In vitro Screening and Induction of Salt Tolerance in Rice

By

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Master of Science
(Agricultural Biotechnology)

DEPARTMENT OF AGRICULTURAL BIOTECHNOLOGY & MOLECULAR BIOLOGY

RAJENDRA AGRICULTURAL UNIVERSITY, BIHAR
PUSA (SAMASTIPUR) – 848125 INDIA

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Regd No. – M/Agri.Biotech/61/2011-12 of R.A.U.

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Rima Kumari

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Dedicated

To

My Loving Parents and Teachers

"Whose perpetual affection and blessings always inspired me for higher ambition in life".

🕮 .....RIMA

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ABSTRACT

Six selected cultivars of rice namely BPT-5204, MTU-7029, Narendra Usar Dhan-3, Rajendra Bhagwati, CSR-30 and Pusa Basmati-1 were screened for salinity tolerance on the basis of seed germination *in vivo* and *in vitro*, seedling growth and callus growth under different salt stress (0-2.5%) created by a salt mixture of NaCl, $CaCl_2$, Na_2SO_4 in 7:2:1 ratio. Cultivars CSR-30 and Narendra Usar Dhan-3 were found to be the most salt tolerant, cvs. MTU-7029 and BPT-5204 to be moderately tolerant and cvs. Rajendra Bhagwati and Pusa Basmati-1 were found to be salt sensitive respectively. The formation of callus and their continued growth at higher levels of salt stress indicated the induction and formation of salt tolerant cells and calluses. The cultivars of rice, their normal calluses and salt tolerant calluses were further screened by 14 salt tolerance linked SSR primer pairs for evaluation of their salt tolerance and for detection of induced salt tolerant callus. On the basis of SSR marker, cultivars CSR-30 and Narendra Usar Dhan-3 can be considered as salt tolerant while cv. Pusa Basmati-1 can be considered as salt sensitive. The other three cvs. MTU-7029, BPT-5204 and Rajendra Bhagwati can be considered as moderately salt tolerant. Molecular marker studies also confirmed induction of salt tolerant calluses in cvs. MTU-7029, Rajendra Bhagwati and BPT-5204.

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ABBREVIATIONS

ANGRAU	:	Acharya NG Ranga Agricultural University	MSS	:	Mature seed scutellum
A	:	Adenine	µl	:	Microlitre
BLB	:	Bacterial leaf blight	µM	:	Micromolar
bps	:	Base pairs	µg	:	Microgram
BA	:	Benzyl adenine	MgCl2	:	Magnesium chloride
BME	:	ß-merceptoethanol	Na	:	Sodium
BAP	:	6- Benzyl amino purine	NAA	:	Naphthalene Acetic Acid
CaCl2	:	Calcium chloride	NaCl	:	Sodium chloride
CV	:	Cofficient of variance	Na2SO4	:	Sodium sulfate
CD	:	Critical difference	NDUAT	:	Narendra Dev University of Agriculture & Technology
CTAB	:	Cetyl trimethylammonium bromide	NaOH	:	Sodium hydroxide
CSSRI	:	Central soil salinity research institute	Nm	:	Nanometre
CC		Control callus			
CS		Salt tolerant callus			
CO2	:	Carbon dioxide	Ng	:	Nano gram
C	:	Cytosine	NILs	:	Near isogenic lines
cvs.	:	Cultivars	ngl^{-1}	:	Nano gram per litre
2,4-D	:	2,4-Dichlorophenoxyacetic acid	NTSYSpc	:	Numerical Taxonomy and Multivariate Analysis System
DNA	:	Deoxyribonucleic acid	PGR	:	Plant Growth Regulator
dNTP	:	Deoxyribonucleotide triphosphate	Psi	:	Pounds per square inch
°C	:	Degree Celsius	PCR	:	Polymerase Chain Reaction
et al.	:	et alii	PP	:	Polymorphism percent
EC	:	Electrical conductivity	PIC	:	Polymorphism information content
EtBr	:	Ethidium bromide	PVP	:	Polyvinyl pyrrolidone

EDTA	:	Ethylenediaminetetraacetic acid	pH	:	Power of Hydrogen
EST	:	Expressed sequence tag	%	:	Percentage
F	:	Forward	QTL	:	Quantitative trait loci
Fig.	:	Figure	RTV	:	Rice tungro virus
FAO	:	Food and Agriculture Organization	RM	:	Rice marker
GA_3	:	Gibberellic acid	RAU	:	Rajendra Agricultural University
gm	:	Gram	RNase	:	Ribonuclease
G1		BPT-5204	G4		Rajendra Bhagwati
G2		MTU-7029	G5		CSR-30
G3		Narendra Usar Dhan-3	G6		Pusa Basmati-1
γ	:	Gamma	R	:	Reverse
HCl	:	Hydrochloric acid	rpm	:	Revolution per minute
HgCl2	:	Mercuric chloride	RILs	:	Recombinant inbred lines
IAA	:	Indole-3-acetic acid	Rf	:	Relative front
IARI	:	Indian agricultural research institute	SSR	:	Simple sequence repeat
IBA	:	Indole-3-butyric acid	SSLP	:	Simple sequence length polymorphism
ISSR	:	Inter-simple sequence repeats	Sl. No.	:	Serial number
K	:	Potassium	SEm	:	Standard error of mean
KIN	:	Kinetin	SE	:	Standard error
KCl	:	Potassium chloride	SHAN	:	Sequential Agglomerative Hierchical Non-overlapping
LS	:	Linsmaier and Skoog	TBE	:	Tris Borate EDTA
L		Leaf	T		Temperature
L	:	Ladder	TE	:	Tris ethylenediaminetetraacetic acid
MSM	:	Methylsulfonyl methane	Tm	:	Melting temperature
$MgCl_2$	:	Magnesium chloride	T	:	Thymine

mgl^{-1}	:	Milligram per litre	UV	:	Ultraviolet
min	:	Minute	UPGMA	:	Unweighted Pair Group Method with Arithmetic Mean
ml	:	Millilitre	V	:	Volt
mM	:	Millimolar	V/V	:	Volume/Volume
mm	:	Millimeter	WBPH	:	White backed plant hoppers
MS	:	Murashige and Skoog	W/V	:	Weight/Volume
M	:	Molar	X	:	Concentration

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CHAPTER – I

INTRODUCTION

INTRODUCTION

Rice (*Oryza sativa* L.) (2n=24) is a self-pollinated, monocot crop belonging to the genus *Oryza* of family Poaceae (Graminae) and sub family Oryzoidea . There are 27 species in the genus *Oryza* of which 25 are wild and two viz *Oryza sativa* L. and *Oryza glaberrima* are cultivated. Globally, rice is one of the most important food grains with overall production of 718.34 million tonnes from 163.46 million hectares with an average yield of 4395 kg/ha. World-wide India stands 1st in rice area and 2nd in rice-production after China with overall production of 105.24 million tonnes from 42.41 million hectares with the productivity of 2462 kg/ha. In Bihar, rice is cultivated on 3.25 million hectares with 7.34 million tonnes production and productivity of 2258 kg/ha. Its pericarp and embryo contain 70-80% starch, 7% protein, 1.5% oils, some important vitamins namely A, B and C and some essential minerals. Compared to wheat and maize, it has the highest net protein utilization value. It also produces more calories and carbohydrates per hectare than any other cereal (Lu and Chang, 1980).

The productivity of several commercial crops is limited by major abiotic stresses including salinity, drought, water logging and heat. Damages caused by these stresses are responsible for enormous economic loss world-wide. Salinity is a major abiotic stress affecting crops adversely. It is caused by poor water management, high evaporation, heavy irrigation, previous exposure to seawater and regular use of chemical fertilizers.

Salinity is the biggest problem in rice growing areas of many countries (Senadhira, 1987). The area affected by salinity in the world covers about 400 million hectares of which 54 million hectares are found in South Asia and South East Asia (Akbar and Ponnamperuma, 1982). In India the salt affected area is around 8.6 million hectares of which about 3.0 million heactares are coastal saline. It is estimated that 20% of the irrigated land in the world is affected by salinity (Yamaguchi and Blumwald, 2005). Moreover, it has been predicted that increasing salinization in agricultural fields will reduce the land available for cultivation by 30% within the next 25 years and upto 50% by the year 2050 (Wang *et al*., 2003).

Excess salt in soil interferes with several physiological and biochemical processes resulting in problems such as ion imbalance, mineral deficiency, osmotic stress, ion toxicity and oxidative stress. These conditions ultimately interact with several cellular components including DNA, protein, lipids and pigments in plants (Zhu, 2002) impeding the growth and development of a vast majority of crops. Plants have evolved many biochemical and molecular mechanisms to protect from the detrimental effects of salt-stress. The main biochemical strategies are induction of antioxidative enzymes, ion-homeostasis and synthesis of compatible organic solutes. The development of salt-tolerant crops that can tolerate high levels of salinity in the soils would be a practical solution of such problem (Yamaguchi and Blumwald, 2005).

Novel techniques like tissue culture and resulting somaclonal variation offer great potential for crop improvement through induction of tolerance/resistance to biotic and abiotic stresses including salinity. Molecular markers are important tools for analysis of genetic composition, the detection of desirable traits and somaclonal variation. Amongst the several classes of molecular markers, SSR marker is believed to be the most suitable marker for characterization of salt tolerant callus and regenerated plant because it is highly polymorphic, highly reproducible, co-dominant and multiallelic marker. Because of several advantages offered by SSR markers, such markers have been widely used in rice for varietal identification, diversity analysis, identification of introgression in inter-specific crosses, tracing pedigrees, locating genes and quantitative trait loci and assisting in marker aided selection (Wong *et al*., 2009). Usefulness of SSR markers for germplasm characterization in rice is well established on the basis of the studies on landraces (Thomson *et al.*, 2007), cultivars (Garris *et al.*, 2005) and relatives (Brondani *et al.*, 2003), showing the importance and preference for analysis of genetic differentiation and diversity at molecular level. So genetic diversity analysis by using SSR could be helpful to the breeders for further planning of rice breeding programme to improve grain quality, yield quality and specially for the stress such as salinity tolerant genotype development. Therefore the present investigation was undertaken with the following objectives:

1. *In vitro* screening of seedling and callus under different concentrations of salt mixtures for salt tolerance.
2. Identification of salt tolerant callus and regeneration of plantlets from such callus.
3. Characterization of salt tolerant callus using polymorphic simple sequence repeat (SSR) markers.

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CHAPTER – II

REVIEW OF LITERATURE

REVIEW OF LITERATURE

2.1 PLANT CELL AND TISSUE CULTURE

The term “Plant tissue culture” broadly refers to the *in vitro* cultivation of plants, seeds and plant parts (tissue, organs, embryos, single cell and protoplast) on nutrient medium under aseptic conditions. In other words, it can be defined as a collection of *in vitro* techniques applied to sustain or grow plant cell, tissue, organ under sterile conditions on a nutrient culture medium of known composition. The science of plant tissue culture takes its roots from path breaking research in botany like discovery of cell followed by propounding of cell theory. In 1839, Schleiden and Schwann proposed that cell is the basic unit of organism. They visualized that cell is capable of autonomy and therefore it should be possible for each cell, if given an environment, to regenerate into whole plant. Based on this premise, in 1902, the well known German plant physiologist, Gottlieb Haberlandt did cell culture and his efforts led to the beginnings of plant tissue culture. He developed the concept of *in vitro* cell culture, and regarded as the father of plant tissue culture. He was first to isolate a single fully differentiated individual plant cells from different plant species like palisade cells from leaves of *Laminum purpureum*, glandular hair of *Pulmonaria* and pith cells from petioles of *Eicchornia crassiples* etc. and was first to culture them in Knop’s salt solution enriched with glucose. In his cultures, cells increased in size, accumulated starch but failed to divide. Therefore, Haberlandt’s prediction failed that the cultured plant cells could grow, divide and develop into embryo and then to whole plant. This potential of a cell is known as totipotency, a term coined by Steward in 1968. Taking cue from Haberlandt’s failure, Hannig (1904) chose embryogenic tissue for culture. He excised nearly mature embryos from seeds of several species of Crucifers and successfully grew them to maturity on mineral salts and sugar solution. In 1908, Simon regenerated callus, buds and roots from poplar stem segment and established the basis of callus culture.

An innovative approach to tissue culture was using meristematic cells like root and stem tips was reported by Kotte (1922) and Robbins (1922) working simultaneously. Twelve years later White (1934) established organ culture on tomato root. In 1926, Fritz Went discovered first plant growth regulators (PGR), indole 3-acetic acid (IAA) for the propagation of cell growth. The induction of division in differentiated permanent cells was possible only after the discovery of kinetin by Muller in 1955.

Skoog and Miller (1957) contributed jointly by demonstrating that auxin – cytokinin balance is prerequisite in the regulation of morphogenesis *in vitro*. In 1958, Steward *et al.* initiated work on cultures of carrot explants and used coconut milk as a nutrient that ultimately led to the discovery of embryogenesis. There was lot of dispute among scientist on composition of culture media. Murashige and Skoog (1962) developed and formulated the most accepted and useful culture medium for any type of plant or culture. Morel and Martin (1952) recovered virus free Dahlia plants.

The role of tissue culture in plant biotechnology was first exemplified by Kanta *et al.* (1962). They developed a technique of test tube fertilization. In 1970, tissue culture headed towards a new research area. The totipotent plant cell could now be altered by insertion of specific foreign genes giving rise to genetically modified crop.

With the advancement in technique, the field of cell and tissue culture has become competent to solve various problems in basic and applied research. It has been used to isolate mutants (Malinga *et al.*, 1973), crop improvement through embryo culture (Smith and Drew, 1990; Lippmann and Lippmann, 1993). Tissue culture has been used for selection of useful somaclones (Heinz *et al.*, 1977; Evans, 1989).

Courtois (1988) reviewed various techniques in tissue culture like anther culture, embryo culture and somaclonal or gametoclonal variation for extending the range of genetic variability and *in vitro* screening for mass selection. He also explained the future prospects of protoplast fusion, gene manipulation and hybrid propagation of hybrids via‘artificial seeds’.

Zapata and Torrizo (1988) reviewed the innovative breeding techniques involving tissue culture like anther culture, pollen culture, somatic cell culture and protoplast culture to complement conventional breeding techniques in the production of new rice varieties. The foundation research included the improvement of callus production and plant regeneration efficiencies through proper use of culture condition and genetic factors, while applied research involved generation of somaclonal, gametoclonal varients and immediate fixation of homozygosity in F1 hybrid for faster production of varieties.

2.1.1 TISSUE CULTURE IN RICE

The tissue culture in rice was initiated with the culture of roots (Fujiwara and Ojima, 1955) and immature embryos (Amemiya *et al.,* 1956). These pioneering works were followed by other reports that led to the all around development in rice tissue culture and their application in the rice crop improvement. Some important aspects of rice cell and tissue culture are anther and pollen culture, protoplast culture, root culture, shoot culture, regeneration of plants and somaclonal variation.

2.1.2 *IN VITRO* REGENERATION OF PLANTS IN RICE

Abe and Futsuhara (1989) studied the isozyme pattern in rice tissue culture and selected higher regenerative callus on this basis, while in same year Gupta and coworkers did cytokinin mediated induction of embryogenic calli and plant regeneration in *indica* rice. Further, in same year Lu and coworkers studied scutellum callus formation and plantlet regeneration in 10 rice cultivars in the response to the different media.

Maheswaran and Rangasamy (1989) showed the effect of 2,4-D and kinetin on callus induction plant regeneration in somatic cell cultures of rice. They induced callus from genotypes IR-50, IR-155 and CO 43 on medium containing 2,4-D (2.0 mg/l) and KIN (0.5 mg/l) and found that the level of regeneration varied with genotype and concentration of growth regulators.

Abe and Futsuhara (1991) did diallel analysis of callus growth and plant regeneration in rice, while Lynch and Benson (1991) gave a method for maintaining plant regeneration capability of rice cell suspension cultures during cryopreservation. Further, Soliman and coworkers (1991) studied the survival mechanism of dried callus and regeneration of plants from such callus in rice, while, Zhang (1991) showed effects of sucrose concentrations, phytohormones and medium types on induction of embryogenic callus in rice. Benson and coworkers (1992) found variation in free radical in rice cell suspensions with different embryogenic potentials, while Kobayashi and coworkers (1992) found enhancement of plantlet regeneration by medium exchange in liquid culture of rice plants.

Bano *et al.* (2005) inoculated seeds of rice (*Oryza sativa* cv. Swat-II) onto MS medium containing various concentrations of auxins and cytokinins. Excellent callus resulted on MS containing 2,4-D and Kn. However, this callus was non-embryogenic. Addition of tryptophan (TPN) @ 50 mg/l induced embryogenic callus. Increase in callus mass was obtained by a change in auxin and cytokinin level. Plantlet regeneration occurred on MS containing BAP at 0.5 mg/l in combination with IAA at a concentration of 0.2 mg/l. The plantlets were transferred to the field after hardening.

Pravin *et al*. (2011) reported high frequency callus induction and *in vitro* regeneration from mature seed derived embryogenic calli of two recalcitrant *indica* rice genotypes Swarna and Mahsuri after partial desiccation treatment. Embryogenic and nodular callus was initiated on MSM medium supplemented with 3% maltose, 0.5 g/l each of L-proline, casein hydrolysate and kinetin gelled with 0.3% of gel rite. Callus induction in MSM medium showed 49% (Swarna) and 71% (Mahsuri) higher than the MS basal medium. By considering gelling agents as variables, it was observed that gel rite @3.0 g/L showed 14.23% and 25.03% increased callus induction over the agar medium in Swarna and Mahsuri varieties respectively. Partial desiccation has shown the significant effect on shoot induction on M2 medium supplemented with MSM salts, 2.5 g/l Kinetin, 0.5 g/l NAA and 0.4% gel rite, irrespective of the rice varieties used. The transformed rice plants using *cry*IAc were transferred to the transgenic green house for bioassay.

Alam *et al.* (2012) conducted an experiment to observe the regeneration potential and also to establish a suitable *in vitro* plantlet regeneration protocol from mature seed derived embryogenic calli of four *indica* rice varieties *viz* BRRI dhan28, BRRI dhan29, BRRI dhan47 and Binadhan-7. The percentage of callus induction frequency was the highest (86.00) in BRRI dhan47 and the lowest

(56.50) in Binadhan-7. Among the culture media the performance of MS +500 mg/l L-Proline + 2.0 mg/l 2, 4-D + 0.8 mg/l BAP was better than any other media for callus induction frequency (%), rapid callusing, size of the callus (mm), texture of callus and colour of callus. Among the four varieties, shoot regeneration was highest in BRRI dhan29 (84.33%) which required the minimum (14.80) days and the lowest was in Binadhan-7 (39.67%) which required the maximum (15.47) days. Among the treatments, the highest (65.75%) shoot regeneration was observed with MS + 6.0 mg/l Kn + 0.5 mg/l NAA which required the minimum days (13.75) to develop green bud formation and the lowest shoot regeneration (56.50) was observed with MS + 2.0 mg/l Kn + 0.5 mg/l NAA. BRRI dhan29 produced more number of shoots (4.67) per callus while Binadhan-7 showed the minimum number of shoots (2.87) per callus. The highest number of shoot producing roots (3.66) was observed in BRRI dhan29 which showed the maximum number of root per plant and the lowest (3.11) in Binadhan-7. Among the three treatments MS + 0.6 mg/l IBA showed the highest percentage 86.67 of root followed by MS+ 0.6 mg/l IBA (70%) and the lowest (66.7%) was in MS+ 0.4 mg/l IBA. The establishment rate of the plantlet in the pot was the highest (67.67%) in BRRI dhan29 and Binadhan-7 showed the lowest establishment rate (51.22%) in pot.

Hoque *et al.* (2013) developed an efficient protocol for the best callus induction and complete plant regeneration in rice varieties such as pakhi biroin, hati baromashi, kacha biroin, badal boro, porichok amon, khoiya boro, joria aman and BRRI dhan53. For callus induction from mature dehusked rice seeds, MS (Murashige & Skoog, 1962) medium supplemented with different concentrations i.e. 1.5, 2.0, 2.5, 3.0 mg/l of 2,4-D were used. Khoiya boro and joria aman did not show efficiency in callus induction. The maximum percentage of callus induction that is 100% was noted for pakhi biroin, hati baromashi and BRRI dhan53, but 75% was the highest percentage of callus for kacha biroin. For plantlet regeneration of pakhi biroin, hati baromashi and kacha biroin, MS media with 0.5 mg/l NAA, 0.5 mg/l Kinetin and various concentrations of BA (1.0, 2.0, 3.0, 4.0 mg/l) were used. On the other hand, MS media with 1.0 mg/l NAA, 1.0 mg/l Kinetin and various concentrations of BA (1.0, 2.0, 3.0, 4.0 mg/l) were used for BRRI dhan53 regeneration.

Mannan *et al.* (2013) conducted an experiment with two traditional rice genotypes (Kalijira and Chinigura) for callus induction and plant regeneration on MS basal medium with different concentrations and combinations of plant growth regulators. It was found that both genotypes exhibited the highest callus induction frequency at 2 mgl^{-1} 2,4-D and plant regeneration at 1 mgl^{-1} BAP and 1 mgl^{-1}IBA.

2.1.3 SOMACLONAL VARIATION IN RICE

Nishi *et al.* (1968) regenerated phynotypically variant plants of rice from derived callus. Zakri (1986) discussed the significance of somaclonal variation for the rice improvement, while Ling and coworkers (1988) developed male sterile somaclones from the indica varieties of rice.

Oono (1988) found somatic mutation in rice tissue culture. Some of the mutations obtained had involved the genome and others had occurred in the genes. Some of the most useful mutations found, included those affecting chlorophyll deficiency, plant height and heading date. Homozygous mutants were also studied. He showed DNA amplification during callus induction and differentiation and dedifferentiation processes at the DNA level.

Zheng and coworkers (1989) obtained somaclonal variation in some grain and quality characters in somatic cultures of rice cultivars. They observed that γ ray treatment inhibited callus formation and studied somaclonal variation in some grain and quality characters in progenies of regenerated plants. They found that about half of the somaclones were higher than the maximum height of the original variety. The variability of most of the traits was increased by combining low dosage of γ radiation with tissue culture.

2.2 SALINITY TOLERANCE

Abiotic stresses impose a major threat to agriculture. Out of them salinity is one. Low salt concentrations suppress growth but higher concentrations can cause death. Faulty irrigation, scarcity of water and hot dry climates frequently result in salinity concentration that limit or prevent crop production. Salinity stress is the major factor to limit plant growth and productivity in many area of the world (Asharf, 2009). Excessive amount of salts in the soil causes both osmotic and ionic stresses (Khelil *et al*., 2007). Tissue culture techniques have been widely used for breeding purpose, especially in selection for stress tolerance. This is a source of genetic variability that rises through genetic modifications during the process of in vitro culture. Salt stress tolerance of plant denotes its ability to prevent, reduce or overcome the possible injurious effects caused directly or indirectly by the excessive presence of soluble salts and toxic ions in its root zone (Sandha *et al*., 1996).

2.2.1 SALINITY TOLERANCE IN RICE

Verma (1985) and Verma and Srivastava (1988) studied physiological effect of salt stress resistance in rice during germination and early seedling growth.While Krishnamurthy and coworkers (1987) found the relationship of salt tolerance with leaf ascorbic acid content and titratable acid in rice varieties. They suggested that these parameters can be used as indicators of salt tolerance in rice varieties. Sahu and coworkers (1993) observed proline accumulation in senescing leaf segments of rice of a salt sensitive (Ratna) and a salt tolerant (Getu) cultivars subjected to sodium chloride stress and it was suggested that the mechanism of proline accumulation is probably different in unstressed and stressed leaves.

Balakrishna and Swaminathan (1994) discussed the screening of salt tolerant rice cultivars for overall performance. They found that the plant growth, total shoot sodium accumulation and plant vigour can be correlated to the salt tolerance. A new source of genes for salt tolerance was found in the traditional cultivar ‘Oor Mundukan’ which can be exploited in breeding for salt tolerance. Ritambhara and coworkers (1995) found the influence of sodium chloride salinity on the behaviours of malate, isocitrate and glucose-6 phosphate dehydrogenases in growing rice seedlings in relation to salt tolerance. Further, Choudhary and Long (1996) studied growth response of rice seedlings to elevated levels of carbon dioxide and salinity and found that rice seedling growth was increased by elevated CO_2 irrespective of the degree of salinity stress.

Ali and coworkers (1996) found the effect of NaCl on seed germination, growth and photosysnthesis of rice, while in the same year Garg and coworkers characterized rice genotypes on basis of morpho-physiological and biochemical responses to salt stress and found that salt tolerance cannot be attributed to any single variable and suggested to use as many indices as possible to identify the ideal genotypes for salt tolerance. Mishra and coworkers (1996) discussed the genetics of salinity tolerance and ionic uptake in rice. They suggested the involvement of both major and minor genes with both additive and non-additive effects.

2.3 TISSUE CULTURE AND SALINITY TOLERANCE

Scowcroft and Larkin (1985) reviewed the somaclonal variation, cell selection and genotype improvement. They have reported the use of cell selection to recover salt tolerant genotypes with the regeneration of plants from the selected cell lines surviving on higher salt concentration.

Jain and coworkers (1990) accomplished *in vitro* selection of salt tolerant plants of *Brassica juncea* by screening highly morphogenic cotyledon explants cultures on high NaCl media. Chowdhary and coworkers (1993) reviewed the biotechnological approaches for developing salt tolerance in field crops. They have discussed the use of plant tissue culture and recombinant DNA technology for the development of salt tolerance.

Gupta and Singhal (1996) showed the effect of sodium and calcium chlorides on callus cultures of *Arachis hypogea*, while Barakat and Abdel Latif (1996) did the *in vitro* selection of wheat callus tolerant to high levels of salt and regenerated plants from such callus. Further, Olmos and Hellin (1996) studied the cellular adaptation of salt tolerant cell lines in *Pisum sativum*, while Gossett and coworkers (1996) showed the antioxidant response to NaCl tolerant cotton cell line grown in the presence of paraquat, Buthionine sulfoximine and exogenous glutathione. Chowdhary and coworkers (1996) reviewed the *in vitro* selection for salt tolerance and disease resistance in some major field crops including Indian mustard, chick-pea and rice.

2.3.1 TISSUE CULTURE AND SALINITY TOLERANCE IN RICE

Oono (1984) for the first time reported selection of salt resistant calli in a number of rice varieties using sea water as stressing agent. Dykes and Nabors (1986), however, selected salt tolerant cells using sodium salts. Further, Kavi Kishore and Reddy (1986) reported the improvement of rice for salt tolerance through tissue culture, while Bhushan and Grover (1993) analysed the effect of calcium and potassium salts on young seedlings and callus tissue of rice cultivars raised in NaCl medium. Miah and co-workers (1996) produced the salt tolerant rice breeding lines through tissue culture. Thach and Pant (1999) studied salt tolerance in rice under in vitro conditions. They investigated that callus can be used as a system for screening salt tolerance in rice and also found that proline plays some role in salt tolerance mechanism in callus, but not in seedling stage.

Forkan *et al*. (2005) developed *in vitro* callogenesis and regeneration systems for some salt tolerant rice cultivars. Mature seed scutellum (MSS) cultured on LS and MS culture media supplemented with 2.5 mg/l 2,4-D resulted in formation of embryonic calli. All cultivars responded well on both RM-1 and RM-2 media for plant regeneration but the cultivars showed varied responses from medium to medium. It was concluded that culture media and plant genotypes affect embryonic callus formation and efficient plant regeneration.

Khaleda *et al.* (2007**)** concluded an experiment to identify responsiveness of some deep water rice cultivars to callus induction and plant regeneration in the presence of NaCl in in vitro conditions. LS basal medium was supplemented with 0.1 to 0.3% (w/v) NaCl and it was found that the callus proliferation and plant regeneration decreased with increase in concentration of NaCl.

Shanthi *et al*. (2010) conducted an experiment for salt tolerance in 7 genotypes of rice and studied their performance under *in vitro* conditions. Callus formation was initiated using MS medium under different salt concentrations. Statistical analysis showed significant differences among all the genotypes and treatments and their interaction effects. It was concluded that this *in vitro* screening technique could be used for screening for salt tolerance and it takes less time, less space, less labour and gives very accurate result as compared to field screening.

2.4 MOLECULAR MARKER FOR SALINITY TOLERANCE IN RICE

Islam *et al*. (2011) reported the detection of QTLs for salinity tolerance at the seedling stage in a F2 breeding population derived from the cross between BRRI dhan40, a moderately tolerant female parent with IR61920-3B-22-2-1 (NSIC Rc106), a highly tolerant male parent. Out of total 300 F2 segregating plants, 93 plants with extreme phenotype for salinity stress response, i.e. tolerant and sensitive, were used for selective genotyping based on of visual seedling stage salt tolerance symptom. A total of 260 SSR and two EST markers evenly spread throughout the whole rice genome at 5 Mb intervals were used for parental polymorphism survey. The 90 polymorphic makers were used for QTL mapping for salinity tolerance at seedling stage. QTL analysis using single marker, interval mapping and composite interval mapping detected three major QTLs on chromosome 1, 8 and 10 with phenotypic variances (R2) of 12.50, 29.0 and 20.20%, respectively. The position of QTL on chromosome 1 was flanked by RM8094 and RM3412 marker which is in the same region as a previously identified major QTL designated as *saltol*. However, two other QTLs with relatively large effects were flanked by RM25 and RM210 on chromosome 8, and

RM25092 and RM25519 on chromosome 10, and appear to be novel QTLs. The markers flanking these QTLs should be useful for molecular marker assisted breeding for salinity tolerance.

Faridul Islam *et al*. (2012) studied the genetic analysis of stress tolerance using 40 SSR markers. All of the used markers were found polymorphic among the 14 rice genotype. The amplicon size ranged from 75bp (RM436) to 330bp (RM26360). A total of 168 alleles were detected, the number of allele per locus ranged from 2 (RM252, RM803120) to 6 (RM570, S12055, S11033) with an average of 4.2 alleles per locus.

Shanthi *et al*. (2012) conducted an experiment of DNA finger printing of salt tolerant and susceptible genotypes using SSR markers in rice. For this a total 50 SSR primers which covered 12 chromosomes were taken up out of these 50 primers. Out of them 37 were polymorphic. The average number of allele per locus was 5.69 indicating a greater magnitude of diversity among the plant material included in this investigation. The PIC value was 0.732, which confirmed that the markers used were highly informative. The cluster analysis grouped the 27 genotypes into nine clusters. The maximum similarity value of 0.786 was observed between the varieties of IR36 and IR64 indicated that these were more closely related.

2.4.1 SSR based polymorphism in salt tolerant rice

Panaud *et al.* (1996) worked on the development of microsatellite markers and characterization of simple sequence length polymorphism (SSLP) in rice. Based on this study, they described 20 new microsatellite markers that were assigned to positions along the rice chromosomes, characterized for their allelic diversity in cultivated and wild rice, and tested for amplification in distantly related species. Their results indicated that the genomic distribution of microsatellites in rice appears to be random, with no obvious bias for, or clustering in particular regions. Sequence analysis of SSLP alleles in three related *indica* varieties demonstrated the clustering of complex arrays of SSR motifs in a single 300 bp region with independent variation in each. Two microsatellite markers amplified multiple loci that were mapped onto independent rice chromosomes, suggesting the presence of duplicated regions within the rice genome.

Blair *et al*. (1999) analysed microsatellite motif frequency in the rice genome by using a total of 32 primers containing different simple sequence repeat (SSR) motifs. The primers were tested for amplification in 59 varieties of cultivated rice. The analysis provided insights into the organization, frequency and levels of polymorphism of different simple sequence repeats in rice. The more common di-nucleotide motifs were more amenable to analysis than the more infrequent tri-nucleotide, tetra-nucleotide and penta-nucleotide motifs. The results suggested that within the di-nucleotide class, the poly (GA) motif was more common than the poly (GT) motif and that the frequency and clustering of specific tri-nucleotide and tetra- nucleotide simple sequence repeats was variable and motif-specific. Furthermore, tri-nucleotide markers were found to be less polymorphic than either di-nucleotide or certain tetra-nucleotide markers.

Temnykh *et al*. (2001) determined the frequency and distribution of different simple sequence repeats (SSRs) in the rice genome. SSR loci were categorized into two groups based on the length of the repeat motif. Class I or hypervariable markers consisted of SSRs of 20 bp and Class II or potentially variable markers consisted of SSRs of 12 bp. GC-rich tri-nucleotide repeats

(TNRs) were most abundant in protein-coding portions of ESTs, whereas AT-rich TNRs showed no such preference, and di-nucleotide and tetra-nucleotide repeats were most frequently found in non-coding, inter-genic regions of the rice genome. Microsatellites with poly (AT)n repeats represented the most abundant and polymorphic class of SSRs but were difficult to amplify.

McCouch *et al*. (2002) developed and validated a total of 2414 new di-nucleotide, tri-nucleotide and tetra-nucleotide non-redundant SSR primer pairs representing 2240 unique marker loci for rice. Duplicate primer pairs were reported for 7% (174) of the loci. The majority (92%) of primer pairs were developed in regions flanking perfect repeats (≥ 24 bp in length). Additional information based on genetic mapping and "nearest marker" information provided the basis for locating a total of 1825 (81%) of the newly designed markers along rice chromosomes. Fifty-six SSR markers (2.8%) appeared to be multiple copy. The largest proportion of SSRs in this data set correspond to poly(GA) motifs (36%), followed by poly(AT) (15%) and poly(CCG) (8%) motifs. AT-rich microsatellites had the longest average repeat tracts, while GC-rich motifs were the shortest.

Neeraja *et al*. (2005) evaluated 29 tall landraces of rice using SSR markers and reported that GC-rich tri-nucleotide motif containing SSRs in the open reading frames (ORFs) had low level of polymorphism than poly GA or AT-rich di-nucleotides and tri-nucleotides containing SSRs located in the introns. Chen *et al*. (2005) analyzed SSR polymorphism by genome-scale comparison between varieties in rice. A total of 1453 loci flanked by 100 bp nucleotide sequences on either side of the simple sequence repeat (SSR) were identified in one *indica* and one *japonica* rice cultivars using bioinformatic tools. Among the sequence hits, 1449 and 1451 SSRs were observed in these two cultivars, respectively. Three hundred seventy one SSRs had the same repeat unit and count, whereas 804 SSRs had special motifs with different repeat counts. Di-nucleotide repeats were the most abundant, whereas hexa-nucleotide repeats were the least abundant.

Lapitan *et al*. (2007) studied the nature of polymorphism in 24 rice cultivars using 164 SSR markers. They reported that the loci amplifying di-nucleotide repeat motif were found to be more polymorphic than those with tri-nucleotide and tetra-nucleotide repeat motif. Among the loci with perfect and compound di-nucleotide repeat motifs, markers with a GA repeat motif showed the largest variability. The result suggested that larger number of repeat motif containing marker detects larger number of alleles.

Bhowmik *et al*. (2009) suggested that selecting salt tolerant genotypes of rice based on phenotypic performance alone is less reliable and will delay progress in breeding and can be more effectively managed with recent advent of molecular markers. They used three selected SSR markers RM7075, RM336, and RM253 to evaluate rice genotypes for salt tolerance. Both phenotypic and genotypic evaluation was done at the seedling stage. Phenotypic evaluation of 11 genotypes was done in hydroponic system using salinized (EC 12ds/m) nutrient solution. The markers showed polymorphism and were able to discriminate salt tolerant genotypes from susceptible. The genotypes having similar banding pattern with pokkali were considered as salt tolerant. The SSR markers (RM7075, RM336 and RM253) identified 8, 9 and 7 salt tolerant genotypes, respectively. Through phenotypic and genotypic studies, three genotypes viz. Pokkali, TNDB-100 and THDB were identified as salt tolerant rice cultivars. These SSR markers might have sequence homology with salt tolerant rice genotypes and consequently the markers could able to identify salt tolerant rice genotypes from susceptible.

Singh *et al.* (2009) established the relationship between SSR length and level of polymorphism in a set of eight diverse rice genotypes using 201 random SSR loci of different repeat motifs and lengths, representing both genic and inter-genic sequences from the 12 rice chromosomes. There was a positive correlation between SSR length and average number of alleles per locus but linearity of this relationship was limited to the SSR length range of 10 to 70 bp. The highest level of polymorphism was in the SSR length range of 51 to 70 bp, beyond which there was stabilization and then decline of polymorphism in SSRs longer than 70 bp. Proportion of polymorphic loci in the different SSR length groups also followed similar pattern with even sharper decline of polymorphism in the highest size range. A genome wide set of 436 validated highly variable SSR (HvSSR) markers was described with repeat lengths of 51 to 70 bp for their consistent amplification and high polymorphism.

Singh *et al.* (2010) estimated genetic diversity among 20 rice genotypes possessing different physiological mechanisms contributing to salt tolerance. A total of 11 clear and repeatable bands were amplified from ten selected SSR primers pairs and 43 fragments were detected from nine ISSR primers. The level of polymorphism was 1.1% with SSR compared to 90.7% with ISSRs. Mean genetic similarity of 0.88 based on SSRs and 0.85 using ISSRs was observed. A total of 43 (39 polymorphic) and 11 bands were detected using 9 ISSR primers and 10 well distributed mapped SSR markers, respectively. Estimates of genetic similarity of ISSRs based on the 39 polymorphic markers between 20 rice cultivars ranged from 0.55 for PR108/CSR19 to 0.94 for Pokkali/CSR20 with an average of 0.81. The estimates revealed by the 11 polymorphic SSR bands showed the average value (0.94) and also the range of genetic similarity (from 0.86 to 1.00 for CSR22/CSR18 and CSR24/CSR20, respectively) reflecting their hyper variability and their high resolution power. The findings are likely to expedite breeding new salt tolerant cultivars by involving parents from diverse molecular clusters.

Senguttuvel *et al*. (2010) investigated molecular mechanism of salt tolerance using microsatellite markers in a set of twenty five genetically divergent genotypes and phenotyping for Na^+/K^+ ratio grown under Yoshida solution with 60 and 120 mM NaCl. The polymorphic SSR markers already reported for major saltol QTLs were utililized in these studies and the results were highly reproducible. The cluster and principal component analysis allowed a clear grouping of 25 genotypes grouped into 8 distinct clusters of resistant and susceptible genotypes with high and low level of Na^+/K^+ ratio. There was a highly significant correlation (R^2 =88.75; P = 5%) between the SSR markers and physiological trait based on ion analysis. The association of SSR markers viz. RM23, 493 and 8053 for the trait linked to Na^+/K^+ ratio can be regarded as the most reliable markers for marker assisted-selection to identify salinity tolerance in rice. The study revealed that the selection of genetically diverse and resistant genotypes based on association of Na^+/K^+ ratio with molecular markers is reliable.

Thomson *et al*. (2010) characterized the saltol quantitative trait locus for salinity tolerance in rice. An analysis of 100 SSR markers on 140 IR29/Pokkali recombinant inbred lines (RILs) confirmed the location of the saltol QTL on chromosome 1 and identified additional QTLs associated with tolerance. Analysis of a series of backcross lines and near-isogenic lines (NILs) developed to better characterize the effect of the saltol locus revealed that saltol mainly acted to control shoot Na+/K+ homeostasis. Multiple QTLs were required to acquire a high level of tolerance. Unexpectedly, multiple pokkali alleles at saltol were detected within the RIL population and between backcross lines, and representative lines were compared with seven pokkali accessions to better characterize this allelic variation. Thus, while the saltol locus presents a complex scenario, it provides an opportunity for marker assisted backcrossing to improve salt tolerance of popular varieties followed by targeting multiple loci through QTL pyramiding for areas with higher salt stress.

Ahmadi and Fotokian (2011) identified the quantitative trait loci associated with salinity tolerance in rice using SSR markers. 62 advanced backcross-inbred lines (BILs), at the BC2F5 generation, derived from the cross of Tarome-Molaei (salt tolerant) and Tiqing (salt sensitive), were used to identify the QTLs involved in salinity stress tolerance, using SSR markers. Advanced backcross inbred lines along with their parents were evaluated for six parameters *viz.* sodium (Na+) and potassium (K+) in roots and shoots and the Na+/K+ ratio, using the modified Yoshida’s nutrient solution at an electrical conductivity of 6 and 12 dS/m. A total of 114, out of 235 simple sequence repeats (SSRs) markers that showed polymorphism in the parents, were used to characterise the BILs. Using the composite interval mapping (CIM) and a minimum logarithm of the odds (LOD) threshold of 3.0, a total of 14 QTLs were detected. The phenotypic variation explained by these QTLs ranged from 9 to 30% of the total variation. A QTL (*QKr1.2*) for K+ content in the root was identified with the highest LOD score (7.8), on chromosome 1. This QTL explicated 30% of the total variation and was identified as a major QTL conferring salt tolerance in rice.

Dhar *et al*. (2012) used twenty six rice genotypes to evaluate salinity tolerance at the seedling stage. Phenotyping for salinity screening of the rice genotypes was done using salinized (EC level 12 dS /m) nutrient solution in hydroponic system following IRRI standard protocol. Genotypes were evaluated individually for salinity tolerance on 1-9 scale on the basis of seedling growth parameters following modified SES (Standard Evaluatin Score) of IRRI. At the seedling stage, sixteen moderately tolerant and ten susceptible genotypes were identified using SES. On the basis of SES score and phenotypic performance, out of 26 rice germplasm, two (BINAdhan-8 and AYT SL-3) were selected. For genotypic salt tolerance of 26 rice germplasms, DNA was extracted from leaf samples using CTAB mini-prep method. Then six selected SSR markers viz., RM10701, RM304, RM11757, RM336, RM7075, and RM152 were used for identification of salt tolerant genotypes. In DNA profiling, a total of 60 alleles were detected with an average number of alleles of 10 per locus (range 8 to 12 per locus) and the PIC values ranged from a low of the 0.7459 (RM152) to a high of 0.8908 (RM10701) and averaged 0.857.

Chattopadhyay *et al.* (2013) identified the phenotyping and QTL-linked marker-based genotyping of rice lines with varying level of salt tolerance at flowering stage. In this investigation, a set of eight genotypes were phenotyped for grain yield and various yield attributing traits as influenced by salt-stress (EC= 8 dS m-1) at flowering stage and subsequently genotyped by using microsatellite markers associated with known QTLs for salt tolerance in rice. One Pokkali and two Chettivirippu accessions were found tolerant to moderately tolerant. These genotypes were genetically distant from the susceptible variety, swarna indicating the scope of deducing novel QTLs for salt tolerance at flowering stage.

Davla *et al.* (2013) conducted an experiment with microsatellites or simple sequence repeats (SSRs) to find out salt tolerant rice genotypes. In view of this, the mapping and marker-assisted selection for salt tolerance genes in rice have been conducted. The present study was carried out with the objective of evaluation of genetic diversity among 19 rice genotypes, representing highly tolerant as well as susceptible rice cultivars using SSR markers. Among 39 SSR markers used, 26 SSR marker loci generated polymorphic patterns and a total of 185 alleles were detected. From these 26 SSR markers, 16 SSR markers are located on the *saltol* region on chromosome 1 of rice. The number of alleles per locus ranged from 3-11 with a mean of 7.1 alleles per locus. The PIC values for 26 SSR markers varied from 0.50 (RM6737) to 0.89(RM3412) with an average PIC of 6.7. Hence, from the present study, it can be proved that SSR markers can detect high polymorphism and are very useful in studying variation among different genotypes.

Sudharani *et al.* (2013) identified the genetic diversity in rice genotypes using microsatellite markers for salinity tolerance. In view of this, a set of eight rice genotypes with different adaptations to salinity stress were characterized for their genetic diversity using microsatellite markers. A total of 85 primers distributed in all the 12 chromosomes of rice were used to assess the genetic diversity. The eight genotypes were grouped into 4 major clusters. Cluster I consists of RP Bio-226 and CSR-27 and these are showing 100 per cent similarity with each other, cluster II consists of CSRC(S) 5-2-2-5 and SR26B, which showed 38 per cent of similarity. Cluster III consists of CST 7-1 and CSRC(S) 5-2-2-5 and showed 61 per cent of similarity with each other, they in turn combined with cluster I and II showing 20 per cent of similarity. Cluster IV comprised of Swarna and CSR-30, which are showing 29 per cent of similarity and they combined showing 16 per cent similarity with remaining samples in the cluster. Salt tolerants genotypes found to be mixed with salt sensitive ones. Cluster I and II consisted of salt sensitive and moderately tolerant varieties.

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CHAPTER – III

MATERIALS AND METHODS

MATERIALS AND METHODS

3.1 MATERIALS

Rice forms the principal food of nearly half of the world’s population. India is the largest rice growing country in the world. Botanically rice is known as *Oryza sativa* L. and it is a self-pollinated, monocot crop belonging to the genus *Oryza* of family Poaceae (Graminae) and subfamily *Oryzoidea*. The genus *Oryza* has 27 species. Out of these 25 are wild species. Among the two cultivated species, *O. sativa* is cultivated in almost entire area of Asian, European, American, and Australian countries, while the cultivation of *O. glaberrima* is limited only to West Africa.

The rice is an annual grass plant growing upto a normal height of 2-4 ft. In place of bearing an ear, rice produces panicle inflorescence composed of a number of fine branches each terminating in a single grain surrounded by a husk. The grains are easily detached together with their husk and in this condition it is known as paddy. Plants are tillering freely and forming clumps. The stem is erect, leaves are long and 2-partite, auriculate. The auricules are hairy and clasping the stem tightly.

The species *O. sativa* has 3 geographical races indica, japonica and javanica. Cultivation of indica varieties is mainly done in tropical and subtropical countries. This race has tall plant type. It has low tillering capacity and it lodges even at moderate fertilizer doses. Japonica cultivation is mainly done in temperate countries. It has profuse tillering capacity and does not lodge under high fertility condition. However the cooked rice of japonica race is sticky in nature. Javanica is intermediate in characteristics between japonica and indica. It has good tillering capacity and could tolerate fertilizer doses upto a considerable limit.

TAXONOMIC CLASSIFICATION OF RICE

There are 7 accepted species of rice:

Kingdom: Plantae – Plants

Subkingdom: Tracheobionta - Vascular plants

Superdivision: Spermatophyta - Seed plants

Division: Magnoliophyta - Flowering plants

Class: Liliopsida - Monocotyledons

Subclass: Commelinidae

Order: Cyperales

Family: Poaceae - Grass family

Genus: *Oryza* L. - rice

Species: *Oryza barthii* - Barth's rice

Species: *Oryza glaberrima* - African rice

Species: *Oryza latifolia* - broadleaf rice

Species: *Oryza longistaminata* - longstamen rice

Species: *Oryza punctata* - red rice

Species: *Oryza rufipogon* - brownbeard rice

Species: *Oryza sativa* – rice

Seeds of rice cultivars were obtained from Department of Plant Breeding and Genetics, Rajendra Agricultural University, Pusa, Bihar. Six rice cultivars were selected for the present experiment namely

a. Narendra Usar Dhan-3

b. CSR-30

c. Pusa Basmati-1

d. Rajendra Bhagwati

e. MTU-7029

f. BPT-5204

Important characteristic features of selected cultivars of rice were tabulated (Table-3.1)

Table-3.1 Important characteristic features of selected cultivars of rice

Characters	Cultivars					
	NarendraUsar Dhan-3	**CSR-30 (YAMINI)**	**Pusa Basmati-1**	**Rajendra Bhawati**	**MTU-7029**	**BPT-5204**
1. Parentage	Leungyai148 x IR 9129-209-2-2-1 xx IR 18272-27-3-1	BR4-10 x Basmati 370	Pusa150 x karnal local	IR36 x Type3	Vasistha x Mahsuri	GEB24 x TNI x Mahsuri
2. Special features	Medium grains	Superfine grain	Super fine aromatic grains	Highly scented	Having medium slender grains, golden husk colour	Medium slender grains
3. Height	Medium	Tall (155 cm)	Semidwarf (85-95 cm)	Semidwarf, erect with dark grain foliage	Short height	Short height
4. Maturity period	130-135 days	150-155 days	135 days	100-105 days	145-150 days	140-145 days
5. Yield	4.5-5.0 t/ha	3.0 t/ha in normal soils & 2.0 t/ha in salt affected soils	4.5t/ha	5-6 t/ha	4-5 t/ha	5-6 t/ha
5. Suitability/Ecosystem	Irrigated saline & alkaline soils	Irrigated saline soils	Irrigated areas	Recommended for irrigated upland ecology	Recommended for rainfed shallow lowland condition	Suitable for rainfed shallow low land ecosystem
6. Resistant to disease	BLB	Resistant to leaf blast, sheath rot, RTV & WBPH	Resistant to blast	Moderately resistant to BLB & Brown spot	Resistant to BLB, Blast & Brown spot	BLB & Blast
8. Resistance to salt	Salt tolerant	Salt tolerant	Salt sensitive	Salt sensitive	Moderately salt tolerant	Moderately salt tolerant
9. Year of release	1999	2001	1989	2008	1979	1986
10. Developed at	NDUAT, Faizabad	CSSRI, Karnal	IARI, New Delhi	RAU, Pusa	ANGRAU, Hydrabad	ANGRAU, Hydrabad

3.2 METHODS

The experiments were conducted in following parts :

- Screening rice cultivars for salt tolerance
- Tissue culture studies
- Induction and selection of salt tolerance
- SSR (Simple sequence repeat) analysis

3.2.1 SCREENING FOR SALT TOLERANCE

Rice is moderately salt sensitive crop (Gupta *et al.*, 1994) but many genotypes are known to be tolerant, moderately tolerant and sensitive. Screening rice genotypes for salinity tolerance is generally done in the field condition. However, laboratory screening can help in saving time, labour and other resources. To screen rice cultivars for salinity tolerance, salt mixture (NaCl, $CaCl_2$, Na_2SO_4 in 7:2:1 ratio) was used at different concentrations.

3.2.1.1 Germination under salt stress

Stock solution of salt mixture (NaCl, $CaCl_2$, Na_2SO_4 in 7:2:1 ratio) was prepared by dissolving 100 gm in about 500 ml of distilled water taken in a 1000 ml measuring flask. The final volume of the solution was made 1000 ml by adding distilled water. From this stock solution, 6 different solutions of salt mixture were prepared with salt concentrations ranging from 0 to 2.5% in six 100 ml conical flasks (table 3.2).

Table-3.2 Preparation of salt solutions

Sl. No.	Salt concentration (%)	Salt stock solution amount (ml)	Distilled water added (ml)
1.	Control (0)	0	100
2.	0.5	5	95
3.	1.0	10	90
4.	1.5	15	85
5.	2.0	20	80
6.	2.5	25	75

50 seeds of each rice cultivar were taken per petridish with germination paper submerged in salt solution. Observations of seed germination were taken at regular intervals. The final observations were recorded after 30 days.

3.2.1.2 Seedling growth under salt stress

Seedlings of selected cultivars of rice of about 1-1.5 cm length were subjected to salt stress by growing them in different concentrations of mixture of salt solution (table-3.2). Final root and shoot length were measured after 30 days. Then fresh and dry weights of root and shoot were taken. The salinity tolerance index, which was the ratio of the performance of a genotype at a particular level of salinity to that of control, was calculated on the basis of shoot and root dry weights following the method of Reddy and Baidyanath (1982).

3.2.2 TISSUE CULTURE

The methodology of tissue culture studies included:

- Selection of explants
- Selection of media
- Preparation of media
- Collection and preparation of explants
- Inoculation
- Incubation
- Subculture
- Observations
- Photography
- Statistical analysis

3.2.2.1 Selection of explants

Dehusked rice seed was used as explant (plate-3.1).

3.2.2.2 Selection of media

Murashige and Skoog (MS) medium (1962) was selected as the basal medium throughout the experiment, as it was the most favorable medium particularly for callusing and plant regeneration. MS medium is also widely accepted medium for the tissue culture of rice. For further studies, an array of media was generated using MS basal medium supplemented with different combinations and concentrations of phytohormones

3.2.2.3 Preparation of media

Composition of media

MS basal medium consisted of inorganic salts and organic components. The inorganic salts were salts of major and minor elements including iron. The organic component contained nutrients like amino acids, vitamins and sucrose as a carbon source. Agar was used as a gelling agent. The medium was further supplemented with different combinations and concentrations of phytohormones.

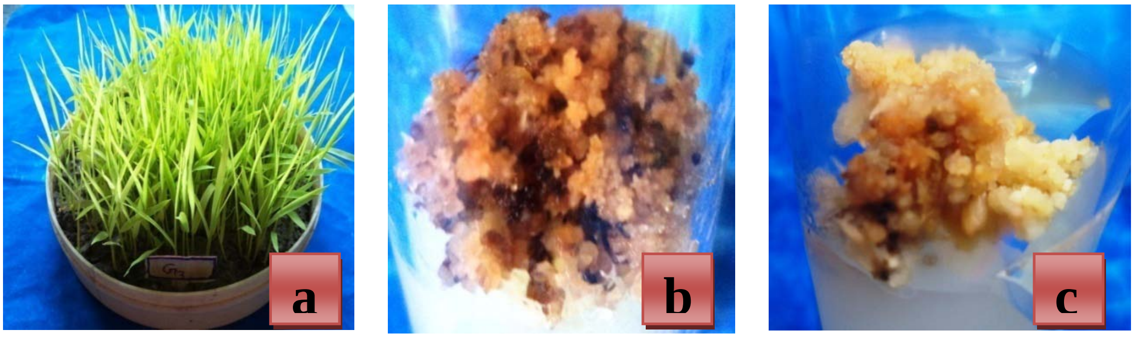
Preparation of stock solutions

Stock solutions of major, minor, iron and vitamins were prepared separately by dissolving the required amount of component constituents in distilled water. In case of the preparation of stock solution of major salts, $CaCl_2$ was added at the end (table 3.3). Similarly, separate stock solutions of auxins (2,4-D and NAA) and cytokinins (Kinetin) were also prepared. The stock solution of auxin was prepared by dissolving the required amount of the constituent first in 2-3 ml of ethanol and then in distilled water to make the desired volume. Similarly, the stock solution of cytokinin was prepared by dissolving it in 5-10 ml N/10 NaOH and distilled water was added to make the required volume (table 3.4). The stock solutions were stored at 4°C in refrigerator and brought to room temperature before use.

Plate-3.1 Rice explant

Rice Seed **Dehusked rice**

Plate-3.2 Sources of DNA



a. rice seeds grown in pot

b. normal callus

c. salt tolerant callus

Table 3.3 Composition of MS medium and preparation of different stock solutions

Sl. No.	Stocks	Constituent	Amount (lit/mg)	Amount in stock solution (mg)	Dissolved in distilled water and made	Conc.	Amount used/lit
	Inorganic						
1.	Major elements	Ammonium nitrate (NH_4NO_3)	1650	16500	500 ml	20x	50 ml
		Potassium nitrate (KNO_3)	1900	19000			
		Calcium chloride ($CaCl_2.2H_2O$)	440	4400			
		Magnesium sulphate ($MgSO_4.7H_2O$)	370	3700			
		Potassium dihyro- orthophosphate (KH_2PO_4)	170	1700			
2.	Minor elements	Boric acid (H_3BO_3)	6.20	620	500 ml	200x	5 ml
		Manganese sulphate ($MnSO_4H_2O$)	22.3	2230			
		Zinc sulphate ($ZnSO_4.7H_2O$)	8.6	860			
		Potassium iodide (KI)	0.83	83			
		Sodium molybdate (Na_2MoO_4)	0.25	25			
		Cupric sulphate ($CuSO_4.5H_2O$)	0.025	2.5			
		Cobalt chloride ($CoCl_26H_2O$)	0.025	2.5			
3.	Iron sources	EDTA- di sodium salt ($Na_2EDTA.7H_2O$)	37.3	373	250 ml	40x	25ml
		Ferrous sulphate ($FeSO_4.7H_2O$)	27.8	278			
	Organic						
4.	Amino acids & vitamins	Glycine ($C_2H_5NO_2$)	2.0	200	250 ml	400x	2.5 ml
		Inositol ($C_6H_{12}O_6$)	100	10,000			
		Nicotinic acid ($C_6H_5NO_2$)	0.5	50			
		Pyridoxine hydrochloride ($C_8H_{11}NO_3HCl$)	0.5	50			
		Thiamine hydrochloride ($C_{12}H_{17}ClN_4O_5.HCl$)	0.1	10			
5.	Carbon Source	Sucrose ($C_{12}H_{22}O_{11}$)	30,000				
6.	Gelating Agent	Agar	8000				

Table- 3.4 Preparation of stock solutions of different phytohormones

Sl. No.	Phyto-hormones	Weight (mg)	Initially dissolved in	Made up with distilled water	Strength
1.	2,4-D	25	2-3 ml ethanol	250 ml	1mg/10 ml
2.	NAA	25	2-3 ml ethanol	250 ml	1mg/10 ml
3	KIN	25	5-10 ml slightly heated N/10 NaOH solution	250 ml	1mg/10 ml

Preparation of 1 litre medium

For preparation of 1 lit medium at first a volumetric flask of 1 litre was taken. Then the required amount of different stock solutions of major, minor, iron, amino acids and vitamins were added into it. Phytohormones were added from their stock solutions as per their requirement and 30 gm of sucrose was also added into it. Sucrose was dissolved gradually by adding distilled water. Further, distilled water was added and volume was made slightly less than 1 litre. The pH of the medium was checked on pH meter and adjusted to 5.8 by adding drop wise dilute NaOH or dilute HCl as needed. The medium was then brought to its precise volume i.e. 1.0 litre. This medium was poured into 1.0 litre conical flask and 8.0 gm agar was added as gelling agent. The whole contents of conical flask was heated to mix the gelling agent properly for 10-15 minutes or till the medium became transparent. The 10 - 20 ml of the medium was poured into each of culture tubes measuring 18mm X 150mm/ 25 X 150. The tubes were plugged with non absorbent cotton plugs, covered with butter paper or aluminium foil and autoclaved at 121°C at 105kPa/15lb/inch^2 for 20 minutes. The tubes were taken out and cooled at room temperature. Though, initially many media were prepared and used differing in phytohormones, but finally 4 media were selected for most of the experiments (table 3.5).

Table-3.5 Composition of selected media

Sl.No.	Media name	Composition
1.	M1	MS + 2.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} KIN
2.	M2	MS + 2.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} KIN
3.	M3	MS + 2.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} KIN
4.	M4	MS + 1.5 mg l^{-1} NAA + 1.5 mg l^{-1} KIN

3.2.2.4 Collection and preparation of explants

- **Soaking of seeds**

Before surface sterilization, rice seeds were soaked in water in one night.

- **Surface sterilization of explants**

Soaked seeds of the selected 6 rice cultivars were washed in running tap water for 15-20 minutes and then treated with 70% ethyl alcohol for 30 sec and then washed with distilled water. Thereafter seeds were treated with 0.1% mercuric chloride ($HgCl_2$) for 10-15 minutes. Such seeds were 3 times rinsed with sterile distilled water under laminar air flow.

3.2.2.5 Inoculation

Sterilization of laminar air flow

All inner sides of laminar air flow, the working chamber, including floor was properly wiped with ethyl alcohol using cotton before use. Then the inoculating instruments like forceps, needles, scalpels, sterilized petridishes, spirit lamps and media were kept inside the chamber. The chamber was closed and the UV lamp was put on for 25-30 min. and the ozone was flown for 20 min. to sterilize the chamber.

Inoculation of explants

Inoculation of explants was done in sterilized laminar air flow. At first, hands were wiped with alcohol, the instruments like forceps, scalpels were soaked in alcohol followed by flaming on a burner in the laminar air flow hood. The process was carried out repeatedly during aseptic inoculation work. Tube light and air flow should be on during inoculation period. Thereafter, surface sterilized seeds were carefully inoculated with the help of forceps into medium over the flame of the sprit lamp.

3.2.2.6 Incubation

The inoculated culture tubes were transferred to the tissue culture room having controlled environment conditions such as temperature 25 °C ± 2 °C and relative humidity (RH) 50 to 80 %. The continuous light of about 2 kilo lux intensity was maintained through fluorescent tubes.

3.2.2.7 Subculture

Established cultures were routinely transferred every 5 -6 weeks.

3.2.2.8 Observation

The main responses of 6 cultivars of rice were observed at regular intervals with respect to the frequencies of establishment of aseptic cultures, callus formation, differentiation of shoots and roots. Observations were also made for the nature and colour of callus, number of differentiated shoots per culture. The growth of callus, existing shoots or differentiated shoots and differentiated roots were observed as per the table provide (table 3.6 and 3.7).

Table-3.6 Observation parameter for tissue culture responses

Symbol used	**Explanation**	**Measurement of growth**		
		Callus (diameter in cm)	**Shoot (height in cm)**	**Root (length in cm)**
-	No growth	-	-	-
+	Low growth	Less than 0.5	Less than 0.5	Less than 0.5
++	Medium growth	0.5 – 1.0	0.5 – 1.0	0.5 – 1.0
+++	Good growth	1.0 – 2.0	1.0 – 2.0	1.0 – 2.0
++++	Excellent growth	More than 2.0	More than 2.0	More than 2.0

Table-3.7 Modified standard evaluation score (SES) of visual salt injury at seedling stage

Observation	Tolerance
Normal growth on leaf symptoms	Highly tolerant
Nearly normal growth, but leaf tips or few leaves whitish and rolled	Tolerant
Growth severely retarded; most leaves rolled; only a few are elongating	Moderately tolerant
Complete cessation of growth; most leaves dry; some plants dying	Susceptible

Source: Gregorio *et al*., (1997)

3.2.2.10 Photography

Photographs of cultured tubes were taken by using Sony Cyber-shot, 16.1 Mega Pixels; 5X Optical Zoom digital camera (Model No. DSC-W710).

3.2.2.11 Statistical analysis

3.2.3 INDUCTION AND SELECTION OF SALT TOLERANCE

Calliclonal selection for salt tolerance

Callus cells are known to possess genetic variant which are generally the result of *in vitro* conditions. Such genetic variants can be easily selected if the appropriate selection pressure can be provided in the medium or in the medium or in the culture environment. Selection for salt tolerance among callus called calliclonal variation, can be done by adding salt mixture in the media and selecting salt tolerant cell lines.

Six different media were prepared by adding different salt mixture (7:2:1) at the level of 0, 0.5, 1, 1.5, 2 and 2.5% in each of them. Healthy control calluses of the size 4 mm diameter of all the 6 cultivars were inoculated on to the above media. After 30 days the final observations were taken and the fresh and dry weights of the calluses were measured.

3.2.4 SSR (SIMPLE SEQUENCE REPEAT) ANALYSIS

SSR is a PCR based molecular technique. In present investigation SSR markers were used for study of genetic variation of DNA isolated from leaf of rice (grown in pots) and also from normal callus as well as salt tolerant callus grown under *in vitro* condition (plate-3.2).

3.2.4.1 Extraction of genomic DNA

3.2.4.2 Preparation of stock solution for genomic DNA extraction

a) 1M Tris-HCl (pH 8.0): 3.938 gm of Tris-HCl was dissolved in 10 ml double distilled water and pH was adjusted to 8.0 using NaOH pellets and 5N NaOH. Final volume was made to 25 ml using double distilled water.

b) 5N NaOH: 5gm NaOH was dissolved in 15 ml double distilled water. After dissolving NaOH, final volume was made to 25 ml by adding double distilled water.

c) 0.5M EDTA (pH 8.0): 4.653 gm of sodium salt was dissolved in 15 ml double distilled water and pH was adjusted to 8.0 using 5 N NaOH and 1 N HCl. Final volume was adjusted to 25 ml using double distilled water.

d) 8 M NaCl: 11.70 gm of NaCl was dissolved in 12 ml double distilled water and final volume was adjusted to 25 ml using double distilled water.

e) 10% CTAB: 5 gm was dissolved in 30 ml double distilled water and final volume was adjusted to 50 ml using double distilled water.

f) 10% PVP: 2.5 gm PVP was dissolved in 15 ml double distilled water and final volume was adjusted to 25 ml using double distilled water.

g) RNAase A solution: 25µl (10mg/ml) RNaseA was dissolved in 975µl nuclease free water

Composition of extraction buffer

(i) CTAB : 3 %(W/V)
(ii) NaCl : 2 M
(iii) Tris-HCl : 100mM
(iv) EDTA : 20mM
(v) PVP : 2 % (W/V)
(vi) β-mercaptoethanol : 1 %

3.2.4.3 Procedure of DNA isolation

Total Genomic DNA was isolated from leaves of 15-20 days old rice leaves (grown in pots), control callus as well as salt tolerant callus (plate-3.2) using CTAB method as described by Doyle and Doyle (1990). The isolation of genomic DNA was completed following the protocol with some modifications by adopting the following steps:

1) 300 mg of 15-20 days old fresh leaves were crushed in 1ml extraction buffer by using glass rod in 2 ml fresh centrifuge tube.
2) The homogenate was incubated at 65^0C for 35 minutes in water bath.
3) After that centrifuged at 13500 rpm for 10 minutes.
4) The supernatant was transferred to a fresh 1.5 ml tube and 650 µl of Chloroform: Isoamyl alcohol mixture (24:1) was added.
5) The content was then mixed by the tubes upside-down and subsequently the mixture was centrifuged at 13500 rpm for 10 minutes.
6) After centrifugation the supernatant was transferred into fresh 1.5 ml tube.
7) Chloroform: Isoamyl alcohol (600 µl) was again added and then centrifuged at 13500 rpm for 10 minutes and supernatant was transferred to a fresh tube.
8) DNA was precipitated by mixing 0.6 volume of chilled isopropanol and then pelleted by centrifugation at 13500 rpm for 10 minutes.
9) The supernatant was removed, the pellet was washed with 250 µl 70% ethanol and centrifuged at 10000 rpm for 5 minutes to remove the salts and then pellets was dried in air. Pellet was then dissolved in 30 µl of T.E buffer.
10) Finally, 2 µl of RNase was added and incubated at 37^0C in water bath for 30 minutes.
11) The DNA was stored at -20^0C for further use.

3.2.4.4 Determination of quality of isolated DNA

The quality of isolated DNA was assessed to judge the suitability of isolated DNA for further work. The assessment of the quality of isolated DNA was completed by adopting the following steps:

- Agarose gel (0.8%) was prepared in 0.5X TBE buffer. During the preparation of agarose gel, agarose was dissolved by heating the solution and then allowing it to cool around 60^0C.
- Ethidium bromide was added and solution was poured into sealed gel casting unit arranged on gel casting plate. The comb was placed at the top of the gel and allowed to solidify at room temperature for 30 minutes.
- After solidification, the comb was removed carefully and gel plate (along with gel) was placed in migration chamber containing 0.5X TBE buffer. In each well, DNA sample along with the gel 1x loading dye was loaded carefully.
- Electrophoresis was carried out at 70 V for 45 minutes so that the dye moved to approximately five cm from the well.
- The gel was viewed under UV (332nm) light to derive the inference regarding the quality of isolated DNA.

3.2.4.5 Amplification of genomic DNA

The amplification of DNA was carried out using standard protocol of polymerase chain reaction (PCR) adjusted to laboratory conditions with known 16 pairs of forward and reverse simple sequence repeat (SSR) primers specific to the unique flanking sequences of the simple sequence repeats distributed among twelve chromosomes present in the genome of rice. The panel of primers was based on the ability of the SSR primers to differentiate the rice varieties used in the earlier studies conducted by the research workers for this purpose.

The amplification was carried out in a thermo-cycler using 15 µl reaction mixture prepared by varying the components involved in composition of reaction mixture (table-3.8) in the cases of each of the 14 primer pairs used in the study (table-3.9). Negative and positive controls were incorporated in PCR amplification conducted to verify the absence of contamination and the efficiency of amplification reaction.

Table- 3.8 Master mixture for PCR reaction (15 µl)

Sl. No.	Working concentration of Components	Volume (µl)
1.	Water (Protease and Nuclease free)	2.8
2.	5X PCR buffer (With 0.75mM KCl and 10 mM $MgCl_2$)	3.0
3.	$MgCl_2$ (10 mM)	1.3
4.	dNTPs mix. (1 mM)	3.0
5.	Primer F (5 µM)	1.2
6.	Primer R (5 µM)	1.2
7.	Taq polymerase (1 unit)	0.5
8.	DNA template (20 ng)	2.0

Note: Before starting the reaction, a short spin was required for proper mixing of reaction components.

The polymerase chain reaction was performed with the help of known 14 pairs of forward and reverse simple sequence repeat (SSR) primers covering the 14 chromosomes (table- 3.9). The primers were obtained from Eurofins mwg/operon. The primer vials were centrifuged before and after the addition of 1X TE buffer to the vials. After dilution, the concentration of each primer stock solution was obtained as 100 µM.

Table- 3.9 List of 14 salt tolerance linked primers pairs

Locus	Chromosome No.	Primer sequence (5’-3’)	Repeat Motif	Annealing temp. (^{0}C)
RM2	7	(F) ACGTGTCACCGCTTCCTC (R) ATGTCCGGGATCTCATCG	(GA)$_{13}$	55
RM4	11	(F)TTGACGAGGTCAGCACTGAC (R)AGGGTGTATCCGACTCATCG	GA)$_{16}$	54
RM11	7	(F)TCTCCTCTTCCCCCGATC (R)ATAGCGGGCGAGGCTTAG	(GA)$_{17}$	53
RM14	1	(F)CCGAGGAGAGGAGTTCGAC (R)GTGCCAATTTCCTCGAAAAA	(GA)$_{18}$	55
RM20	11	(F)ATCTTGTCCCTGCAGGTCAT (R)GAAACAGAGGCACATTTCATTG	(ATT)$_{14}$	53
RM24	1	(F)GAAGTGTGATCACTGTAACC (R)TACAGTGGACGGCGAAGTCG	(AG)$_{22}$	56
RM204	6	(F)GTGACTGACTTGGTCATA (R)GCTAGCCATGCTCTCGTACC	(AG)$_{45}$	55
RM223	8	(F)GAGTGAGCTTGGGCTGAAAC (R)GAAGGCAAGTCTTGGCACTG	(AG)$_{20}$	56
RM242	9	(F)GGCCAACGTGTGTATGTCTC (R)TATATGCCAAGACGGATGGG	(AG)$_{11}$	55
RM253	6	(F)TCCTTCAAGAGTGCAAAACC (R)GCATTGTCATGTCGAAGCC	(AG)$_{22}$	56
RM292	1	(F)ACTGCTGTTGCGAAACGG (R)TGCAGCAAATCAAGCTGGAA	GT)$_{10}$-G-(TGA)$_2$	55
RM302	1	(F)TCATGTCATCTACCATCACAC (R)ATGGAGAAGATGGAATACTTGC	(AT)$_{13}$	56
RM333	10	(F)GTACGACTACGAGTGTCACCAA (R)GTCTTCGCGATCACTCGC	(TAT)$_{19}$(CTT)$_{19}$	54
RM336	7	(F)CTTACAGAGAAACGGCATCG (R)GCTGGTTTGTTTCAGGTTCG	(AAG)$_{18}$	56

The stock solution was diluted to 5 µM to make it a working solution and diluted primer was stored at -20^{0}C. The annealing temperature for different primer pairs was kept approximately at 5^{0}C lesser than their melting temperature (Tm). For different primer pairs used in the study, annealing temperature varied depending upon AT and GC content of the primer pair in question. The information regarding the melting temperature was provided by the company that supplied the primers. It was further verified by calculating the melting temperature of the primers on the basis of sequence information regarding the nucleotide composition of the primers. The melting temperature was calculated by using the following formula:

$$\mathbf{Tm = 4\,(G+C) + 2\,(A+T)}$$

The amplification of specific regions was achieved by using the SSR primers specific to the unique flanking sequences of the simple sequence repeats distributed among twelve chromosomes in the genome of rice. The PCR was carried out using the program (table-3.10) listed below:

Table 3.10 Programme of polymerase chain reaction (PCR)

SL No.	Reaction condition	Time and temperature
1.	Initial denaturation	4 min. at 94^{0}C
2.	35 cycles of	
	a) Denaturation	1 min. at 94^{0}C
	b) Annealing	1 min. at 53-56^{0}C
	c) Extension	2 min. at 72^{0}C
3.	Final extension	10 min. at 72^{0}C

The products of amplification were stored at 4^{0}C till separation and resolution through electrophoresis. The amplified products with expected product size above 100 bp were subjected to agarose gel (2%) electrophoresis at 100 volts for one and half hours and then visualized and documented with the help of gel documentation system. The size of amplified fragment was estimated with the help of 50 bp ladder (Fermentas).

3.2.4.6 Separation of amplified DNA

Preparation of gel

During the preparation of 2% gel, 3.0 gm of agarose was taken and poured into the 150 ml TBE (0.5X) buffer present in 250 ml volumetric flask and swirled to mix. The solution was boiled inside microwave oven to dissolve the agarose properly. A transparent solution indicated that the agarose dissolved properly. Ethidium bromide was then added. The solution was kept for cooling. The gel casting plates were set in the casting unit in such a way so that the material could not runoff. Also the comb was inserted. The gel was slowly poured in the gel casting plate, air bubbles were avoided. If any air bubble was observed, it was pushed away to the side with help of micro tip. The gel was leaved for 30 min. to set it properly. After setting of gel, the comb was carefully removed. The gel was transferred to the electrophoretic tank with well towards negative end. The running buffer was then poured to completely submerge the gel. The 0.5X TBE buffer was used as gel buffer and also for running the gel. 50X stock solution of TBE buffer was diluted to 100 times to make it 0.5X TBE working solution.

Preparing the sample

The gel loading buffer was added to the DNA samples. An appropriate amount of loading buffer was always added. The microtips were left into the tubes in which samples were present which were again used to load them into the gel.

Loading the gel

The samples were loaded into the well carefully with the help of microtips. Along with samples, 50 bp DNA ladder was loaded for the analysis of the samples.

Running the gel

Once the samples were loaded in the gel, the power supply was switched on and the gel was run at 100 volts. The power source was checked by observing at the electrode whether the bubbles were evolving or not. Initially electrophoresis was done at lower voltage to allow the DNA to move into the gel slowly and evenly. The gel was allowed to run until the blue color tracking dye reached 3/4th of the gel, thereafter only the power supply was disconnected.

Visualization of DNA

The gel was carried to the dark room to look on the UV transilluminator supplying radiation at 312 nm and > 2500 µWm^{-2}. The amplified DNA fragments were visible as the orange band because the ethidium bromide intercalated into DNA and fluorescenced under UV light. The amplified products were documented with the help of gel documentation system (Alpha Innotech, USA) and the size of amplified fragments was estimated with the help of 50 bp ladder (Fermentas).

3.2.4.7 Analysis of amplified products

The size of amplified products was determined in relation to the size of markers in 50 bp DNA ladder. The position of the amplified products visualized in the form of position of bands on the gel corresponded to the location of the bands along Y-axis (ranging from 0 to 1030). The Rf value for each band was also determined assuming the location of well as initial position (Rf=0) and the position of migrated dye as final position (Rf=1) as a frame of reference. The different bands produced by each one of the twelve primer pairs were compared and classified into the two different categories of shared and unique bands and then expressed in percentage.

3.2.4.8 Analysis of SSR markers based polymorphism

The information pertaining to allelic diversity and the suitability of the SSR based polymorphism for identification of polymorphic and informative markers to characterize and differentiate the locally adapted rice varieties was generated on the basis of comparison of polymorphism information content (PIC) of the SSR primer pairs. The data on polymorphism information content (PIC) of the SSR markers was obtained by calculating the value according to the formula as described by Aderson *et al*. (1993). The polymorphism information content (PIC) of the SSR primer pairs was calculated as follows.

$$\textbf{PIC} = 1-\sum_{j=1}^{k} P^2 ij$$

Where, k is the total number of alleles detected for a marker,

P_{ij} is the frequency of the j^{th} allele for i^{th} marker and summation extends over k alleles.

3.2.4.9 Computation of polymorphism coefficient

The information regarding informativeness of the marker was obtained by computing the polymorphism per cent as follows:

PP = (Number of unique alleles/Total number of alleles) x 100

3.2.4.10 Analysis of SSR markers based divergence

The polymorphism in respect of SSR was recorded on the basis of presence or absence of the SSR bands in different varieties under investigation in the present study. All the varieties were scored for the presence and absence of the SSR bands. The data were entered into binary matrix as discrete variables and this data matrix was subjected to further analysis. Genetic similarities among varieties were calculated on the basis of presence and absence of common bands. The genetic associations among varieties were analyzed by calculating the similarity coefficient (Dice, 1945) for pair-wise comparisons based on the proportions of shared bands produced by primers.

Similarity coefficient = 2a/(2a+b+c)

Where,

a = Number of bands between J^{th} and K^{th} genotypes

b = Number of bands present in J^{th} genotype but absent in K^{th} genotype

c = Number of bands absent in j^{th} genotype but present in K^{th} genotype

Cluster analysis was performed using the data on similarity coefficients. The method used for tree building in the cluster analysis involved sequential agglomerative hierarchical non-overlapping (SAHN) clustering based on similarity coefficients. The dendrogram based on similarity indices was obtained by un-weighted pair-group method using arithmetic mean (UPGMA). Analysis was performed with the help of NTSYS-pc software (Rohlf, 1997). The nature of diversity between rice varieties under evaluation in the present investigation was assessed by identifying the clusters at appropriate phenon levels.

☆☆☆

CHAPTER – IV

EXPERIMENTS AND OBSERVATIONS

EXPERIMENTS AND OBSERVATIONS

Tissue culture experiments were conducted in six selected rice cultivars namely CSR-30, Narendra Usar Dhan-3, MTU-7029, BPT-5204, Pusa Basmati-1 and Rajendra Bhagwati for screening their salt tolerance and to induce salt tolerance by exploiting calliclonal and somaclonal variations. The *in vitro* developed salt tolerant calli were tested for salt tolerance using molecular marker (SSR). Thus the experiments and observations were conducted as follows:

- Germination studies
- Tissue culture studies
- Molecular marker studies

4.1 Germination studies

Six selected rice cultivars were screened for salt tolerance in the laboratory using following techniques.

- Seed germination under salt stress
- Seedling growth under salt stress

4.1.1 Seed germination under salt stress

The seeds of six selected rice cultivars were put to germination under different levels of salt concentrations (table-3.2) ranging from 0-2.5%. The germination of seeds was initiated within the first week. The first sign of germination was observed by the appearance of the plumule followed by radical. Only in control (0% salt) the seeds of all the cultivars showed germination in the first week. Some seeds of cvs. Narendra Usar Dhan-3 and CSR-30 also germinated in 0.5% salt solution in the first week. The seeds of all other cultivars germinated in 1.0 and 1.5% salt solutions in the second week. Only seeds of cvs. Narendra Usar Dhan-3 and CSR-30 were germinated in 2% salt solution. There was no germination of seeds in all cultivars in 2.5% salt solution.

The germination percentage in control (0% salt) in all the cultivars was at par with the mean 93.66%. In 0.5% salt concentration the best seed germination was found in cultivar G3 (82.0%) followed by cvs. G5 (81.33%) and G2 (76.66%) which were at par. Minimum germination was found in cv. G6 (63.33%). Similarly in 1% salt, the highest and at par germination was found in cvs. G5 (42.66%), G3 (42.66%) and lowest in cv. G6 (22.00%). At 1.5% salt the highest and at par germination was found in cvs. G5 (22.66%), G3 (22.00%) and lowest in cv. G6 (2.66%). The germination percentage gradually decreased with increased salt concentrations which were presented in table-4.1 and graphically represented in fig-4.2. Overall the best germination was found in cv. G5 (41.94%) followed by cvs. G3 (41.61%), G2 (36.22%), G1 (35.44%), G4 (30.99%) and G6 (30.10%) respectively (Fig-4.1).

Table-4.1 Germination (%) of seeds of rice cultivars on different salt concentrations

Salt (%)	**Cultivars**									
	G1	G2	G3	G4	G5	G6	Mean	CD	CV	SE (m)
0%	93.33±1.76	94.66±0.66	94.00±1.15	92.66±1.76	94.66±0.66	92.66±1.33	93.66	-	2.41	1.30
0.5%	74.66_b±2.90	76.66_a±2.66	82.00_a±1.55	65.33_c±2.40	81.33_a±1.33	63.33_c±2.40	73.88	6.99	5.26	2.24
1%	35.33_b±2.90	36.00_b±3.05	42.66_a±0.66	24.66_c±0.66	42.66_a±1.33	22.00_c±1.15	33.88	5.93	9.73	1.90
1.5%	9.33_b±0.66	10.00_b±1.15	22.00_a±1.15	3.33_c±0.66	22.66_a±1.33	2.66c±0.66	11.66	3.05	14.56	0.98
2%	-	-	9.00_b±0.57	-	10.33_a±0.33	-	3.33	0.84	14.63	0.27
2.5%	-	-	-	-	-	-				
Mean	35.44	36.22	41.61	30.99	41.94	30.10				
CD	5.75	5.42	2.78	3.97	3.08	3.88				
CV	9.02	8.33	3.71	7.13	4.09	7.17				
SE (m)	1.84	1.74	0.89	1.27	0.99	1.24				

Values followed by the same letter in row are not significantly different using Duncan’s Multiple Range Test (DMRT) at 5%

Fig- 4.1 Effect of cultiv mination (%) of seeds

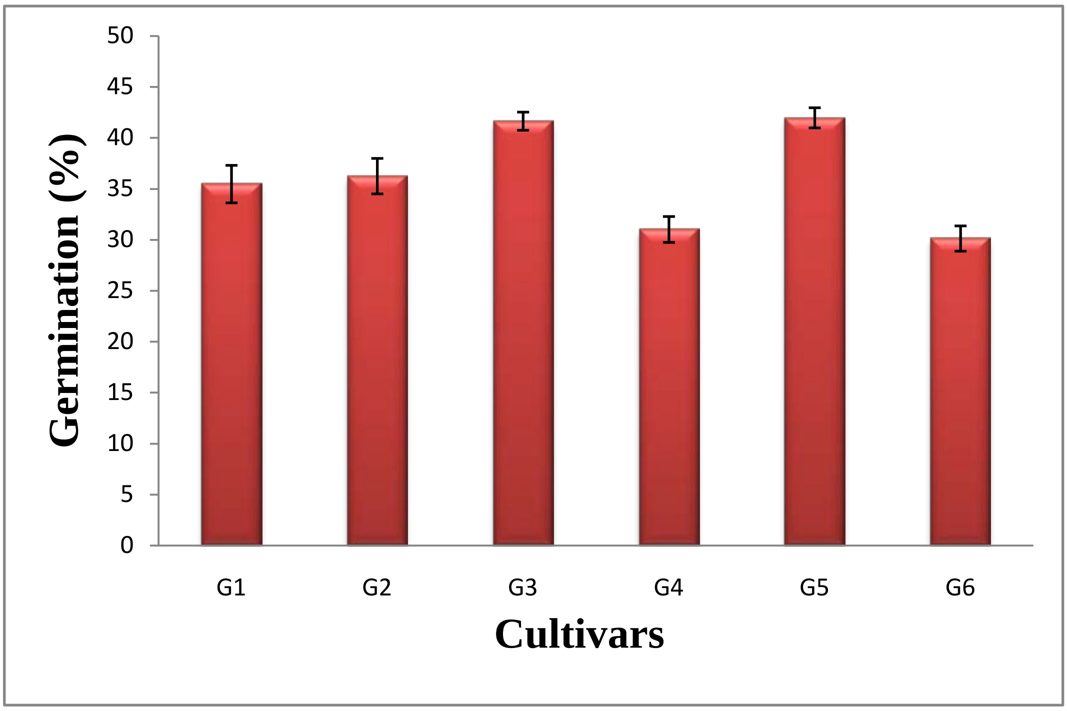
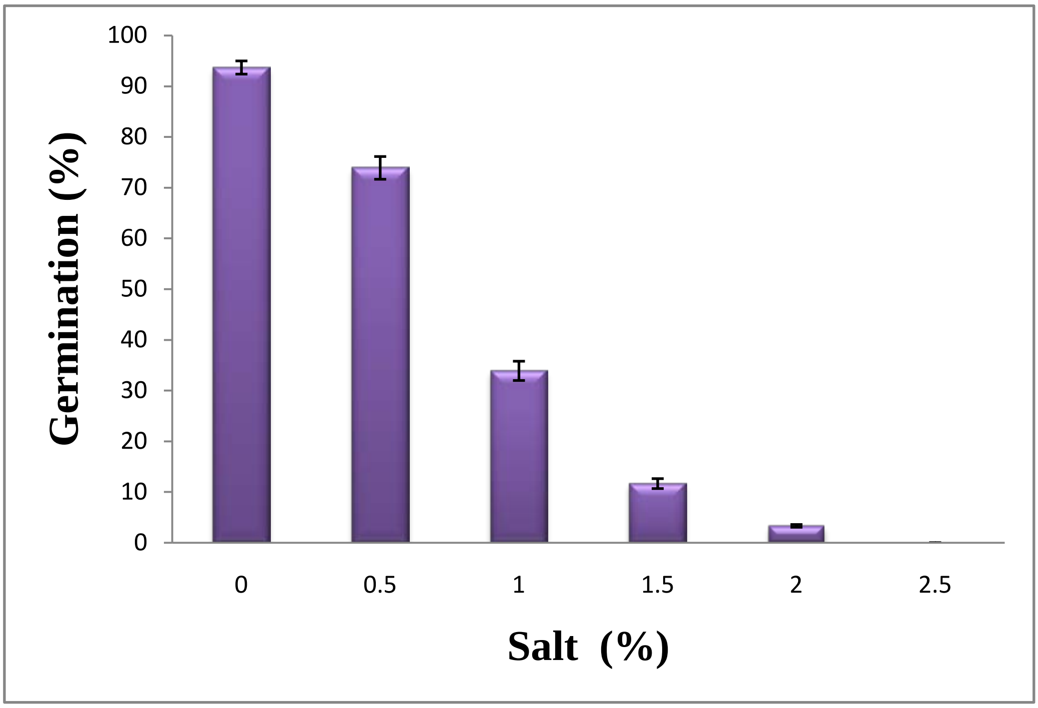


Fig- 4.2 Effect of salt on germination (%) of seeds



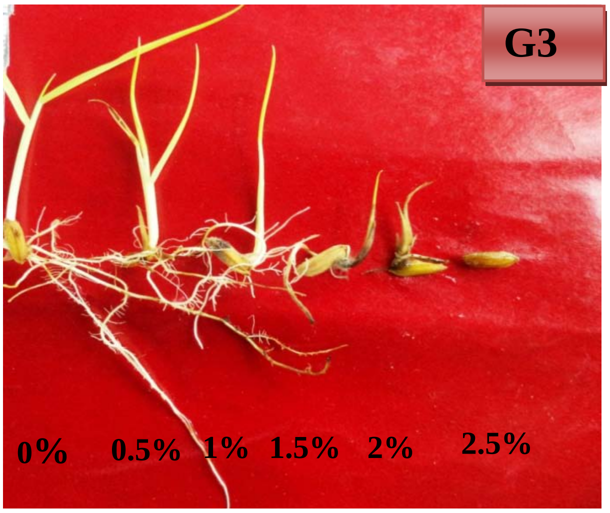
4.1.2 Seedling growth under salt st

Seedling of all six selected cultivars was grown in salt solutions with the salt concentrations ranging from 0-2.5%. The seedling growth was inversely proportional to the salt concentrations. The shoot growth was more affected than the root growth. The fresh weight and dry weight of seedling were measured to evaluate the seedling growth under different salt stress. Salinity tolerance index (STI) was calculated based on shoot and root dry weights at different levels of salt stresses of all selected six cultivars, which were represented in table-4.2 and graphically represented in fig-4.3. Cultivar G5 (57.07%) showed the highest mean STI followed by cvs. G3 (51.32%), G2 (37.09%), G1 (36.48%), G4 (34.07%) and G6 (21.64%) respectively.

Table-4.2 Seedling growth of rice cultivars under different salt concentrations

Cultivar	**Salt concentrations (%)**	**Seedling growth**					
		Shoot		Root		Salinity tolerance index (STI)	
		Fresh wt	Dry wt	Fresh wt	Dry wt	STI	**Mean STI**
G1	0	15.4	2.13	14.86	1.60		36.48
	0.5	14.07	1.61	9.53	0.92	66.54	
	1.0	6.57	1.28	3.71	0.57	47.85	
	1.5	4.0	0.8	2.0	0.4	31.55	
	2.0	-	-	-	-	-	

	0	16.33	3.75	10.91	2.0		
	0.5	15.5	2.9	6.5	1.2	68.66	
	1.0	14.37	2.25	5.12	0.87	51.75	
	1.5	6.66	1.16	4.16	0.5	27.96	
	2.0	-	-	-	-	-	
G3	0	25	3	26.91	3.91		51.32
	0.5	13.66	2.1	18.83	2.25	63.77	
	1.0	3.63	2.09	11.45	2.0	60.40	
	1.5	3.55	1.66	9.44	1.2	43.01	
	2.0	2.5	1.5	3.5	1.0	38.11	
G4	0	19.38	2.94	11.77	1.5		34.07
	0.5	10.66	2.2	8.5	0.94	68.74	
	1.0	8.16	1.91	6.75	0.83	60.14	
	1.5	2.55	0.22	0.88	0.11	7.40	
	2.0	-	-	-	-	-	
G5	0	20.91	3.53	14.25	2.75	-	57.07
	0.5	18.00	3.4	13.63	2.71	97.42	
	1.0	17.57	2.85	9.0	1.42	66.18	
	1.5	10.25	1.0	7.50	1.5	41.43	
	2.0	6.5	0.5	6.0	0.5	23.25	
G6	0	18.46	2.46	9.30	1.30		21.64
	0.5	9.88	1.22	5.66	0.88	58.64	
	1.0	3.16	0.5	2.33	0.33	22.85	
	1.5	0.8	0.1	0.6	0.08	5.10	
	2.0	-	-	-	-	-	



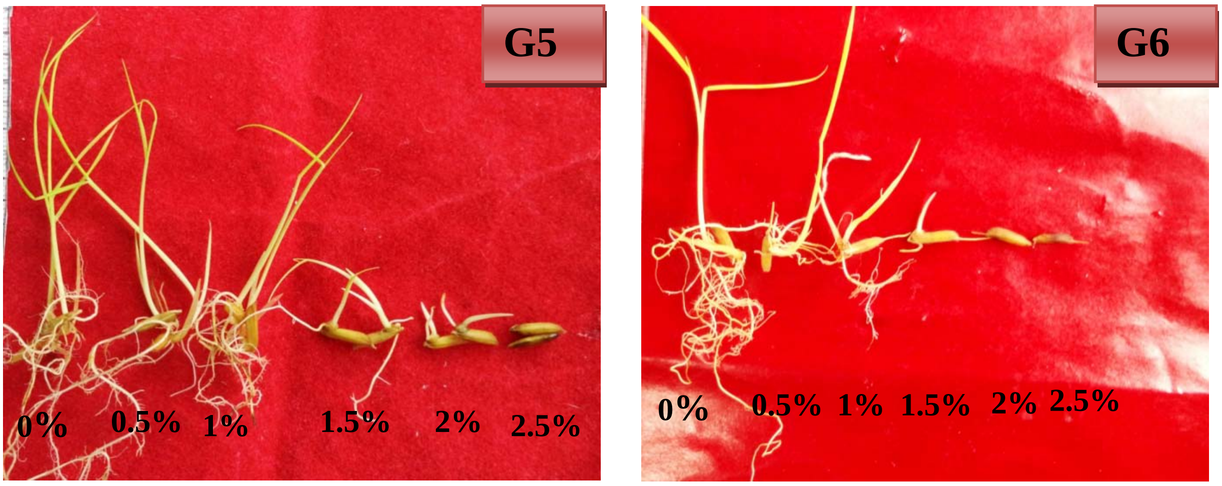
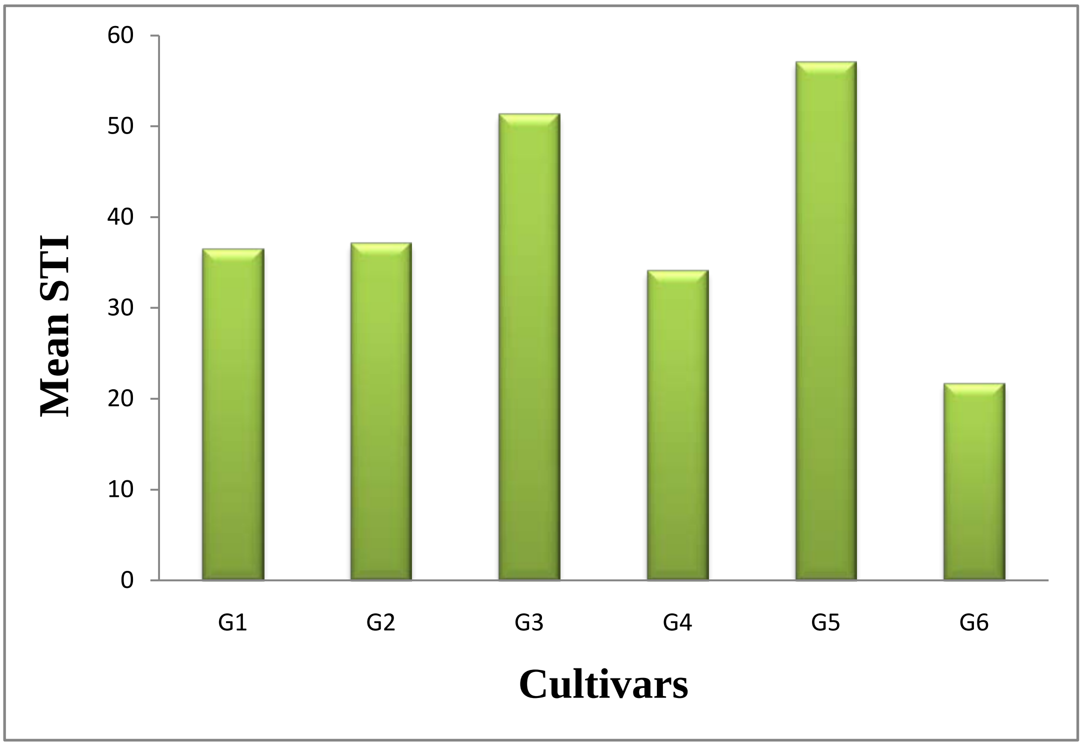


Plate-4.1 ***In vivo*** **seedling growth under salt concentrations**

Photos showing growth of seedlings of rice cultivars G3, G4, G5 and G6 under different salt concentrations ranging from 0-2.5%

Fig-4.3 Salinity tolerance index (STI) of rice cultivars based on seedling growth under different salt concentrations



4.2 Tissue culture studies

The tissue culture technique was used to study the callusing response of cultured seeds of all selected cultivars and regeneration of plants from such callus. The observations were recorded at progressive stages of tissue culture responses. The tissue culture experiments were done under following headings:

- Selection of explant
- Selection of media
- Seed culture
- Seed culture under salt stress
- Callus growth under salt stress
- Regeneration of plant from callus

4.2.1 Selection of explant

Dehusked rice seed was used as explant.

4.2.2 Selection of media

Medium is the most important factor for the success of tissue culture experiment and thus selection of appropriate medium is the first step of any tissue culture experiment. Four different media were generated using MS basal medium supplemented with different combinations and concentrations of phytohormones (2, 4-D, NAA and KIN) as mentioned in materials and methods (table-3.5). Out of four, first three media M1, M2 and M3 were used for callusing while the last one M4 was used for regeneration of plant.

4.2.3 Seed culture

Dehusked rice seed was cultured on three selected callusing media, which showed the following responses.

- Establishment
- Germination
- Callus formation

4.2.3.1 Establishment of cultured seeds

Establishment of cultured explants depended upon pre-treatment and surface sterilization. The establishment of cultured seeds was evaluated with respect to the effect of medium and cultivars which were represented in table-4.3 and graphically represented in fig 4.4 and 4.5.

The cultured seeds showed no variations in establishment responses on different media among six cultivars. The establishment frequency was at par among the six cultivars and on the three media on which seeds were cultured. The overall mean frequency of establishment of all the cultivars was the maximum in medium M3 (89.99%) followed by M2 (89.16%) and M1 (88.05%) respectively (fig-4.5). Considering all the three media, the overall mean establishment was the maximum in cv. G1 (91.10%) followed by cvs. G3 (89.99%), G4 (89.44%), G5 (88.88%), G2 (88.33%), G6 (86.66%) respectively (table- 4.3 and fig-4.4).

Table-4.3 Establishment (%) of cultu f rice cultivars on different media

Media	Cultivars									
	G1	G2	G3	G4	G5	G6	Mean	CD	CV	SE(m)
M1	88.33±1.66	85±5.77	88.33±4.41	91.66±3.33	90±2.88	85±2.88	88.05	-	7.33	3.72
M2	91.66±1.66	90±2.88	93.33±1.66	85.00±5.00	86.66±3.33	88.33±1.66	89.16	-	5.76	2.96
M3	93.33±1.66	90.00±5.00	88.33±4.41	91.66±1.66	90.00±5.00	86.66±1.66	89.99	-	6.92	3.6
Mean	91.10	88.33	89.99	89.44	88.88	86.66				
CD	-	-	-	-	-	-				
CV	3.16	9.24	7.17	6.97	7.5	4.3				
SE(m)	1.66	4.71	3.72	3.6	3.84	2.15				

Values are expressed as mean±SE

Fig-4.4 Effect of rice cultivars on establishment (%) of cultured seeds

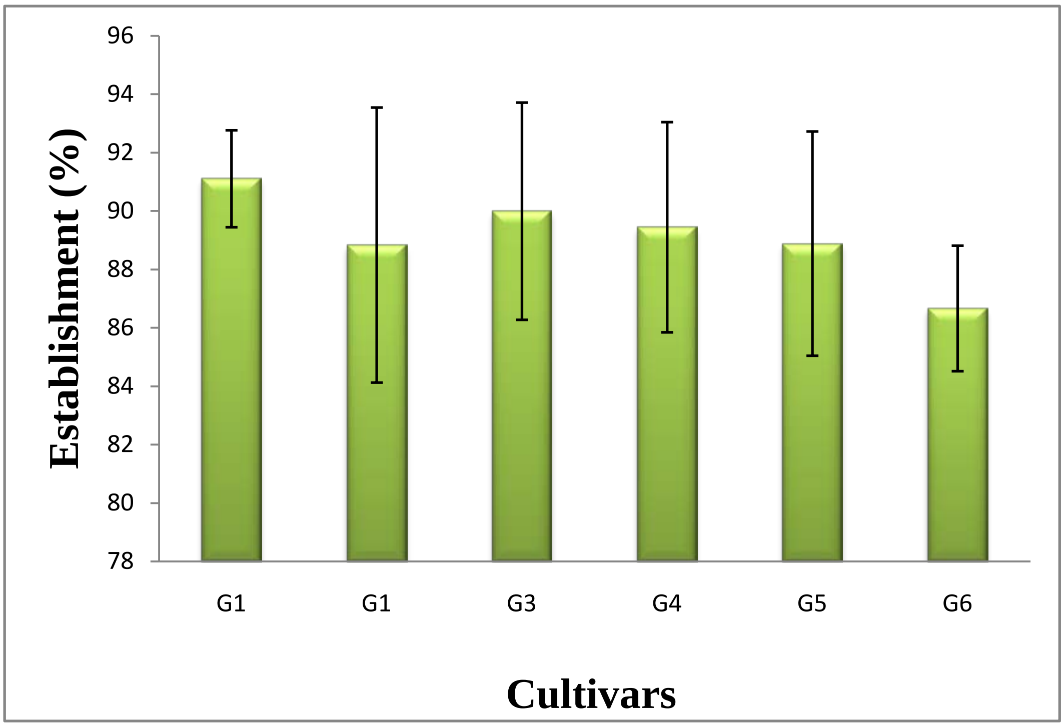
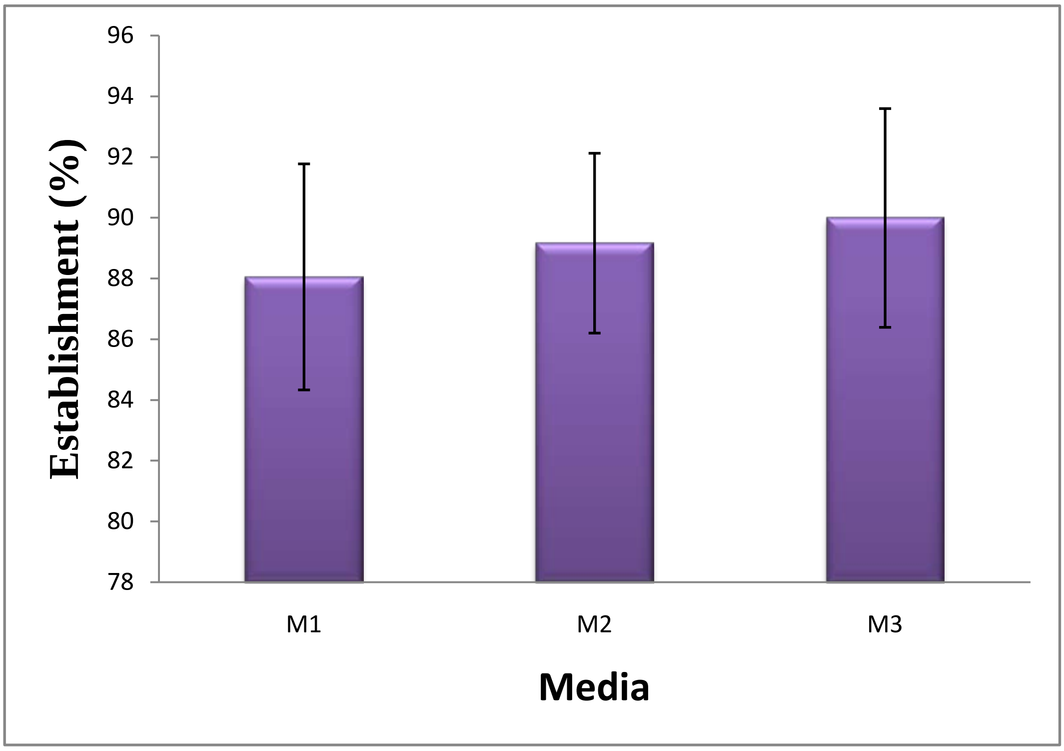


Fig-4.5 Effect of medium on ıent (%) of cultured seeds



4.2.3.2 Germination of cultured seeds

The cultured seeds started germinating within the first week. Majority of cultures showed either the development of plumule leading to formation of shoot or the development of radical leading to formation of root. Some cultures showed development of both root and shoot.

The germination percentage of cultured seeds of all the cultivars on the three callusing media was at par. Similarly the effect of the three media on germination frequency was at par among all cultivars. However medium M1 (94.04%) showed the best germination frequency among all cultivars followed by media M2 (92.22%) and M3 (91.50%). In effect of cultivar, the germination percentage of all cultivars was at par. The overall best germination frequency was observed in cv. G1 (93.59%) followed by cvs. G3 (92.67%), G1 (92.61%), G5 (92.41%), G6 (92.28%), G4 (91.91%) respectively (table-4.4 and fig-4.6 and 4.7).

Table 4.4 Germination (%) of cultured seeds of rice cultivars on different media

Media	Cultivars									
	G1	G2	G3	G4	G5	G6	Mean	CD	CV	SE(m)
M1	92.37±2.07	94.05±0.40	94.63±3.04	94.52±0.20	96.39±1.80	92.13±1.96	94.01	-	3.43	1.86
M2	92.68±1.90	94.42±0.17	92.78±1.95	90.39±2.16	90.62±1.74	92.46±2.12	92.22	-	3.42	1.81
M3	92.78±1.95	92.32±2.41	90.61±2.06	90.83±1.95	90.23±4.49	92.26±2.01	91.50	-	5.00	2.64
Mean	92.61	93.59	92.67	91.91	92.41	92.28				
CD	-	-	-	-	-	-				
CV	3.69	2.61	4.49	3.17	5.57	3.81				
SE(m)	1.97	1.41	2.40	1.68	2.97	2.03				

Values are expressed as mean±SE

Fig-4.6 Effect of rice cultivars on germination (%) of cultured seeds

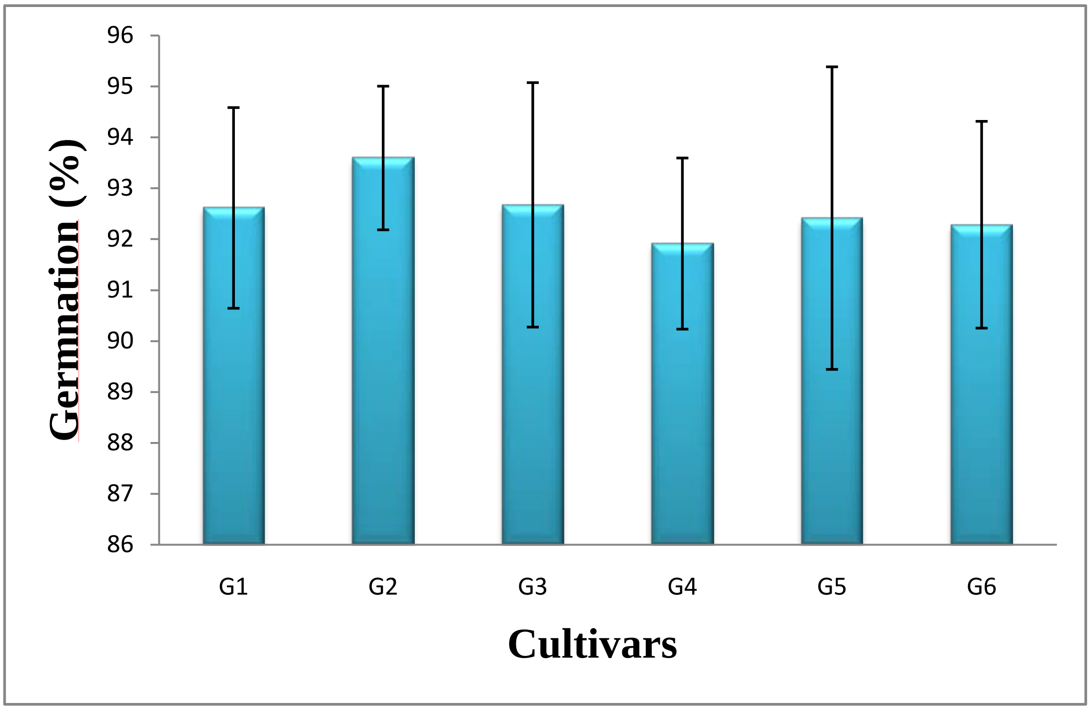
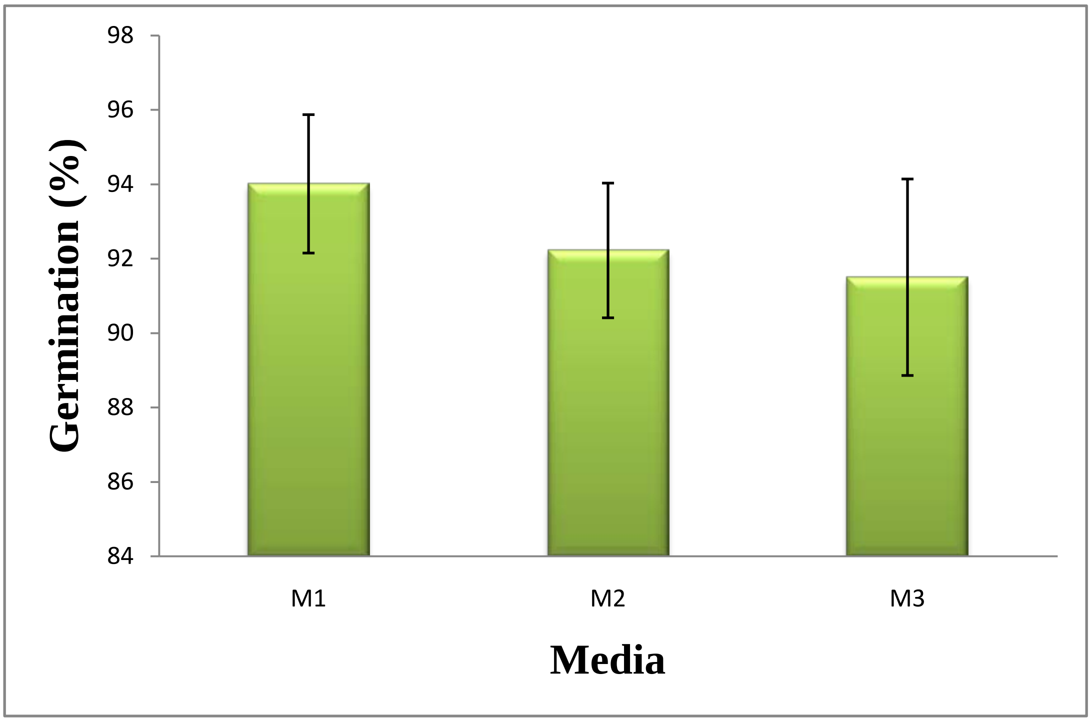


Fig-4.7 Effect of mediur nation (%) of cultured seeds



4.2.3.3 Callus formation of cultured seeds

Cultured seeds of rice cultivars showed callus formation from germ pore in the first week in some, while in others the base of initiating shoot swelled and formed callus in the second week. The frequency of cultures showing callus formation from shoot base was more than from the germ pore of cultured seeds. The callus colour ranged from cream to brown with the majority of the calli being cream. The nature of callus was friable but there were some compact regions also. The callus growth was good to excellent in cvs. G4, G5 and G6, average to good in cvs. G2 and G3 and low to good in cv. G1 (plate-4.2).

The overall frequency of callus formation was the best on medium M1 (82.08%) followed by M2 (80.94%) and M3 (80.67) respectively considering all six cultivars. The callus formation percentage among six cultivars and on three media were at par. The overall best callus formation frequency was found in cv. G5 (91.72%) followed by cvs. G6 (91.44%), G4 (81.08%), G3 (79.08%), G2 (73.355) and G1 (70.70%) respectively considering the three media (table-4.5 and fig-4.8 and 4.9).

Table-4.5 Callus formation (%) of cultured seeds of rice cultivars on different media

Media	**Cultivars**									
	G1	G2	G3	G4	G5	G6	Mean	CD	CV	SE (m)
M1	$73.29_{c}\pm2.70$	$75.54_{b}\pm3.39$	$77.72_{b}\pm0.71$	$83.71_{b}\pm2.87$	$88.48_{a}\pm3.92$	$93.75_{a}\pm3.60$	82.08	9.52	6.44	3.05
M2	$70.75_{b}\pm4.09$	$72.25_{b}\pm2.82$	$76.60_{b}\pm5.19$	$83.17_{a}\pm2.71$	$88.55_{a}\pm3.49$	$94.33_{a}\pm3.21$	80.94	11.49	5.89	3.68
M3	$68.08_{c}\pm4.49$	$72.27_{c}\pm2.91$	$82.93_{b}\pm0.87$	$76.36_{c}\pm1.51$	$98.14_{a}\pm1.85$	$86.24_{b}\pm1.98$	80.67	7.95	5.48	2.55
Mean	70.70	73.35	79.08	81.08	91.72	91.44				
CD	-	-	-	-	-	-				
CV	9.40	7.21	6.72	5.22	6.07	5.71				
SE(m)	3.84	3.05	3.07	2.44	3.21	3.01				

Values followed by the same letter in row are not significantly different using Duncan’s Multiple Range Test (DMRT) at 5%

Fig-4.8 Effect of cultivars on callus formation (%) of cultured seeds]

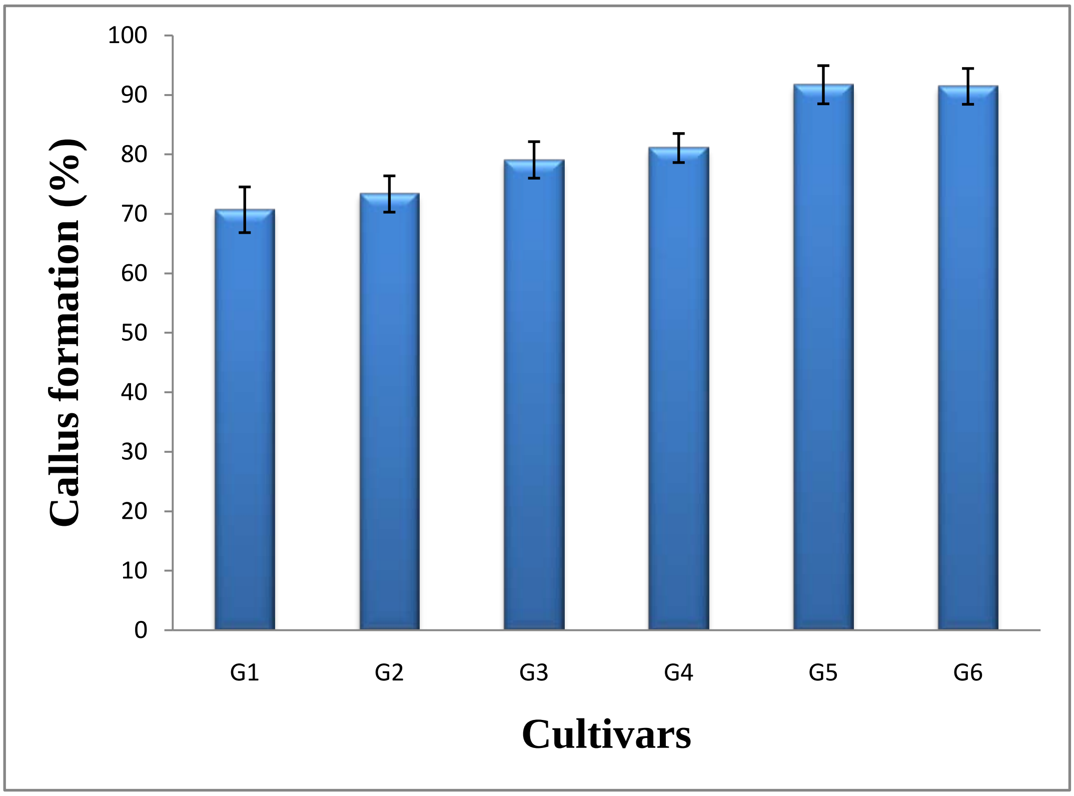
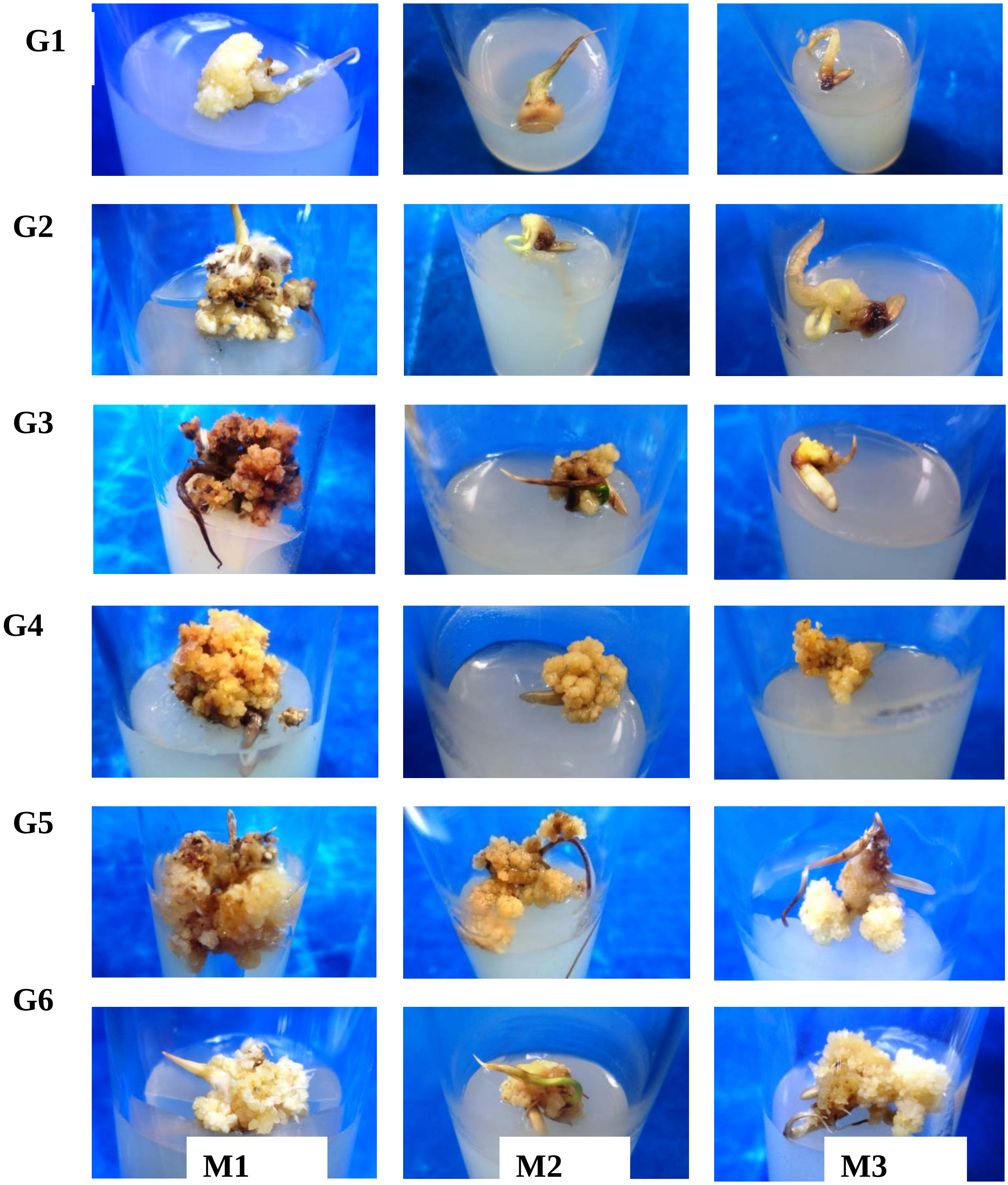
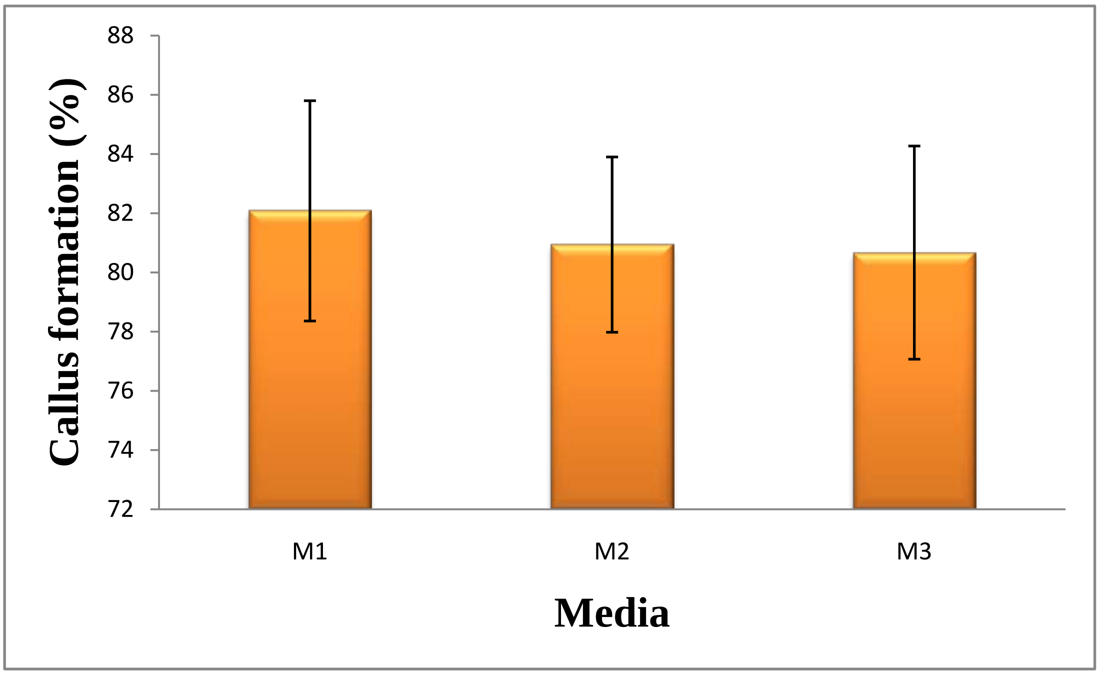


Plate-4.2 Callus formation from cultured seeds on selected medium



Photos of different types of callus formed by cultured seeds of six selected rice cultivars on three media

Fig-4.9 Effect of medium on callus formation (%) of cultured seeds



4.2.4 Seed culture under salt stress

Dehusked rice seed was cultured on medium M1 supplemented with different salt concentrations ranging from 0-2.5%, which showed the following responses:

- Establishment
- Germination
- Callus formation

4.2.4.1 Establishment of cultured seeds under salt stress

The establishment of explant was an index to show the frequency of culture remaining sterile and viable. The establishment of cultured seeds was evaluated with respect to the effect of salt concentrations and cultivars which were represented in table-4.6 and graphically represented in fig 4.10 and 4.11.

The cultured seeds showed no variations in establishment responses on different salt concentrations among six cultivars. The establishment frequency was at par among the six cultivars and on different salt concentrations. The overall mean frequency of establishment of all the cultivars was the maximum in 0% salt (91.38%) followed by 2.5% salt (90.83%), 1 and 2% salt (90.55%), 0.5% salt(89.71%) and 1.5% salt (89.44) respectively (fig-4.10). Considering all the salt concentrations, the overall mean establishment was the maximum in cv. G6 (92.21%) followed by cvs. G4 (91.66%), G5

(91.38%), G3 (90.83%), G1 (88.88%), G2 (87.49%) respectively (table- 4.6 and fig-4.9).

Table 4.6 Establishment (%) of cultured seeds of rice cultivars on different salt concentrations

Salt (%)	Cultivars									
	G1	G2	G3	G4	G5	G6	Mean	CD	CV	SE(m)
0%	90.00±0.00	86.66±6.00	91.66±1.66	95.00±0.00	93.33±1.66	91.66±1.66	91.38	-	5.15	2.72
0.5%	86.66±1.66	85.00±5.00	90.00±2.88	91.66±1.66	91.66±1.66	93.33±1.66	89.71	-	5.25	2.72
1%	86.66±3.33	88.33±4.41	91.66±3.33	93.33±1.66	91.66±3.33	91.66±3.33	90.55	-	6.37	3.33
1.5%	88.33±4.41	86.66±3.33	90.00±0.00	90.00±2.88	90.00±0.00	91.66±3.33	89.44	-	6.37	3.33
2%	91.66±1.66	88.33±3.33	90.00±5.00	88.33±3.33	90.00±2.88	95.00±0.00	90.55	-	5.96	3.11
2.5%	90.00±0.00	90.00±2.88	91.66±1.66	91.66±1.66	91.66±1.66	90.00±2.88	90.83	-	3.89	2.041
Mean	88.88	87.49	90.83	91.66	91.38	92.21				
CD	-	-	-	-	-	-				
CV	4.78	8.51	5.50	4.06	4.07	6.04				
SE(m)	2.45	4.30	2.88	2.15	2.15	3.19				

Values are expressed as mean±SE

Fig-4.10 Effect of cultivars on establishment (%) of cultured seeds

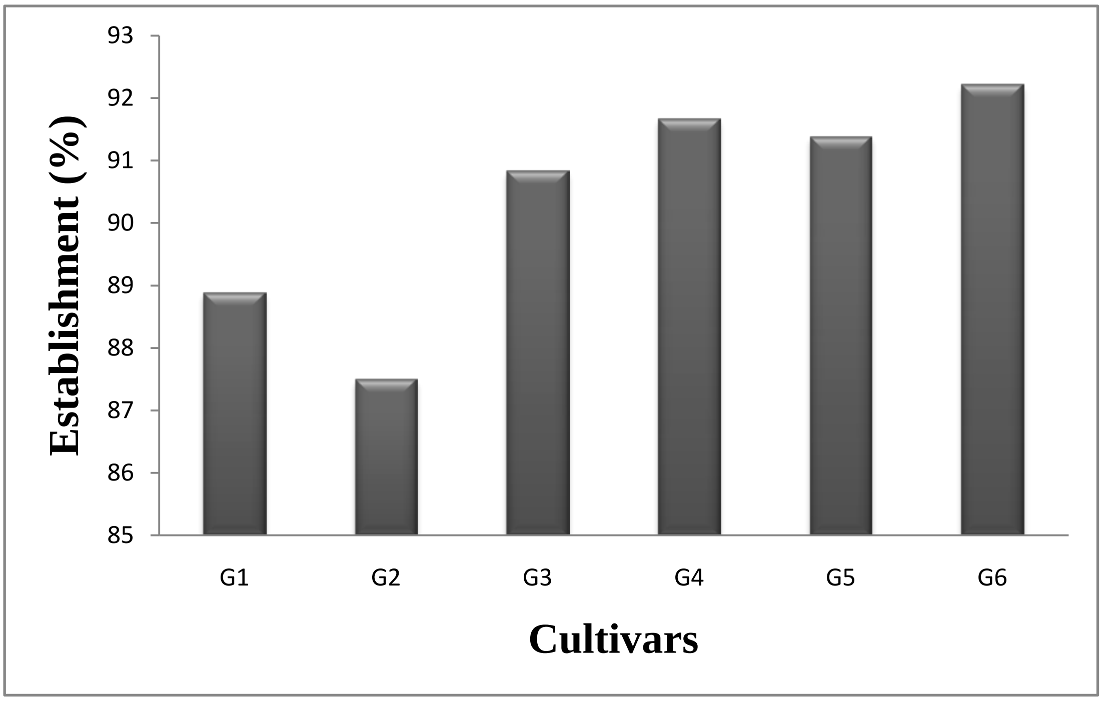
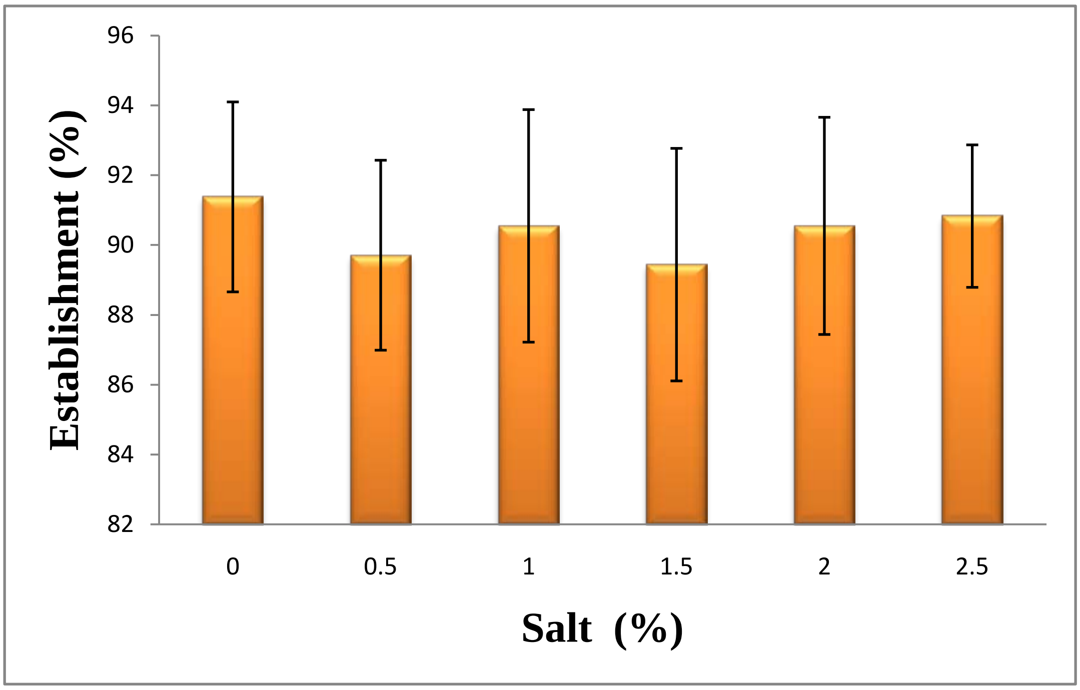


Fig 4.11 Effect of salt on establishment (%) of cultured seeds



4.2.4.2 Germination of cultured seeds under salt stress

The germination of cultured seeds was initiated within the first week of six selected rice cultivars on medium M1 supplemented with different salt concentrations ranging from 0-2.5%.

The germination percentage in control (0% salt) in all the cultivars was at par with the mean 92.17%. In 0.5% salt concentration the best germination of cultured seeds was found in cv. G5 (81.86%) followed by cv. G3 (81.44%) which were at par. Minimum germination was found in cv. G6 (41.12%). Similarly in 1% salt, the highest and at par germination was found in cv. G5 (38.06%) and cv. G3 (36.52%), and the lowest in cv. G6 (11.06%). At 1.5% salt the highest and at par germination was found in cvs. G5 (18.51%) and G3 (16.29%) and the lowest in cv. G6 (3.33%). The germination percentage gradually decreased with increased salt concentrations which were represented in table-4.7 and graphically represented in fig-4.13. Overall, cv. G5 (39.49%) showed the best germination followed by cvs. G3 (38.75%), G2 (31.29%), G1 (29.33%), G4 (26.55%) and G6 (24.09%) respectively (fig-4.12).

Table 4.7 Germination (%) of cultured seeds of rice cultivars on different salt concentrations

Salt (%)	**Cultivars**									
	G1	G2	G3	G4	G5	G6	Mean	CD	CV	SE(m)
0%	90.73±1.85	94.63±3.04	92.68±1.90	92.97±1.75	92.98±4.64	89.08±3.21	92.17	-	5.49	2.92
0.5%	53.80_b±3.50	55.18_b±2.89	81.44_a±1.91	49.02_b±2.41	81.86_a±1.46	41.12_c±2.24	60.40	7.77	7.15	2.49
1%	24.99_b±4.81	29.96_b±2.53	36.52_a±2.77	14.03_c±1.49	38.07_a±2.06	11.06_c±0.37	25.77	7.76	16.75	2.49
1.5%	6.50_b±0.80	7.97_b±0.21	16.29_a±0.66	3.33_c±0.33	18.51_a±1.85	3.33_c±0.66	9.32	2.87	17.16	0.92
2%	-	-	5.59_a±0.33	-	5.56_a±0.17	-	1.85	0.47	14.28	0.15
2.5%	-	-	-	-	-	-	-	-	-	-
Mean	29.33	31.29	38.75	26.55	39.49	24.09				
CD	7.99	6.24	3.80	5.78	7.12	6.19				
CV	15.15	11.09	5.45	12.09	10.03	13.58				
SE(m)	2.56	2.00	1.22	1.85	2.28	1.98				

Values followed by the same letter in row are not significantly different using Duncan's Multiple Range Test (DMRT) at 5%

Fig 4.12 Effect of cultivars on germination (%) of cultured seeds

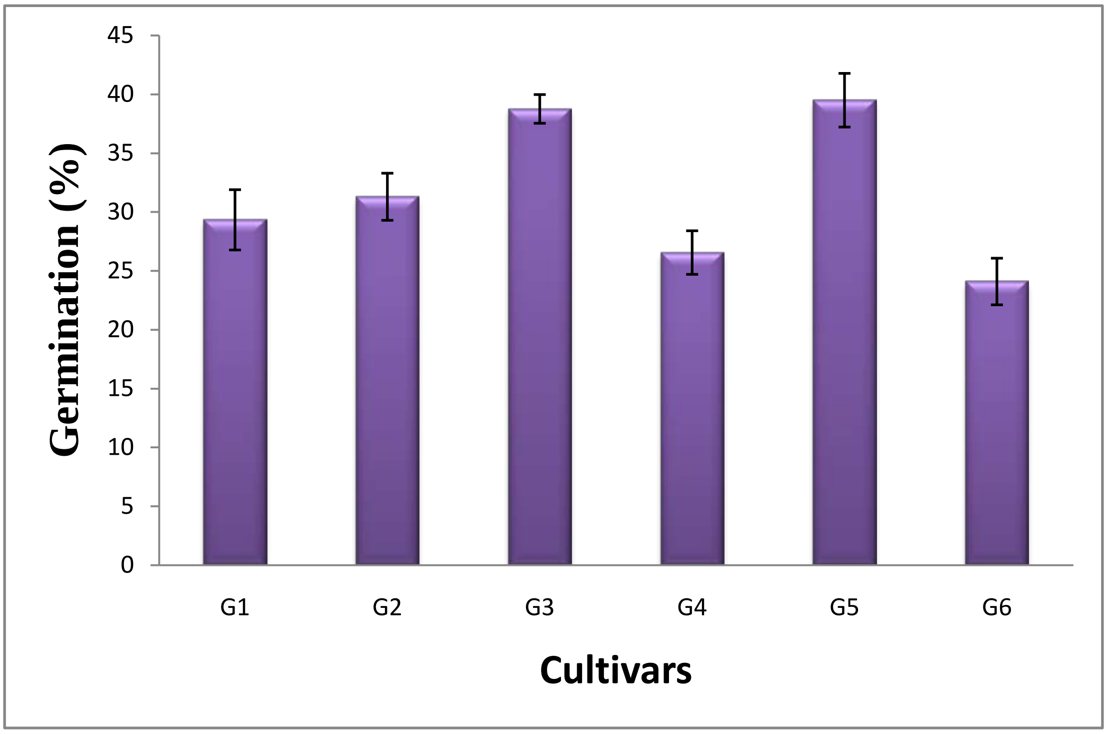
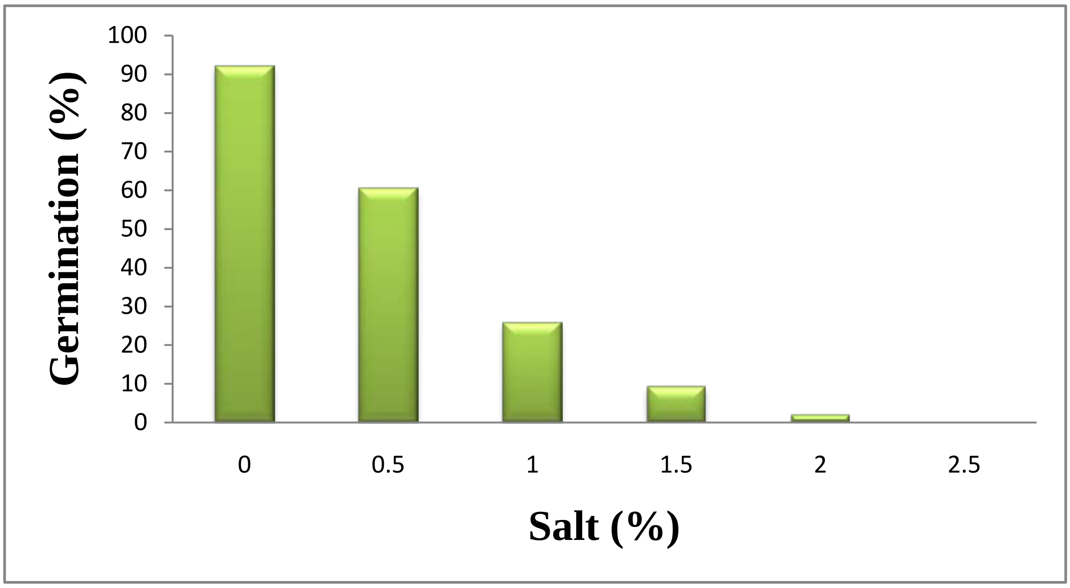


Fig 4.13 Effect of salt on germination (%) of cultured seeds



4.2.4.3 Callus formation of cultured seeds under salt stress

The callus formation from cultured seeds of six selected rice cultivars on medium M1 supplemented with different salt concentrations ranging from 0-2.5% was initiated within the first week. The callus colour ranged from cream to brown with the majority of the calli being cream. The nature of callus was friable but there were some compact regions also. Compared to control in which callus growth was average to excellent, in 0.5% salt it was average to good. At 1% salt callus growth was low to average and at 1.5%, it was low and 2% salt there was very little callus growth (plate-4.3a and 4.3b). The callus formation percentage gradually decreased with increased salt concentrations which were represented in table-4.8 and graphically represented in fig-4.15.

In control (0% salt), the best callus formation was found in cv. G5 (96.48%). The minimum callus formation was found in cv. G1 (68.51%) followed by cv. G2 (70.85%) which were at par. In 0.5% salt concentration, the best callus formation was found in cv. G5 (63.64%) and the minimum in G6 (33.81%). In 1% salt concentration, the best callus formation was found in cv. G5 (25.17%) followed by cvs. G2 (22.42%) and G3 (21.87%) which were at par and the minimum in G6 (9.13%). Similarly, in 1.5% salt concentration, the best callus formation was found in cv. G5 (13.29%) and the minimum in cv. G6 (2.50%) followed by cv. G4 (3.36%), which were at par. Overall, cv. G5 (34.02%) showed the best callus formation followed by cvs. G3 (28.66%), G2 (24.34%), G1 (23.4.9%), G4 (23.05%) and G6 (22.41%) respectively, which was graphically represented in fig-4.14.

Table 4.8 Callus formation (%) of cultured seeds of rice cultivars on different salt concentrations

Salt mixtures	Cultivars									
	G1	G2	G3	G4	G5	G6	Mean	CD	CV	SE(m)
0%	$68.51_{d}\pm1.85$	$70.85_{d}\pm2.13$	$85.37_{b}\pm2.04$	$80.69_{c}\pm1.75$	$96.48_{a}\pm1.75$	$89.07_{b}\pm0.19$	81.82	5.45	3.70	1.75
0.5%	$49.78_{b}\pm1.51$	$47.03_{b}\pm1.61$	$49.89_{b}\pm1.61$	$41.80_{b}\pm1.61$	$63.64_{a}\pm1.62$	$33.81_{c}\pm3.02$	47.65	5.94	6.93	1.90
1%	$16.99_{b}\pm1.08$	$22.42_{a}\pm1.03$	$21.87_{a}\pm0.82$	$12.47_{b}\pm1.66$	$25.17_{a}\pm4.06$	$9.13_{c}\pm0.86$	18.00	6.09	18.80	1.95
1.5%	$5.68_{c}\pm0.29$	$5.78_{c}\pm0.23$	$9.25_{b}\pm0.45$	$3.36_{d}\pm0.74$	$13.29_{a}\pm0.60$	$2.50_{d}\pm0.38$	6.64	1.51	12.64	0.48
2%	-	-	$5.59_{a}\pm0.33$	-	5.56_{a}	-	1.85	0.47	14.28	0.15
2.5%	-	-	-	-	-	-				
Mean	23.49	24.34	28.66	23.05	34.02	22.41				
CD	3.36	3.66	3.54	3.81	6.04	5.81				
CV	7.96	8.36	6.88	9.20	9.88	14.45				
SE(m)	1.08	1.17	1.13	1.22	1.94	1.86				

Values followed by the same letter in row are not significantly different using Duncan's Multiple Range Test (DMRT) at 5%

Fig 4.14 Effect of cultivars on callus formation of cultured seeds

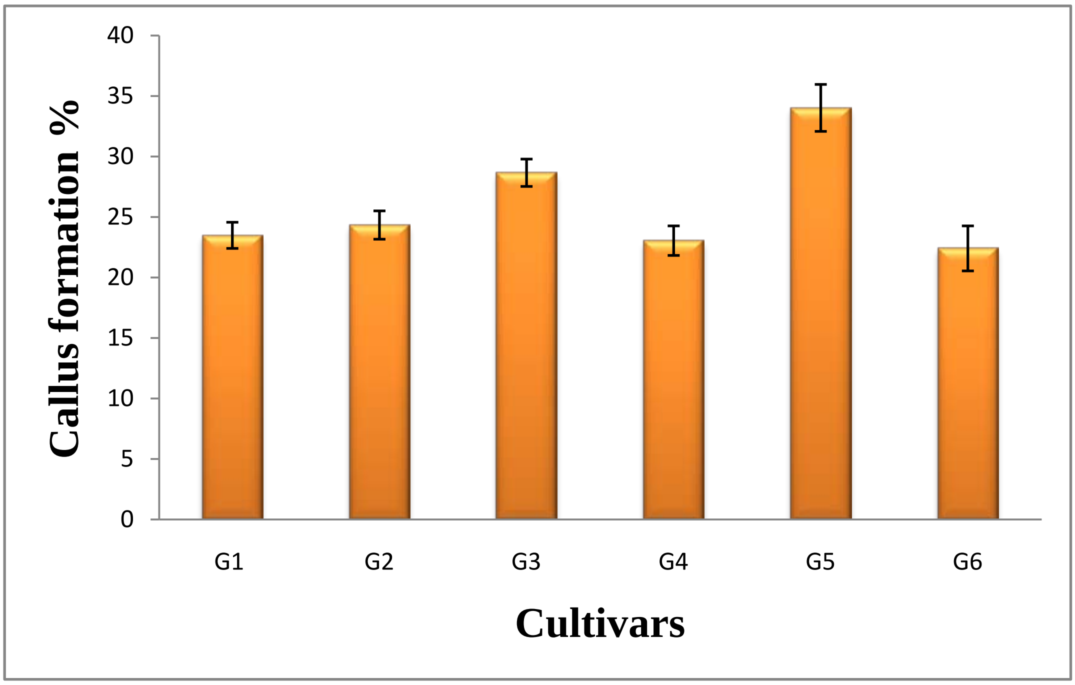
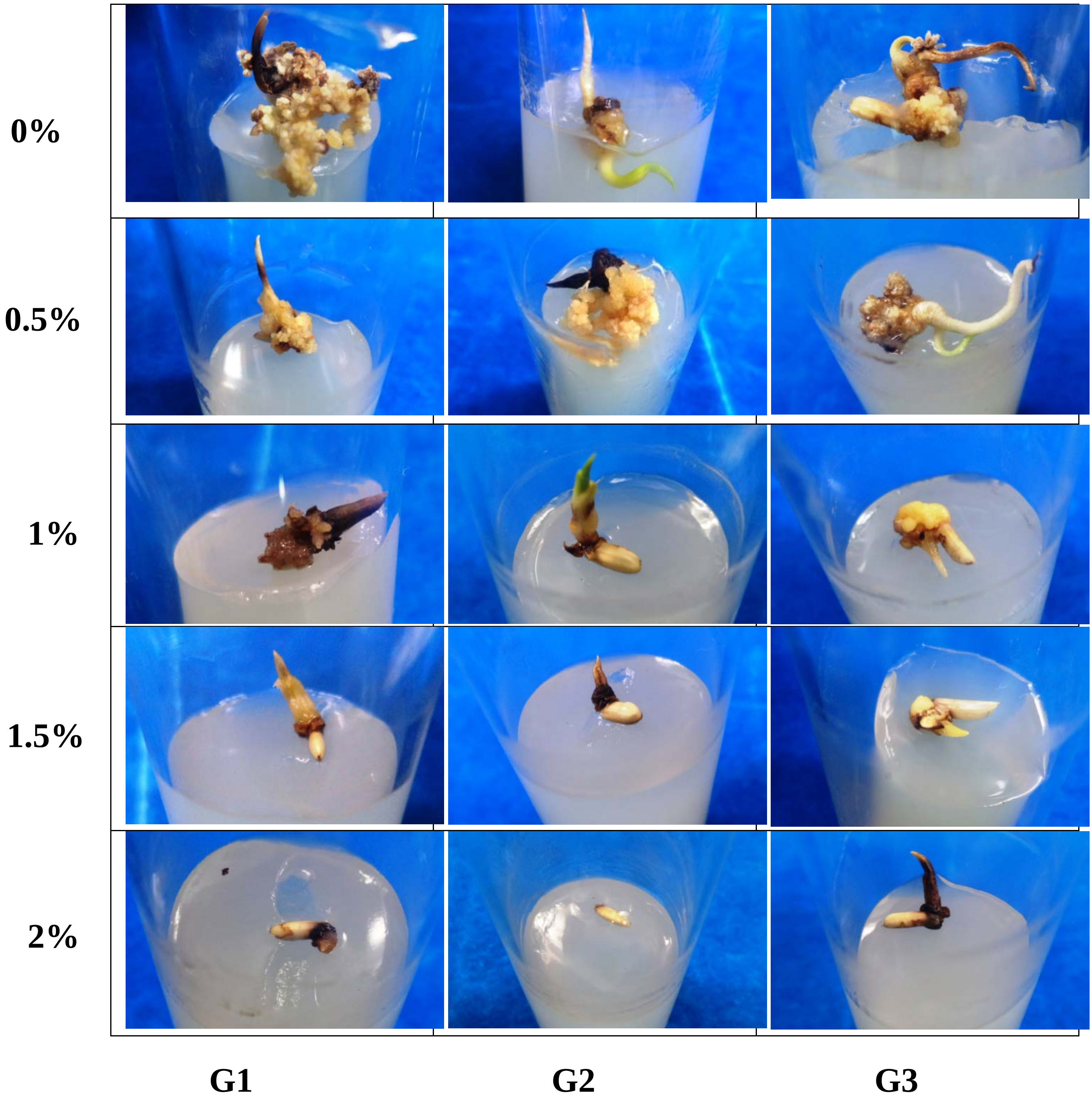
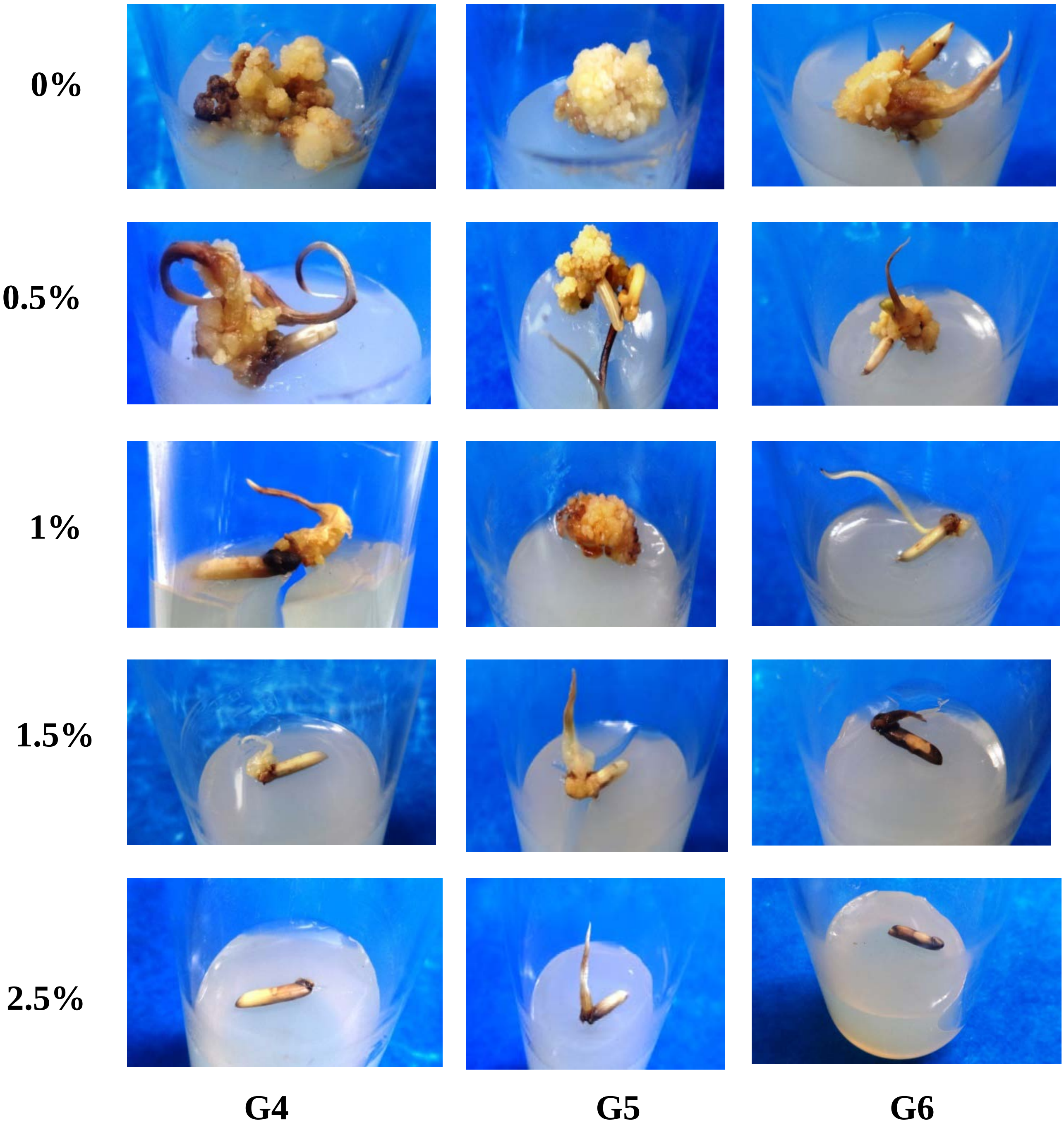


Plate-4.3a Callus formation from cultured seeds under different salt concentrations



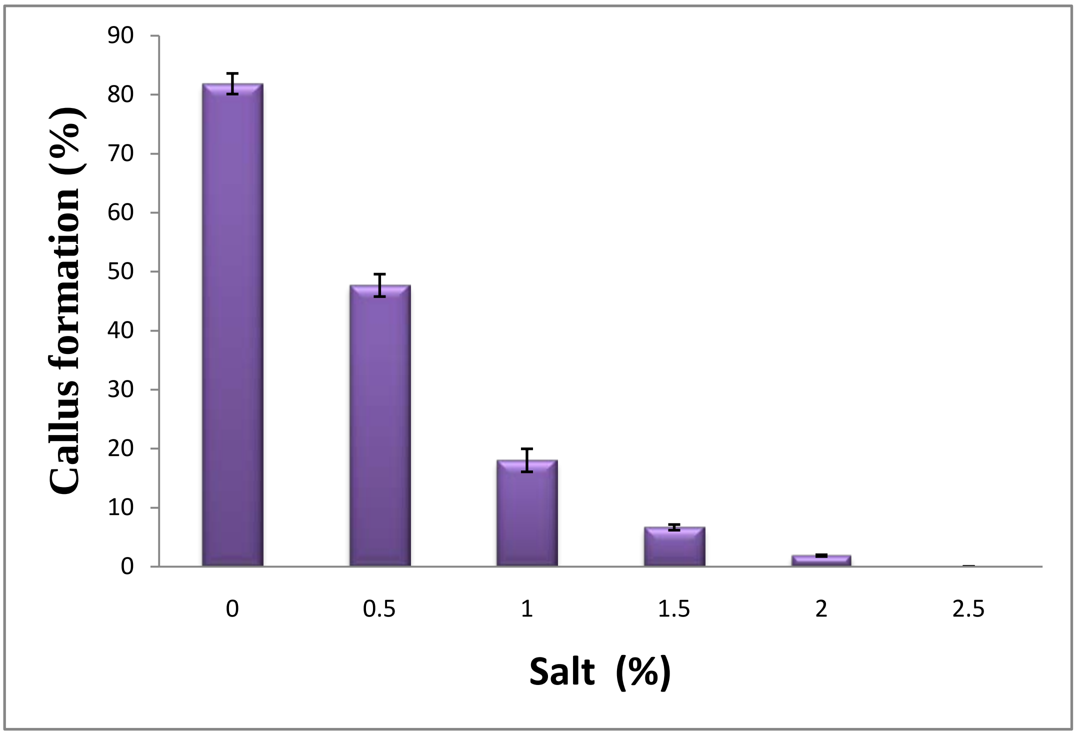
Photos of different types of callus formed by cultured seeds of three selected rice cultivars G1, G2 and G3 under different salt concentrations ranging from 0-2%

Plate-4.3b Callus formation from cultured seeds under different salt concentrations



Photos of different types of callus formed by cultured seeds of three selected rice cultivars G4, G5 and G6 under different salt concentrations ranging from 0-2.5%

Fig 4.15 Effect of salt on callus formation of cultured seeds



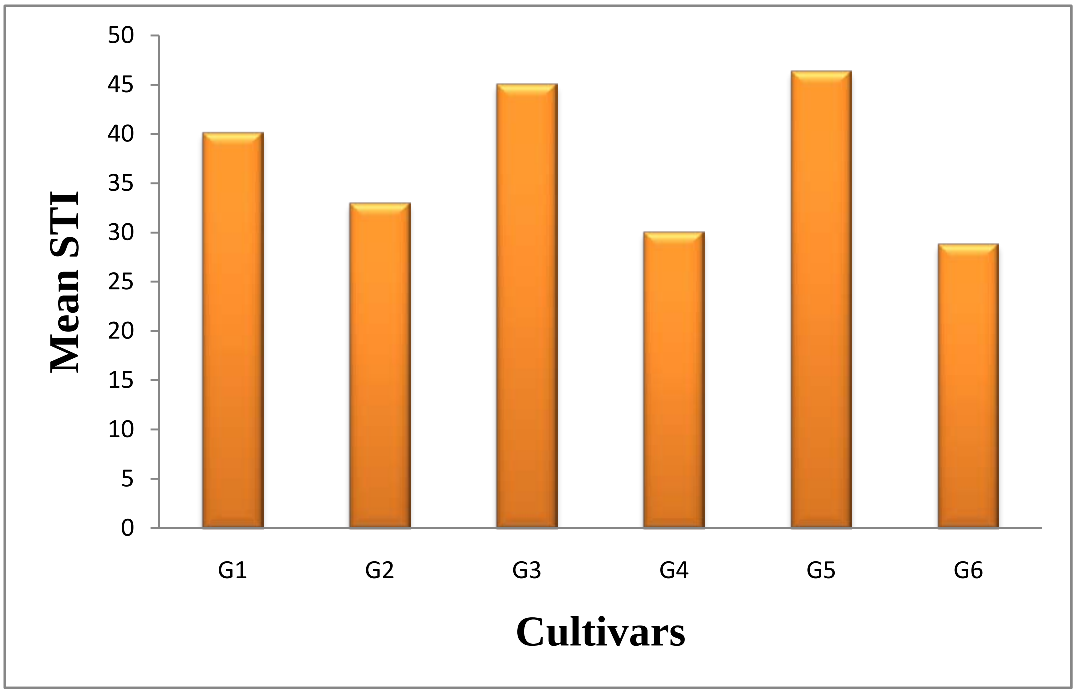
4.2.5 Callus growth under salt stress

To develop salt tolerance, calluses of all six selected cultivars were cultured on M1 medium with different salt concentrations ranging from 0-2.5%. The callus growth measured as increase in fresh and dry weights gradually decreased with increased salt concentrations which were represented in table-4.9 and graphically represented in fig-4.16. On the basis of increase in dry weight, salinity tolerance index was calculated. Cultivar G5 (46.33%) showed the highest mean STI followed by cvs. G3 (45.06%), G1 (40.10%), G2 (32.97%), G4 (30.03%) and G6 (28.80%) respectively.

Table-4.9 Callus growth of rice cultivars on medium MS + 2.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} KIN medium supplemented with different salt concentrations

Cultivar	**Salt concentrations (%)**	**Callus growth**			
		Final callus weight (mg)		Salinity tolerance index (STI)	
		Fresh wt	Dry wt	STI	**Mean STI**
G1	0	1296	129		40.10
	0.5	1012	101	78.29	
	1.0	576	60	46.48	
	1.5	356	46	35.65	
	2.0	-	-	-	
G2	0	1388	166		32.97
	0.5	1086	143	86.14	
	1.0	484	56	33.73	
	1.5	189	20	12.04	
	2.0	-	-		
G3	0	1610	162		45.06
	0.5	1308	140	86.42	
	1.0	808	99	61.11	
	1.5	305	30	22.84	
	2.0	165	16	9.87	
G4	0	1363	149		30.03
	0.5	1027	118	79.19	
	1.0	421	42	28.18	
	1.5	182	19	12.75	
	2.0	-	-	-	
G5	0	1731	225		46.33
	0.5	1513	181	80.44	
	1.0	1077	140	62.66	
	1.5	663	72	32.00	
	2.0	201	24	11.42	
G6	0	1187	118		28.80
	0.5	795	83	70.33	
	1.0	351	36	30.50	
	1.5	157	17	14.40	
	2.0	-	-	-	

Fig-4.16 Salinity tolerance index (STI) of rice cultivars based on medium MS + 2.0 mg l^{-1} 2,4-D +1.0 mg l^{-1} KIN supplemented with different salt concentrations



4.2.6 Regeneration of plant from callus

The calluses of all cultivars developed at all concentrations of salt ranging from 0-2.5% were subcultured on regenerating medium M4 (MS + 1.5 mg l^{-1} NAA + 1.5mg l^{-1} KIN). The calluses developed without salt stress only regenerated plants on the selected medium (plate-4.4). The calluses developed in presence of salt stress failed to differentiate plants.

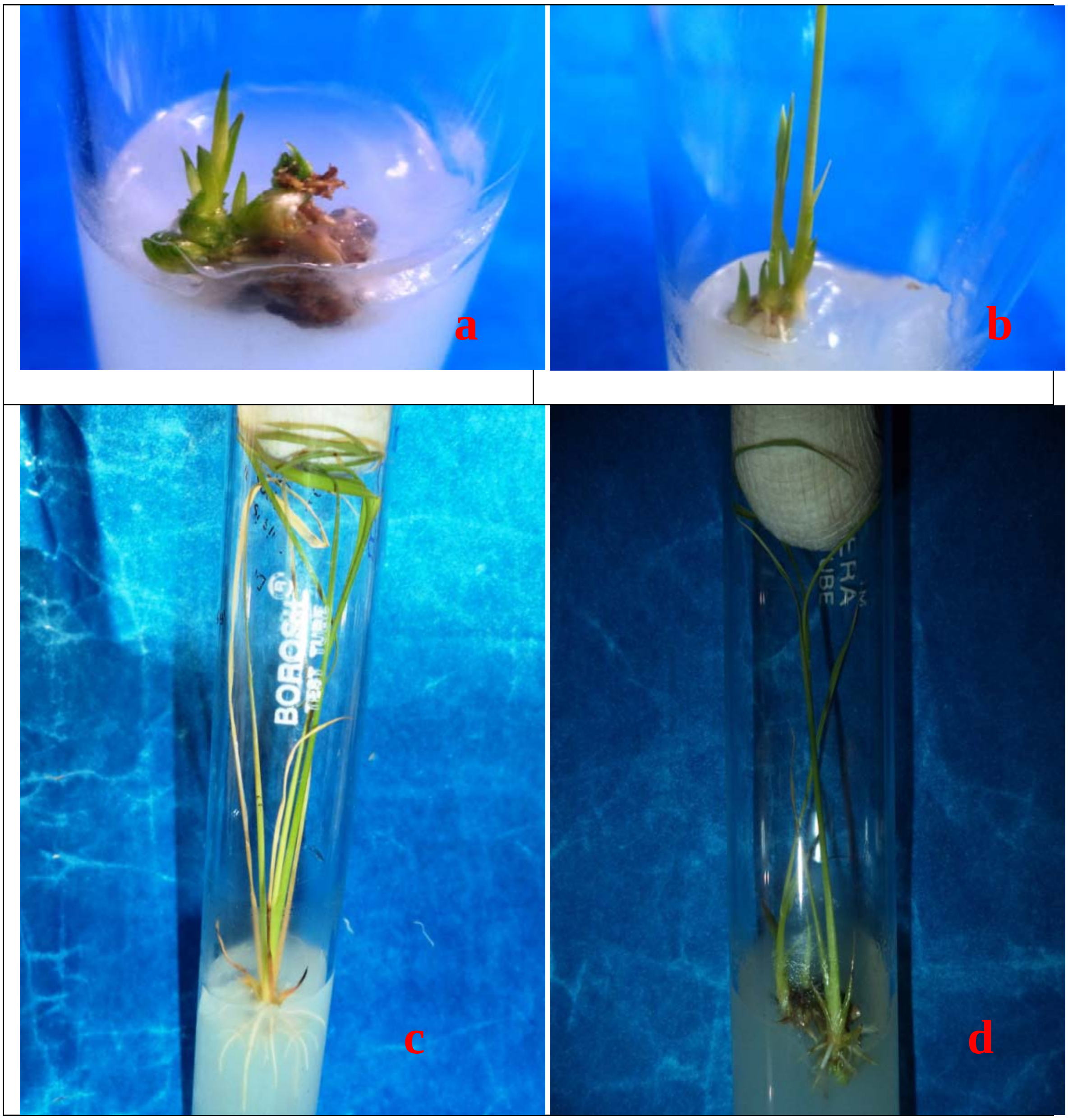
4.3 Molecular marker studies (SSR marker)

SSR is a PCR based molecular technique. In present investigation SSR markers were used for study of genetic variation of DNA isolated from leaf of rice (grown in pots) and also from normal callus as well as salt tolerant callus grown under *in vitro* condition (plate-3.2).

4.3.1 Molecular characterization of salinity tolerance

To characterize the selected cultivars of rice, the calluses grown without salt and salinity tolerant calluses grown in presence of salt were done using 14 SSR primers. These primers were linked to salt tolerance (table-3.9). The amplification of simple sequence repeat loci with different primers were described as follows:

Plate-4.4 Regeneration of plants from callus



Photos of subcultured callus formed from cultured seeds of rice on medium M4 (MS + 1.5 mg l^{-1} NAA + 1.5mg l^{-1} KIN) showing shoot differentiation in cvs. G2 and G3 (a and b), and shoot and root differentiation in cvs. G4 and G5 (c and d)

4.3.1.1 Amplification using primer pair RM 2

All the entries under characterization in the present study yielded amplified products due to primer pair RM 2 directed amplification of simple sequence repeat locus known to be located on Chromosome No. 7 in rice (plate-4.5). The overall size of the amplified products approximately ranged from 150.00-bp (.BPT-5204 (L), MTU-7029(L), Narendra Usar Dhan3(L), Rajendra Bhagwati (L), CSR-30 (L), CSR-30 (L) and BPT-5204 (CC)) to 172.00 bp (CSR-30 (CS)). Single band was visualized in the cases of all entries. Altogether six types of amplified products were generated including three unique bands and three shared bands. The position of the bands corresponding to the location of bands along the Y-axis varied from 616 in CSR-30 (CS) to 638 in (.BPT-5204 (L), MTU-7029(L), Narendra Usar Dhan3 (L), Rajendra Bhagwati (L), CSR-30 (L), CSR-30 (L) and BPT-5204 (CC)) (table 4.10). The Rf value obtained for different bands ranged from 0.684 for the amplified product of approximately 150.00 bp to 0.713for the amplified product of approximately 172.00 bp.

Table-4.10 Analysis of bands produced by primer pair RM2 based amplification of genomic DNA extracted from eighteen entries of rice

Lane	Band	Position	Mol.Weight (bp)	Rf
1	1	638	150.00	0.713
2	1	638	150.00	0.713
3	1	638	150.00	0.713
4	1	638	150.00	0.713
5	1	638	150.00	0.713
6	1	638	150.00	0.713
7	1	638	150.00	0.713
8	1	634	154.00	0.708
9	1	630	158.00	0.702
10	1	630	158.00	0.702
11	1	626	162.00	0.697
12	1	626	162.00	0.697
13	1	626	162.00	0.697
14	1	626	162.00	0.697
15	1	626	162.00	0.697
16	1	622	166.00	0.692
17	1	616	172.00	0.684
18	1	638	150.00	0.713

4.3.1.2. Amplification using primer pair RM 4

The primer pair RM 4 directed amplification of simple sequence repeats locus known to be located on chromosome No.11 in rice, was successfully achieved and amplified products were generated in all the entries under characterization in the present study (plate-4.6). The overall size of the amplified products approximately ranged from 161.36 bp (Pusa Basmati-1 (CC)) to 181.82 bp (Pusa Basmati-1 (CS)). A single band was visualized in all the cases. Altogether four types of amplified products were generated including two unique bands and two shared bands. The position of the bands corresponding to the location of bands along the Y-axis varied from 678 in Pusa Basmati-1 (CS) to 696 in Pusa Basmati-1 (CC) (table-4.11). The Rf value obtained for different bands ranged from 0.693 for the amplified product of approximately 181.82 bp to 0.717 for the amplified product of approximately 161.36 bp.

Table-4.11 Analysis of bands produced by primer pair RM4 based amplification of genomic DNA extracted from eighteen entries of rice

Lane	Band	Position	Mol. Weight (bp)	Rf
1	1	694	163.64	0.714
2	1	694	163.64	0.714
3	1	694	163.64	0.714
4	1	694	163.64	0.714
5	1	694	163.64	0.714
6	1	694	163.64	0.714
7	1	694	163.64	0.714
8	1	694	163.64	0.714
9	1	694	163.64	0.714
10	1	694	163.64	0.714
11	1	696	161.36	0.717
12	1	694	163.64	0.714
13	1	682	177.27	0.698
14	1	694	163.64	0.714
15	1	694	163.64	0.714
16	1	682	177.27	0.698
17	1	694	163.64	0.714
18	1	678	181.82	0.693

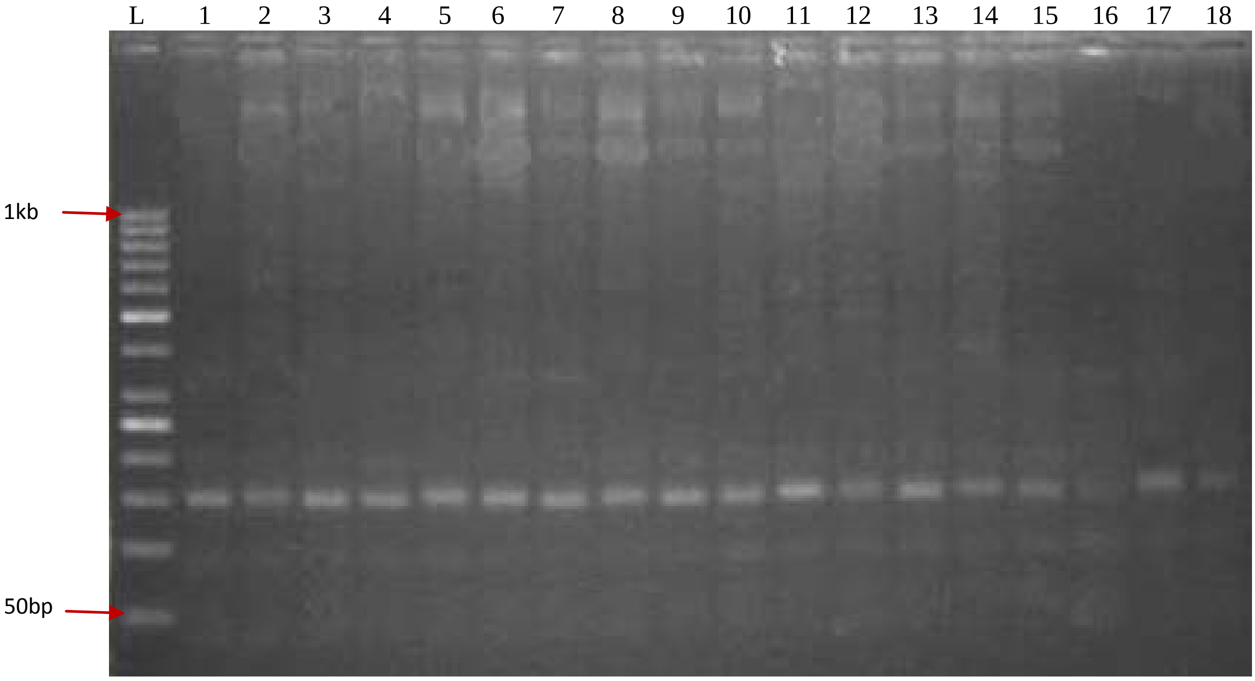


Plate-4.5 Amplification of region of genomic DNA extracted from the leaves, control callus and salt tolerant callus in rice cultivars by primer RM 2 used in the study

1.BPT-5204 (L)
2..MTU-7029(L)
3.N.Usar Dhan3(L)
4.R.Bhagwati (L)
5.CSR-30 (L)
6.P.Basmati-1 (L)
7.BPT-5204 (CC)
8..MTU-7029(CC)
9.N.Usar Dhan-3(CC)
10.R.Bhagwati (CC)
11.CSR-30 (CC)
12.P.Basmati-1 (CC)
13.BPT-5204 (CS)
14..MTU-7029(CS)
15.N.Usar Dhan3(CS)
16.R.Bhagwati(CS)
17.CSR-30 (CS)
18.P.Basmati-1(CS)

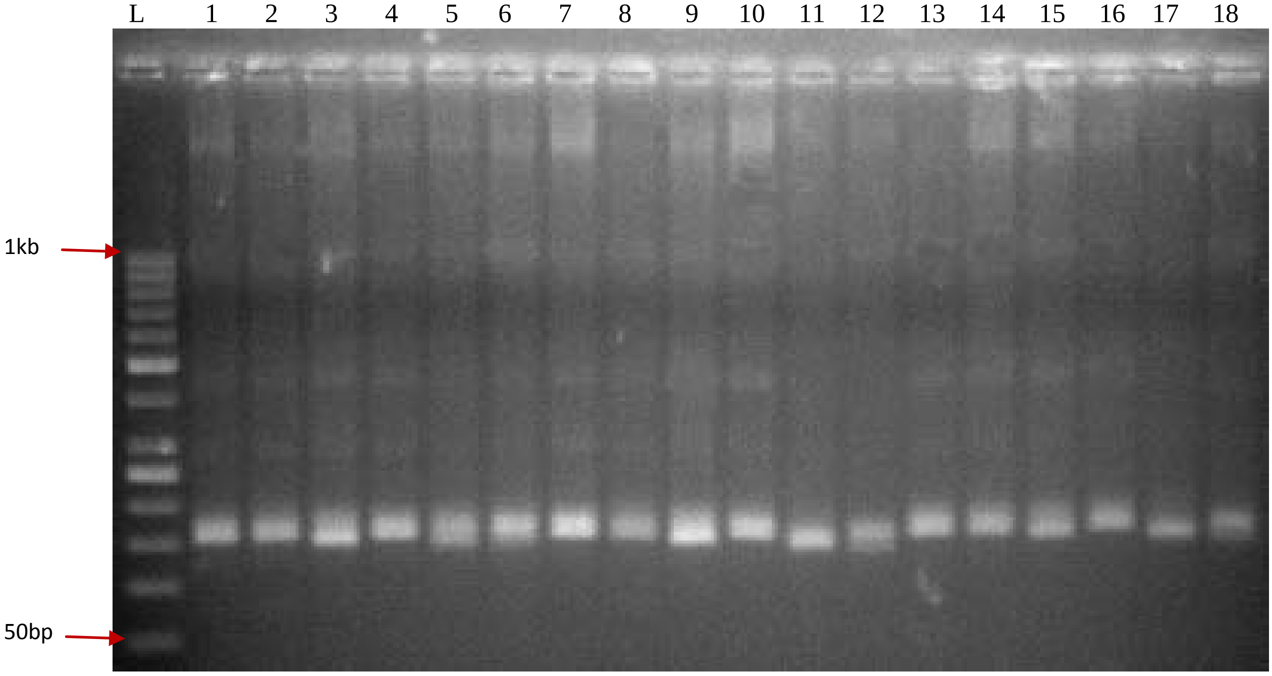


Plate-4.6 Amplification of region of genomic DNA extracted from the leaves, control callus and salt tolerant callus in rice cultivars by primer RM 4 used in the study

1.BPT-5204 (L)
2..MTU-7029(L)
3.N.Usar Dhan3(L)
4.R.Bhagwati (L)
5.CSR-30 (L)
6.P.Basmati-1 (L)
7.BPT-5204 (CC)
8..MTU-7029(CC)
9.N.Usar Dhan-3(CC)
10.R.Bhagwati (CC)
11.CSR-30 (CC)
12.P.Basmati-1 (CC)
13.BPT-5204 (CS)
14..MTU-7029(CS)
15.N.Usar Dhan-3(CS)
16.R.Bhagwati(CS)
17.CSR-30 (CS)
18.P.Basmati-1 (CS)

4.3.1.3. Amplification using primer pair RM 11

All (18) entries under characterization in the present study yielded amplified products due to primer pair RM 11 directed amplification of simple sequence repeat locus known to be located on Chromosome No.7 in rice (plate-4.7). The overall size of the amplified products approximately ranged from 143.48 bp in BPT-5204 (L), MTU-7029(L), Rajendra Bhagwati (L), CSR-30 (L) to 185.00 bp in Narendra Usar Dhan-3 (CS). A single band was visualized in the cases of 16 out of 18 entries. In the case of two entries, namely, CSR-30 (L) and Pusa Basmati-1 (L) two bands were observed. Altogether eight types of amplified products were generated including three unique bands and five shared bands. The position of the bands corresponding to the location of bands along the Y-axis varied from 608 in Narendra Usar Dhan-3 (CS) to 642 in BPT-5204 (L), MTU-7029(L), Rajendra Bhagwati (L), CSR-30 (L) (table 4.12). The Rf value obtained for different bands ranged from 0.709 for the amplified product of approximately 185.00 bp to 0.760 for the amplified product of approximately 143.48 bp.

Table-4.12 Analysis of bands produced by primer pair 11 based amplification of genomic DNA extracted from eighteen entries of rice

Lane	Band	Position	Mol. Weight (bp)	Rf
1	1	642	143.48	0.760
2	1	642	143.48	0.760
3	1	628	160.00	0.739
4	1	642	143.48	0.760
5	1	620	170.00	0.727
5	2	642	143.48	0.760
6	1	620	170.00	0.727
6	2	636	150.00	0.751
7	1	620	170.00	0.727
8	1	624	165.00	0.733
9	1	616	175.00	0.721
10	1	624	165.00	0.733
11	1	628	160.00	0.739
12	1	628	160.00	0.739
13	1	616	175.00	0.721
14	1	610	182.50	0.712
15	1	608	185.00	0.709
16	1	620	170.00	0.727
17	1	624	165.00	0.733
18	1	624	165.00	0.733

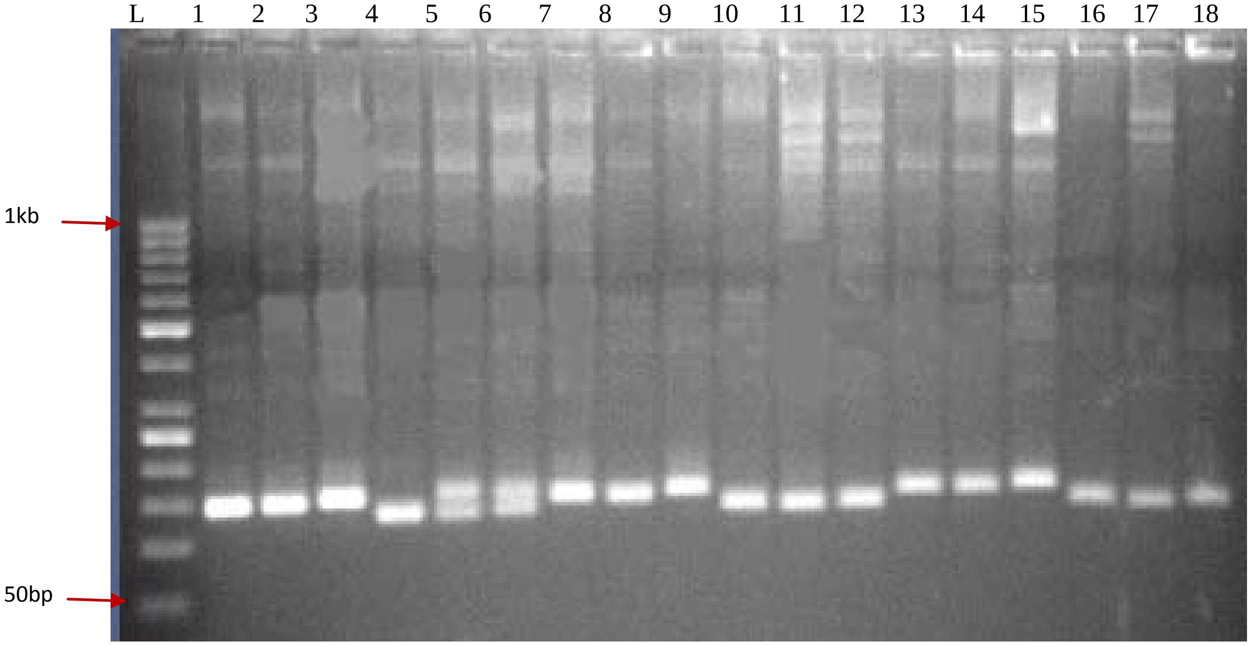


Plate-4.7 **Amplification of region of genomic DNA extracted from the leaves at seedling stage in rice by primer RM 11 used in the study**

1.BPT-5204 (L)
2..MTU-7029(L)
3.N.Usar dhan3(L)
4.R.Bhagwati (L)
5.CSR-30 (L)
6.P.Basmati (L)
7.BPT-5204 (CC)
8..MTU-7029(CC)
9.N.Usar.dhan-3(CC)
10.R.Bhagwati (CC)
11.CSR-30 (CC)
12.P.Basmati (CC)
13.BPT-5204 (CS)
14..MTU-7029(CS)
15.N.Usar.dhan3(CS)
16.R.Bhagwati(CS)
17.CSR-30 (CS)
18.P.Basmati (CS)

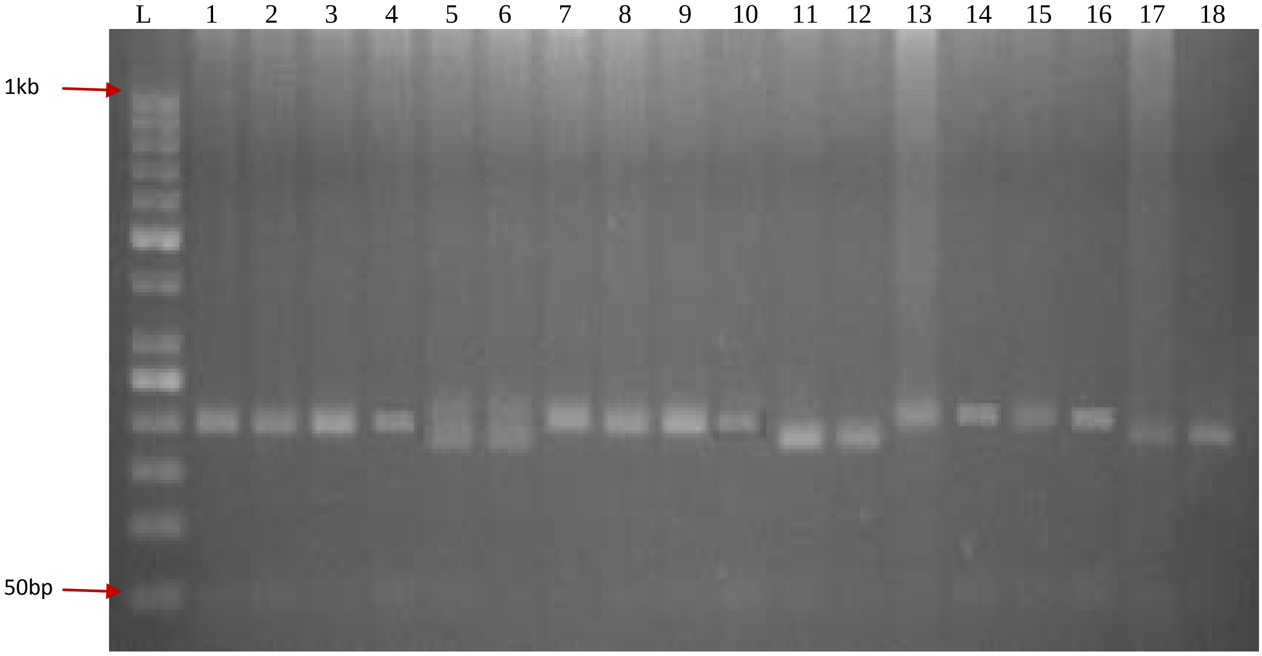


Plate-4.8 **Amplification of region of genomic DNA extracted from the leaves, control callus, salt tolerant callus in rice cultivars by primer RM 14 used in the study.**

1.BPT-5204 (L)
2..MTU-7029(L)
3.N.Usar Dhan3(L)
4.R.Bhagwati (L)
5.CSR-30 (L)
6.P.Basmati-1 (L)
7.BPT-5204 (CC)
8..MTU-7029(CC)
9.N.Usar Dhan-3(CC)
10.R.Bhagwati (CC)
11.CSR-30 (CC)
12.P.Basmati-1(CC)
13.BPT-5204 (CS)
14..MTU-7029(CS)
15.N.Usar Dhan3(CS)
16.R.Bhagwati(CS)
17.CSR-30 (CS)
18.P.Basmati-1(CS)

4.3.1.4 Amplification using primer pair RM 14

All the entries under characterization in the present study yielded amplified products due to primer pair RM 14 directed amplification of simple sequence repeat locus known to be located on Chromosome No.1 in rice (plate-4.8). The overall size of the amplified products approximately ranged from 184.78 bp in CSR-30 (CC), Pusa Basmati-1 (CC), Pusa Basmati-1 (CS) to 206.25 bp in CSR-30 (L), Pusa Basmati-1 (L). A single band was visualized in 16 entries, two bands appear in the case of CSR-30 (L) and Pusa Basmati-1. Altogether six types of amplified products were generated including three unique bands and three shared bands. The position of the bands corresponding to the location of bands along the Y-axis varied from 184.78 in CSR-30 (CC), Pusa Basmati-1 (CC), .Pusa Basmati-1 (CS) to 206.25 bp in CSR-30 (L), Pusa Basmati-1 (L) to 206.25 in CSR-30 (L), Pusa Basmati-1 (L) (table 4.13). The Rf value obtained for different bands ranged from 0.665 for the amplified product of approximately 206.25 bp to 0.689 for the amplified product of approximately 184.78 bp.

Table-4.13 Analysis of bands produced by primer pair 14 based amplification of genomic DNA extracted from eighteen entries of rice

Lane	Band	Position	Mol. Weight (bp)	Rf
1	1	626	197.83	0.673
2	1	626	197.83	0.673
3	1	626	197.83	0.673
4	1	626	197.83	0.673
5	1	620	206.25	0.665
5	2	636	186.96	0.687
6	1	620	206.25	0.665
6	2	638	184.78	0.689
7	1	626	197.83	0.673
8	1	626	197.83	0.673
9	1	626	197.83	0.673
10	1	626	197.83	0.673
11	1	638	184.78	0.689
12	1	638	184.78	0.689
13	1	622	203.13	0.668
14	1	622	203.13	0.668
15	1	622	203.13	0.668
16	1	622	203.13	0.668
17	1	634	189.13	0.684
18	1	638	184.78	0.689

4.3.1.5. Amplification using primer pair RM 20

Using primer pair RM 20, amplification was successfully achieved at simple sequence repeat locus known to be located on Chromosome No.11 in rice. All the entries under characterization in the present study yielded amplified products due to primer directed amplification of this locus (plate-4.9). The overall size of the amplified products approximately ranged from 205.94 bp in Narendra Usar Dhan-3(CC) and CSR-30 (CC) to 288.24 bp in CSR-30 (CS) and Pusa Basmati-1 (CS). Double bands were visualized in the 16 entries.While triple bands were appeared in case of CSR-30 (L) and Pusa Basmati-1(L). Altogether sixteen types of amplified products were generated including six unique bands and ten shared bands due to amplification of this locus. The position of the bands corresponding to the location of bands along the Y-axis varied from 582 in CSR-30 (CS) and Pusa Basmati-1 (CS) to 640 in Narendra Usar Dhan-3(CC) and CSR-30 (CC) (table-4.14). The Rf value obtained for different bands ranged from 0.614 for the amplified product of approximately 288.24 bp to 0.697 for the amplified product of approximately 205.56 bp.

Table-4.14 Analysis of bands produced by primer pair 20 based amplification of genomic DNA extracted from eighteen entries of rice

Lane	Band	Position	Mol. Weight (bp)	Rf
1	1	586	282.35	0.62
1	2	608	250.00	0.651
2	1	604	255.88	0.646
2	2	624	227.78	0.674
3	1	604	255.88	0.646
3	2	638	208.33	0.694
4	1	604	255.88	0.646
4	2	624	227.78	0.674
5	1	594	270.59	0.631
5	2	608	250.00	0.651
5	3	634	213.89	0.689
6	1	594	270.59	0.631
6	2	606	252.94	0.649
6	3	628	222.22	0.68
7	1	594	270.59	0.631
7	2	624	227.78	0.674
8	1	610	247.22	0.654
8	2	634	213.89	0.689
9	1	608	250.00	0.651
9	2	640	205.56	0.697
10	1	610	247.22	0.654
10	2	630	219.44	0.683
11	1	590	276.47	0.626
11	2	640	205.56	0.697
12	1	586	282.35	0.62
12	2	624	227.78	0.674
13	1	586	282.35	0.62
13	2	622	230.56	0.671
14	1	604	255.88	0.646
14	2	622	230.56	0.671
15	1	604	255.88	0.646
15	2	634	213.89	0.689
16	1	604	255.88	0.646
16	2	622	230.56	0.671
17	1	582	288.24	0.614
17	2	634	213.89	0.689
18	1	582	288.24	0.614
18	2	614	241.67	0.66

4.3.1.6. Amplification using primer pair RM 24

All the entries under characterization in the present study yielded amplified products due to primer pair RM 24 directed amplification of simple sequence repeat locus known to be located on Chromosome No.1 in rice (plate-4.10). The overall size of the amplified products approximately ranged from 152.94 bp in BPT-5204 (L), MTU-7029(L), Narendra Usar Dhan3 (L), Rajendra Bhagwati (L), CSR-30 (L), Pusa Basmati-1 (L) to 197.06 bp in Rajendra Bhagwati (CS), CSR-30 (CS), Pusa Basmati-1 (CS). A single band was visualized in all the cases of entries. Altogether 8 types of amplified products were generated including three unique bands and five shared bands. The position of the bands corresponding to the location of bands along the Y-axis varied from 606 in Rajendra Bhagwati(CS), CSR-30 (CS), Pusa Basmati-1 (CS) to 632 in BPT-5204 (L), MTU-7029(L), Narendra Usar Dhan3(L), Rajendra Bhagwati (L), CSR-30 (L), Pusa Basmati-1 (L) (table-4.15). The Rf value obtained for different bands ranged from 0.715 for the amplified product of approximately 197.06 bp to 0.755 for the amplified product of approximately 152.94 bp.

Table-4.15 Analysis of bands produced by primer pair 24 based amplification of genomic DNA extracted from eighteen entries of rice

Lane	Band	Position	Mol. Weight (bp)	Rf
1	1	632	152.94	0.755
2	1	632	152.94	0.755
3	1	632	152.94	0.755
4	1	632	152.94	0.755
5	1	632	152.94	0.755
6	1	632	152.94	0.755
7	1	616	176.47	0.734
8	1	614	179.41	0.731
9	1	616	176.47	0.734
10	1	612	182.35	0.728
11	1	614	179.41	0.731
12	1	610	185.29	0.726
13	1	608	188.24	0.723
14	1	608	188.24	0.723
15	1	606	191.18	0.72
16	1	602	197.06	0.715
17	1	602	197.06	0.715
18	1	602	197.06	0.715

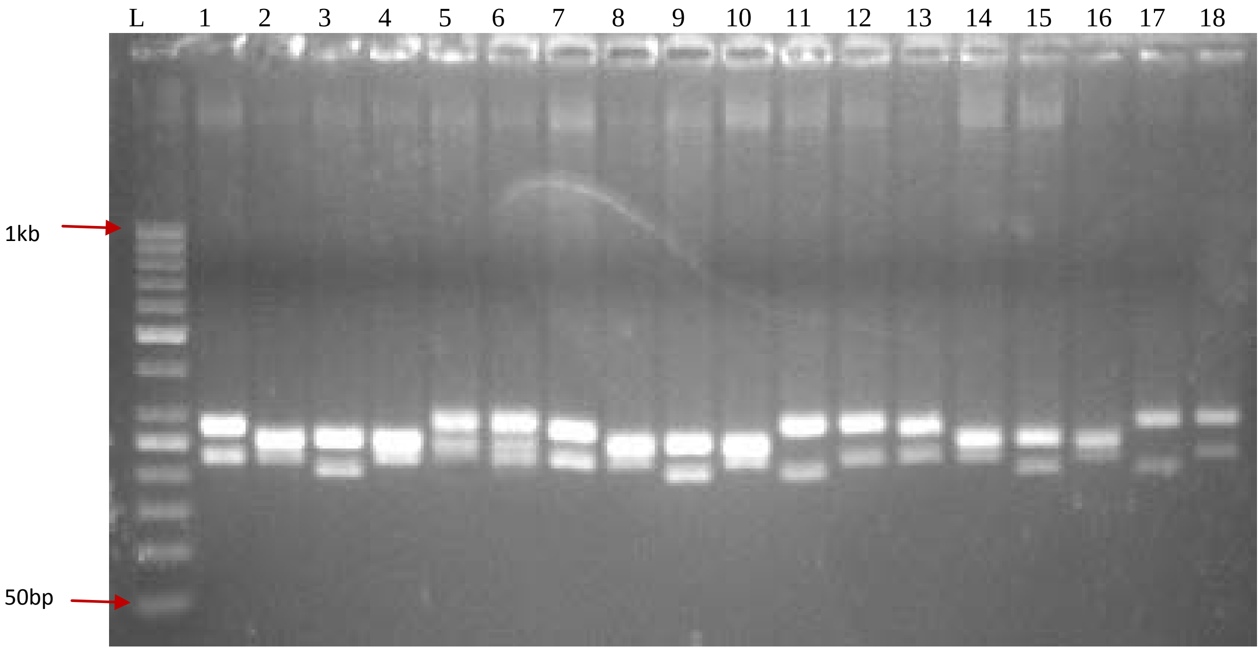


Plate-4.9 Amplification of region of genomic DNA extracted from the leaves, control callus and salt tolerant callus in rice cultivars by primer RM 20 used in the study

1.BPT-5204 (L)
2..MTU-7029(L)
3.N.Usar Dhan3(L)
4.R.Bhagwati (L)
5.CSR-30 (L)
6.P.Basmati -1(L)
7.BPT-5204 (CC)
8..MTU-7029(CC)
9.N.Usar Dhan-3(CC)
10.R.Bhagwati (CC)
11.CSR-30 (CC)
12.P.Basmati1 (CC)
13.BPT-5204 (CS)
14..MTU-7029(CS)
15.N.Usar Dhan3(CS)
16.R.Bhagwati(CS)
17.CSR-30 (CS)
18.P.Basmati-1(CS)

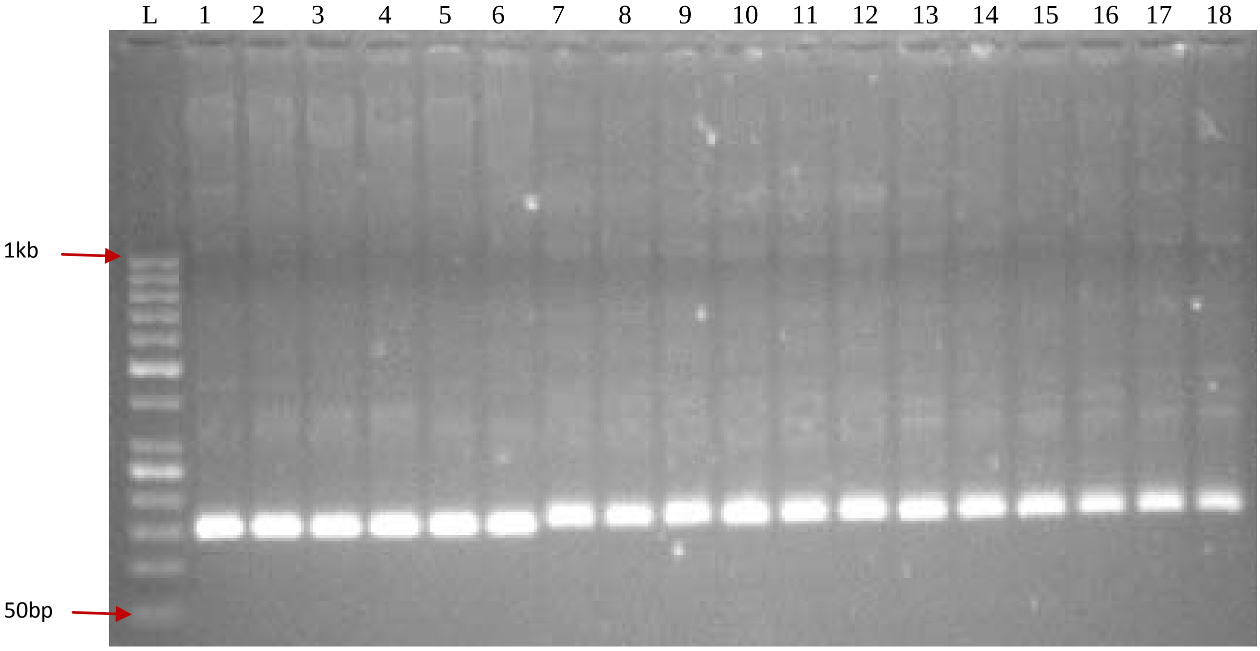


Plate-4.10 Amplification of region of genomic DNA extracted from the leaves, control callus and salt tolerant callus in rice cultivars by primer RM 24 used in the study

1.BPT-5204 (L)
2..MTU-7029(L)
3.N.Usar Dhan-3(L)
4.R.Bhagwati (L)
5.CSR-30 (L)
6.P.Basmati-1 (L)
7.BPT-5204 (CC)
8..MTU-7029(CC)
9.N.Usar Dhan-3(CC)
10.R.Bhagwati (CC)
11.CSR-30 (CC)
12.P.Basmati-1(CC)
13.BPT-5204 (CS)
14..MTU-7029(CS)
15.N.Usar Dhan-3(CS)
16.R.Bhagwati(CS)
17.CSR-30 (CS)
18.P.Basmati-1(CS)

4.3.1.7 Amplification using primer pair RM 204

All (18) the entries under characterization in the present study yielded amplified products due to primer pair RM 204 directed amplification of simple sequence repeat locus known to be located on Chromosome No.6 in rice (plate-4.11). The overall size of the amplified products approximately ranged from 126.00 bp in BPT-5204 (L), MTU-7029 (L), Rajendra Bhagwati (L), Narendra Usar Dhan-3(CC) MTU-7029(CS) to 660 in BPT-5204 (L), MTU-7029(L), Rajendra Bhagwati (L), Narendra Usar Dhan-3 (CC) and Narendra Usar Dhan3 (CS) and Narendra Usar Dhan3 (CS) to 182.64 bp in MTU-7029 (CS). A single band was visualized in all the cases. Altogether eight types of amplified products were generated including four unique bands and four shared bands. The position of the bands corresponding to the location of bands along the Y-axis varied from 606 in (table-4.16). The Rf value obtained for different bands ranged from 0.676 for the amplified product of approximately 182.61 bp to 0.744 for the amplified product of approximately 126.00 bp.

Table-4.16 Analysis of bands produced by primer pair 204 based amplification of genomic DNA extracted from eighteen entries of rice

Lane	Band	Position	Mol. Weight (bp)	Rf
1	1	660	126.00	0.744
2	1	660	126.00	0.744
3	1	608	180.43	0.678
4	1	660	126.00	0.744
5	1	638	148.00	0.716
6	1	638	148.00	0.716
7	1	650	136.00	0.732
8	1	610	178.26	0.681
9	1	660	126.00	0.744
10	1	640	146.00	0.719
11	1	640	146.00	0.719
12	1	650	136.00	0.732
13	1	656	130.00	0.739
14	1	606	182.61	0.676
15	1	660	126.00	0.744
16	1	638	148.00	0.716
17	1	640	146.00	0.719
18	1	640	146.00	0.719

4.3.1.8. Amplification using primer pair RM 223

The primer pair RM 223 directed amplification of simple sequence repeat locus known to be located on Chromosome No.8 in rice was achieved in the cases of all the entries (plate-4.12). The size of the amplified products approximately ranged from 143.10 bp in Rajendra Bhagwati (L), CSR-30 (CS), Rajendra Bhagwati (CS), CSR-30 (CS) and Pusa Basmati-1 (CS) to 156.52 bp in BPT-5204 (L), MTU-7029 (L), Pusa Basmati-1 (L) and BPT-5204 (CS). A single band was visualized in all the cases. Altogether four types of amplified products were generated including one unique band and three shared bands. The position of the bands corresponding to the location of bands along the Y-axis varied from 554 in BPT-5204 (L), MTU-7029(L), Pusa Basmati-1 (L) and BPT-5204 (CS) to 568 in Rajendra Bhagwati (L), CSR-30 (CS), Rajendra Bhagwati (CS), CSR-30 (CS) and Pusa Basmati-1 (CS) (table-4.17). The Rf value obtained for different bands ranged from 0.644 for the amplified product of approximately 156.52 bp to 0.663 for the amplified product of approximately 143.10 bp.

Table-4.17 Analysis of bands produced by primer pair 223 based amplification of genomic DNA extracted from eighteen entries of rice

Lane	Band	Position	Mol. Weight (bp)	Rf
1	1	554	156.52	0.644
2	1	554	156.52	0.644
3	1	558	152.17	0.649
4	1	568	143.10	0.663
5	1	558	152.17	0.649
6	1	554	156.52	0.644
7	1	558	152.17	0.649
8	1	558	152.17	0.649
9	1	558	152.17	0.649
10	1	558	152.17	0.649
11	1	562	148.28	0.655
12	1	558	152.17	0.649
13	1	554	156.52	0.644
14	1	558	152.17	0.649
15	1	558	152.17	0.649
16	1	568	143.10	0.663
17	1	568	143.10	0.663
18	1	568	143.10	0.663

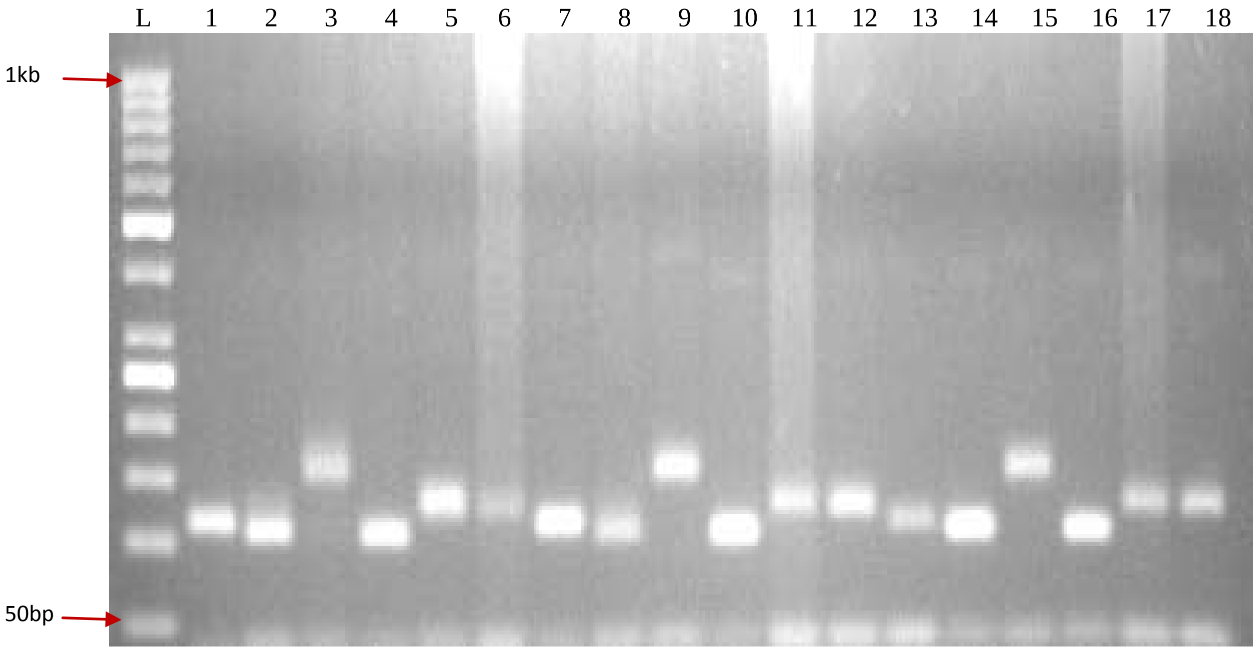


Plate-4.11 Amplification of region of genomic DNA extracted from the leaves at seedling stage in rice by primer RM 204 used in the study

1.BPT-5204 (L)
2..MTU-7029(L)
3.N.Usar dhan-3(L)
4.R.Bhagwati (L)
5.CSR-30 (L)
6.P.Basmati (L)
7.BPT-5204 (CC)
8..MTU-7029(CC)
9.N.Usar.dhan-3(CC)
10.R.Bhagwati (CC)
11.CSR-30 (CC)
12.P.Basmati (CC)
13.BPT-5204 (CS)
14..MTU-7029(CS)
15.N.Usar.dhan-3(CS)
16.R.Bhagwati(CS)
17.CSR-30 (CS)
18.P.Basmati (CS)

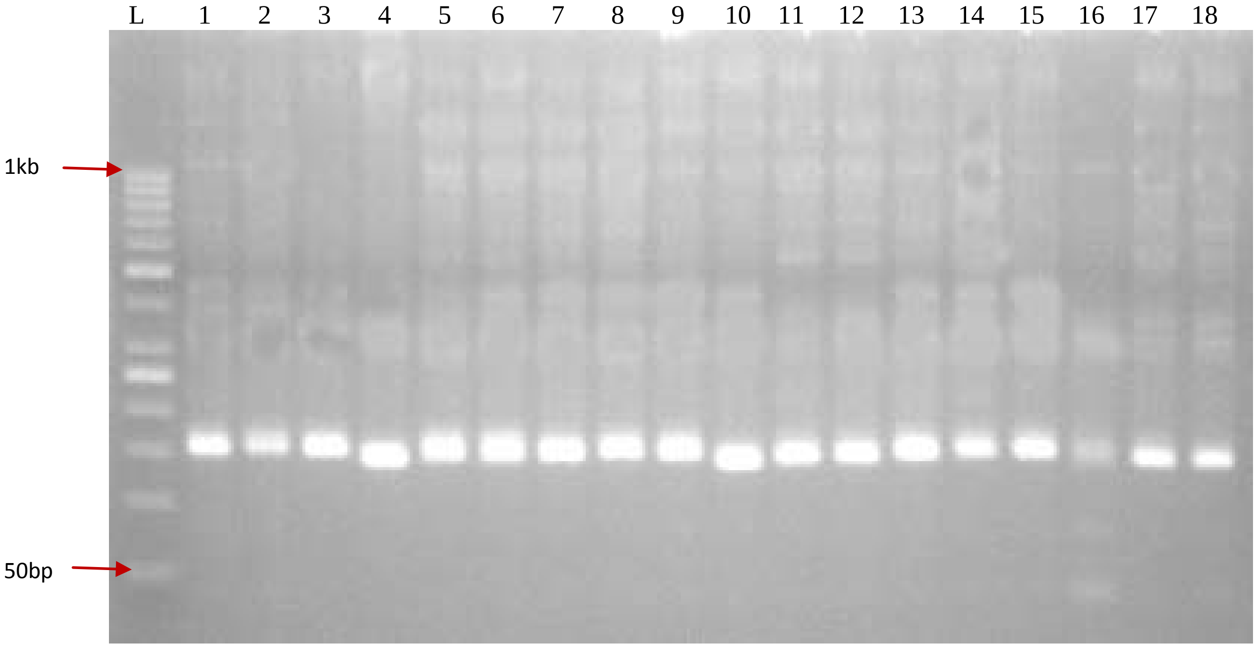


Plate-4.12 Amplification of region of genomic DNA extracted from the leaves, control callus and salt tolerant callus in rice by primer RM 223 used in the study.

1.BPT-5204 (L)
2..MTU-7029(L)
3.N.UsarDhan3(L)
4.R.Bhagwati (L)
5.CSR-30 (L)
6.P.Basmati -1(L)
7.BPT-5204 (CC)
8..MTU-7029(CC)
9.N.Usar Dhan-3(CC)
10.R.Bhagwati (CC)
11.CSR-30 (CC)
12.P.Basmati-1 (CC)
13.BPT-5204 (CS)
14..MTU-7029(CS)
15.N.Usar Dhan3(CS)
16.R.Bhagwati(CS)
17.CSR-30 (CS)
18.P.Basmati-1(CS)

4.3.1.9. Amplification using primer pair RM 242

All the entries under characterization in the present study yielded amplified products due to primer pair RM 242 directed amplification of simple sequence repeat locus known to be located on Chromosome No. 9 in rice (plate-4.13). The overall size of the amplified products approximately ranged from 227.78 bp in Pusa Basmati (L) to 281.25 bp in Narendra Usar Dhan3 (CS) and Rajendra Bhagwati (CS). A single band was visualized in the 16 cases. Two bands aperar in case of CSR-30 (L) and Pusa Basmati (L). Altogether nine types of amplified products were generated including three unique bands and six shared bands. The position of the bands corresponding to the location of bands along the Y-axis varied from 582 in Narendra Usar Dhan3 (CS) and Rajendra Bhagwati (CS) to 618 in Pusa Basmati (L) (table-4.18). The Rf value obtained for different bands ranged from 0.656 for the amplified product of approximately 281.25 bp to 0.705 for the amplified product of approximately 227.78 bp.

Table-4.18 Analysis of bands produced by primer pair 242 based amplification of genomic DNA extracted from eighteen entries of rice

Lane	Band	Position	Mol. Weight (bp)	Rf
1	1	614	233.33	0.699
2	1	614	233.33	0.699
3	1	604	247.22	0.686
4	1	604	247.22	0.686
5	1	590	268.75	0.667
5	1	614	233.33	0.699
6	1	590	268.75	0.667
6	2	618	227.78	0.705
7	1	596	259.38	0.675
8	1	604	247.22	0.686
9	1	590	268.75	0.667
10	1	590	268.75	0.667
11	1	610	238.89	0.694
12	1	610	238.89	0.694
13	1	596	259.38	0.675
14	1	592	265.63	0.669
15	1	582	281.25	0.656
16	1	582	281.25	0.656
17	1	604	247.22	0.686
18	1	608	241.67	0.691

4.3.1.10. Amplification using primer pair RM 253

All the entries under characterization in the present study yielded amplified products due to primer pair RM 253 directed amplification of simple sequence repeat locus known to be located on Chromosome No. 6 in rice (plate-4.14). The overall size of the amplified products approximately ranged from 116.07 bp MTU-7029 (L) and MTU-7029 (CC) to 152.27 in CSR-30 (CS). A single band was visualized in the 15 cases. Two bands appeared in case of MTU-7029 (L), MTU-7029 (CC) and MTU-7029 (CS). Altogether seven types of amplified products were generated including three unique bands and four shared bands. The position of the bands corresponding to the location of bands along the Y-axis varied from 538 in CSR-30 (CS) to 552 in MTU-7029 (L) and MTU-7029 (CC) (table-4.19). The Rf value obtained for different bands ranged from 0.663 for the amplified product of approximately 152.27 bp to 0.683 for the amplified product of approximately 116.07 bp.

Table-4.19 Analysis of bands produced by primer pair 253 based amplification of genomic DNA extracted from eighteen entries of rice

Lane	Band	Position	Mol.Weight (bp)	Rf
1	1	552	139.29	0.683
2	1	552	139.29	0.683
2	2	578	116.07	0.72
3	1	552	139.29	0.683
4	1	556	135.71	0.689
5	1	556	135.71	0.689
6	1	556	135.71	0.689
7	1	556	135.71	0.689
8	1	556	135.71	0.689
8	2	578	116.07	0.72
9	1	556	135.71	0.689
10	1	556	135.71	0.689
11	1	548	142.86	0.677
12	1	548	142.86	0.677
13	1	548	142.86	0.677
14	1	548	142.86	0.677
14	2	572	121.43	0.711
15	1	548	142.86	0.677
16	1	548	142.86	0.677
17	1	538	152.27	0.663
18	1	542	148.21	0.669

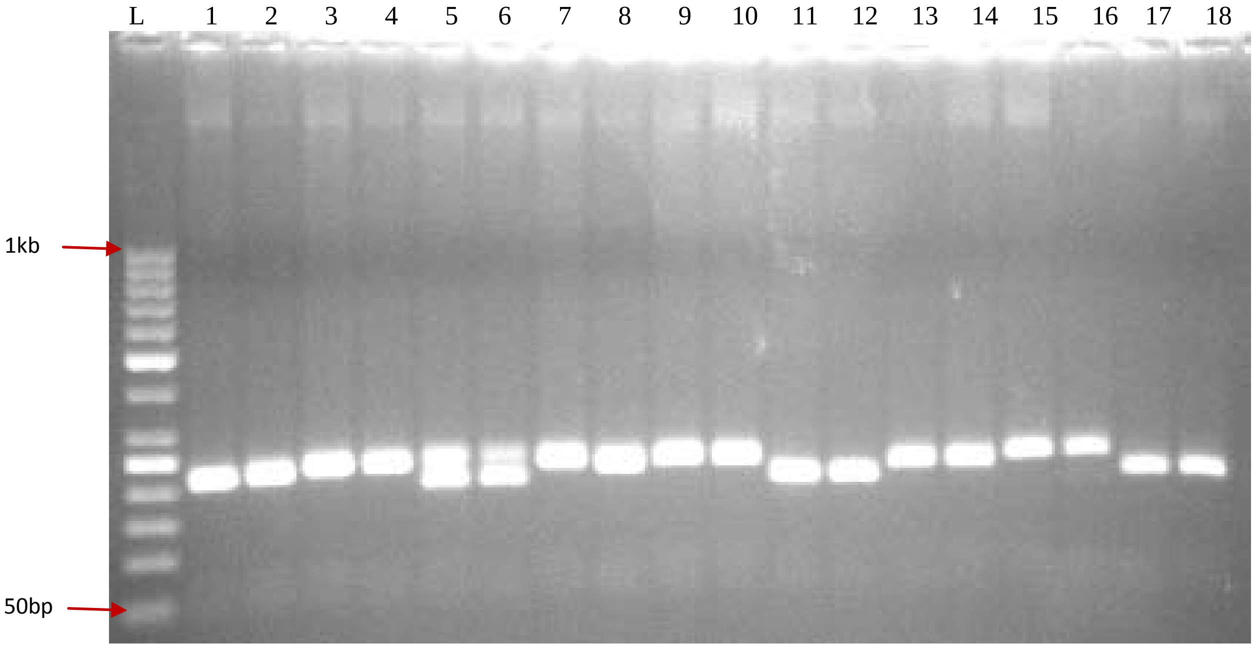


Plate-4.13 Amplification of region of genomic DNA extracted from the leaves, control callus and salt tolerant callus in rice cultivars by primer RM 242 used in the study

1.BPT-5204 (L)
2..MTU-7029(L)
3.N.UsarDhan3(L)
4.R.Bhagwati (L)
5.CSR-30 (L)
6.P.Basmati -1(L)
7.BPT-5204 (CC)
8..MTU-7029(CC)
9.N.Usar Dhan-3(CC)
10.R.Bhagwati (CC)
11.CSR-30 (CC)
12.P.Basmati-1 (CC)
13.BPT-5204 (CS)
14..MTU-7029(CS)
15.N.Usar Dhan3(CS)
16.R.Bhagwati(CS)
17.CSR-30 (CS)
18.P.Basmati-1CS)

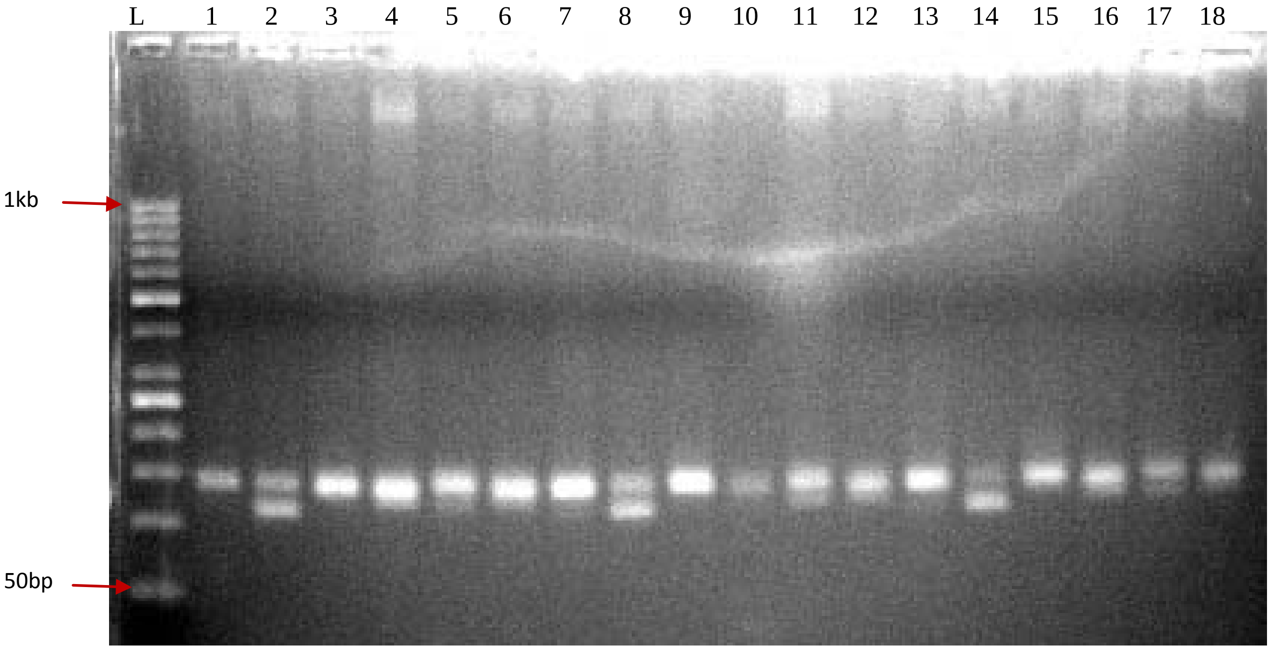


Plate-4.14 Amplification of region of genomic DNA extracted from the leaves at seedling stage in rice by primer RM 253 used in the study

1.BPT-5204 (L)
2..MTU-7029(L)
3.N.Usar dhan3(L)
4.R.Bhagwati (L)
5.CSR-30 (L)
6.P.Basmati (L)
7.BPT-5204 (CC)
8..MTU-7029(CC)
9.N.Usar.dhan-3(CC)
10.R.Bhagwati (CC)
11.CSR-30 (CC)
12.P.Basmati (CC)
13.BPT-5204 (CS)
14..MTU-7029(CS)
15.N.Usar.dhan3(CS)
16.R.Bhagwati(CS)
17.CSR-30 (CS)
18.P.Basmati (CS)

4.3.1.11. Amplification using primer pair RM 292

The primer pair RM 292 directed amplification of simple sequence repeat locus known to be located on Chromosome No.1 in rice was achieved in all the entries under characterization in the present study (plate-4.15). The overall size of the amplified products approximately ranged from 156.52 bp in CSR-30 (L) to 167.39 bp in Rajendra Bhagwati (CS), CSR-30 (CS) and Pusa Basmati-1 (CS). A single band was visualized in the cases of all the entries. Altogether four types of amplified products were generated including one unique band and three shared bands. The position of the bands corresponding to the location of bands along the Y-axis varied from 646 in Rajendra Bhagwati (CS), CSR-30 (CS) and Pusa Basmati-1 (CS) to 656 in CSR-30 (L) (table 4.20). The Rf value obtained for different bands ranged from 0.656 for the amplified product of approximately 167.39 bp to 0.668 for the amplified product of approximately 156.52 bp.

Table-4.20 Analysis of bands produced by primer pair 292 based amplification of genomic DNA extracted from eighteen entries of rice

Lane	Band	Position	Mol. Weight (bp)	Rf
1	1	652	160.87	0.663
2	1	652	160.87	0.663
3	1	652	160.87	0.663
4	1	652	160.87	0.663
5	1	656	156.52	0.668
6	1	652	160.87	0.663
7	1	652	160.87	0.663
8	1	652	160.87	0.663
9	1	652	160.87	0.663
10	1	650	163.04	0.661
11	1	652	160.87	0.663
12	1	652	160.87	0.663
13	1	652	160.87	0.663
14	1	652	160.87	0.663
15	1	650	163.04	0.661
16	1	646	167.39	0.656
17	1	646	167.39	0.656
18	1	646	167.39	0.656

4.3.1.12. Amplification using primer pair RM 302

All the entries under characterization in the present study yielded amplified products due to primer pair RM 302 directed amplification of simple sequence repeat locus known to be located on Chromosome No.1 in rice (plate-4.16). The overall size of the amplified products approximately ranged from 120.37 bp in Pusa Basmati-1 (L), BPT-5204 (CC), MTU-7029 (CC), Narendra Usar Dhan-3 (CC) Rajendra Bhagwati (CC) and BPT-5204 (CS) to 207.89 bp in MTU-7029 (L). A single band was visualized in the 14 entries. Two bends were appeared in case of MTU-7029(L), Narendra Usar Dhan3 (L), Narendra Usar Dhan-3 (CC) and Narendra Usar Dhan3 (CS). Altogether six types of amplified products were generated including two unique bands and four shared bands. The position of the bands corresponding to the location of bands along the Y-axis varied from 490 in MTU-7029 (L) to 572 in Pusa Basmati-1 (L), BPT-5204 (CC), MTU-7029 (CC), Narendra Usar Dhan-3 (CC), Rajendra Bhagwati (CC) and BPT-5204 (CS) (table-4.21). The Rf value obtained for different bands ranged from 0.616 for the amplified product of approximately 207.89 bp to 0.734 for the amplified product of approximately 120.37 bp.

Table-4.21 Analysis of bands produced by primer pair 302 based amplification of genomic DNA extracted from eighteen entries of rice

Lane	Band	Position	Mol. Weight (bp)	Rf
1	1	568	124.07	0.728
2	1	490	207.89	0.616
2	2	564	127.78	0.723
3	1	526	165.91	0.668
3	2	568	124.07	0.728
4	1	568	124.07	0.728
5	1	568	124.07	0.728
6	1	572	120.37	0.734
7	1	572	120.37	0.734
8	1	572	120.37	0.734
9	1	526	165.91	0.668
9	2	572	120.37	0.734
10	1	572	120.37	0.734
11	1	568	124.07	0.728
12	1	568	124.07	0.728
13	1	572	120.37	0.734
14	1	568	124.07	0.728
15	1	524	168.18	0.665
15	2	568	124.07	0.728
16	1	564	127.78	0.723
17	1	564	127.78	0.723
18	1	564	127.78	0.723

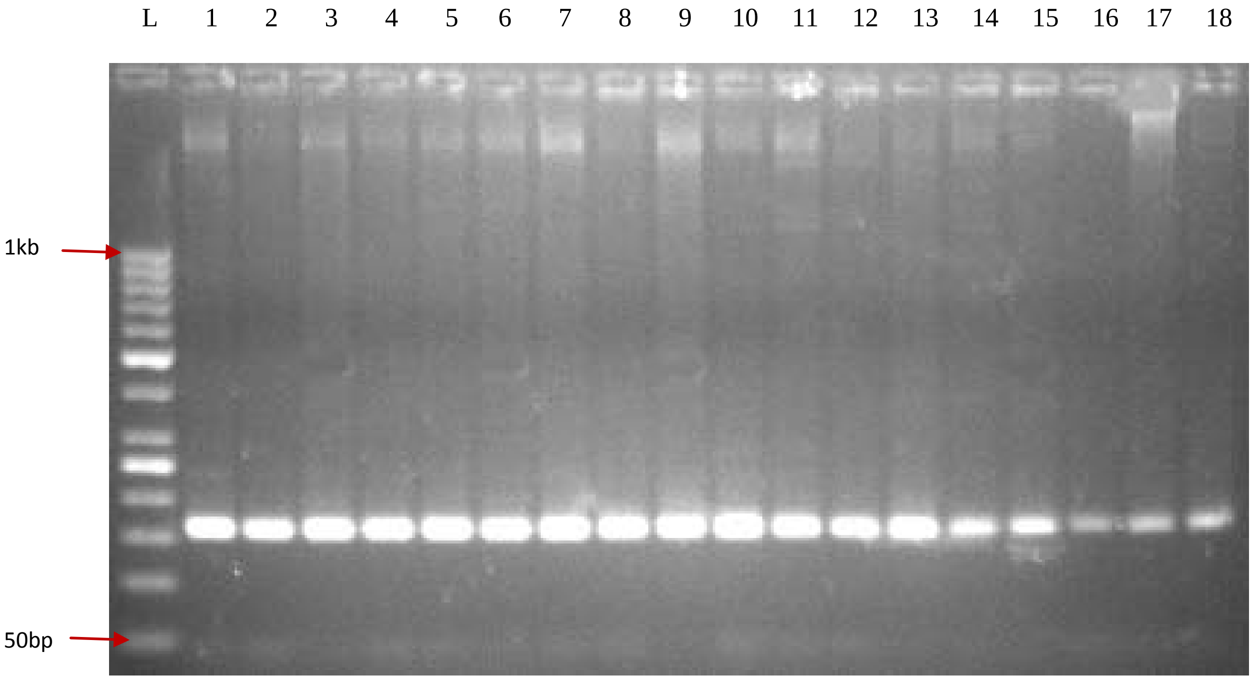


Plate-4.15 Amplification of region of genomic DNA extracted from the leaves at seedling stage in rice by primer RM 292 used in the study

1.BPT-5204 (L)
2..MTU-7029(L)
3.N.Usar dhan3(L)
4.R.Bhagwati (L)
5.CSR-30 (L)
6.P.Basmati (L)
7.BPT-5204 (CC)
8..MTU-7029(CC)
9.N.Usar.dhan-3(CC)
10.R.Bhagwati (CC)
11.CSR-30 (CC)
12.P.Basmati (CC)
13.BPT-5204 (CS)
14..MTU-7029(CS)
15.N.Usar.dhan3(CS)
16.R.Bhagwati(CS)
17.CSR-30 (CS)
18.P.Basmati (CS)

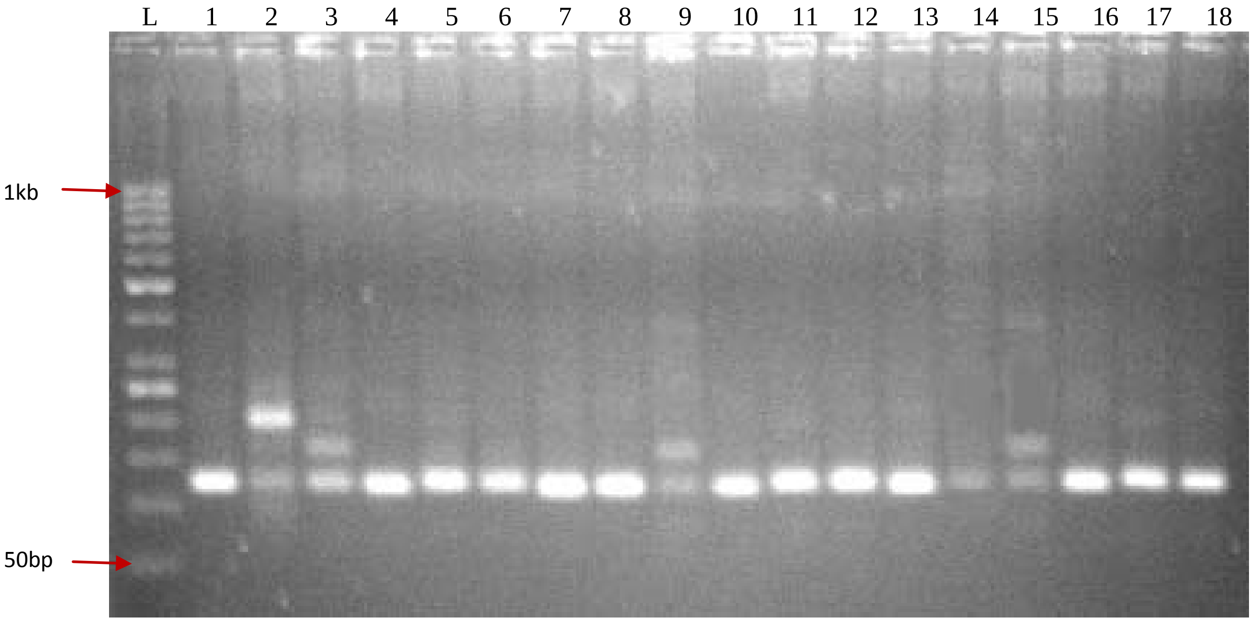


Plate-4.16 Amplification of region of genomic DNA extracted from the leaves, control callus and salt tolerant callus in rice by primer RM 302 used in the study

1.BPT-5204 (L)
2..MTU-7029(L)
3.N.UsarDhan3(L)
4.R.Bhagwati (L)
5.CSR-30 (L)
6.P.Basmati -1(L)
7.BPT-5204 (CC)
8..MTU-7029(CC)
9.N.Usar.dhan-3(CC)
10.R.Bhagwati (CC)
11.CSR-30 (CC)
12.P.Basmati-1 (CC)
13.BPT-5204 (CS)
14..MTU-7029(CS)
15.N.Usar Dhan3(CS)
16.R.Bhagwati(CS)
17.CSR-30 (CS)
18.P.Basmati-1(CS)

4.3.1.13 Amplification using primer pair RM 333

All the entries under characterization in the present study yielded amplified products due to primer pair RM 333 directed amplification of simple sequence repeat locus known to be located on Chromosome No. 10 in rice (plate-4.17). The overall size of the amplified products approximately ranged from 185.71 bp in BPT-5204 (L) to 207.90 bp in CSR-30 (CS) and Pusa Basmati-1 (CS). A single band was visualized in all entries. Altogether six types of amplified products were generated including two unique bands and four shared bands. The position of the bands corresponding to the location of bands along the Y-axis varied from 592 in CSR-30 (CS) and Pusa Basmati-1 (CS) to 610 in BPT-5204 (L) (table-4.22). The Rf value obtained for different bands ranged from 0.673 for the amplified product of approximately 207.90 bp to 0.698 for the amplified product of approximately 185.71 bp.

Table-4.22 Analysis of bands produced by primer pair 333 based amplification of genomic DNA extracted from eighteen entries of rice

Lane	Band	Position	Mol.Weight (bp)	Rf
1	1	610	185.71	0.698
2	1	608	188.10	0.695
3	1	606	190.48	0.692
4	1	606	190.48	0.692
5	1	600	197.62	0.684
6	1	600	197.62	0.684
7	1	600	197.62	0.684
8	1	600	197.62	0.684
9	1	600	197.62	0.684
10	1	600	197.62	0.684
11	1	600	197.62	0.684
12	1	598	200.00	0.681
13	1	598	200.00	0.681
14	1	600	197.62	0.684
15	1	598	200.00	0.681
16	1	598	200.00	0.681
17	1	592	207.50	0.673
18	1	592	207.50	0.673

4.3.1.14 Amplification using primer pair RM 336

Using primer pair RM 336, amplification of simple sequence repeat locus known to be located on Chromosome No. 7 in rice was achieved. All the entries under characterization in the present study yielded amplified products due to primer pair RM 336 directed amplification of simple sequence repeat locus (plate-4.18). A single band was visualized in the cases of 17 entries. Altogether eight types of amplified products were generated including six unique bands and two shared bands. The position of the bands corresponding to the location of bands along the Y-axis varied from 644 in CSR-30 (L) to 716 in MTU-7029(L) (table-4.23). The Rf value obtained for different bands ranged from 0.693 for the amplified product of approximately 20526 bp to 0.784 for the amplified product of approximately 131.48 bp.

Table-4.23 Analysis of bands produced by primer pair 336 based amplification of genomic DNA extracted from eighteen entries of rice.

Lane	Band	Position	Mol. Weight (bp)	Rf
1	1	678	168.75	0.736
2	1	716	131.48	0.784
3	1	678	168.75	0.736
4	1	656	191.67	0.708
5	1	644	205.26	0.693
5	1	678	168.75	0.736
6	1	678	168.75	0.736
7	1	678	168.75	0.736
8	1	656	191.67	0.708
9	1	664	183.33	0.718
10	1	652	195.83	0.703
11	1	678	168.75	0.736
12	1	678	168.75	0.736
13	1	678	168.75	0.736
14	1	688	158.33	0.749
15	1	668	179.17	0.723
16	1	656	191.67	0.708
17	1	678	168.75	0.736
18	1	678	168.75	0.736

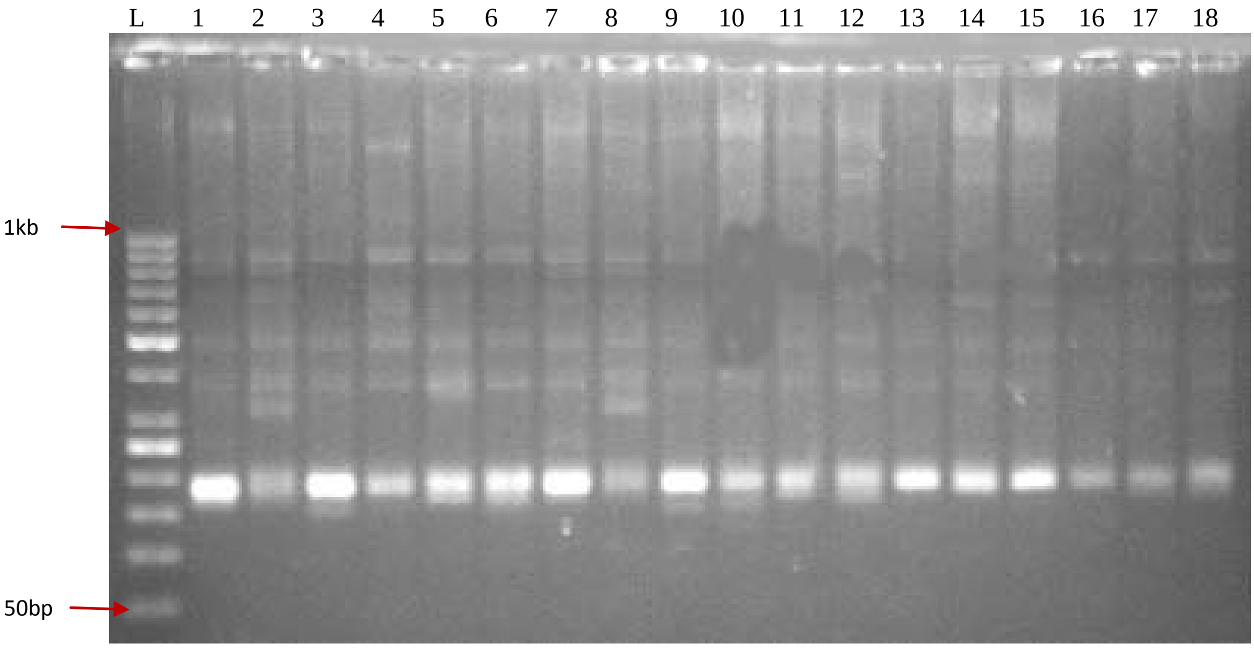


Plate-4.17 Amplification of region of genomic DNA extracted from the leaves control callus and salt tolerant callus in rice cultivars by primer RM 333 used in the study

1.BPT-5204 (L)
2..MTU-7029(L)
3.N.UsarDhan3(L)
4.R.Bhagwati (L)
5.CSR-30 (L)
6.P.Basmati -1(L)
7.BPT-5204 (CC)
8..MTU-7029(CC)
9.N.Usar Dhan-3(CC)
10.R.Bhagwati (CC)
11.CSR-30 (CC)
12.P.Basmati-1(CC)
13.BPT-5204 (CS)
14..MTU-7029(CS)
15.N.Usar Dhan3(CS)
16.R.Bhagwati(CS)
17.CSR-30 (CS)
18.P.Basmati-1(CS)

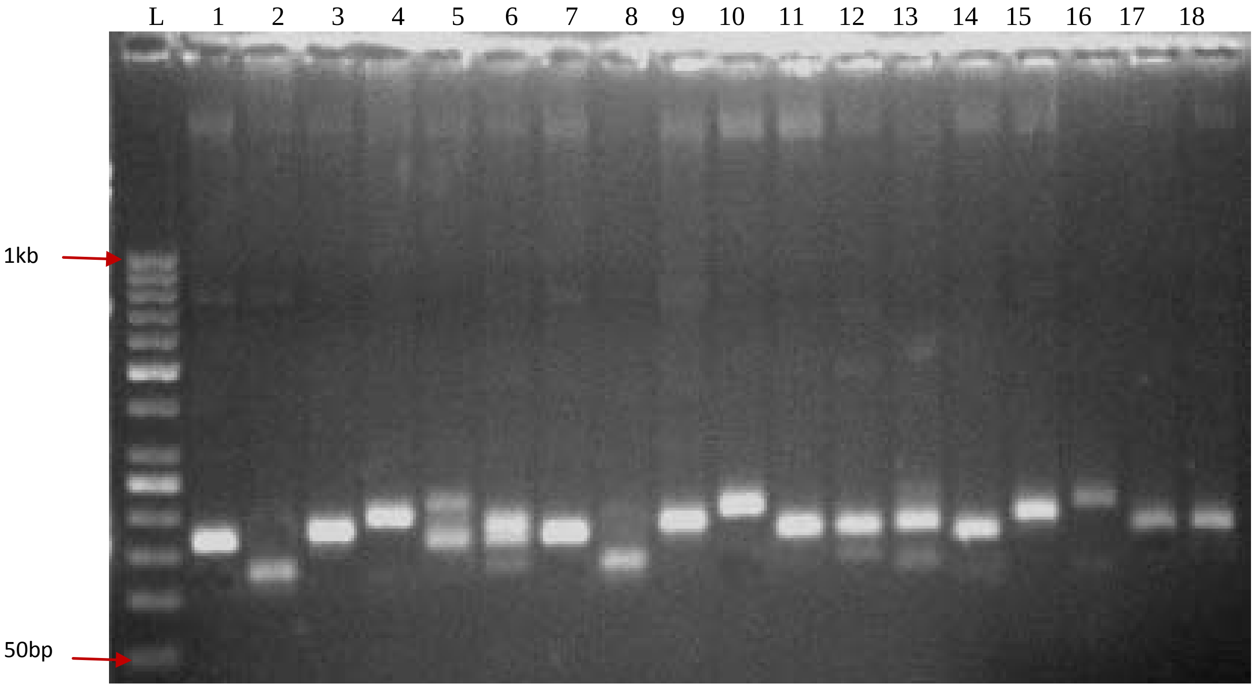


Plate-4.18 Amplification of region of genomic DNA extracted from the leaves, control callus and salt tolerant callus in rice cultivars by primer RM 336 used in the study

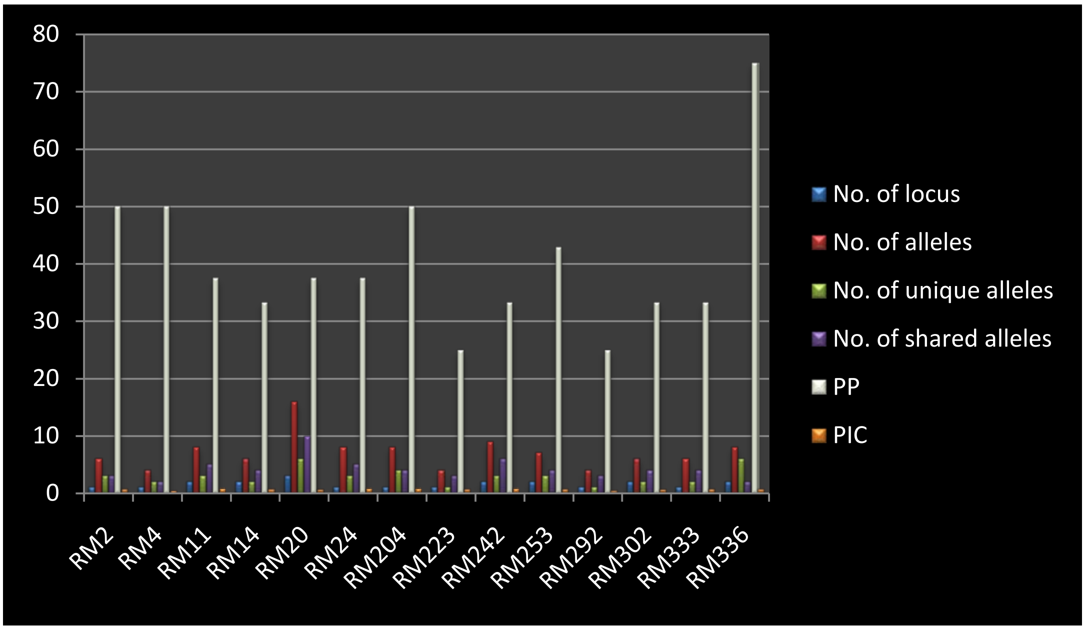
1.BPT-5204 (L)
2..MTU-7029(L)
3.N.UsarDhan3(L)
4.R.Bhagwati (L)
5.CSR-30 (L)
6.P.Basmati -1(L)
7.BPT-5204 (CC)
8..MTU-7029(CC)
9.N.Usar Dhan-3(CC)
10.R.Bhagwati (CC)
11.CSR-30 (CC)
12.P.Basmati-1(CC)
13.BPT-5204 (CS)
14..MTU-7029(CS)
15.N.Usar Dhan3(CS)
16.R.Bhagwati(CS)
17.CSR-30 (CS)
18.P.Basmati-1(CS)

Table-4.24 Analysis of primer pairs used for the amplification of genomic DNA extracted from eighteen entries

Primer	No. of locus	Size of alleles (bp)	No. of alleles	No. of unique alleles	No. of shared alleles	PP	PIC
RM2	1	150.00-172.00	06	03	03	50.0	0.703
RM4	1	161.36-181.82	04	02	02	50.0	0.376
RM11	2	143.48-185.00	08	03	05	37.5	0.802
RM14	2	184.78-206.25	06	02	04	33.33	0.685
RM20	3	205.94-288.24	16	06	10	37.5	0.623
RM24	1	152.94-197.06	08	03	05	37.5	0.814
RM204	1	126.00-182.64	08	04	04	50.0	0.820
RM223	1	143.10-156.52	04	01	03	25.0	0.648
RM242	2	227.78-281.25	09	03	06	33.33	0.827
RM253	2	116.07-152.27	07	03	04	42.85	0.688
RM292	1	156.52-167.39	04	01	03	25.00	0.512
RM302	2	120.37-207.89	06	02	04	33.33	0.623
RM333	1	185.71-207.90	06	02	04	33.33	0.722
RM336	2	131.48-205.26	08	06	02	75.00	0.645

PP: Polymorphism per cent; PIC: Polymorphism information content;

Fig-4.17 Analysis of primer pairs used for the amplification of genomic DNA extracted from eighteen entries



The number of alleles per locus ranged from three in the cases of RM 14, and RM 302 to eight in the case of RM 24 and RM 204. The primer pairs RM 11, RM 14, RM 20, RM 242, RM 253, RM 302 and RM 336 generated amplified products due to amplification of more than one locus. The total alleles identified in the eighteen entries were classified into two categories; (a) unique alleles and (b) shared alleles. A total of 41 unique and 59 shared allelic variants were generated in the form of amplified products by using 14 primer pairs. The polymorphism per cent revealed in the form of percentage of unique allele was recorded to be the maximum in the case of RM 336 and the minimum in the case of RM 223 and RM292 with an average value of 40.21 per cent.

The level of polymorphism exhibited amongst the entries under evaluation in the present study using fourteen primer pairs was assessed by calculating polymorphism information content (PIC) of each of the primer pairs (table 4.24). The PIC values revealing allele diversity and frequency among the entries varied from 0.376 in the case of RM 4 to 0.827 in the case of RM 242 with an average of 0.677 across the primer.

4.3.2 Similarity coefficients between entries

The similarity coefficients were computed amongst eighteen entries on the basis of presence and absence of the amplified products generated by using fourteen primer pairs specific to the unique flanking sequences of the simple sequence repeats distributed among twelve chromosomes of the rice genotypes under evaluation (table-4.25). The magnitude of similarity coefficient between BPT-5204 (CC) and BPT-5204, BPT-5204 (CS) and BPT-5204, Narendra Usar Dhan-3 (CC) and Narendra Usar Dhan-3 (L), Narendra Usar Dhan-3 (CS) and Narendra Usar Dhan-3 (L), Pusa Basmati-1(CS) and Pusa Basmti-1 (L), BPT-5204 (CS) and BPT-5204 (CC), Narendra Usar Dhan-3 (CS)and Narendra Usar Dhan-3 (CC), Pusa Basmati-1 (CS) and Pusa Basmti-1 (CC) (1.00) was found to be the maximum amongst pair-wise combinations of entries. This was followed by remarkably higher magnitude of similarity coefficient between the entries which are mention in table. The magnitude of similarity coefficient between entries was equal to 0.111. The results revealed ample diversity at the molecular level amongst the eighteen entries under evaluation in the present study.

4.3.3 Clustering of entries based on molecular markers

Using similarity coefficient as a measure of similarity in numerical taxonomic approach of classification, an assessment of the nature and extent of differentiation and divergence was made. The method used for the tree building in the analysis involved sequential agglomerative hierarchical nested clustering based on similarity matrix. The dendrogram was generated following unweighted pair group method using arithmetic mean (Fig.4.18) and the clusters were identified at appropriate phenon level.

Considering broad classification of entries, as indicated by dendrogram, basically the entries were divided into three groups. The first multi-genotypic group consisted of nine entries, namely, CSR-30 (L), CSR-30 (CC), CSR-30 (CS) , Narendra Usar Dhan-3 (L), Narendra Usar Dhan-3 (CC), Narendra Usar Dhan-3 (CS), MTU-7029 (L), MTU-7029 (CC) and MTU-7029 (CS), whereas the second multi-genotypic group consisted of Rajendra Bhagwati (L)**,** Rajendra Bhagwati (CC) and Rajendra Bhagwati (CS), third multi-genotypic group consisted BPT-5204 (L), BPT-5204 (CC), BPT-5204 (CS), Pusa Basmati (L), Pusa Basmati-1 (CC) and Pusa Basmati-1 (CS).

Table -4.25 Estimates of 16 SSR primer pairs based Dice's similarity coefficients among 18 rice entries used in the present study

	CSR30(L)	MTU-7029(L)	N.Usar Dhan-3 (L)	R.Bhagwati (L)	BPT-5204 (L)	P.Basmti-1 (L)	CSR-30 (CC)	MTU-7029 (CC)	N.UsarDhan-3 (CC)	R.Bhagwati (CC)	BPT-5204 (CC)	P.Basmti-1(CC)	CSR-30 (CS)	MTU-7029 (CS)	N.Usar Dhan-3 (CS)	R.Bhagwati (CS)	BPT-5204 (CS)	P.Basmati-1
CSR-30(L)	1.000																	
MTU-7029(L)	0.428	1.0000																
N.UsarDhan-3(L)	0.500	0.285	1.000															
R.Bhagwati(L)	0.125	0.125	0.333	1.000														
BPT-5204(L)	0.222	0.100	0.111	0.111	1.000													
P.Basmti-1(L)	0.250	0.111	0.285	0.285	0.375	1.000												
CSR-30(CC)	1.000	0.428	0.500	0.125	0.222	0.250	1.000											
MTU-7029(CC)	0.428	1.000	0.285	0.125	0.100	0.111	0.428	1.000										
N.UsarDhan-3(CC)	0.500	0.285	1.000	0.333	0.100	0.285	0.500	0.2857	1.000									
R.Bhagwati(CC)	0.111	0.111	0.285	0.800	0.100	0.250	0.111	0.111	0.285	1.000								
BPT-5204(CC)	0.222	0.100	0.111	0.111	0.750	0.375	0.222	0.100	0.111	0.100	1.000							
P.Basmti-1(CC)	0.250	0.111	0.285	0.285	0.375	1.000	0.250	0.222	0.285	0.250	0.375	1.000						
CSR-30(CS)	1.000	0.428	0.500	0.125	0.222	0.250	1.000	0.428	0.500	0.111	0.222	0.250	1.000					
MTU-7029(CS)	0.428	.666	0.285	0.125	0.100	0.111	0.428	0.666	0.285	0.111	0.100	0.111	0.428	1.000				
N.UsarDhan-3(CS)	0.500	0.285	1.000	0.333	0.111	0.285	0.500	0.285	1.000	0.285	0.111	0.285	0.500	0.285	1.000			
R.Bhagwati(CS)	0.100	0.100	0.250	0.666	0.090	0.222	0.100	0.100	0.250	0.571	0.090	0.222	0.100	0.222	0.250	1.000		
BPT-5204(CS)	0.181	0.083	0.200	0.200	0.750	0.300	0.181	0.083	0.200	0.181	0.750	0.300	0.181	0.083	0.200	0.166	1.000	
P.Basmati-1(CS)	0.250	0.111	0.285	0.285	0.375	1.000	0.2500	0.111	0.285	0.250	0.375	1.000	0.250	0.111	0.285	0.222	0.300	1.000

Fig-4.18 Dendrogram based on Dice similarity coefficient among 6 rice cultivars evaluated using 14 SSR primer pairs

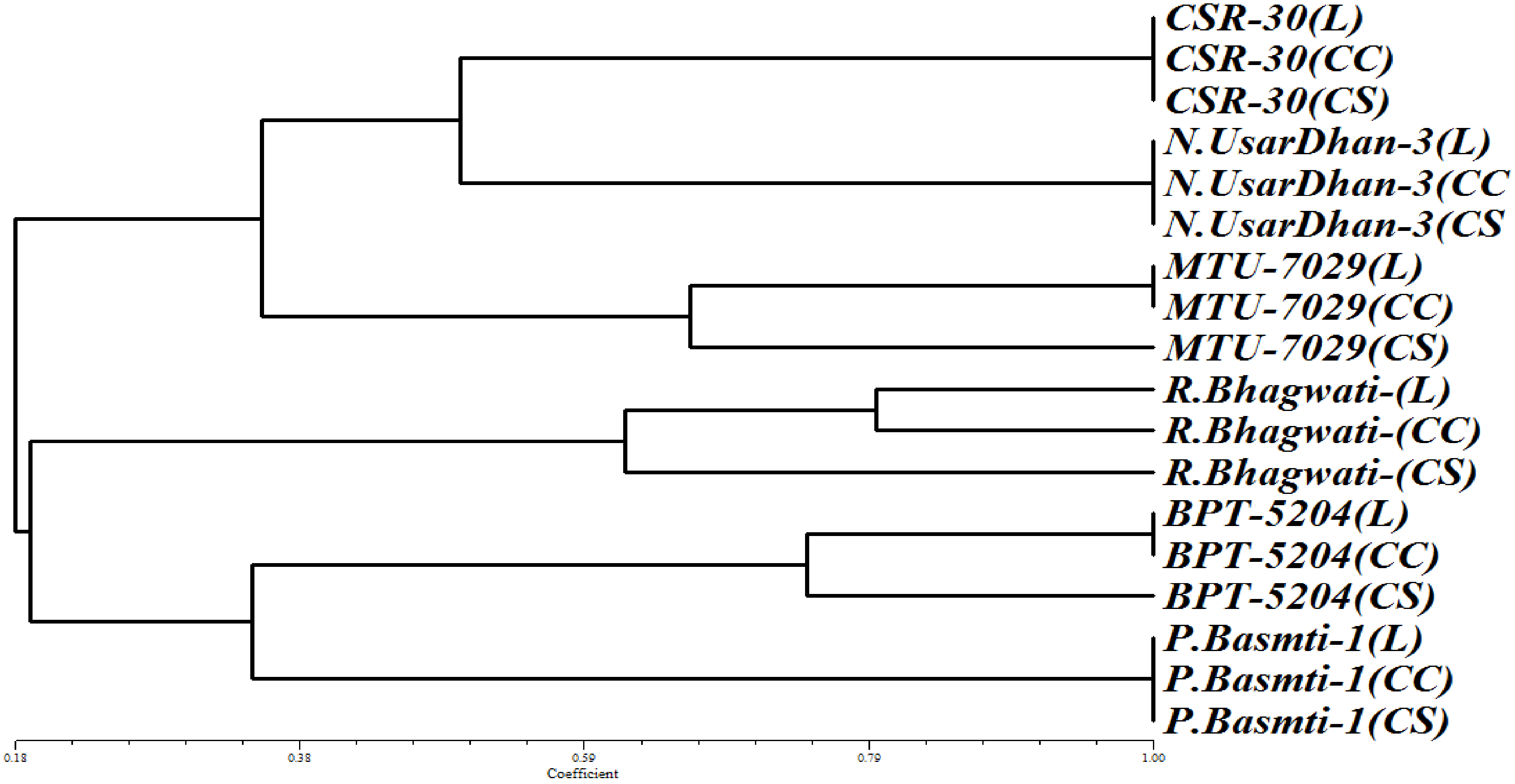


Table-4.26 Composition of clusters based on similarity coefficient in numerical taxonomic approach of cluster analysis

Clusters identified at different phenon levels			Entries included in each clusters
25	**50**	**75**	
A (6)	AI (3)	AI (3)	CSR-30(L), CSR-30(CC), CSR-30(CS)
	AII (3)	AII (3)	Narendra Usar Dhan-3 (L), Narendra Usar Dhan-3 (CC) Narendra Usar Dhan-3 (CS)
B (3)	B (3)	BI (2)	MTU-7029 (L), MTU-7029 (CC)
		BII (1)	MTU-7029 (CS)
C (3)	CI (2)	CIa (1)	Raje,ndra Bhagwati (L)
		CIb (1)	Rajendra Bhagwati (CC)
	CII (1)	CII (1)	Rajendra Bhagwati (CS)
D (3)	D (3)	DI (2)	BPT-5204 (L), BPT-5204 (CC)
		DII (1)	BPT-5204 (CS)
E (3)	E (3)	E (3)	Pusa Basmati-1 (L), Pusa Basmati-1(CC), Pusa Basmati-1 (CS)

Figures in parenthesis indicates number of entries in respective clusters

Cluster-1 was divided into two clusters (table-4.26). Di-genotypic cluster A consisted of two groups. Group AI consisted of CSR-30 (L), CSR-30 (CC) and CSR-30 (CS). All showing genetic similarity of 1.0, indicating no variation between leaf, normal callus and salt tolerant callus in cv. CSR-30. Group AII consisted of Narendra Usar Dhan-3 (L), Narendra Usar Dhan-3 (CC) and Narendra Usar Dhan-3 (CS). All showing genetic similarity of 1.0, indicating no variation between leaf, normal callus and salt tolerant callus in cv. Narendra Usar Dhan-3. These two groups AI and AII showed a genetic similarity of 0.50. Di-genotypic cluster B further divided into two groups. Group BI consisted of MTU-7029 (L) and MTU-7029 (CC), showing a genetic similarity of 1.0, indicating no variation between leaf and normal callus in cv. MTU-7029. Group BII consisted of only one entry MTU-7029 (CS) showing a genetic similarity of 0.66 with group BI. Thus, the salt tolerant callus (BII) showed variation of 0.34 compared to leaf and normal callus.

Cluster-2 was tri-genotypic and divided into two groups CI and CII (table-4.26). Di-genotypic group CI consisted of Rajendra Bhagwati (L) and Rajendra Bhagwati (CC) showing a a genetic similarity of 0.80, indicating tissue culture induced variation of 0.20 between leaf and normal callus. Mono-genotypic group CII consisted of only one entry Rajendra Bhagwati (CS). This group showed a genetic similarity of 0.57 with group CI indicating further variation in salt tolerant callus of cv. Rajendra Bhagwati.

Cluster-3 consisted of two groups D and E (table-4.26). The D group, consisted of di-genotypic group DI and mono-genotypic group DII. Group DI consisted of BPT-5204 (L) and BPT-5204 (CC) showing a genetic similariy of 1.0, indicating no variation between leaf and normal callus in cv. BPT-5204. Group DII consisted of only one entry BPT-5204 (CS) showing a genetic similarity of 0.75 with group DI, Thus, the salt tolerant callus (DII) showed variation of 0.25 compared to leaf and normal callus. Group E consisted of Pusa Basmati-1 (L), Pusa Basmati-1 (CC) and Pusa Basmati-1 (CS). All showing genetic similarity of 1.0, indicating no variation between leaf, control callus and salt tolerant callus in cv. Pusa Basmati-1.

Thus, based on salt tolerant linked 14 SSR marker studied there was no variation in leaf, normal callus and salt tolerant callus of cvs. CSR-30, Narendra U sar Dhan-3 and Pusa Basmati-1. There was no variation between leaf and normal callus in case of cvs. MTU-7029 and BPT-5204. However, salt tolerant calli showed variation in both the cultivars. In cultivar Rajendra Bhagwati, variations were observed between leaf, normal callus and salt tolerant callus. Salt tolerant index (STI) of the cultivars based on seedling dry weight and callus dry weight under salt stress indicated that cv. CSR-30 was the most salt tolerant followed by cvs. Narendra Usar Dhan-3, MTU-7029, BPT-5204, Rajendra Bhagwati and Pusa Basmati-1.

The status of cultivars for their relative salt tolerance was substantiated by the study of 14 salt tolerant linked SSR markers. The most salt tolerant cvs. CSR-30 and Narendra Usar Dhan-3 were grouped together in group A and were again grouped with moderately tolerant cv. MTU-7029. In the other group, salt sensitive cvs. Rajendra Bhagwati and Pusa Basmati-1 were clubed together with moderately tolerant cv. BPT-5204. Thus, the results of *in vivo, in vitro* screening for salt tolerance of rice cultivars largely coincides with the screening based on salt tolerant linked SSR marker. The experiment also resulted in induction of salt tolerant in cvs. MTU-7029, Rajendra Bhagwati and BPT-5204 through tissue culture induced somaclonal variation as confirmed molecular marker studied.

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CHAPTER – V

DISCUSSION

DISCUSSION

The world's human population is expected to become more than 9 billion upto 2050 and to feed this population an increase of more than 70% is required in global food production (FAO, 2011). This will require an average yearly increase in food production of 44 million tonnes per year for next 40 years (Tester and Langridge, 2010). Achieving this target is very difficult considering large scale soil degradation and decreasing availability of arable land. An important factor of soil degradation is soil salinity, which is increasing day by day and about 7% of earth land surface have been affected with the soil salinity. The area of salt affected soil and its continuous expansion is the highest in some of the most populous countries namely India, Bangladesh and Pakistan, which is posing a serious threat to sustainable agricultural production.

Rice, the most loved cereal of Asia, feeds the majority of the world's population. More than 90% of the world's rice is grown and consumed in Asia where 60% of the earth's people and about two-third of the world's poor live including India, Bangladesh and Pakistan (Khush and Virk, 2000). Salinity, a serious problem affecting one third of all the irrigated land in the world (Mass and Hoffman, 1977), impairs normal growth and limits the realization of yield potential of modern cultivars of rice. It is one of the major obstacles in increasing production in rice growing areas worldwide. Rice is rated as an especially salt-sensitive crop (Shannon *et al.*, 1998). It is differentially affected by salinity at different stages. The effects of salinity on the growth of rice were found to be related to the stage of plant development, salt concentration, type of salt, duration of exposure to salt, soil pH, water regime, temperature, humidity and solar radiation (Akbar, 1986). Rice crop is relatively tolerant to salinity and alkalinity during germination stage.

Salinity stress at higher level results in multiple effects on plant growth and development such as disturbed cell expansion, impaired metabolism, necrosis, limited protein synthesis and eventually the enhancement of cell death (Ayala-Astorga and Alcaraz-Melendez, 2010). Soil salinity is one of the major limitation factors that alone causes 5% yield loss (Tabatabaei, 2006). To counteract salinity stresses, plants utilize number of defence mechanisms, ultimately leading to stress tolerance (Bostock, 2005;

Chehab *et al*., 2007). This comprises a range of physiological and biochemical adjustments in plants including leaf wilting, leaf area reduction, leaf abscission, root growth stimulation, and alterations in relative water content (Bartels and Sunkar, 2005). Molecular responses to abiotic stress on the other hand include perception of the particular stress, signal transduction, gene expression and ultimately metabolic changes in the plant thus providing stress tolerance (Agarwal *et al*., 2006; Mondal and Ganie, 2014).

Developing salt tolerant plant through marker assisted breeding has added advantage over either conventional breeding or through transgenic technology. Due to their dense distribution throughout the genome, high reproducibility, co-dominant alleles and highly variable nature, microsatellites or simple sequence repeats (SSRs) meet the requirements of an ideal genetic marker. Subsequently, they have become markers of choice for genome mapping,fingerprinting and population analysis as well as in evolutionary studies (McCouch *et al*., 1997; Garland *et al*., 1999). Though SSR markers have been extensively used in the studies of genetic diversity of rice yet, few of them are only reported to be trait specific. Since SSRs were found to be either intergenic or intragenic (Tóth *et al*., 2000), the variable length of repeat motifs at the SSR could be related to differential function or activity of the segments of chromosomes in which they reside. This in-turn may represent the differential potential of expression of a particular trait e.g. salt tolerance, in contrasting genotypes differing in that very trait and exhibiting variability at SSR loci. Although SSR were considered as evolutionary neutral markers, the functional significance of SSRs had been corroborated (Gur-Arie *et al*., 2000; Li *et al*., 2002; Rocha *et al*., 2002). They have been reported to play a crucial role in the regulation of gene activity, regulation of DNA metabolic processes and chromatin organisation (Li *et al*., 2002). Further, SSRs act as hot spots for the recombination (Jeffreys *et al*., 1998; Templeton *et al*., 2000; Li *et al*., 2002) and that dinucleotide repeats are the sites of choice for recombination because of their high affinity for recombinase. Thus, SSRs may also influence recombination directly by their effects on DNA structure (Biet *et al*., 1999).

The salinity tolerance is a complex trait with many factors like soil, nutrients and ion uptake affecting the performance of a genotype. The degree of biomass production under non-saline and different saline conditions is generally taken as a measure of tolerance. Salt tolerant genotypes are the best alternatives for utilization of salinity affected lands. Salt tolerance can be either screened in existing genotype of a crop plant or can be induced through intervention of conventional and biotechnological tools. Rice, which is largely sensitive to salinity, can be made salt tolerant through these methods and their salinity tolerant status can be confirmed utilizing specific salt tolerance linked molecular markers.

5.1 Germination studies

The germination of seeds of six selected cultivars of rice did not show any variation in absence of salinity stress but in the presence of salt concentrations, the germination of seeds differed in different cultivars. The best germination was observed in cultivar CSR-30 followed by cv. Narendra Usar Dhan-3, which may be considered as salt tolerant based on germination performance in the presence of salt. Cultivar Pusa Basmati-1 showed the least germination and can be considered as salt sensitive. Similarly, cv. Rajendra Bhagwati can also be considered as salt sensitive, while cvs. BPT-5204 and MTU-7029 can be considered as moderately tolerant (fig-4.1).

There was decrease in seed germination with increase in salt concentration in all the six cultivars of rice. At 2% salt, germination was only observed in cultivars CSR-30 and Narendra Usar Dhan-3, while at 2.5 % salt no seed germination was observed. Bhumba and Singh (1965) have shown the inhibitory effect of salt stress on seed germination, while Paliwal and Gandhi (1968) found in paddy the inhibitory effect of salt due to anions. Ali *et al.* (1996) observed in rice varieties of different groups, a concentration dependent decrease in seed germination under sodium chloride stress as found in present investigation. They also observed differences in salinity tolerance among cultivars based on germination frequency and considered it significant because decline in germination also leads to significant reduction in seedling length, early seedling vigour, speed of germination and dry matter production per plant (Reddy *et al.*, 1994). Kazemi and Eskandari (2011) observed delayed and decreased germination of rice seeds under salt stress. High concentration of salt reduces the water potential and hinders water absorption by germinating seeds and thus reduces germination (Jamil *et al.*, 2006). Salinity may affect seed germination by decreasing the ease with which the seeds take up water because the activity and events normally associated with germination get either delayed or proceed at a reduced rate (Abogadallah and Quick, 2009).

Seedling survival and growth were also important indicator of the salt tolerance of a cultivar (Garg *et al*., 1996). As observed with seed germination, seedling growth was also adversely affected with the increase of salt concentration. The shoot growth was more affected than root growth. A similar observation on adverse effect on seedling growth due to salt stress and shoot growth being more affected than root growth was observed in rice (Welfare *et al*., 1996). Adverse effect of salinity on seedling growth of rice has also been reported by Djanaguiraman *et al*. (2003) and Hakim *et al*. (2010). The adverse effect of salt stress is reflected in the dry matter accumulation of the seedling. Thus the dry matter accumulation of seedling shoot and root indicated by their dry weight can be used as an index to measure the salinity tolerance of the cultivar. The salt tolerance index (STI) of the six cultivars of rice based on accumulation of dry weight of seedling shoot and root suggested cv. CSR-30 to be most salt tolerant followed by cvs. Narendra Usar Dhan-3, MTU-7029, BPT-5204 and Rajendra Bhagwati. The cv. Pusa Basmati-1 showed the least tolerance to salt and considered to be salt sensitive (fig-4.3). Thus, on the basis of seed germination and seedling growth of the rice cultivars in the presence of salinity stress, the cultivars can be screened for their relative salinity tolerance.

5.2 Tissue culture studies

Plant tissue culture is an important aspect of plant biotechnology and it has led to the development of many varieties of crop plant particularly rice, that are being grown in large areas of the world. One of the important mechanism by which these varieties were developed, is somaclonal variations, which are the variations among regenerated plants in tissue culture (Larkin and Scowcroft, 1981). It has provided a novel method for producing variations and selecting the variations at the cell, tissue, organ or the plant level for the desired characters required ultimately for the plant improvement (Larkin, 1985). Tissue culture technique thus can be used for developing stress tolerance, particularly salt tolerance in crop plants including rice (Gamborg *et al*., 1985; Raghava Ram and Nabors, 1985; Kabir *et al*., 2008). Somaclonal variations are more when the plants are regenerated through callus phase.

Callus represents clumps of unorganized parenchymatous tissue formed by the vigorous proliferation by cell division showing no polarity. It is generally formed under the influence of phytohormones content of the explants and the growth hormone content of the medium, on which, the explant is cultured. The transformation of an explant into a proliferating callus mass under culture conditions, is reflected in a change in the basic architectural pattern of the tissue by cell division. Loss of certain cell types, development of new cell types and quiescent cells becoming metabolically active are some examples (Thorpe, 1980; Wagle *et al*., 1987). Callus induction also depends on the plant genotype, the source or origin of the explant and the physiological state of the tissue at culture (Murashige, 1974).

The three media, namely M1 (MS + 2.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} KIN), M2 (MS + 2.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} KIN), and M3 (MS + 2.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} KIN), selected for callus formation from the cultured seeds of rice cultivars, showed at par callusing response. All the three media had higher concentrations of 2,4-D (2.0-2.5 mg l^{-1}) with lower concentrations of KIN (0.5-1.0 mg l^{-1}) and were suitable for callus formation as they contained 2,4-D the most potent callus inducer (Nishi *et al*., 1973; Sekiya *et al*., 1977). However, cultivar played an important role in callus formation and cv. CSR-30 showed the best callusing response followed by cvs. Pusa Basmati-1, Rajendra Bhagwati, Narendra Usar Dhan-3, MTU-7029 and BPT-5204 respectively (fig-4.9). Like any other tissue culture response, callus formation also depends on genotype and some genotype perform better than other genotypes (Liang, 1978; Abe and Futsuhara, 1986; Oard and Rutger, 1988).

Seeds of the six selected cultivars of rice when cultured on medium M1 (MS + 2.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} KIN) with different salt concentrations showed seed germination and callus formation. Seed germination was found at par in different cultivars in absence of any salt. Under salt stress, there was a decrease in seed germination with increase in concentration of salt (fig-4.12) and overall cv. CSR-30 showed the best germination followed by cvs. Narendra Usar Dhan-3, MTU-7029, BPT-5204, Rajendra Bhagwati and Pusa Basmati-1 respectively. Thus, on the basis of germination of cultured seeds under salt stress cvs. CSR-30 and Narendra Usar Dhan-3 can be considered as salt tolerant and cvs. Pusa Basmati-1 and Rajendra Bhagwati can be considered as salt sensitive. Cultivars BPT-5204 and MTU-7029 can be considered as moderately salt tolerant.

The ability to form callus from cultured seeds of rice cultivars under salt stress depended on their relative salt tolerance. Seeds of more tolerant cultivar produced more callus compared to susceptible ones. The best callus formation in presence of salt stress was found in the cultured seeds of cv. CSR-30 followed by cvs. Narendra Usar Dhan-3, MTU-7029, BPT-5204, Rajendra Bhagwati and Pusa Basmati-1 respectively (fig-4.13). Thus, the cultivar CSR-30 can be considered as the most salt tolerant and cv. Pusa Basmati-1 can be considered as the most salt susceptible among the all cultivars based on their ability to form callus in presence of salt stress. Callus formation percentage gradually decreased with increased salt stress and there was no callus formation at 2.5% salt stress (Thach and Pant, 1999; Shanthi *et al*., 2010). Only seeds of cvs. CSR-30 and Narendra Usar Dhan-3 formed low callus in presence of 2% salt. The ability of cultured seeds to form callus at higher concentration of salt, suggested the presence of salt tolerant cells in these callus. These cells might have originated through somaclonal variation and thus, continued to grow in presence of salt.

The developed callus of all the six cultivars of rice without salt stress, were cultured on callusing medium with different levels of salt stress to increase the frequency of induction of salt tolerance. The continued growth of callus even in the presence of high concentrations of salt (upto1.5-2%), indicated the induction of salt tolerance. The callus growth decreased with increased salt stress. This showed that the salt had an inhibitory effect on the growth of callus. The increasing salt concentrations created an osmotic or ionic shock resulting in adverse effect on growth of plant cells and tissues. This was also supported by Senadhira (1987) and Aditya and Baker (2006). Shankhdhar *et al*. (2000) also reported the dramatic decrease in the fresh weight of the callus with increasing salt concentration. The reduction might be as a result of reduced water availability in the culture medium due to increased salt concentration.

Based on callus growth in the presence of salt stress as indicated by their dry weight, the salt tolerance index (STI) of the six selected rice cultivars was estimated. Cultivar CSR-30 showed the highest mean salt tolerance index (STI) followed by cv. Narendra Usar Dhan-3 and can be considered as salt tolerant while Pusa Basmati-1 showed the least mean salt tolerance index (STI) and can be considered as salt susceptible. The other cvs. BPT-5204, MTU-7029 and Rajendra Bhagwati can be considered as moderately tolerant based on their mean salt tolerance index (STI) being in between tolerant and susceptible.

Thus, based on germination studies of seeds *in vivo* and *in vitro*, and seedling and callus growth under different salt stresses, the six selected cultivars were ranked for their salt tolerance level. Cultivars CSR-30 and Narendra Usar Dhan-3 were found to be the most salt tolerant, cvs. MTU-7029 and BPT-5204 to be moderately tolerant and cvs. Rajendra Bhagwati and Pusa Basmati-1 were found to be salt sensitive respectively. The formation of callus and their continued growth at higher levels of salt stress indicated the induction and formation of salt tolerant cells and calluses.

The calluses of all cultivars developed without salt stress regenerated plant on medium M4 (MS + 1.5 mg l^{-1} NAA + 1.5mg l^{-1} KIN). However, the calluses developed in presence of salt failed to differentiate plant indicating the inability of salt tolerant callus for regeneration. Loss of regeneration potential under high salt stress has been reported (Reddy and Vaidyanath, 1986; Priya *et al*., 2011).

5.3 Molecular marker studies

The normal leaf, normal callus and salt tolerant callus of the six selected rice cultivars were analysed by 14 salt tolerance linked SSR primer pairs. The study revealed remarkably higher level of genetic polymorphism which allowed unique genotyping of the entries and somaclonal variants. Altogether 100 allelic variants were detected at 22 SSR loci with an average of 4.54 alleles per locus due to length variation of simple sequence repeats present in entries. A total of 59 shared and 41 unique allelic variants were generated by using 14 primer pairs (table-4.24). All the primer pairs utilized in the present study generated unique alleles. The number and proportion of unique alleles varied considerably with the primer pairs. The number of unique alleles per locus ranged from one in the cases of RM223, RM292 to six in the case of RM 336, RM20. Similarly, the number of shared alleles per locus ranged from two in the cases of RM4, RM336 to ten in the cases of RM20. In general, marker detecting greater number of alleles per locus detected more number of unique alleles in accordance with the earlier reports (Bajracharya *et al*., 2006; Brondani *et al*., 2006; Joshi and Behera, 2006; Lapitan *et al*., 2007; Herrera *et al*., 2008; Ebana *et al*., 2008; Borba *et al*., 2009; Pervaiz *et al*., 2010; Rabbani *et al*., 2010; Vanaza *et al*., 2010; Singh *et al*., 2011). The presence of unique alleles indicated that the materials used in this study are useful as a rich source of genetic diversity for their purposeful and effective utilization in rice breeding for salt tolerance.

The primer pairs differed in their ability to determine variability among different entries based on their polymorphism. The higher polymorphism of a primer indicated greater percentage of unique alleles generated by that primer. The polymorphism per cent revealed in the form of percentage of unique allele was recorded to be the maximum (75) in the case of RM336 and the minimum (25) in the case of RM223 and RM292 with an average value of 40.21 per cent. The primer pairs showing higher polymorphism percent than the average value were RM336, RM2, RM204, RM4 and RM253.

The PIC value of a marker reflects marker allelic diversity and frequency among the cultivars. The higher PIC of a marker indicated higher probability of detecting the number of allele among cultivars. The PIC values revealing allele diversity and frequency among the entries varied from 0.376 in the case of RM 4 to 0.827 in the case of RM 242 with an average of 0.677 across the primer. Polymorphic information content (PIC) value ranged from 0.21 to 0.76 with an average of 0.57 in 14 genotypes of stress tolerant rice with 40 SSR markers (Islam *et al*., 2012). Further, Islam *et al*. (2008) reported a PIC value of 0.55 using 21 rice genotypes, Joshi and Behrea (2006) reported 0.54 using 38 rice genotypes, and Pervaiz *et al*. (2009) reported 0.60 using 35 rice genotypes. Brondani *et al*. (2006) and Giarrocco *et al*. (2005) observed an average PIC value of 0.66, 0.73 and 0.69 for the world collection, traditional Brazilian rice varieties and Argentine rice germplasm, respectively. Findings of these high PIC value might be due to inclusion of more diverse set of rice germplasm as observed in present investigation. The primer pairs having higher polymorphism information content (PIC) than the average value were RM242, RM204, RM11, RM2, RM14, RM24, RM253, RM333. The primer pairs RM2 and RM204 are the only two that have higher polymorphism percent as well as polymorphism information content and these can be considered highly informative for grouping rice cultivars on the basis of their salt tolerance.

The estimates of similarity coefficient clearly indicated the existence of complete similarity and absence of any somaclonal variants in the cases of three entries namely cvs. CSR-30, Narendra Usar Dhan-3 and Pusa Basmati-1. Somaclonal variation was observed in normal callus of cv. Rajendra Bhagwati and salt tolerant calluses of cvs. MTU-7029, Rajendra Bhagwati and BPT-5204.

Analysis of allelic data generated on the basis of amplification profile obtained with SSR primer pairs allowed differentiation of salt tolerant entries from rest of the entries comprising moderately tolerant and susceptible entries. On the basis of SSR marker studies, the cvs. CSR-30 and Narendra Usar Dhan-3 can be considered as most salt tolerant while cv. Pusa Basmati-1 can be considered as most salt sensitive. The other three cvs. MTU-7029, BPT-5204 and Rajendra Bhagwati can be considered as moderately salt tolerant. Faridul Islam *et al*. (2012) differentiated stress tolerant rice cultivars on the basis of SSR marker. However, Sudharani *et al*. (2013) assessed 8 rice genotypes with different salt stress tolerant by 85 primers. They could get salt tolerant genotypes mixed with salt sensitive ones. In the present investigation salt tolerant cultivars were differentiated from salt sensitive ones because only salt tolerant primer pairs were used. The information about the genetic diversity of these rice genotypes on the basis of their salt tolerance will be useful to breeder for proper identification and selection of appropriate parents' in future breeding program for developing new salt stress tolerant rice varieties. This differentiation of cultivars on the basis of their salt tolerance confirmed their status as revealed by germination and tissue culture studies.

Thus, the present research work resulted in induction of salinity tolerant callus in cultivars MTU-7029, Rajendra Bhagwati, BPT-5204 and screening of cultivars for their salinity tolerance in the order of CSR-30, Narendra Usar Dhan-3, MTU-7029, BPT-5204, Rajendra Bhagwati and Pusa Basmati-1 respectively.

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CHAPTER – VI

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

Rice is the principal food of nearly half of the world’s population and India is the largest rice growing country in the world. The crop is adversely affected by salinity, which affects production and productivity. Developing salt tolerant cultivars is the best way to overcome the problem of salinity.

Tissue culture induced somaclonal variations is an important way of inducing salinity tolerance in rice. Six rice cultivars namely BPT-5204, MTU-7029, Narendra Usar Dhan-3, Rajendra Bhagwati and Pusa Basmati-1 were screened for their salinity tolerance through studies of seed germination, seedling growth and growth of callus under different levels of salt stress (0-2.5%) created by a salt mixture of NaCl, $CaCl_2$, Na_2SO_4 in 7:2:1 ratio. The selection of calluses having capability to grown under salt stress led to induction of salinity tolerance. The six cultivars of rice, their normal calluses and salt tolerant calluses were further screened with the help of 14 salt tolerance linked SSR primer pairs for their salinity tolerance and to confirm the induction and formation of salt tolerant callus. The main conclusions of the experiment were:

- There was hardly any difference among cultivars for germination without salt.
- However, in presence of salt, the best germination was found in cvs. CSR-30 and Narendra Usar Dhan-3 followed by cvs. MTU-7029, BPT-5204, Rajendra Bhagwati and Pusa Basmati-1 respectively.
- There was gradual decrease of germination with increasing salt concentration.
- Salinity tolerance index was calculated on the basis of seedling dry weight and callus dry weight which indicated that cvs. CSR-30 and Narendra Usar Dhan-3 were found to be the more salt tolerant followed by cvs. MTU-7029, BPT-5204, Rajendra Bhagwati and Pusa Basmati-1 respectively.
- Plants were regenerated from normal callus of all the six cultivars on medium MS + 1.5 mg l^{-1} NAA + 1.5mg l^{-1} KIN.

- However, callus grown in presence of salt failed to regenerate normal plantlets.
- The use of fourteen SSR primer pairs for the analysis of the entries under evaluation in the present study revealed remarkably higher level of genetic polymorphism which allowed unique genotyping of the entries and somaclonal variants.
- Altogether 100 allelic variants were detected at 22 SSR loci with an average of 4.54 alleles per locus due to length variation of simple sequence repeats present in entries.
- A total of 59 shared and 41 unique allelic variants were generated by using 14 primer pairs. The primers RM2 and RM204 appeared to be more informative.
- The estimates of similarity coefficient clearly indicated the existence of complete similarity and absence of any somaclonal variants in the cases of three entries namely CSR-30, Narendra Usar Dhan-3 and Pusa Basmati-1.
- Somaclonal variation was observed in normal callus of cv. Rajendra Bhagwati and salt tolerant calli of cvs. MTU-7029, Rajendra Bhagwati and BPT-5204.
- Analysis of allelic data generated on the basis of amplification profile obtained with SSR primer pairs allowed differentiation of salt tolerant entries from rest of the entries comprising moderately tolerant and susceptible entries.
- The present research work resulted in induction of salinity tolerant callus in cultivars MTU-7020, Rajendra Bhagwati, BPT-5204 and screening of cultivars for their salinity tolerance in the order of CSR-30, Narendra Usar Dhan-3, MTU-7029, BPT-5204, Rajendra Bhagwati and Pusa Basmati-1 respectively.

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