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In vitro step up NaCl treatment method as inducer of salinity tolerance in Basmati rice varieties

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Abstract

Background: Salt has been recorded to negatively impacts rice crop's seedling and reproductive stages. Globally, soil salinity is 2nd most important abiotic factor, profoundly impacting rice productivity. The purpose of current research is to induce salinity tolerance in salt sensitive varieties of Basmati rice [Basmati-385 (Bas-385), Super Basmati (Sup-Bas) and Basmati-2000 (Bas-2000)] by use of *in vitro* step-up NaCl treatment method.

Methods: The *in vitro* step up NaCl treatment method was used to produce salt-tolerant calli from salt-sensitive Basmati rice varieties. The regeneration of these salt-tolerant calli in a salt-free medium led to the M_1 generation. Subsequently, M_2 and M_3 generations were cultivated and grown under salt stress conditions.

Results: The highest callus induction percentage was achieved for all Basmati rice varieties by using MS medium supplemented with 2.0 mg/L 2,4-D. These calli underwent *in vitro* step up NaCl treatments, with the maximum tolerance level to NaCl being 1.0% across all varieties. Bas-385 and Bas-2000 exhibited regeneration frequencies of 75% and 85% respectively when cultured in MS medium supplemented with 2.0 mg/L BAP and 0.5 mg/L NAA, while Sup-Bas displayed a regeneration frequency of 79% in MS medium comprising 3.0 mg/L BAP with 0.5 mg/L NAA. In subsequent M_2 and M_3 generations, a decline was observed in all selected agronomic and morphological features in all varieties under salinity stress in comparison to parent plants.

Conclusion: This research validates the induction of salt tolerance in salt susceptible varieties of Basmati rice by *in vitro* step up NaCl treatment technique.

Introduction

Rice (*Oryza sativa* L.) is cultivated extensively worldwide, serving as a staple food for over half of the global population and contributing to 21% of global per capita energy consumption [1]. It is rich in dietary energy (27%), protein (20%), and fat (3%) [2]. Rice is grown across all continents except Antarctica, covering more than 167.13 million hectares and yielding approximately 782 million tons [3,4]. However, the majority of rice production occurs in Asia, accounting for over 90.7% of the world's output [5,3]. In Pakistan, rice cultivation occupies about 11% (3,034 thousand hectares) of the total cultivated area, yielding 7,410 thousand tons annually. It is a high-value cash crop, contributing 0.6% to value added in the gross domestic product and 3.1% to the agricultural sector [6].

The Basmati aromatic traditional rice and the short-stemmed and high-yielding IRRI-Pak varieties are the two main types of rice grown in Pakistan. The most prevalent type, aromatic Basmati rice, is found in 62% of the country's rice fields and accounts for 52% of the country's rice production. Basmati varieties are primarily cultivated in the Punjab province, where 94% of the Basmati area and 92% of the production are concentrated [6]. In Punjab, there has been a significant increase of 60% in Basmati cultivation area and a staggering 155% increase in yield from 1982 to 2002 [6,7].

Despite its genetic potential, rice yield has not reached its maximum in most rice-growing regions globally. One of the key reasons for this shortfall is the susceptibility of rice to various biotic (pests, insects, diseases) and abiotic factors (soil salinity, drought, waterlogging, cold) [8,9]. Soil salinity ranks as the second most crucial abiotic factor affecting rice productivity worldwide [10], with rice being recognized as salt-susceptible, leading to adverse effects during both sprouting and reproductive stages [11-15].

Salt-affected soils pose a significant challenge at universal and local farm levels. Out of the total 167.13 million hectares of rice-growing land, approximately 31% is afflicted with high salinity levels, greatly impeding normal rice productivity. In Pakistan, one million hectares of rice-growing region is pretentious by salinity, consequentially lead to a decline of 40–70% in crop yield [16-19].

Recent projections estimate the global population to reach 9 billion by 2050, with emerging countries contributing 97% of this growth. Rice demand is expected to reach 590 million tons by 2050 [20,2]. To meet this escalating demand, developing new rice varieties capable of growing under salt stress by exploiting genetic variability is imperative.

The use of plant tissue culture technology for screening plants tolerant to abiotic stresses offers a promising approach for selecting cell populations on a large scale [21]. In vitro screening methods allow rapid proliferation of cell populations in petri dishes or flasks using well-defined growth media. Cell lines established through tissue culture applications can be vetted *in vitro* for tolerance to various stresses, including salinity, which has been widely employed in rice breeding [22-24] and other plants [25,21].

Soil salinity is now recognized as a significant problem in Pakistan's prime Basmati rice-growing areas [26], highlighting the need for developing rice varieties resistant to salinity effects. The purpose of this study is to use the *in vitro* step-up NaCl treatment method to persuade the salinity tolerance in economically imperative salt susceptible varieties of Basmati rice (Bas-385, Sup-Bas and Bas-2000).

Methods

Explants disinfestation

Licensed seeds of Bas-385, Sup-Bas, and Bas-2000 underwent a cleaning process by washing with cleanser for 15 minutes, followed by several rinses with tap water. Subsequently, the seeds were drenched in distilled water overnight. For surface sterilization, a 0.1% $HgCl_2$ (Mercuric chloride) solution was employed, and manually dehusked seeds were soaked for 15 minutes. Afterwards, the seeds underwent a thorough washing with sterilized purified water, repeated 3-5 times within the sterilized atmosphere of a laminar airflow cabinet.

Callogenesis

For callus induction, disinfected seeds were introduced onto agar-solidified MS [27] medium supplemented with various combinations of plant growth regulators [2,4-Dichlorophenoxy acetic acid (2,4-D), Benzyl aminopurine (BAP), Kinetin (KIN)], with pH attuned to 5.7 - 5.8. The cultures were incubated in darkness at 25 ± 1°C temperature. Callus cultures were allowed to develop for 4 weeks. at the end of the 4th week, information regarding frequency of callus induction and the morphology of callus were recorded. The medium exhibiting the highest prospective for callus formation and proliferation rate was identified as the optimal medium for next experimentation. Non-embryogenic calli were discarded, while vigorous embryogenic calli were carefully chosen and sub-cultured onto new medium for proliferation after every 2 weeks.

In vitro selection of salt tolerance calli

Vigorous embryogenic calli, aged eight weeks, were carefully excised under aseptic conditions and exposed to *in vitro* step-up NaCl treatments method. These treatments involved a range of NaCl concentrations

from 0, 0.5, 1, 1.5, 2 to 2.5%, incorporated into agar coagulated Murashige and Skoog medium, at intermissions of 3 weeks. The calli exhibiting high salt tolerance were maintained on the same medium for three sequential proliferation phases, each phase lasting for six weeks.

Plant regeneration

To initiate regeneration, all NaCl-tolerant calli were transferred to MS medium supplemented with diverse recipes of growth regulators. The cultures were then cultivated in a growth room under a photoperiod of 16 hrs. light and 8 hrs. dark at 25±1°C temperature. After every three weeks, the cultures were shifted to new fresh medium. The appearance of green shoots measuring over 3cm in height was considered for calculating the Regeneration Frequency (RF).

These regenerated calli were transferred to basic Murashige and Skoog medium devoid of any growth regulators to promote root development. Subsequently, they were shifted to a hydroponic solution to facilitate root hardening. Each regenerated plant was allotted a unique plant line number (M_1) and allowed to grow until reaching maturity.

Screening for salt tolerance from M_2 generation

M_2 lines derived from M_1 plants, excluding those exhibiting poor plant characteristics such as droopy leaves and weak culms, were harvested and sequentially numbered from M_2 1 to n. Screening for salt tolerance was conducted by cultivating all M_2 lines under saline field conditions, with an electrical conductivity (EC) equivalent to the maximum percentage of NaCl (14 ds/m) that the salt-tolerant calli had previously survived.

Screening for salt tolerance from M_3 generation

After 140 days, the M_2 generation was harvested, now referred to as the M_3 generation, exhibiting normal grain fertility at a rate of 80% on an individual plant basis. M_3 lines were sequentially numbered from M_3 1 to n. Screening for salinity tolerance in the M_3 lines was conducted by cultivating all lines in a salt stressed field with an electrical conductivity (EC) equal to the maximum percentage of NaCl (14 ds/m) that the salt-tolerant calli had previously endured. Control plants (parent plants) of all varieties were also transplanted alongside the M_3 lines for comparison.

Performance of M_2 and M_3 generations

Data regarding several agronomic and morphological traits of the salt-tolerant M_2 and M_3 lines were meticulously recorded for all varieties. The selected traits included height of plant, length of panicle, number of tillers and the number of plants producing fertile seeds.

Statistical analysis

All experimentation was conducted in a triplicate manner. The obtained data was subjected to Analysis of Variance (ANOVA), and the differences between the means were deliberate through Duncan's Multiple Range Test at a significance level of $P < 0.05$. SPSS version 12 was used to do the statistical analysis.

Results

Callogenesis

Callogenic response of Basmati rice varieties on Murashige and Skoog (MS) medium supplemented with various combinations of plant growth regulators after 4 weeks of inoculation is given in Table 1. No callogenic response was observed in control and the maximum callus induction response in Basmati-385 (78%), Super Basmati (75%) and Basmati-2000 (80%) was achieved on MS medium fortified with 2.0 mg/L 2,4-D. The calli were granular and compact in texture, embryogenic in nature and light yellow to light brown in color (Fig. 1a,1b and 1c). The second-best results were obtained in all the varieties in Murashige and Skoog medium containing 1.0 mg/L 2,4,-Dichlorophenoxy acetic acid while the percentage of callus induction decreased across all varieties as the concentration of 2,4-D increased.

When combining 2,4-D (2.0 mg/L) with different levels of BAP, the highest callus induction was observed on BAP at a level of 2.0 mg/L across all varieties. However, this induction decreased with further increases in BAP concentration. Similarly, when different concentrations of KIN were tested with 2,4-Dichlorophenoxy acetic acid (2.0 mg/L), the optimal retort was obtained at a KIN concentration of 1.0 mg/L in all varieties (refer to Table 1).

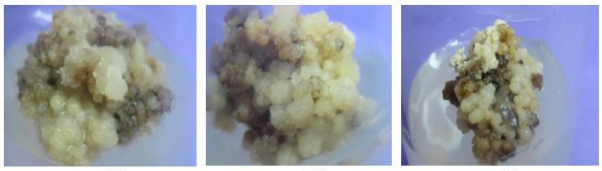

Figure 1: Callogenic response of (a) Basmati-385, (b) Super Basmati and (c) Basmati-2000 on Murashige and Skoog medium invigorated with 2.0 mg/L 2,4-Dichlorophenoxy acetic acid.

In vitro selection of salt tolerance calli

Vigorous embryogenic calli of Bas-385, Sup-Bas and Bas-2000, aged eight weeks, were aseptically cut out. Approximately 50mg (initial weight) of calli from every variety were exposed to *in vitro* step-up NaCl treatments. These treatments ranged from 0, 0.5, 1, 1.5, 2 to 2.5% NaCl, incorporated into agar-solidified Murashige and Skoog medium invigorated with 2.0 mg/L 2,4- Dichlorophenoxy acetic acid. The treatments were administered at 3-week intervals (Table 2).

Relative growth value of calli inl each variety was decreased with increasing NaCl concentration as compared to control. In case of Bas-385, relative growth at 0.5% NaCl was 1.90 and 1.95 at 1.0% NaCl. While, at higher concentration of NaCl (1.5, 2.0 and 2.5%) growth value sharply decreased, indicating that maximum NaCl tolerance level for calli of Bas-385 was 1.0%. Similar trend for percentage NaCl tolerance level was also observed in case of Sup- Bas and Bas-2000 (Table 2).

Medium	Plant Growth regulators (mg/L)	Callus induction(%)		
		Bas-385	Sup-Bas	Bas-2000
MS + 2,4-D	0	00	00	00
	1	66.33 ± 4.25^{b}	60.45 ± 6.66^{bc}	70.65 ± 3.41^{b}
	2	78.41 ± 6.23^{a}	75.77 ± 6.45^{a}	80.88 ± 7.80^{a}
	3	43.26 ± 2.12^{cd}	45.23 ± 4.25^{cd}	50.45 ± 4.25^{bc}
	4	35.32 ± 3.33^{d}	40.42 ± 4.10^{cd}	30.33 ± 3.25^{de}
	5	25.55 ± 2.12^{def}	25.22 ± 2.36^{def}	20.14 ± 3.41^{efg}
	6	15.11 ± 2.41^{ef}	15.11 ± 1.25^{ef}	10.11 ± 1.24^{fg}
	7	10.12 ± 1.14^{ef}	10.12 ± 1.22^{ef}	10.11 ± 1.24^{fg}
	8	08.25 ± 1.36^{efg}	09.11 ± 1.00^{efg}	10.11 ± 1.24^{fg}
MS + 2,4-D (2.0mg/L) + BAP	1	41.14 ± 2.45^{b}	36.25 ± 3.36^{c}	49.44 ± 4.25^{b}
	2	45.45 ± 2.36^{a}	40.14 ± 4.12^{bc}	53.32 ± 3.25^{a}
	3	32.11 ± 2.05^{bc}	31.18 ± 3.41^{cd}	40.41 ± 4.12^{c}
	4	20.22 ± 4.12^{cde}	23.41 ± 2.11^{def}	30.22 ± 3.21^{de}
MS + 2,4-D (2.0mg/L) + KIN	1	43.41 ± 3.22^{ab}	53.23 ± 5.41^{a}	48.41 ± 4.26^{b}
	2	31.33 ± 2.45^{bc}	36.25 ± 3.23^{c}	33.10 ± 3.21^{de}
	3	28.41 ± 1.45^{cd}	30.32 ± 1.25^{cd}	24.32 ± 2.33^{ef}
	4	25.71 ± 1.32^{cd}	26.45 ± 2.25^{de}	10.12 ± 3.02^{fg}

Table 1: Callogenic expression of Basmati rice varieties in MS medium invigorated with different plant growth regulators. 100 seeds of each variety were inoculated in each experiment. Experiments were performed in triplicate.

Basmati Rice variety	NaCl Treatments (1)	Relative growth (2)
Bas-385	Control	2.20 ± 1.02a
	0.5 %	1.90 ± 0.88b
	1.0%	1.95 ± 0.75b
	1.5%	0.85 ± 0.21c
	2.0%	0.71 ± 0.11cd
	2.5%	0.40 ± 0.09d
Sup-Bas	Control	2.40 ± 0.98a
	0.5 %	1.73 ± 0.66b
	1.0%	1.80 ± 0.85b
	1.5%	0.73 ± 0.23cd
	2.0%	0.55 ± 0.41cd
	2.5%	0.29 ± 0.10de
Bas-2000	Control	2.30 ± 1.00a
	0.5 %	1.86 ± 1.04b
	1.0%	1.93 ± 1.11b
	1.5%	0.84 ± 0.32c
	2.0%	0.62 ± 0.11cd
	2.5%	0.36 ± 0.09cde

Means values trailed by the diverse letters are meaningfully different as per DMRT p<0.05.
Table 2: Assortment of NaCl tolerant calli by use of step up NaCl treatment method.

(1) Around 50mg of callus (initial weight) underwent *in vitro* step-up NaCl treatment, spanning concentrations from 0, 0.5, 1, 1.5, 2 to 2.5% NaCl. This treatment was administered in agar coagulated MS medium supplemented with 2 mg/L 2,4-Dichlorophenoxy acetic acid at three week intermissions.

(2) Relative growth: The ratio of the calli's final weight to its initial weight is what determines their relative growth. The outcomes that have been reported are the average of three replicates.C

Plant regeneration

The calli of Basmati rice varieties, which exhibited high tolerance to salt (1.0% NaCl), underwent three consecutive proliferation phases in the same Murashige and Skoog medium invigorated with 2,4-Dichlorophenoxy acetic acid 2.0 mg/L and 1.0% NaCl, with a 6-week interval each time, to fully adapt to this level of salinity stress. For regeneration, all chosen salt-tolerant calli were transferred to Murashige and Skoog medium supplemented with different blends of 6-Benzylaminopurine with 0.5mg/L 1-Naphthaleneacetic acid. A hundred calli from each variety were moved to the regeneration medium. According to Table 3, in Murashige and Skoog medium invigorated with 2.0 mg/L 6-Benzylaminopurine and 0.5 mg/L 1-Naphthaleneacetic acid, Basi-385 and Bas-2000 had regeneration frequencies of 75% and 85%, respectively. Sup-Bas had regeneration frequency of 79% in Murashige and Skoog medium invigorated with 3.0 mg/L 6-Benzylaminopurine and 0.5 mg/L 1-Naphthaleneacetic acid. It was noted that the percentage of regeneration frequency decreased as BAP levels rose (Table 3).

Medium	Regeneration frequency (%)		
	Bas-385	Sup-Bas	Bas-2000
MS + 0.5mg/LNAA + BAP (1.0 mg/L)	60.65±5.22c	58.45±4.36bc	63.36±6.33c
MS + 0.5mg/LNAA + BAP (2.0 mg/L)	75.76±4.57a	60.62±2.12bc	85.14±7.65a
MS + 0.5mg/LNAA + BAP (3.0 mg/L)	70.41±7.22b	79.71±4.65a	74.23±4.56b
MS + 0.5mg/LNAA + BAP (4.0 mg/L)	52.31±2.64d	41.21±5.32c	50.54±5.23d
MS + 0.5mg/LNAA + BAP (5.0 mg/L)	38.25±4.25ef	30.33±2.33cd	24.21±1.23ef

Means values trailed by the different letters are meaningfully different according to DMRT p<0.05.
Table 3: Rejuvenation potential of 1.0% NaCl Tolerant calli.

The shoots grown *in vitro* were shifted to MS basal medium (barren of any growth regulators) to induce root formation (refer to Table 4). Subsequently, the in vitro grown roots were transferred to a hydroponic solution for the purpose of hardening. The plant line number (M_1) was given to each regenerated plant, and it was allowed to grow until it reached maturity. Later M_2 and M_3 generation were also obtained and the number of plants per variety in each generation is given in Table 4.

Basmati varieties	Number of plants		
	M_1	M_2	M_3
Bas-385	70.24±4.35^{b}	50.54±5.54^{b}	35.35±2.35^{a}
Sup-Bas	72.42±6.13^{b}	45.63±4.13^{bc}	20.42±2.24^{b}
Bas-2000	78.75±7.53^{a}	60.65±6.16^{a}	32.23±2.35^{ab}

Means values trailed by the different letters are expressively different according to DMRT p<0.05.
Table 4: Number of salt tolerant plants attained in M_1, M_2 and M_3 generations.

Varieties	Plant height (cm)			Panicle length			No of Tillers			No of plants with fertile seeds			Total weight (g) [1]		
	Parent	M_2	M_3	Parent	M2	M3	Parent	M_2	M_3	Parent	M_2	M_3	Parent	M_2	M_3
Basmati-385	133	85 (36)	73 (45.1)	30	24 (20.0)	20 (33.0)	25	16 (36.0)	12 (52.0)	50	35 (30.0)	21 (58)	420	290 (30.9)	184 (56.1)
Super Basmati	115	67 (41.7)	53 (53.9)	25	19 (24.0)	16 (36.0)	20	13 (35.0)	8 (60.0)	50	20 (60)	10 (80)	432	163 (62.2)	89 (79.3)
Basmati-2000	134	75 (44)	67 (50)	32	25 (21.8)	22 (31.2)	27	18 (33.3)	14 (48.1)	50	32 (36)	19 (62)	450	270 (40.0)	168 (62.6)

[1]Total weight was measured with all plants with fertile seeds.
Table 5: Depiction of salt tolerant M_2 and M_3 generations.

Performance of M_2 and M_3 generations

Morphological and agronomical characteristics of M_2 and M_3 generations were documented and compared with those of parent plants. A decline in all features was noted in all varieties under saline conditions in comparison to the parent plants (refer to Table 5). Results indicate that Sup-Bas exhibited the highest sensitivity to salt stress, displaying a significant decrease in nearly all morphological and agronomical traits considered in the M_2 and M_3 generations. Among the rest of 2 varieties, Bas-385 demonstrated greater salt resistance and exhibited superior field performance compared to Bas-2000.

Discussion

Abiotic stresses are the primary constraint to development and yield of plants in the farming area. Among them, salt stress is a main pressing issue in arid and semi-arid regions of the world. The detrimental effects that salinity has on plant development include osmotic and oxidative stress, mineral deficiency, nutritional imbalance, or a combination of these factors [28, 29].

Tissue culture technique is being routinely employed for screening of stress tolerance cell lines in various plant species. Using salts as a selective agent, the *in vitro* selection pressure method has been successfully used to induce tolerance to salinity in plants, allowing the preferred survival and growth of desired genotypes [30].

In the present work, from mature seed explants, embryogenic calli of three varieties of Basmati rice (Bas-385, Sup-Bas, and Bas-2000) were induced. The MS medium supplemented with 2.0 mg/L 2,4-D was found to be the best medium showing maximum callus induction and proliferation potential for all these Basmati rice varieties as compared to MS medium containing 2,4-D in mix with various concentrations of BAP or KIN. 2,4- Dichlorophenoxy acetic acid at the level of 2.0 mg/L has also been reported by Ullah *et al.* inducer growth regulator for Basmati-385 followed by Basmati-370 [31]. Similar findings were reported by Afrasiab and Jafar working with Super Basmati and Rafique *et al.* working with Basmati-370 [32,33].

Several researchers have also reported that 2,4-D at 2.0 mg/L produced the most desired calli in different varieties of rice [34,35,36,37,38]. Therefore, it can be concluded that that 2,4-D alone is more effective for the induction of callus in rice varieties as compared to combination of hormones in different concentrations. Upadhyaya *et al.* also reported 2,4-D as the only growth regulator effective in callus induction media for rice [39].

In this research work, the *in vitro* step up NaCl treatment method resulted in calli of Basmati rice varieties that were 1% NaCl-tolerant. While at higher concentration of NaCl (1.5, 2.0 and 2.5%) the calli turned brown and eventually died. Same method was also used by Miki *et al.* for induction of salinity tolerance in salt sensitive rice cultivar Nipponbare and vigorously growing shoot bud clumps up to 2% NaCl tolerant were obtained [22]. Our results are well supported by Htwe *et al.* who described that increasing the concentration of NaCl into culture medium resulted in necrosis of callus in five rice genotypes [40]. Likewise, Wu *et al.* explained that the browning of callus may be due to necrosis or tissue damage as result of oxidation of phenolic compounds [41]. *In vitro* step up NaCl treatment method is an alternative approach for induction of salinity tolerance in calli or plantlets as compared to single step salt treatment [22].

The salt tolerant calli were transferred to regeneration medium and best response was found to be 85% for Basmati-2000 and 75% for Basmati-385 in MS medium containing NAA (0.5 mg/l) with BAP (2.0 mg/l) and 79% for Super Basmati in MS medium having NAA(0.5 mg/l) with BAP (3.0 mg/l). Similar combination of hormones have been reported by Singh

et al. for plant regeneration of four varieties of rice from callus [34]. Regenerates of these salt tolerant calli were grown as M_1 generation in salt free field conditions. M_2 and M_3 generation were grown in saline field with EC 14 dS/m which was equal to maximum % of salt which was tolerated by the calli of different rice varieties.

All the agronomical and morphological characters studied showed a significant decrease as compared to control plants. Reduced growth parameters due to salt stress are a common occurrence in plants, observed in cultured cells, tissues, or organs grown on NaCl-supplemented medium. Slower growth is often considered an adaptive mechanism for plant survival under stress, with salt tolerance being inversely related to growth rate [42]. Khorami and Safarnejad found that increased NaCl levels elevated Na+ and Cl- concentrations in the cell cytoplasm, causing toxicity that hampers plant growth [43]. Saffan noted that osmotic effects from salinity stress can disrupt the plant's water balance, leading to reduced turgor and inhibiting plant growth [44]. Similar reduction in agronomic characters of NaCl treated plants of rice varieties have been reported by many researchers [45,46,47].

Osmotic stress can result from high salinity, and high salt concentrations in soils make it more difficult for plants to absorb water and nutrients [48]. At the flowering stage, high salt makes pollens less viable, which affects grain yield [49,50].

In the present research work, we induced salt tolerance in salt sensitive varieties of Basmati rice using *in vitro* step up NaCl treatment technique and M_2 and M_3 generations of these Basmati varieties were harvested at 14 ds/m EC. According to IRRI, soil salinity beyond 4ds/m is considered as moderate salinity while more than 8 ds/m is high salinity for rice [12]. With increasing NaCl concentration, various agronomic and morphological characteristics of these salt-tolerant M_2 and M_3 generations significantly declined. Results of this research work led us to conclude that this induction of salt tolerance in these varieties of Basmati rice was due to the adaptation of calli to saline environment by step up salt treatment method.

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Author Contributions

Supervised, checked and proofreading by Dr. Humera Afrasiab.

Study design and conception, material preparation, data collection and statistical analysis, interpretation of the results and first draft was written by Naveeda Batool.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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