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Induction of Somatic Embryo and Plantlet Regeneration from Mature Caryopsis Culture under NaCl-Salt Stress Conditions in Traditional Indian Black Rice (*Oryza sativa* L.)

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This study was conducted to establish an efficient in vitro regeneration technique for the induction of somatic embryo and plantlet regeneration under NaCl-salt stress conditions in black rice (*Oryza sativa*. L., cv Co57), a traditional Indian cereal food. Embryogenic calli were obtained from mature caryopsis culture on Murashige and Skoog (1962) medium fortified with 2,4-dichlorophenoxyacetic acid (2.0mg/L) either alone or in combination with various concentrations of NaCl (10mM, 25mM, 50mM, and 100mM) in order to induce the salt tolerant somatic embryo differentiation. Furthermore, embryogenic calli were found to show shoot regeneration on MS-medium fortified with 2.0mg/L of 6-benzylaminopurine (BAP) in combination with 0.5mg/L of α-naphthaleneacetic acid (NAA) and in presence of NaCl (10mM, 25mM, 50mM, and 100mM). Significantly, the high concentration (150mM) of NaCl was proved to be lethal for both somatic embryogenesis as well as plantlets regeneration. Moreover, the low frequency (40.3%) of somatic embryogenesis and minimum number of salt tolerant somatic embryos per callus (3.2±0.1) was recorded with the caryopsis explants that were treated with the high concentration (100mM) of NaCl-salt added embryo induction medium. Significantly, low frequency of salt-tolerant plantlets regeneration (25%) and minimum number of plantlets per embryogenic callus (1.5±0.1) was recorded in the embryogenic callus that was treated with 100mM of NaCl in regeneration medium containing BAP (2.0mg/L) in combination with NAA (0.5mg/L). Further, salinity tolerant plantlets were transferred to soil and gradually acclimatized under growth chamber conditions. This study thus offers a suitable technique for production of salt tolerant black rice, an alternative approach for the traditional Indian black rice crop improvements.

Key words: Mature caryopsis, Plant growth regulators, Somatic embryogenesis, Plant regeneration, Salinity

Rice is the most important food crop world-wide and in Asia more than 90% of rice is being cultivated and consumed as the main food source. Black rice is known as a good source of antioxidant (Yawadio *et al*., 2007) and proteins, vitamins such as (niacin, vitamin B, riboflavin) (Ichikwa *et al*., 2001; Sompong *et al*., 2011; Jang *et al*., 2012). The black color of the rice kernel is due to containing high amount of anthocyanin pigment which is present in the aleurone layer, pericarp layer and seed coat (Bolea and Vizireanu, 2017).

Salinity in soil is one of the major abiotic stresses and it thoroughly affects the metabolic activities in plant (Zinnah *et al*., 2013). Unfortunately, high salt concentration in the soil and water particularly near the coastal land restricts the production of rice. In recent past, different strategies such as traditional breeding program, tissue culture stress selection approach and genetic engineering have been in practice to produce the salt tolerant crop variety (Ahmad *et al*., 2007; Khaleda *et al*., 2007; Tariq *et al*., 2008; Evangelista *et al*., 2009; Abiri *et al*., 2015; Rattana and Bunnag, 2015).

Additionally, literature reveals the production of salt tolerant crop varieties through in vitro culture technique has been reported in sugarcane (Mallikarjun *et al*., 2008), wheat (Benderradji *et al*., 2007), rice (Aditya and Baker, 2006; Prajuabmam *et al*., 2009). Moreover, reports on callus formation and plantlet regeneration using immature embryos and mature seeds (Cai *et al*., 2013; Azizi *et al*., 2015; Kumar *et al*., 2017; Binte Mostafiz and Wagiran, 2018), leaf base segments (Ramesh *et al*., 2009), microspores (Shariatpanahi *et al*., 2006), immature inflorescence (Kavas *et al*., 2008), and anthers (Maharani *et al*., 2020) are also available.

Plant development through in vitro culture technique depends on some of the factors such as amount and type of plant growth regulators (PGRs) treatments, culture medium components, explant type, solidifying agents, and culture condition etc. (Ge *et al*., 2006; Feng *et al*., 2011; Parmar *et al*., 2012; Ahmad *et al*., 2016; Kumar *et al*., 2017; Bente Mostafiz and Wagiran, 2018; Repalli *et al*., 2019).

This study deals with the induction of somatic embryos under salt stress conditions in black rice food crop followed by regeneration of salinity stress tolerant plantlets using mature caryopsis as explant. Regeneration of salt tolerant rice could be a meaningful approach to achieve production of black rice under salinity conditions of soil and water prevailing near the coastal land. Moreover, this study based on regeneration of salt tolerant black rice is of its first kind of report and thus it would be significant step in rice crop improvement program.

MATERIALS AND METHODS

Seed collection and Explant Sterilization

To begin with, healthy and dry seeds of black rice (*Oryza sativa* L., cv. Co57) were collected from TNAU, Coimbatore (India). The seeds were dehusked and washed by sterile distilled water 2-3 times followed by further sterilization with Tween-20 for 8-10 mins. Rice seeds were washed further with distilled water repeatedly 3-4 times and treated with (70%) of ethanol (v/v) for 1 min followed by rinsing with three to four times with sterile distilled water (SDW).

Seeds were then treated with mercuric chloride (0.1%) for 8-10 mins and rinsed 3-4 times with sterile distilled water. Further, sterilized seeds were dried on autoclaved Whatman paper for 5 mins under laminar air flow cabinet to minimize the chance of water born contamination. The seeds containing embryonic axis were kept away from the medium and scutellum region which is inoculated up position in the nutrient medium.

Nutrient Medium for Embryogenic Callus Induction

Sterilized seeds or caryopses were inoculated in MS (Murashige and Skoog, 1962) medium supplemented with 3% sucrose, 0.8 % agar supplemented with various concentrations of 2,4–D (0.5, 1.0, 2.0, 3.0, and 4.0mg /L). The pH of the nutrient medium was adjusted 5.5 to 5.8 with 1N (NaOH or HCl) and further nutrient medium was autoclaved at 121^0C for 20 mins. The culture tubes were incubated under fluorescent light at 5000 Lux with an ambient temperature of 25 $\pm 2^0$ C and maintenance of 16 hours light and 8 hours dark.

Nutrient Medium for Plantlet Regeneration

Embryogenic callus was transferred to MS-medium fortified with sucrose (30g/L) + agar (0.8%) + BAP (0.5,

1.0, 2.0, 3.0, and 4.0mg/L) + 0.5mg/L of (α-naphthaleneacetic acid (NAA) as constant. Moreover, for shoot initiation, non-embryogenic part of callus was removed to obtain the embryogenic part of the callus. After 4-weeks of incubation time, shoot regeneration was obtained from the embryogenic callus. Interestingly, regenerated shoots were later found to show root formation in the same regeneration medium as mentioned above.

Salinity Stress Treatments

In order to induce somatic embryogenesis and identification of the salt tolerant embryogenic callus, mature seeds were inoculated in the MS-nutrient medium fortified with 2,4-D (2.0 mg /L) in combination with various concentrations of (10, 25, 50, 100, and 150 mM) NaCl (Hi – media). Somatic embryogenesis was recorded after 6-weeks period. Due to nutrient deficiency, embryogenic callus was further sub-cultured on the same MS-medium for proliferation and also to check the salt tolerance level of the embryogenic callus.

Salt tolerant embryogenic calli were later transferred to regeneration medium supplemented with various concentrations (10, 25, 50, 100, and 150mM) of NaCl along with (2.0 mg/L of BAP + 0.5 mg/L of NAA) for the selection of the salt tolerant plant regeneration.

Transplantation to Soil

Regenerants grown under control and salt-treated conditions were transferred to the cup soil containing vermiculite, vermicompost and sand (20%, 20%, and 60%) respectively and gradually acclimatized in growth chamber.

Statistical Analysis

Frequency of Somatic embryogenesis (%)= $\frac{No.\ of\ embryogenic\ callus}{Total\ No.\ of\ explants} \text{ X } 100$

Frequency of Plantlets Regeneration (%)= $\frac{No.\ of\ embryogenic\ callus\ with\ plantlet\ regeneration}{Total\ No.\ of explant\ with\ embryogeneic\ callus} \text{ X } 100$

During callus induction, somatic embryogenesis, and plantlets regeneration, 25-30 seeds were used for each experiment and 3 replicates were conducted for each experiment to calculate the mean (%) and (SE) by applying SPSS software.

RESULTS

Induction of Callus and Somatic Embryogenesis

In the control experiment, mature caryopsis was seen to germinate and develop into the mature seedlings within 10 days of culture initiation. Moreover, callus formation was apparent from the base of the germinated seedlings in explants that were treated with various concentrations (0.5mg/L, 1.0mg/L, 2.0mg/L, and 4.0mg/L) of 2,4-D (**Fig. 1A-D**) respectively. The texture of the callus was recorded after 4-weeks of culture initiation. In case of calli obtained from the explants that were growing with 2,4-D (2.0 mg/L), the texture of the calli was appeared to be compact, nodular, and embryogenic (**Fig. 1C**) while lower concentrations (0.5mg/L and 1.0mg/L) of 2,4-D were proved to be less effective for the induction of compact and nodular callus (**Fig. 1A** & **B**) respectively. Moreover, the colour of the callus was creamy white after 4-weeks of culture initiation, globular structure was formed in the compact callus.

Significantly, when the 2,4-D concentration was increased up to (4.0mg/L), it gradually decreases the embryogenic potential of the callus and the texture of the calli was seen as friable and brownish in colour (**Fig. 1D**). After 4-weeks of culture initiation, embryogenic calli were sub-cultured into the same medium for the proliferation of the embryogenic callus. The highest frequency (90%) of embryogenic callus formation and maximum number of somatic embryos per callus (9.3±0.4) was recorded with the 2,4-D (2.0mg/L) whereas the low frequency (23.3%) of embryogenic callus and minimum number of somatic embryos per callus (1.1±0.1) was obtained with the 2,4-D (4.0mg/L) **(Table 1**).

Somatic embryogenesis under Salinity Stress

In this study, mature caryopsis was used as the explant for the establishment of somatic embryogenesis and the culture was initiated with the concentration of 2,4-D (2.0mg/L) in combination with various concentrations (10mM, 25mM, 50mM, 100mM, and 150mM) of NaCl and after 4-weeks of culture initiation, explants were found to show the embryogenic callus formation.

Embryogenic callus was sub-cultured into the same medium for another 2-weeks for the proliferation of the embryogenic callus and also to obtain the salt tolerant embryogenic callus. Embryogenic callus formation was recorded with the (10mM-100mM) of NaCl treatments.

Moreover, explants that were treated with the low concentration (10mM) of NaCl salt solution, callus texture was seen to be compact and the calli were apparent white in colour **(Fig. 1E)**. However, with the increase in NaCl concentrations, callus texture was found to be affected by salinity stress and, therefore, with 50mM of NaCl treatments, induced callus was turned out to be less embryogenic (**Fig. 1F**) in nature. Moreover, when the NaCl concentration was increased up to 100mM, it decreases the embryogenesis events **(Fig. 1G)**. Significantly, the explants that were treated with the very high concentration (150mM) of 2,4-D into NaCl, were emerged as poorly effective to show callus formation including embryogenesis (**Fig. 1H**).

The highest frequency (85.3%) of embryogenic callus was obtained from the concentration 10mM of NaCl and also the maximum number of somatic embryos per callus was found to be (9.2±0.3) while the minimum frequency (40.3%) of embryogenic callus along with number of somatic embryos per callus (3.2±0.1) was obtained with the high concentration (100mM) of NaCl-treatments. Moreover, when NaCl concentration was increased up to 150mM, it was proved to be lethal and the induced callus was failed to show differentiation of somatic embryos **(Table 2)**.

Regeneration of Plantlets

In order to achieve the plantlets regeneration, embryogenic calli were transferred after 6- weeks of culture initiation into the regeneration medium supplemented with various concentrations (0.5mg/L, 1.0mg/L, 2.0mg/L, and 3.0mg/L) of BAP with constant NAA (0.5mg/L). After 2-weeks of transfer, shoot initiation was visible from the embryogenic callus. The lowest concentration of BAP (0.5mg/L) with NAA (0.5mg/L) gives the minimum number of shoot initiation (merely in form of green spots) from the embryogenic callus **(Fig. 2A)**, while with the further increased concentration of BAP (1.0mg/L) along with NAA (0.5mg/L), green spots were found to be converted into young elongated emerging shoots (**Fig. 2B**).

However, the maximum number of shoot initiation per callus and the length of the regenerated shoots were obtained with the calli which were growing with BAP (2.0mg/L) and NAA (0.5mg/L) concentration **(Fig. 2C)**. Significantly, when the concentration of BAP was further increased (3.0mg/L) with NAA (0.5mg/L), callus was found to show inhibitions in the shoot initiation **(Fig. 2D)**.The highest frequency (80.3%) of plantlet formation was recorded with the BAP (2.0 mg/L) with NAA (0.5 mg/L) and the maximum number of shoot initiation per embryogenic callus (7.7±0.3) was obtained while the lowest frequency (40%) of plantlet regeneration was seen with the BAP (3.0mg/L) in combination with NAA (0.5mg/L) and also the minimum number (1.2±0.1) of shoot initiation was recorded **(Table 3).** Interestingly**,** same nutrient medium was proved to be effective for root initiation as well.

Plantlets regeneration under salinity stress

In present study, seeds that were growing with basal medium were found to grow into complete seedlings (**Fig. 3A**) after 2-weeks of culture initiation while the embryogenic calli that were growing in regeneration medium (2.0mg/L of BAP + 0.5mg/L of NAA) were seen to exhibit plantlet regeneration (**FIG. 3B**) after 4-5 weeks of culture initiation.

Moreover, embryogenic calli that were grown under salinity stress conditions and further were transferred to regeneration medium (2.0mg/L of BAP + 0.5mg/L of NAA) added with respective concentrations (10mM, 25mM, 50mM, and 100mM) of NaCl salt solutions, also could show the regeneration of plantlets. Embryogenic callus was sub-cultured once in every 2-weeks in order to obtain multiple-shoot and root formation on the same regeneration medium (2.0mg/L of BAP + 0.5mg/L of NAA) added with respective concentrations of NaCl.

The lowest concentration (10mM) of NaCl was proved to be the least inhibitory for plantlets regeneration and therefore, maximum number of green plantlets formation was observed (**Fig. 3C)**. However, highest concentration (100mM) of NaCl in regeneration medium proved to be significantly inhibitory for both shoot and root regeneration **(Fig. 3D)**.

The highest frequency (79.2%) of embryogenic calli exhibited plantlet regeneration in the nutrient medium containing (10mM) of NaCl and the also the maximum number of plantlets regeneration per embryogenic callus (7.3±0.2) was recorded. Moreover, in contrast, the lowest frequency (25%) of plantlet regeneration was obtained with the calli growing in presence of high concentration (100mM) of NaCl and thus the minimum number (1.5±0.1) of plantlet regeneration per embryogenic callus **(Table 4)** was recorded.

Transplantation of Regenerants

Regenerated plantlets were washed with sterile distilled water until the agar removed from the rooting part and the plantlets were transferred to the sterile cup containing vermiculite, vermi-compost and sand (20%:20%:60%) and plantlets growing under control and salt-treated conditions were gradually acclimatized under growth chamber conditions **(Fig. 3E & 3F)** respectively.

Table 1. Effect of various concentrations of 2,4-D on the frequency of somatic embryogenesis from mature caryopsis culture in black rice (*Oryza sativa* L.).

Concentration of 2,4-D (mg/L)	Mean frequency of somatic embryogenesis (%)	No. of Somatic Embryos/Callus (Mean ± SE)
0.5	30.0	2.0 ±0.2
1.0	56.6	3.5±0.3
2.0	90.0	9.3±0.4
3.0	80.0	5.7±0.3
4.0	23.3	1.1±0.1

Table 2. Effect of various concentrations of NaCl with 2,4-D (2.0mg/L) on the frequency of somatic embryogenesis from mature caryopsis culture in black rice (*Oryza sativa* L.).

Concentration of NaCl (mM) with 2,4-D (2.0 mg/L)	Mean frequency of somatic embryogenesis (%)	No. of Somatic Embryos/Callus (Mean ± SE)
Control	90.0	9.3±0.4
10	85.3	9.2±0.3
25	77.2	7.8 ±0.2
50	63.3	6.1±0.2
100	40.3	3.2±0.1
150	0.0	0.0

Table 3. Effect of various concentrations of BAP in combination with NAA (0.5mg/L) on the frequency of plantlets regeneration from the embryogenic callus induced during mature caryopsis culture in black rice (*Oryza sativa* L.).

Concentration of BAP (mg/L) with NAA (0.5 mg/L)	Mean frequency of somatic embryogenesis (%)	No. of Regenerated Plantlets/Embryogenic Callus (Mean ± SE)
0.5	51.7	3.1±0.1
1.0	70.0	5.6 ±0.2
2.0	80.3	7.7±0.3
3.0	40.0	1.2±0.1
4.0	0.0	0.0

Table 4. Effect of various concentrations of NaCl in combination with BAP (2.0 mg/L) and NAA (0.5 mg/L) on plantlet regeneration from the embryogenic callus induced during mature caryopsis culture in black rice (*Oryza sativa* L.)

Concentration of NaCl (mM) with BAP (2.0 mg/L) and NAA (0.5 mg/L)	Mean frequency of somatic embryogenesis (%)	No. of Regenerated Plantlets/Embryogenic Callus (Mean ± SE)
Control	80.3	7.7± 0.3
10	79.2	7.3 ±.0.2
25	70.4	5.7± 0.2
50	50	3.0± 0.1
100	25	1.5±0.1

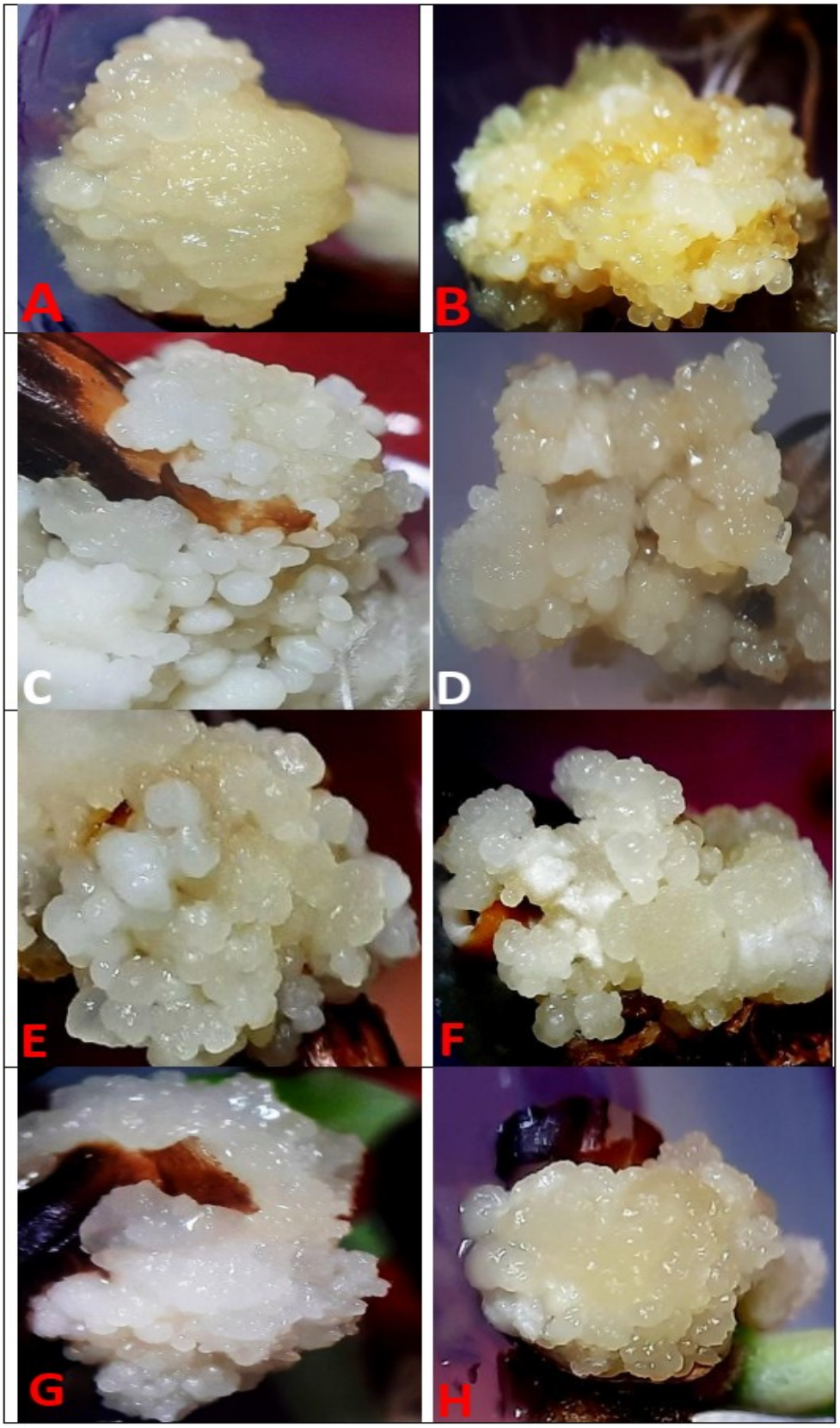

Figure 1. Black rice **(***Oryza sativa* L.**);** Mature caryopsis culture showing effects of salinity stress on somatic embryogenesis; **(A)** 2,4-D (0.5 mg/L) **(B)** 2,4-D (1.0 mg/L) **(C)** 2,4-D (2.0 mg/L) **(D**) 2,4-D (4.0 mg/L) **(E**) 2,4-D (2.0 mg/L) + 10mM of NaCl **(F**) 2,4-D (2.0 mg/L) + 50mM of NaCl **(G)** 2,4-D (2.0 mg/L) +100mM of NaCl **(H)** 2,4-D (2.0 mg/L) +150mM of NaCl salt treatments (after 6-weeks of culture initiation).

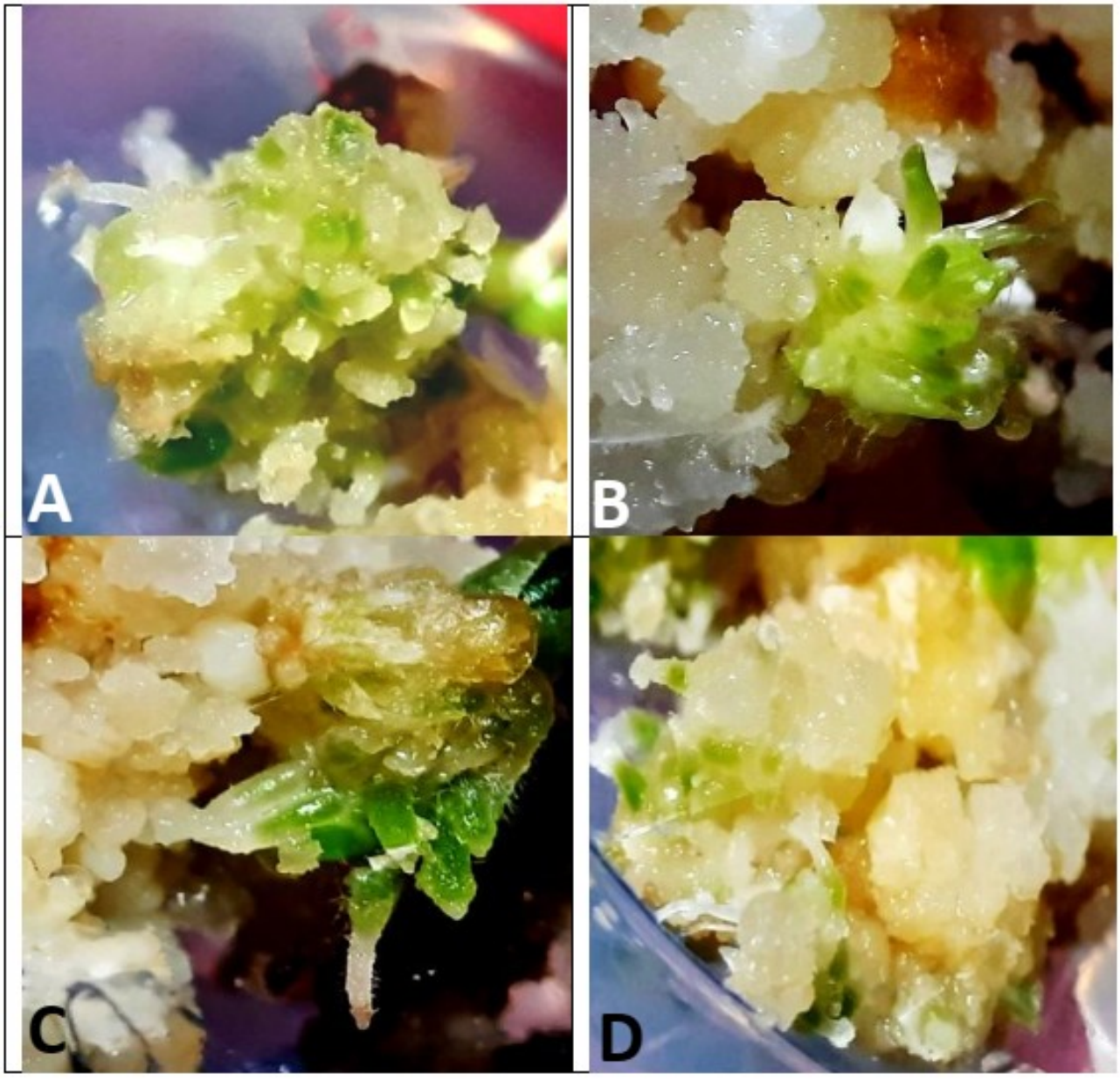

Figure 2. Black rice (*Oryza sativa* L.)- Mature caryopsis culture showing effects of various concentrations of BAP in combination with NAA (0.5mg/L) on plantlets regeneration; **(A)** 0.5mg/L **(B)** 1.0mg/L **(C)** 2.0mg/L **(D)** 3.0mg/L (after 2-weeks on transfer of embryogenic callus to regeneration medium).

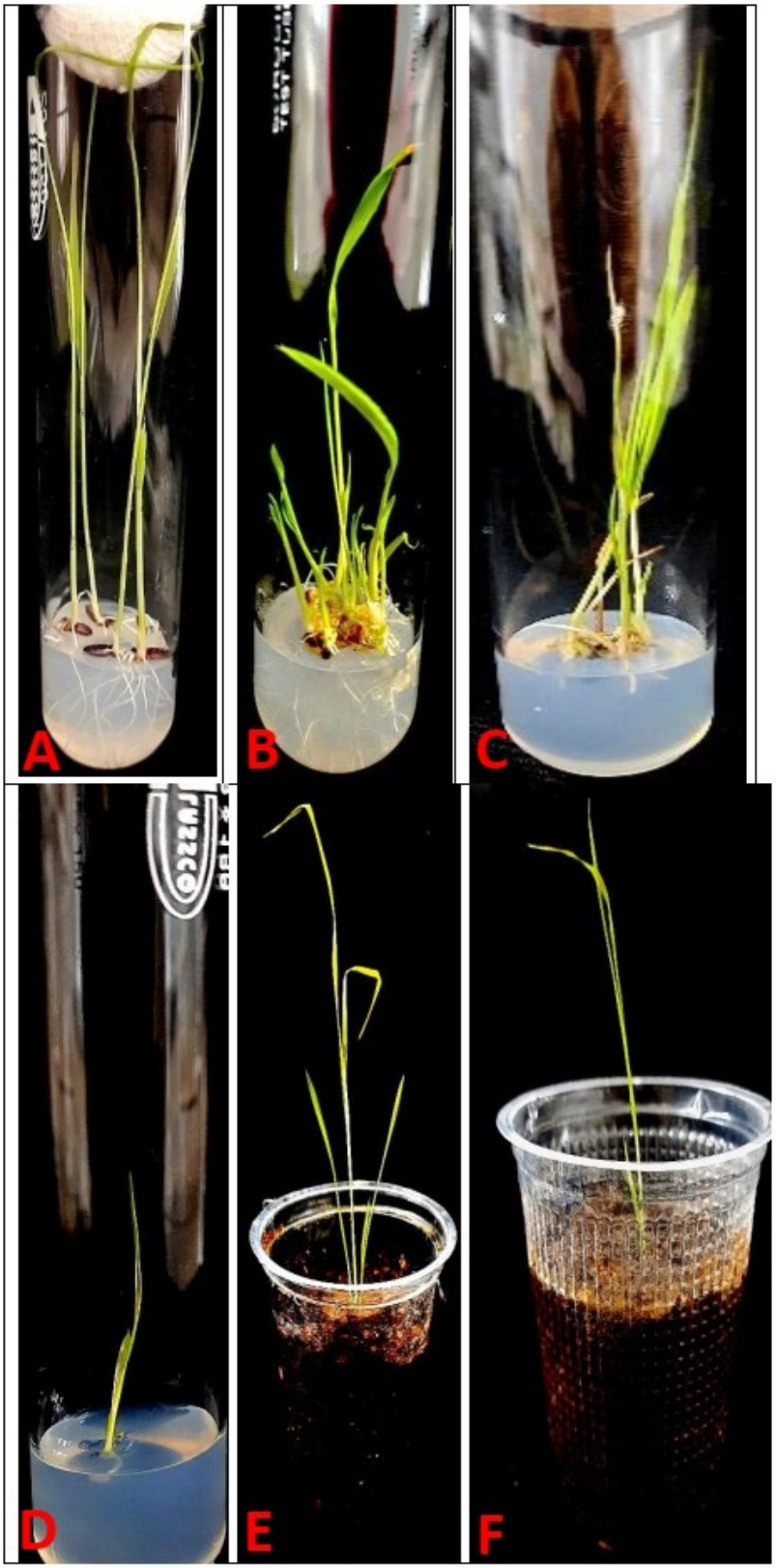

Figure 3. Black rice (*Oryza sativa* L.)- Mature caryopsis culture showing effects of various concentrations of NaCl in combination with BAP (2.0mg/L) and NAA (0.5mg/L) on plantlets regeneration; **(A)** Normal seedling development from mature caryopsis culture on basal medium **(B)** 2.0mg/L of BAP + 0.5mg/L of NAA **(C)** 10mM of NaCl **(D)** 100mM of NaCl **(E)** Hardening of normal regenerated plantlet **(F)** Hardening of salt tolerant regenerated plantlet (After 4-weeks of plantlets regeneration).

DISCUSSION

It is expected that world population would to reach about 9.1 billion by the year 2050, so the production of food is required to be increased by 70% to meet the requirements and therefore, food production world-wide needs to be increased by 60-110% (Tilman *et al*., 2011; FAO, 2009; 2012).

Due to drought, salinity, and submergence tolerance stress in rice field is a primary challenge for the production of rice yield therefore stress tolerant rice production is major priority for the world population (Grover *et al*., 2000). Amongst various ionic species (Na, Ca, Cl, SO_4, and HCO_3), NaCl is the most dominant ion present in the saline soil condition (Akbar and Ponnam-peruma, 1982). Moreover, the salt added to water or soil gives the secondary osmotic stress to the plant (kirst, 1977).

Moreover, when the soil contains very high concentration of NaCl, it leads to the hyperosmotic stress to the plant and it consequently leads to reduction in nutrient absorption by the plant (Wani *et al*., 2010).

Effect of Plant Growth Regulators on Embryogenic Callus Induction

In previous study, callus formation could be observed with 2mg/L of 2,4-D in combination of 1mg/L of NAA supplemented nutrient medium and moreover, the callus was induced after 21 days of culture initiation (Evangelista *et al*., 2009). Significantly, the high percentage (75%) of callus induction during mature embryo culture was obtained with 2,4-D (3.0mg/L) for Chini kanai (local variety) of rice while in other cultivar of rice (BRRI Dhan38) same frequency of callus induction was obtained with high 2,4-D (5.0mg/L) (Zinnah *et al*., 2013).

As per only solitary report available in black rice**,** embryogenic callus was obtained from the anther in the combination of the hormone (2.0mg/L of NAA + 0.5 mg/L of kinetin + 20µM of putrescine) in Indonesian black rice and the callus was kept in the dark condition for 4-6 weeks culture induction period (Maharani *et al*., 2019).

Significantly, results reveal in present study indicate that 2.0mg/L of 2,4-D alone was good enough to induce very high frequency (90%) of embryogenic callus formation and also the maximum number of somatic embryos per callus (9.3±0.4) in Indian black rice (cv. Co57). Moreover, high concentration (4.0mg/L) and low concentration (0.5mg/L) of 2,4-D in present study were turned out to be strongly inhibitory and therefore, 23.3% and 30% respectively of mature caryopses could show callus formation with differentiation of somatic embryos.

Somatic embryogenesis under salinity stress

Previous study in rice, callus formation was obtained in presence of 2,4-D (2.5 mg/L) + KIN (0.5 mg/L) and after 4-weeks of culture, induced callus was sub-cultured into the different concentrations of NaCl to identify the weight of the callus and after another 4-weeks, callus was transferred into the regeneration medium. Once the NaCl concentration present in the medium increases it sharply decreases the fresh weight of the callus (Wani *et al*., 2010).

Moreover, earlier study on mature embryo culture, callus was induced after 3 weeks with 2,4-D (2.0mg/L) in the dark conditions and after 3-weeks, the callus was sub-cultured into the different concentrations of NaCl for 4-weeks in order to check the proline content (Shankhdhar *et al*., 2000).

In case of mature seed culture in rice cultivars (BRRI dhan32, BR10, and BRRI dhan47) for the formation of the salt tolerant somatic embryo formation and the embryos were formed with the concentrations (2.5 mg/L of 2, 4-D and 1.0 mg/L of KIN) and 4 to 5 weeks old embryogenic callus were transferred into different concentrations of the NaCl (2.9, 5.9, 8.8, 11.7g/L) (Siddique *et al*., 2014).

However, present study involves the application of NaCl (10mM-100mM) with the beginning of the callus initiation in callus induction medium containing (2mg/L) of 2,4-D. Moreover, somatic embryogenesis was recorded maximum (85.3%) in presence of 10mM of NaCl. Significantly, 100mM of NaCl resulted the induction of salt tolerant embryogenic callus (40.3%) while further increase in NaCl (150mM) concentration was proved to be lethal for both induction of callus and

somatic embryos. Interestingly, report on induction of somatic embryos under NaCl-salinity stress conditions in black rice is completely lacking.

In another study, salt tolerant callus formation 2, 4-D (2.0 mg/L) was possible keeping in the dark for 30 days and the callus was sub-cultured into the same medium with different concentration of the NaCl. Moreover, salt tolerant callus was again sub-cultured into the same NaCl concentrations in order to check the salt tolerant line and the callus was sub-cultured into the regeneration medium (Kalhori *et al*., 2017). Of late, mature seed of black rice (cv. CHAK HAO-AMUBI) was used for *in vitro* mutagenesis by using EMS (0.0-0.015%) in regeneration medium (Tripathy *et al*., 2022)

Plantlet Regeneration from Embryogenic callus under Salinity Stress

In previous study, after 4-weeks shoot induction formed from the concentration 2.0mg/L (KIN) + 1.0 mg/L (BAP) and the percentage of shoot induction in control (82%) was found to be higher than 50mM (44%) and it was followed by 100 mM NaCl (15%) and after 4-weeks root induction formed from the concentration (0.5mg/L of BAP, 1.0mg/L of KIN, 1.0 mg/L of IBA, and 0.5mg/L of NAA) (Kalhori *et al*., 2017).

To obtain plant regeneration, in earlier study, 4-week-old salt tolerant callus was transferred into the regeneration medium with the different concentrations of kinetin with 1.0mg/L NAA+2.0mg/L BAP added with different concentrations of NaCl. After 4-weeks, BRRI 38 cultivar was found to support regeneration (20%) with 50mM of NaCl and (0%) in 150mM NaCl concentration and similarly, Chini kanai cultivar gives (20%) regeneration with 100mM while with 200mM of NaCl, there was no plantlet regeneration (Zinnah *et al*., 2013).

Furthermore, plant regeneration from salt tolerant callus was obtained at the concentration BAP (0.5 mg/L) with different concentrations of NaCl (0.5, 1.0, 1.5, and 2.0%) after 3-4 weeks (Shankhdhar *et al*., 2000). After mention as 3-months, plantlet regeneration formed from the embryogenic calli that were transferred into the Linsmaier and Skoog (LS) medium added with 2.0 mg/L BA and 10mg/L NAA and the embryogenic calli were further sub-cultured into the fresh medium to obtain regenerated plantlets (Evangelista *et al*., 2009)

An earlier study, PAU201 and PR116 cultivars were used for shoot initiation formed with the concentrations (2.0mg/L of BAP + 0.5 mg/L of KIN + 0.5 mg/L of NAA) after 4-weeks of incubation period and the rooting was initiated from the basal medium. When the NaCl concentration in the medium was increased then the fresh weight of the callus in the medium was found to be decreased (Wani *et al*., 2010).

Moreover, present study reveals plantlet regeneration were formed from the concentration (2.0mg/L of BAP + 0.5 mg/L of NAA) with different concentrations of NaCl (10mM-100mM). However, plantlet regeneration was recorded maximum (79.2%) in the presence of 10mM of NaCl. Significantly, minimum frequency of plantlet regeneration was recorded (25%) in the presence of 100mM of NaCl. During present study, regenerated plantlets under control and salt-treated conditions were gradually acclimatized in growth chamber.

CONCLUSION

In vitro tissue culture technique could be proved effective to produce the salt tolerant black rice crop (Co57) variety. In this study, salt tolerant callus induction, somatic embryogenic formation, and plantlet regeneration were achieved in order to grow salt tolerant black rice crop for the coastal land. Additionally, developed protocols of somatic embryogenesis and plantlets regeneration under salinity stress conditions in present study could be significant for the selection of salt-tolerant transgenic black rice.

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CONFLICTS OF INTEREST

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

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