli was determined by keeping the samples for an hour in an oven preset at 100–110 °C for the purpose of killing. Thereafter, it was placed in an oven set as 60 ± 2 °C till to get constant dry weight. Proline, nitrate reductase and SOD activity were measured in 5 replication each containing 50 mg calli for proline (dry mass) and 100 mg (fresh mass) of calli for each nitrate reductase and SOD activity, obtained from primed and non-primed seeds, by using the standard methods of Bates et al. (1973), Srivastava (1974) and Dhindsa et al. (1981), respectively.

Plant regeneration

The 45-day-old embryogenic calli were fragmented into small pieces of approximately 100 mg were transferred to MS-based regeneration medium (MSR) modified with combination of BAP (4.44 µM) (6-benzylaminopurine), 4.66 µM of kinetin and NAA (5.37 µM) (naphthaleneacetic acid) @ 4:1:1 mgL^{-1}, as previously determined in preliminary experimentations (Mondal and Bose 2013). The medium was induced with different concentrations of NaCl like 0, 50, 100, 200 and 300 mM. The pH of the medium was adjusted to 5.8 before autoclaving; maltose and agar concentration of the medium were 3% (w/v) and 0.8% (w/v), respectively. The media was autoclaved at a temperature of 121 °C and pressure of 15 psi for 20 min. Inoculation was carried out under a sterilized environment in a laminar air flow cabinet. The tubes were kept at 16 h light/8 h dark at 2000 lx light intensity of cool white fluorescent light at 25 ± 2 °C temperature. Somatic embryo formation and the greening of the embryogenic calli started 10–15 days after transferred. The mean number of shoots per regenerating callus and the rooting ability of regenerated shoots expressed as percentage of shoots showing at least one well developed root were then estimated for each treatment prior to transfer into the solarite soil without any NaCl treatment; then they were placed in laboratory for hardening purposes up to 10 days. The frequencies of total calli and embryogenic calli induction, regeneration percentages, number of regenerating calli, recovery of plantlets, number of shoots per calli and proline content (25 mg dry weight of leaf (followed the method of Bates et al. (1973)) were determined in vitro. The experiment was conducted with 5 replications except proline content (in this regard 3 replications were taken into consideration) and each consists of 5 culture tubes. Subculturing was done after the exhaustion of the media along with the respective concentrations of salt stress. Data were collected after 2 months of culture on regeneration medium, and percentage were estimated from 25 calli per treatment.

All the glasswares and chemicals used in the present investigation were of Borosil Glass Works Limited and HiMedia Laboratories Pvt. Ltd., India, respectively.

Statistical analysis

Mean values were taken from each treatment of three to five independent replications based on the data; and Statistical Package for Social Science (SPSS Version 23.0) was used for the analysis of variance. Significant differences among various treatments were determined by using Duncan's multiple range test (DMRT).

Results

The data presented in Table 1 reveal the effect of seed priming with distilled water (D.W.) and different concentrations of $Mg(NO_3)_2$/kinetin alone or in combination on total callus induction and its percentage, total embryogenic calli and its percentage, fresh and dry weights, proline content, nitrate reductase activity and superoxide dismutase activity of rice var. Swarna by using maltose as carbon source and casein hydrolysate. However, 4 mM $Mg(NO_3)_2$ + 2.5 ppm kinetin primed seeds (T_9) showed maximum number of callus induction [Mean value-10.0; with F(df1-8, df2-36) = F_{val}-9.675 at $p < 0.01$] and its percentage (100%), number of embryogenic calli [Mean value-8.0; with F(df1-8, df2-36) = F_{val}-15.07 at $p < 0.01$] and its percentage (80%) which is followed by treatment T_6 & T_7. Whereas, the treatment T_9

Table 1 Effect of seed priming with distilled water (D.W.) and different concentrations of $Mg(NO_3)_2$/Kinetin alone or in combination on total number of callus induction and embryogenic calli, fresh and dry weights, proline content, nitrate reductase and SOD activity of rice var. Swarna by using maltose as carbon source and casein hydrolysate

Treatments	Total number of callus induction (Mean ± SD)	Percentage of callus induction	Total number of embryogenic calli (Mean ± SD)	Percentage of embryogenic calli	Fresh weight of callus (g) (Mean ± SD)	Dry weight of callus (g) (Mean ± SD)	Proline content (mg g^{-1} dry weight) (Mean ± SD)	Nitrate reductase activity (Mean ± SD) (nmol $NO^{2-}h^{-1}$ g^{-1} calli fresh weight)	SOD activity (Mean ± SD) (unit × 10^2 g^{-1} min^{-1} fresh weight of leaf)
Control (T_1)	8.00 ± 0.71^{b}	80	3.80 ± 0.84^{c}	38	1.12 ± 0.04^{f}	0.180 ± 0.006^{d}	0.994 ± 0.011^{f}	336.40 ± 5.41^{h}	8.59 ± 0.078^{g}
Hydro (T_2)	9.20 ± 0.84^{a}	92	5.80 ± 0.84^{b}	58	1.19 ± 0.02^{e}	0.193 ± 0.010^{d}	1.012 ± 0.076^{f}	397.20 ± 12.07^{g}	10.44 ± 0.505^{f}
2.5 ppm (T_3)	9.40 ± 0.55^{a}	94	7.00 ± 0.71^{ab}	70	1.41 ± 0.02^{b}	0.266 ± 0.008^{b}	1.111 ± 0.007^{de}	450.20 ± 5.54^{f}	10.88 ± 0.506^{e}
5 ppm (T_4)	9.20 ± 0.45^{a}	92	6.60 ± 0.55^{ab}	66	1.35 ± 0.03^{bc}	0.232 ± 0.009^{c}	1.115 ± 0.017^{de}	476.80 ± 3.27^{e}	11.45 ± 0.078^{d}
2 mM (T_5)	9.40 ± 0.55^{a}	94	6.80 ± 0.84^{ab}	68	1.26 ± 0.04^{d}	0.223 ± 0.011^{c}	1.101 ± 0.017^{e}	525.20 ± 5.54^{d}	11.28 ± 0.080^{de}
4 mM (T_6)	10.00 ± 0.00^{a}	100	7.80 ± 0.84^{a}	78	1.38 ± 0.03^{bc}	0.253 ± 0.009^{b}	1.164 ± 0.023^{cd}	617.80 ± 7.29^{c}	11.64 ± 0.047^{cd}
6 mM (T_7)	10.00 ± 0.00^{a}	100	7.60 ± 0.55^{a}	76	1.33 ± 0.07^{c}	0.229 ± 0.007^{c}	1.200 ± 0.020^{bc}	634.00 ± 10.15^{b}	12.00 ± 0.065^{bc}
8 mM(T_8)	10.00 ± 0.00^{a}	100	7.00 ± 0.71^{ab}	70	1.34 ± 0.02^{bc}	0.237 ± 0.004^{c}	1.221 ± 0.015^{b}	645.80 ± 4.87^{b}	12.35 ± 0.91^{b}
2.5 ppm + 4 mM(T_9)	10.00 ± 0.00^{a}	100	8.00 ± 0.71^{a}	80	1.48 ± 0.02^{a}	0.314 ± 0.004^{a}	1.296 ± 0.127^{a}	673.40 ± 7.09^{a}	13.77 ± 0.070^{a}
SEm(±)	0.30		0.47		0.02	0.005	0.019	4.61	0.16
CD at 0.05	0.50		0.79		0.04	0.009	0.033	7.79	0.26
CD at 0.01	0.73		1.14		0.06	0.012	0.047	11.24	0.38

Data were collected after 45 days of culture in callus induction medium, and percentage were estimated from 5 replications (each containing 10 culture tubes) per treatment

Data presented are means from five replicates (each containing 10 culture tubes) with standard deviation. Within each treatment, different letters indicate significant differences by Duncan's multiple range test at $p < 0.01$

performed best and showed statistically significant result in comparison with other treatments in improving the fresh and dry weights [Mean value-1.48 g and 0.314 g; with F(df1-8, df2-36) = F_{val}-45.91 and 121.97, respectively, at $p < 0.01$], proline content [Mean value-1.296 mgg^{-1} dry weight; with F(df1-8, df2-36) = F_{val}-50.66 at $p < 0.01$], nitrate reductase activity [Mean value-673.4 nmol $NO_2^{-}h^{-1}$ g^{-1} calli fresh weight; with F(df1-8, df2-36) = F_{val}-1376.69 at $p < 0.01$] and superoxide dismutase activity [Mean value-13.77 unit × 10^2 g^{-1} min^{-1} fresh weight of leaf; with F(df1-8, df2-36) = F_{val}-163.85 at $p < 0.01$]. The data were collected after 45 days of callus culture. Figure 1a–i presents the callus initiation after 45 days in the callus induction medium obtained from the various treatments: T_1 (non-primed seeds), T_2 (hydro-primed seeds), T_3 and T_4 (2.5 and 5 ppm kinetin primed seeds), T_5 to T_8 (2 to 8 mM magnesium nitrate primed seeds) and T_9 (2.5 ppm kinetin + 4 mM magnesium nitrate primed seeds).

Likewise, in the present experiment, distilled water, kinetin and $Mg(NO_3)_2$ alone or in combination primed seeds were used in somatic embryogenesis and get more responsive embryogenic callus. Those calli were placed in NaCl-induced regeneration medium supplemented with 0, 50, 100, 200 and 300 mM concentrations of NaCl to check the regeneration efficiency as well as salt tolerance ability of rice calli. Tables 2 and 3 present the effect of NaCl doses on number of regenerating calli (in parentheses), regeneration percentages, recovery of plantlets (percentage value given in parentheses), number of shoots regenerated per calli and proline content (mg g^{-1} dry weight of leaf), and the observation was recorded after 2 months of culture on regeneration medium of 45-day-old calli obtained from mature embryos of rice var. Swarna of primed and non-primed seeds. The poorest performance was shown by 300 mM NaCl supplied medium in respect to regeneration percentages, number of regenerating calli and recovery percentage, as it failed to regenerate any plantlets from all the treatments. In control and all NaCl treated sets except 300 mM NaCl, T_9 performed best in respect of regeneration percentage (84, 56, 28 and 16 percent at 0, 50, 100 and 200 mM of NaCl concentration in regeneration medium, respectively); recovery of plantlets (17, 10, 5, 3 calli from 0, 50, 100 and 200 mM of NaCl concentration in regeneration medium, respectively) and number of shoots regenerated per callus (mean value—7.2, 2.6 and 1.6 from 0, 50 and 100 mM of NaCl concentration in regeneration medium, respectively), which was statistically significant at $p < 0.01$ with F(df1-8, df2-36) = F_{val}-4.47, 3.41, 2.72, 1.24 at 0, 50, 100 and 200 mM of NaCl concentration, respectively, followed by T_3 & T_6. The percentage were estimated from 25 calli per treatment. The control non-primed seeds (T_1) failed to regenerate any plantlets from 100 to 300 mM concentrations of NaCl. Maximum proline content was achieved by T_9 (1.3, 3.03, 4.43 and 5.07 mgg^{-1} dry weight of leaf; with F(df1-8, df_2-18) = F_{val}-30.83, 221.01, 1704.51 and 6476.49 at 0, 50, 100 and 200 mM of NaCl concentration in regeneration medium, respectively) except

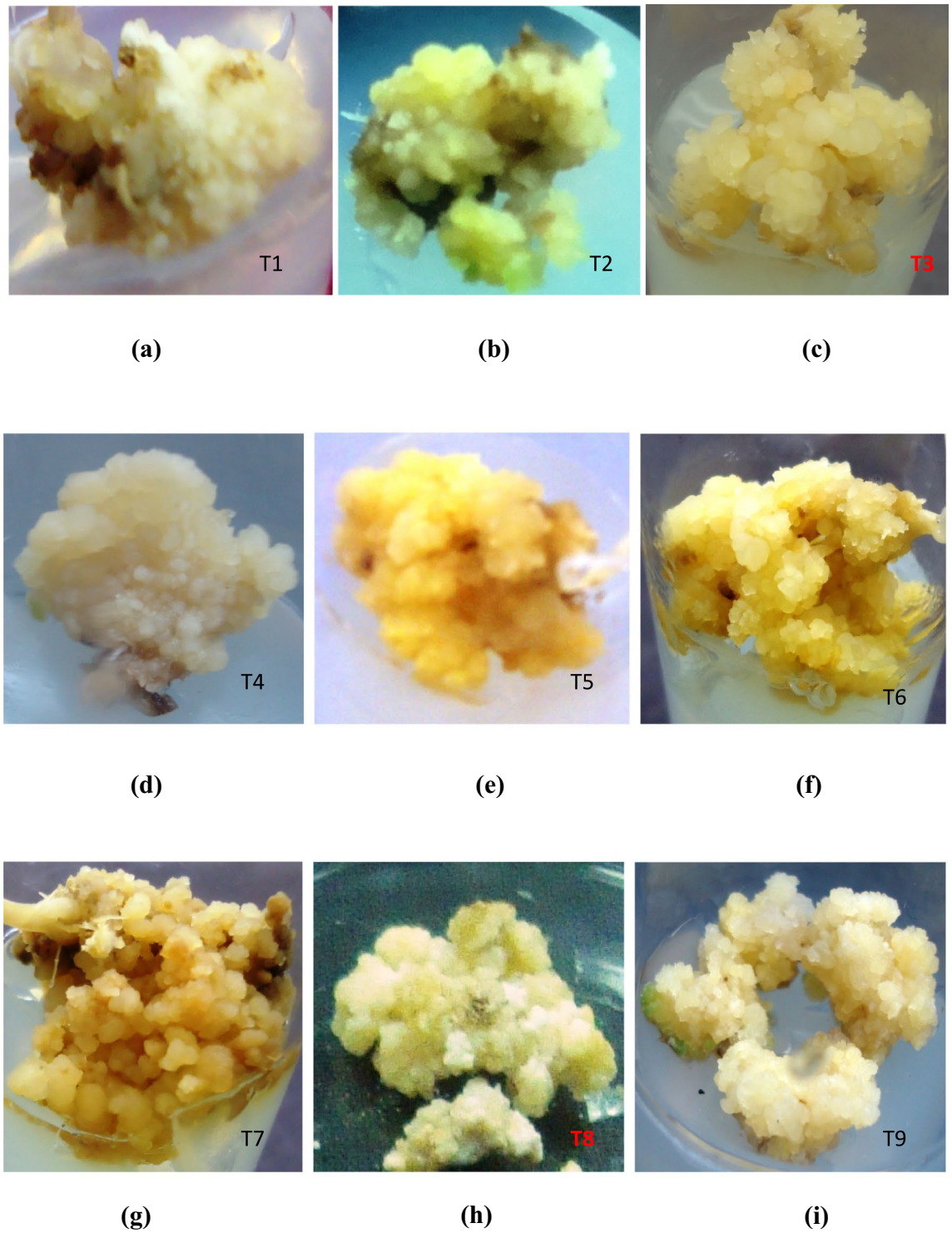

Fig. 1 a–i 45 days after callus initiation in the callus induction medium obtained from the various treatments: T_1 (non-primed control seeds), T_2 (hydro-primed seeds), T_3 and T_4 (2.5 and 5 ppm kinetin primed seeds), T_5 to T_8 (2 to 8 mM magnesium nitrate primed seeds) and T_9 (2.5 ppm kinetin + 4 mM magnesium nitrate primed seeds)

300 mM concentration of NaCl in regeneration medium, which was statistically significant at $p < 0.01$ and that was followed by other primed seeds calli. For every case, proline content was also increased with increasing concentration of NaCl except the control one as it failed to regenerate any plantlets above 50 mM NaCl concentration. Whereas, Fig. 2

Table 2 Effect of NaCl doses on regeneration percentages, number of regenerating calli (in parentheses) and recovery of plantlets (percentage value given in parentheses) from 45-day-old calli obtained from mature embryos of rice var. Swarna of primed and non-primed seeds

Treatments	Regeneration percentages and number of regenerating calli					Recovery of plantlets (%)				
	Concentrations of salt (mM NaCl)									
	0	50	100	200	300	0	50	100	200	300
Control (T_1)	60 (15)	8 (2)	0 (0)	0 (0)	0 (0)	5 (20)	1 (4)	0 (0)	0 (0)	0 (0)
Hydro (T_2)	68 (17)	20 (5)	0 (0)	0 (0)	0 (0)	8 (32)	2 (8)	0 (0)	0 (0)	0 (0)
2.5 ppm (T_3)	72 (18)	44 (11)	20 (5)	12 (3)	0 (0)	12 (48)	7 (28)	3 (12)	1 (4)	0 (0)
5 ppm (T_4)	72 (18)	36 (9)	16 (4)	4 (1)	0 (0)	11 (44)	5 (20)	1 (4)	0 (0)	0 (0)
2 mM (T_5)	68 (17)	32 (8)	8 (2)	0 (0)	0 (0)	9 (36)	4 (16)	1 (4)	0 (0)	0 (0)
4 mM (T_6)	80 (20)	40 (10)	20 (5)	8 (2)	0 (0)	14 (56)	6 (24)	3 (12)	1 (4)	0 (0)
6 mM (T_7)	76 (19)	36 (9)	20 (5)	8 (2)	0 (0)	13 (52)	4 (16)	2 (8)	1 (4)	0 (0)
8 mM (T_8)	72 (18)	28 (7)	12 (3)	4 (1)	0 (0)	13 (52)	3 (12)	1 (4)	1 (4)	0 (0)
2.5 ppm + 4 mM (T_9)	84 (21)	56 (14)	28 (7)	16 (4)	0 (0)	17 (68)	10 (40)	5 (20)	3 (12)	0 (0)

Data were collected after 2 months of culture on regeneration medium, and percentage were estimated from 25 calli per treatment

presents the plantlets obtained from 50 mM NaCl-induced regeneration medium of the various treatments, i.e., T_1 to T_9, and Fig. 3 presents the plantlets obtained from 100 to 200 mM NaCl-induced regeneration medium of the various treatments T_3, T_6 and T_9.

Likewise, in the present case, 50 mM NaCl stressed primed calli showed better regeneration frequency with good number of green plantlets followed by 100 and 200 mM NaCl stressed primed calli but as the concentrations of NaCl increased the regeneration frequency and seedling vigor were decreased simultaneously proline content were significantly increased in increasing concentration of NaCl. Calli obtained from primed seeds having more stress tolerance ability.

Discussion

In the present experiment, along with 2,4-D, maltose as carbon source and casein hydrolysate rather than sucrose for better embryogenic callus induction. Similarly, Mondal et al. (2013) standardize the callus induction medium for rice and stated that addition of adequate levels of synthetic auxins, such as 2,4-D into basal medium resulted in prolific callus formation from a variety of rice explants. The results of the present study also showed that the presence of 2,4-D in culture medium is crucial for rice callus induction from mature seeds of both primed and non-primed one. However, it is a well-established fact that carbohydrate not only functions as a potential carbon source for metabolism but also plays an important role in the regulation of osmotic potential as reported by Naqvi et al. (2006). The frequency of green somatic embryos was greatly influenced by carbon sources and the best results achieved with the presence of 10 g/L maltose in culture medium, in two varieties, i.e., MR 219 and MR 232, while kept in light condition for 3 weeks in pre-regeneration medium as reported by Zuraida et al. (2011). In spite of all these factors related to somatic embryogenesis, primed seeds showed better results in the callus inducing medium containing maltose + CH than the sucrose + CH for total and embryogenic callus induction (%) as reported by Mondal and Bose (2013). They also observed that calli obtained from primed seeds having more fresh and dry weights and proline content as compared to non-primed control seeds. In addition, Mondal and Bose (2013) concluded that the use of $Mg(NO_3)_2$ primed rice seeds in somatic embryogenesis can regenerate plantlets having more stress tolerance ability as it regenerated more number of roots with increased amount of proline. In addition, cytokinins play important roles in the interaction of plants with both biotic and abiotic factors, including salinity and many others (Taiz et al. 2015). Likewise, kinetin increases the cell division and greening of the calli in the presence of light (Taiz et al. 2015).

Similarly, Mondal et al. (2014) stated that kinetin as a priming agent in rice seeds was used in somatic embryogenesis, and they were getting better regeneration percentage with stress ameliorating capacity which elucidates a path to develop environmental stress resistance varieties. Likewise, in the present experiment, distilled water, kinetin and $Mg(NO_3)_2$ alone or in combination primed seeds were used in somatic embryogenesis and get more responsive embryogenic callus. Those calli were placed in NaCl-induced regeneration medium supplemented with 0, 50, 100, 200 and 300 mM concentrations of NaCl to check the regeneration efficiency as well as salt tolerance ability of rice calli.

Mondal et al. (2014) found that BAP at 4 mg L^{-1}, Kinetin 1 mg L^{-1} and NAA 1 mg L^{-1} were optimum for complete plantlet regeneration from mature seed derived 45-day-old callus of rice variety Swarna. Hence in the present

Table 3 Effect of NaCl doses on number of shoots regenerated per callus and proline content obtained from 45-day-old calli of rice var. Swarna of primed and non-primed seeds

Treatments	Number of shoots regenerated per callus (Mean ± SD)					Proline content (mg g^{-1} dry weight of leaf) (Mean ± SD)				
	Concentrations of salt (mM NaCl)									
	0	50	100	200	300	0	50	100	200	300
Control (T_1)	3.6 ± 0.89^{b}	0.4 ± 0.89^{c}	0.0 ± 0.0^{b}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	1.0 ± 0.020^{e}	1.58 ± 0.023^{f}	0.00 ± 0.000^{g}	0.00 ± 0.000^{f}	0.00 ± 0.00^{a}
Hydro (T_2)	3.6 ± 1.14^{b}	0.8 ± 1.10^{bc}	0.0 ± 0.0^{b}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	1.1 ± 0.050^{de}	1.69 ± 0.026^{f}	0.00 ± 0.000^{g}	0.00 ± 0.000^{f}	0.00 ± 0.00^{a}
2.5 ppm (T_3)	4.0 ± 1.58^{b}	2.0 ± 0.71^{abc}	1.0 ± 1.0^{ab}	0.4 ± 0.9^{a}	0.0 ± 0.0^{a}	1.1 ± 0.035^{bc}	1.86 ± 0.026^{e}	2.56 ± 0.060^{f}	3.40 ± 0.075^{e}	0.00 ± 0.00^{a}
5 ppm (T_4)	5.6 ± 1.67^{ab}	1.4 ± 0.55^{abc}	0.4 ± 0.9^{ab}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	1.2 ± 0.008^{b}	2.00 ± 0.035^{de}	3.11 ± 0.055^{e}	0.00 ± 0.000^{f}	0.00 ± 0.00^{a}
2 mM (T_5)	5.2 ± 1.30^{ab}	1.2 ± 0.84^{abc}	0.4 ± 0.9^{ab}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	1.1 ± 0.017^{cd}	2.01 ± 0.057^{d}	3.46 ± 0.055^{d}	0.00 ± 0.000^{f}	0.00 ± 0.00^{a}
4 mM (T_6)	5.2 ± 0.84^{ab}	2.8 ± 0.84^{a}	1.4 ± 1.3^{ab}	0.4 ± 0.9^{a}	0.0 ± 0.0^{a}	1.2 ± 0.010^{bc}	2.51 ± 0.099^{c}	3.83 ± 0.060^{c}	4.15 ± 0.071^{d}	0.00 ± 0.00^{a}
6 mM (T_7)	6.0 ± 1.00^{ab}	1.6 ± 1.14^{abc}	0.6 ± 0.9^{ab}	0.2 ± 0.4^{a}	0.0 ± 0.0^{a}	1.2 ± 0.015^{b}	2.60 ± 0.065^{bc}	3.91 ± 0.093^{bc}	4.55 ± 0.061^{c}	0.00 ± 0.00^{a}
8 mM(T_8)	4.6 ± 1.14^{b}	1.4 ± 1.34^{abc}	0.2 ± 0.4^{ab}	0.2 ± 0.4^{a}	0.0 ± 0.0^{a}	1.2 ± 0.015^{b}	2.74 ± 0.044^{b}	4.07 ± 0.121^{b}	4.74 ± 0.069^{b}	0.00 ± 0.00^{a}
2.5 ppm + 4 mM(T_9)	$\mathbf{7.2 \pm 1.48^{a}}$	$\mathbf{2.6 \pm 0.89^{ab}}$	$\mathbf{1.6 \pm 0.5^{a}}$	0.8 ± 0.8^{a}	0.0 ± 0.2^{a}	$\mathbf{1.3 \pm 0.038^{a}}$	$\mathbf{3.03 \pm 0.097^{a}}$	$\mathbf{4.43 \pm 0.093^{a}}$	$\mathbf{5.07 \pm 0.062^{a}}$	0.00 ± 0.00^{a}
SEm(±)	0.8	0.6	0.5	0.3	0.0	0.015	0.05	0.06	0.04	0.00
CD at 0.05	1.3	1.0	0.9	0.6	0.0	0.025	0.08	0.10	0.07	0.00
CD at 0.01	1.9	1.5	1.2	0.8	0.0	0.036	0.12	0.15	0.11	0.00

Data were collected after 2 months of culture on regeneration medium, and percentage were estimated from 25 calli per treatment

Data presented are means from three replicates for number of shoots regenerated per callus and five replicates for proline content with standard deviation (each containing five culture tubes). Within each treatment, different letters indicate significant differences by Duncan's multiple range test at $p < 0.01$

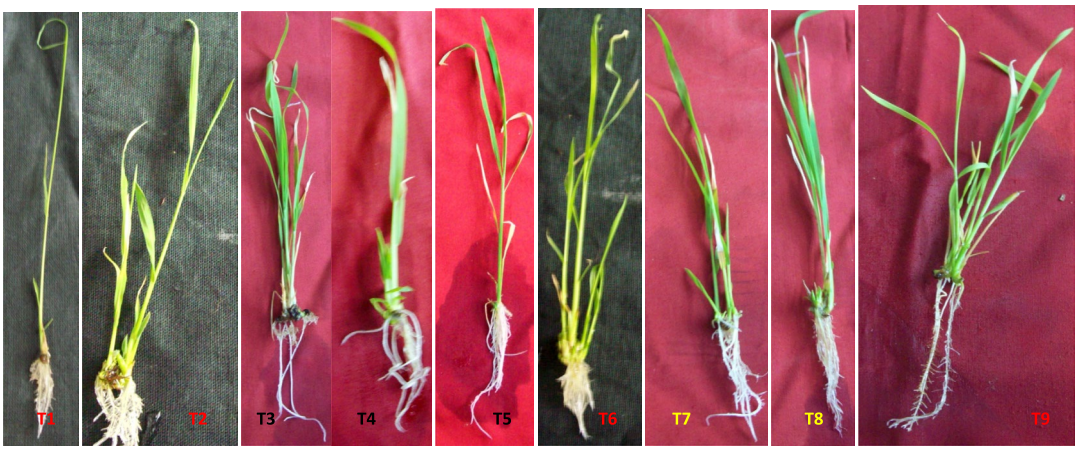

Fig. 2 **a–i** Plantlets obtained from 50 mM NaCl-induced regeneration medium of the various treatments T_1 (non-primed control seeds), T_2 (hydro-primed seeds), T_3 and T_4 (2.5 and 5 ppm kinetin primed seeds), T_5 to T_8 (2 to 8 mM magnesium nitrate primed seeds) and T_9 (2.5 ppm kinetin + 4 mM magnesium nitrate primed seeds)

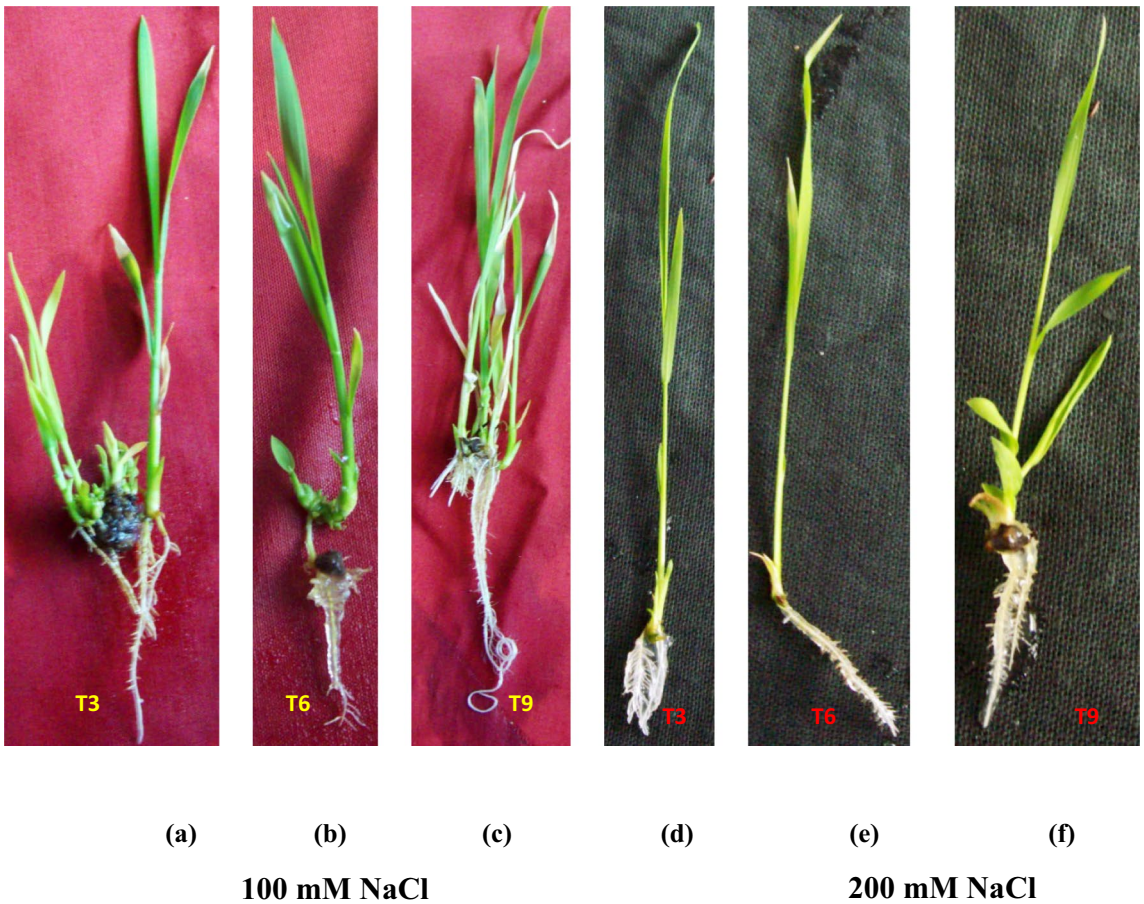

Fig. 3 **a–f** Plantlets obtained from 100 to 200 mM induced regeneration medium of the various treatments T_3 (2.5 ppm kinetin primed seeds), T_6 (4 mM magnesium nitrate primed seeds) and T_9 (2.5 ppm kinetin + 4 mM magnesium nitrate primed seeds)

investigation, auxin (NAA) and cytokinin (BAP and kinetin in combination) were also tested at the concentration of 4:1:1 mgL^{-1} (BAP: kinetin: NAA) supplemented with different concentrations of NaCl for the regeneration of plantlets form the embryogenic calli of rice cultivar Swarna. NaCl in the regeneration medium had a detrimental effect upon most parameters associated with plantlet regeneration. Similar kind of experiment was also reported by Taratima et al. (2022), where they study the in vitro culture of rice calli cv. Luem Pua observed that in increasing NaCl concentration (0, 50, 100, 150 and 200 mM) the survival percentage of calli as well as seedling in soil culture was decreased. Seedling growth under salinity treatment also decreased when NaCl concentration was increased as noted by Taratima et al. (2022). Likewise, Summart et al. (2010) described that the study of physiological functions at cellular level is the primary prerequisite before developing a salt-tolerant line to overcome the adverse effects of soil salinity, it also affects the yield of the crops. The result of the present study indicates that increased concentrations of NaCl over a longer time period in the culture medium (MSR medium) inhibits the regeneration potential which might be due to the reason of osmotic or ionic shock while using the normal control seeds for callus induction. This result was also supported by Taratima et al. (2022). Whereas, in the present case priming regulated osmotic adjustment was also noted and as a result plantlets can withstand in 200 mM NaCl containing culture medium but the regeneration and survival percentage was very low. Literature suggested that improved salt tolerance from primed seeds was observed with high capacity of osmotic adjustment hence plants from primed seeds accumulate Na^+ and Cl^- in roots and more sugars and organic acids (Yunita et al. 2014). In addition, with that seed priming enhanced the resistance capability toward various abiotic stress by modulating various pathways involved in different metabolic adjustments (Jisha et al. 2013). Likewise, Nowak and Shulaev (2003) reported that primed propagule responds quicker to a higher degree than non-prime one and thus cope up better in stressful condition. In addition, with that they introduce the term 'priming' for tissue culture propagation. For instance, halopriming (seed treatment with salt in order to improve seed germination and decrease saline intolerance) significantly increased the proline content, SOD, APX, CAT and non-enzymatic ROS scavenging activity with balanced Na^+/K^+ ratio and as a result seedlings with better salt stress tolerance ability as reported by EI-Serafy et al. (2021).

Moreover, in support of present experiment like calli obtained from primed seeds having more osmotic adjustment capability as it synthesizes more proline than the normal one, Chen and Jiang (2010) and Yunita et al. (2014) were also noted that in cellular level, plants have developed complex mechanisms to adjust hyperosmotic stress and ionic imbalance by osmotic adjustment (OA). These mechanisms accumulate osmotic regulators such as sugars and proline to protect membrane integrity and stabilize enzymes against oxidative stress. In continuation, Kalhori et al. (2017) selected and characterize salt-tolerant Malaysian rice via somatic embryogenesis based on the growth performance and antioxidant capacity including proline content and the plantlets obtained from 50 to 100 mM NaCl selected as salt-tolerant line. The observation depicted that proline content significantly increased under salt stress. Whereas, callus cultured in 200 and 300 mM NaCl containing regeneration medium turn blackish-brown and stiff and acutely necrotic. To counteract the effect of increased accumulation of salt ions in the vacuoles, proline has been reported to increase in the cytoplasm, which might act as an osmoticum (Kalhori et al. 2017).

Conclusion for future biology

A critical scrutiny of the result suggested that the concentrations of NaCl more than 50 mM were inhibitory for the calli obtained from the non-primed seeds and unable to regenerate any plantlets in the medium containing more than 50 mM NaCl. The calli obtained from 2.5 ppm kinetin, 4 mM $Mg(NO_3)_2$ and 2.5 ppm kinetin + 4 mM $Mg(NO_3)_2$ primed seeds were showed better result as compared to the other treatments in the above-mentioned parameters in the different concentrations of NaCl-induced medium and survive up to 200 mM of NaCl. Whereas, the calli obtained from 2.5 ppm kinetin + 4 mM $Mg(NO_3)_2$ primed seeds were showed best regeneration efficiency among all the treatments. In a nutshell, it can be concluded that primed seeds of indica rice may be a better option to increase the regeneration potentiality. Therefore, the present study annotates a path toward the use of seed priming technology in indica rice during somatic embryogenesis to fulfill the goal of better regenerating capability of calli followed by proper commercialization of the stress amelioration capable lines.

However, from last 2–3 decades scientists were trying to develop salt tolerance lines by using in vitro culture technique but the success rate is very low. In addition, incorporation of primed seeds into this abiotic stress tolerance line development by using somatic embryogenetic process can be a path breaking achievement in coming future. As it is very unusual that primed seeds were taken for somatic embryogenesis to check the survival potentiality and salinity tolerance level of the plantlets in NaCl-induced medium. Amalgamation of seed priming technology into tissue culture industry opens the window for plant scientists to develop environmental stress resistance varieties in the future. Further studies will be essential to incorporate molecular biology along with seed priming for the development of stress resistance cultivars by using somatic embryogenesis.

Author contributions Sananda Mondal did the experiment and prepared the manuscript; Bandana Bose help in preparation of manuscript and act as a mentor.

Funding No funding is available.

Data availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Not applicable.

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