IN VITRO DEVELOPMENT OF SALT AND HEAT STRESS TOLERANCE IN RICE (*ORYZA SATIVA* L.)

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BY

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Dedicated

To

My Parents

DECLARATION

I hereby declare that the research work embodied in this thesis entitled **"*IN VITRO* DEVELOPMENT OF SALT AND HEAT STRESS TOLERANCE IN RICE (*ORYZA SATIVA* L.)"** has been carried out by me for the Degree of **Master of Philosophy** under the guidance of **Professor Dr. S. M. Shahinul Islam**, Institute of Biological Sciences, University of Rajshahi, Rajshahi-6205, Bangladesh.

I also declare that the result presented in this dissertation is my own investigation and any part of this thesis work has not been submitted to elsewhere for any degree/diploma or for similar purpose.

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CERTIFICATE

This is to certify that **Toyfiquz Zaman** worked under my supervision as M. Phil Fellow, Roll No. M-2013084503, Session: 2019-2020, Institute of Biological Sciences, University of Rajshahi, Bangladesh. It's my great pleasure to forward his thesis entitled **"*IN VITRO* DEVELOPMENT OF SALT AND HEAT STRESS TOLERANCE IN RICE (*ORYZA SATIVA* L.)"** which is a bonafide record of research at the Institute of Biological Sciences, University of Rajshahi, Bangladesh.

This work is original and has not been submitted so far in part or in full, for the award of any degree or diploma by any other institute in home or abroad. **Toyfiquz Zaman** has fulfilled all the requirements for submission of the thesis for the award of the Degree of **Master of Philosophy**.

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The Author

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

The following abbreviations have been used through the text:

%	:	Percent
ANOVA	:	Analysis of variance
BRRI	:	Bangladesh Rice Research Institute
cm	:	Centre meter
dS/m	:	Deci Sieme per mole
e.g	:	Example
EC	:	Electrical conductivity
EDTA	:	Ethylene diamine tetra acetic acid
et al.	:	et alibi (and others)
etc.	:	Etcetera = and others
ha	:	Hectare
HCl	:	Hydro chloric acid
$HgCl_2$	:	Murcuric chloride
i.e.	:	Id est (that is)
Kin	:	Kinetin
m	:	Metre
mg	:	Miligram
mg/l	:	Milligram per liter
N_6	:	Chu et al. (1975) medium
°C	:	Degree celsius
PGRs	:	Plant growth regulators
P^H	:	Negative logarithm of hydrogen ion (H-) concentration
v/v	:	Volume by volume
Viz	:	Videlicet (L.); namely
w/v	:	Weight by volume

ABSTRACT

Rice is a staple crop that provides food for billions of people worldwide, but it is susceptible to damage from biotic and abiotic stress, particularly in tropical or subtropical areas. On top of that, stress has a negative impact on the productivity and quality of rice. To close the gap between output and demand, significant productivity gains are needed given the rising demand for rice. The study assessed the germination rates and callus induction capabilities of various rice varieties under different conditions in lab and also in the field. An efficient and reproducible *in vitro* regeneration process is required for the production of abiotic stress tolerance in rice through biotechnological approaches. In this study, optimization of culture media, callus induction, and plant regeneration of five Aman rice varieties *viz*. BRRI dhan34, BRRI dhan51, BRRI dhan71, BRRI dhan79, and BRRI dhan95 were considered and mature seeds of those varieties were collected from BRRI Regional Station, Rajshahi. To investigate the effects of different concentrations of 2,4-D and other plant growth regulators on callus induction and regeneration some experiments were conducted and evaluated. Two basal media *viz*. MS and N6 were considered for the experiments out of four (MS, B5, N6 and SK-3) studied previously under this research works. It was observed that all five rice varieties showed higher callus induction on MS medium supplemented with 3.0 mg/l 2,4-D than other concentrations. Based on callus induction data were recorded and BRRI dhan34, BRRI dhan51, BRRI dhan71, BRRI dhan79 and BRRI dhan95 gave 63.4%, 50.4%, 40.4%, 58% and 53% embryogenic calli, induction respectively. In combination with BAP (2.0 mg/l), Kin (2.0 mg/l) and NAA (0.5 mg/l) added to MS medium data showed better performance on regeneration. They can be used as a baseline for demand-driven *in vitro* rice propagation, providing useful information that can be combined with other agronomic features in rice

development to improve the abiotic stress-tolerant cultivars through biotechnological methods in future. In addition of different concentration of NaCl in medium affects on callus growth. Data showed that at 2.0 g/l concentration was better than others. Higher NaCl concentrations correlated with decreased germination rates and increased callus necrosis, although BRRI dhan34 (7.2±0.8%) and BRRI dhan51 (7.2±0.8%) exhibited salt tolerances across all studies concentrations. Another experiment was conducted on the effect of heat stress and results showed that BRRI dhan34, BRRI dhan79 and BRRI dhan95 showed tolerances and it was observed that maintaining robust callus growth even at elevated temperatures at 35°C and 40°C. In contrast, BRRI dhan51 (6%) and BRRI dhan71 (8%), displayed modest heat tolerance (6%), exhibiting diminished performance under higher temperatures. These findings are very importance on selecting appropriate rice varieties based on their germination rates, callus induction, salt and heat tolerance of rice varieties in Bangladesh.

CHAPTER-I
INTRODUCTION

1. INTRODUCTION

Rice (*Oryza sativa* L.) is not just a staple crop; it is a lifeline for billions of people worldwide. Its significance in global food security cannot be overstated, as it serves as a primary source of sustenance for a substantial portion of the global population. With Bangladesh ranking fourth globally in rice production, the cultivation of this grain forms an integral part of the nation's agricultural landscape and societal fabric. The cyclical rhythm of rice farming, spanning Aus (spring-summer), Aman (summer-fall), and Boro (spring-winter) seasons, underscores its significance in ensuring food security for the country's vast population, estimated at 135 million. Its farming is essential to Bangladesh's enormous population's sustenance and well-being (Bhuiyan and Mahmood 2002). The regional landscape further accentuates the prominence of rice, with approximately 60% of global rice production concentrated in South-East Asia (FAO 2020), a region where countries like Bangladesh, Cambodia, Indonesia, Laos, Myanmar, Thailand, and Vietnam exhibit a robust appetite for the grain, with per capita consumption ranging from 130 to 180 kg annually (Khush 2005).

However, as the recent demand of rice in Bangladesh's due to increasing population, projected to reach 194.353 million by 2050, challenges loom large on the horizon. The imperative to augment rice production by nearly 30% to meet future demands underscores the pressing need for agricultural innovation and sustainable practices (Basak 2011). Rice stands as a dependable source of dietary energy for a significant portion of the global population, particularly in regions like Asia, Africa, and Latin America. Its widespread cultivation and consumption bolster food security by

ensuring a consistent supply of calories. Beyond its role as a carbohydrate source, rice also boasts essential nutrients such as vitamins, minerals, and dietary fiber. In areas where rice holds sway as a dietary staple, it plays a pivotal role in meeting nutritional requirements and staving off malnutrition (Khush 2005).

Accessible to diverse socio-economic groups due to its affordability and widespread availability, rice emerges as a vital sustenance source for vulnerable populations, especially those grappling with food insecurity and poverty (Pingali 2012). Moreover, the cultivation and consumption of rice are deeply deep-rooted in the cultural fabric of numerous societies, serving as a beacon of tradition, identity, and communal unity (Mertz et al. 2009). Its cultural significance further solidifies its status as a staple crop across the globe. Economically, the rice industry exerts a significant impact on the global stage, generating income, employment, and foreign exchange earnings (FAO 2021). The backbone of livelihoods for millions of farmers and agricultural workers, particularly in developing nations where agriculture reigns supreme, rice farming forms the bedrock of economic stability (Dasgupta and Roy 2015). The economic importance of rice spans across multiple dimensions, exerting significant influence on local, national, and global economies. It provides livelihoods for millions of farmers, agricultural laborers, and related industries such as milling, transportation, and marketing (Lal 2004). In many developing countries, where agriculture forms the backbone of the economy, rice farming sustains entire communities and rural economies (FAO 2020). Rice is a significant commodity in global trade, with many rice-producing countries exporting surplus production to international markets (Lin 2011). Export earnings from rice contribute to foreign exchange reserves, helping to stabilize national

economies and support economic growth (Fischer et al. 2005). For countries with competitive advantages in rice production, such as Thailand, Vietnam and India, rice exports represent a vital component of their trade balance. The rice industry stimulates the growth of agribusinesses and value-added activities along the rice value chain. This includes input suppliers (seeds, fertilizers, pesticides), machinery manufacturers, storage facilities, processing plants, and distribution networks. The expansion of these ancillary industries creates additional employment opportunities and contributes to economic diversification in rural areas. In instant, the economic importance of rice is multifaceted, encompassing employment generation, income generation, export earnings, food security, agribusiness development, infrastructure investment, and social stability (FAO 2017). As a fundamental staple crop, rice plays a central role in sustaining livelihoods, supporting rural economies, and driving economic growth in rice-producing regions worldwide.

In many developing countries, where agriculture forms the backbone of the economy, rice farming sustains entire communities and rural economies. Rice is a significant commodity in global trade, with many rice-producing countries exporting surplus production to international markets. Export earnings from rice contribute to foreign exchange reserves, helping to stabilize national economies and support economic growth. For countries with competitive advantages in rice production, such as Thailand, Vietnam, and India, rice exports represent a vital component of their trade balance. The rice industry stimulates the growth of agribusinesses and value-added activities along the rice value chain. This includes input suppliers (seeds, fertilizers, pesticides), machinery manufacturers, storage facilities, processing plants, and distribution networks. The expansion of these

ancillary industries creates additional employment opportunities and contributes to economic diversification in rural areas. In summary, the economic importance of rice is multifaceted, encompassing employment generation, income generation, export earnings, food security, agribusiness development, infrastructure investment, and social stability. As a fundamental staple crop, rice plays a central role in sustaining livelihoods, supporting rural economies, and driving economic growth in rice-producing regions worldwide.

In Bangladesh, the importance of rice in the food sector is unparalleled, shaping the nation's economy and food security on multiple fronts (Bhuiyan and Mahmood 2002). Serving as a linchpin of economic activity, the rice sector contributes substantially to GDP, employment, and rural livelihoods (Basak 2011). Engaging a significant portion of the populace, especially in rural areas where agriculture reigns supreme, rice farming serves as the primary income source (FAO 2021). Furthermore, rice reigns supreme as the staple food in Bangladesh, constituting over 95% of daily food consumption (Pingali 2012). Its cultivation is indispensable for ensuring food security and nutritional adequacy, particularly among rural and low-income households (Mertz et al. 2009). Beyond its nutritional value, rice plays a crucial role in fostering social stability and cohesion in Bangladesh, acting as a unifying force across diverse socio-economic and cultural strata (FAO 2020). During times of crisis, rice serves as a bulwark against food shortages and emergencies in Bangladesh, offering a dependable source of sustenance (Khush 2005). Its widespread availability helps cushion the impact of food scarcities and price fluctuations on vulnerable populations, thereby mitigating the adverse effects of crises. Despite its paramount importance, the rice sector in Bangladesh grapples

with myriad challenges, including land constraints, environmental degradation, and the impacts of climate change (Dasgupta and Roy 2015). However, ongoing endeavors in agricultural research, technology adoption, and policy interventions present avenues for bolstering rice productivity, resilience, and sustainability (FAO 2020). In essence, rice assumes a central role in both the economy and food security of Bangladesh, acting as a crucial source of income, nutrition, and social cohesion. Strengthening the rice sector is imperative for ensuring food security, fostering economic development, and advancing the well-being of the population, not only in Bangladesh but also beyond its borders. When compared to other cereal crops like corn, wheat, barley, and rice, rice has less fat and protein (Gregorio and Senadhira 1997). Compared to white rice, brown rice is more nutrient-dense and contains iron, potassium, folic acid, magnesium, thiamine, phosphorus, copper, zinc, and vitamin B6 (FAO 2017). The most significant industry in Bangladesh's economy is agriculture, which accounts for 61.2% of all arable land, generates 19.60% of the country's GDP, and enrolls 63% of its population. According to estimates from the United Nations Population Division, Bangladesh's overall population is expected to reach 194.353 million by 2050, and the country will require 49.07 million tons of rice, which is approximately 30% more than what is currently produced. About 10.5 million hectares of this country's land are used for rice production, a number that has remained constant over the previous three decades. Bangladeshi farmers usually cultivate three main types of rice varieties- Aman, Aus, and Boro.

Rice stands out as a powerhouse of carbohydrates, constituting a prime source of energy for countless individuals worldwide (Pingali 2002). However, the carbohydrate content may vary depending on the rice type, with white rice typically

boasting higher levels compared to its brown counterpart due to differences in processing techniques (FAO 2021). Despite not being classified as a complete protein source; rice still packs a noteworthy punch in terms of protein content (Khush 2005). Brown rice, in particular, tends to offer more protein than its white counterpart, while certain rice varieties like black rice may even boast enhanced protein content and diverse amino acid profiles (Gregorio and Senadhira 1997). Moreover, brown rice emerges as a stellar source of dietary fiber, boasting both soluble and insoluble variants (FAO 2017). This fiber content proves instrumental in promoting digestive health, regulating blood sugar levels, and mitigating the risk of chronic ailments such as heart disease and diabetes. Additionally, rice encompasses a rich array of vitamins, notably B vitamins like thiamine (vitamin B1), niacin (vitamin B3), and vitamin B6 (FAO 2020). These vitamins play indispensable roles in facilitating energy metabolism, sustaining nervous system function, and fostering overall health. Notably, brown rice often retains more vitamins compared to its white counterpart due to the preservation of the outer bran layer during processing. Furthermore, rice serves as a notable reservoir of essential minerals, including iron, magnesium, phosphorus, and zinc, all of which are critical for diverse physiological functions ranging from bone health to immune function and blood oxygen transport (Basak 2011). Certain rice varieties, such as black rice and red rice, boast an added bonus in the form of antioxidants like anthocyanins and flavonoids (Dasgupta and Roy 2015) These antioxidants function as cellular defenders, shielding against oxidative damage, mitigating inflammation, and potentially warding off chronic diseases (FAO 2020). The glycemic index (GI) of rice, influenced by factors such as processing and cooking methods, exhibits variability across different varieties (Karim et al. 2024, FAO 2017). Typically,

brown rice carries a lower GI compared to white rice, resulting in a slower and steadier rise in blood sugar levels. This characteristic renders brown rice an optimal choice for individuals managing diabetes or seeking to regulate blood sugar levels effectively (Lin 2011). Rice emerges as a nutritional powerhouse and a versatile staple food, catering to the dietary needs and energy requirements of millions across the globe. Opting for whole grain varieties like brown rice and diversifying the rice repertoire can further amplify its nutritional benefits, thereby contributing to overall health and well-being.

Bangladesh, being a country with high population density, faces a challenge in expanding cultivable land. Therefore, agricultural genetics plays a crucial role in developing high-yielding varieties and stress-tolerant rice plants (Hossain and Ahmed 2002). The advancements in the rice sector are primarily attributed to genetic enhancements in producing high-yielding rice varieties. Rice production in numerous nations is at risk because of elevated salinity and drought conditions, leading to an inability to attain food self-sufficiency (Boyer 1982). Plant growth and productivity are significantly impacted by major biotic and abiotic stresses, leading to a cascade of morphological, physiological, and biochemical alterations in plants. Plant growth and productivity are undeniably influenced by a myriad of factors, with biotic and abiotic stresses emerging as significant determinants in shaping agricultural outcomes. Biotic stresses, arising from living organisms such as pests, pathogens, and weeds, can inflict substantial damage to crops, leading to yield losses and diminished productivity (Liu et al. 2019). On the other hand, abiotic stresses, including environmental factors like drought, salinity, heat, and cold, pose formidable challenges to plant growth and development, further exacerbating

agricultural vulnerabilities. Numerous published studies corroborate the profound impact of biotic and abiotic stresses on plant physiology, productivity, and crop yields. For instance, research conducted by Liu et al. (2019) highlights the detrimental effects of biotic stresses, particularly pest infestations, on crop performance and yield outcomes (Khan et al. 2015). Similarly, studies by Reddy et al. (1994) underscore the adverse effects of abiotic stresses such as drought and salinity on plant growth, water use efficiency, and overall productivity.

Salinity poses a significant threat to rice production, affecting plant growth, development, and overall productivity. Published research provides insights into the multifaceted effects of salinity on rice, shedding light on the physiological, biochemical, and molecular responses of rice plants to saline conditions. Studies conducted by Munns and Tester (2008) and Ismail and Horie (2017) have elucidated the detrimental effects of salinity on rice growth and yield. High soil salinity inhibits root growth, reduces water uptake, and impairs nutrient absorption, ultimately leading to stunted plant growth and diminished grain yield. Research by Flowers and Flowers (2005) and Hussain et al. (2017) highlights the physiological adaptations of rice plants to saline stress. These adaptations include osmotic adjustment, ion homeostasis, and the activation of antioxidant defense mechanisms to mitigate the adverse effects of salt-induced oxidative stress. Studies by Parida and Das (2005) and Cuin et al. (2008) have investigated the biochemical changes induced by salinity in rice plants. Salinity triggers alterations in metabolic pathways, including the accumulation of compatible solutes such as proline and glycine betaine, as well as the synthesis of osmoprotectant and stress-related proteins. Molecular studies conducted by Negrão et al. (2011) and Mishra et al.

(2014) have provided insights into the genetic and molecular mechanisms underlying rice tolerance to salinity. These studies have identified key genes and regulatory pathways involved in salt stress perception, signal transduction, and the activation of stress-responsive genes. Research by Gregorio et al. (1997) has explored varietal differences in rice tolerance to salinity. Certain rice cultivars exhibit inherent tolerance to saline conditions, attributed to genetic diversity and allelic variation in genes associated with salt tolerance traits. Studies by Islam et al. (2018) and Rahman et al. (2016) have investigated agronomic and molecular approaches to enhance rice tolerance to salinity. These approaches include the use of salt-tolerant rice varieties, agronomic practices such as water management and soil amendments, as well as genetic engineering techniques to introduce salt tolerance genes into rice germplasm. Since, salinity exerts profound effects on rice physiology, biochemistry and molecular biology, posing a significant challenge to rice production in saline-affected areas. By understanding the complex mechanisms of salt stress tolerance and employing integrated management strategies, stakeholders can work towards enhancing rice resilience to salinity and ensuring sustainable rice production in salt-affected regions.

The impact of heat stress on rice cultivation is a pressing concern in agricultural research, as rising global temperatures poses significant challenges to crop productivity and food security. Many articles provide valuable insights into the effects of heat stress on rice, elucidating various physiological, biochemical, and molecular responses of rice plants to elevated temperatures. Effect on Growth and Yield: Studies by Jagadish et al. (2015) have highlighted the detrimental effects of heat stress on rice growth and yield. High temperatures during critical growth stages

can lead to reduced grain filling, decreased pollen viability, and ultimately, diminished yield potential where physiological adaptations of rice plants is required. These adaptations include increased leaf temperature, accelerated leaf senescence, and alterations in photosynthetic activity, which can impact overall plant growth and productivity. Heat stress triggers the accumulation of reactive oxygen species (ROS), leading to oxidative damage to cellular components such as lipids, proteins, and nucleic acids. Molecular studies conducted by Larkindale and Huang (2005) and Nakashima et al. (2007) have provided insights into the genetic and molecular mechanisms underlying rice tolerance to heat stress. These studies have identified key heat shock proteins (HSPs), transcription factors, and signaling pathways involved in heat stress response and thermostolerance. Research by Pradhan et al. (2020) has explored varietal differences in rice tolerance to heat stress. Certain rice cultivars exhibit inherent resilience to high temperatures, attributed to genetic diversity and allelic variation in genes associated with heat tolerance traits.

Krishnan et al. (2021) have investigated agronomic and molecular approaches to enhance rice tolerance to heat stress. These approaches include the use of heat-tolerant rice varieties, agronomic practices such as mulching and supplemental irrigation, and genetic engineering techniques to introduce heat tolerance genes into rice germplasm. Heat stress poses significant challenges to rice production, affecting plant growth, development, and overall productivity. By understanding the complex mechanisms of heat stress response and employing integrated management strategies, stakeholders can work towards enhancing rice resilience to heat stress and ensuring sustainable rice production in a changing climate. Moreover,

advancements in breeding technologies, genetic engineering, and precision agriculture hold immense potential for developing stress-tolerant crop varieties capable of withstanding biotic and abiotic challenges (Mickelbart et al. 2015). By harnessing the power of innovation and interdisciplinary collaboration, stakeholders can work towards enhancing agricultural resilience, ensuring food security, and sustaining global food production systems in the face of mounting biotic and abiotic pressures.

The study was assessed to evaluate the germination rates and callus induction capabilities of various rice varieties under different stress conditions in laboratory and field conditions. An efficient and reproducible *In vitro* regeneration process was assayed for the production of abiotic stress tolerance in rice through biotechnological approaches.

CHAPTER-II

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 Background of rice

More than 115 countries around the world cultivate around 100,000 different types of rice. The most intensive cultivation of rice occurs in tropical and sub-tropical regions globally. More than 85% of the global rice production occurs in China, Japan, India, Bangladesh, Thailand, Indonesia, Southeast Asia, and the nearby islands in the Pacific Ocean. China and India produce around 50% of the rice supply, while another 25% are produced in Japan, Bangladesh, Thailand, Indonesia and Burma. Brazil is the leading rice producing country. During the last two decades, dramatic increase in rice production has been witnessed in many developing countries. Bangladesh stands third in respect of area among rice producing country of world after India and China and ranks fourth among rice producing countries of the world after China, India and Japan (BBS 1995). The area of harvest account for about 93% of the total cereals and 70% of the gross temporary cropped area.

2.2 Taxonomy of rice

The taxonomic status of rice is listed here, based on the Integrated Taxonomic Information System (ITIS 2006).

Taxon	**Name of Taxon**
Kingdom	: Plantae
Sub-Kingdom	: Viridiplantae
Division	: Tracheophyta
Sub-Division	: Spermatophytina
Class	: Magnoliopsida
Order	: Poales
Family	: Poaceae
Genus	: *Oryza* L.
Species	: *Oryza sativa* L.

Oryza sativa L. Taxonomic Serial No. TSN 41976 (http://www.itis.gov/servlet).

2.3 Genome of rice

The entire genetic makeup of rice, one of the most significant cereal crops in the world, is known as the rice genome. A staple food for almost half of the world's population, especially in Asia, is rice (*Oryza sativa* L.). An international team of researchers finished sequencing the rice genome in 2002, marking an important scientific milestone. This accomplishment offered insightful information about the genes, regulatory components, and evolutionary background of rice. The rice genome is divided into 12 chromosomes and contains about 430 million base pairs. Comprehending the rice genome has enabled progress in crop breeding, genetic engineering, and farming methods targeted at enhancing crop productivity, disease resistance, and adaptability to environmental stressors including salinity and drought.

According to Goff et al. (2002), the genome of rice (*Oryza sativa* L. japonica subspecies) is 420 Mb in size and may contain between 32,000 and 50,000 genes. According to IRGSP (2005), the rice genome is 40 times smaller than the wheat genome and makes up one-sixth of the maize genome. The *Oryza* genus's species were categorized based on how well their genomes fit together. Because *Oryza sativa's* genome is AA-type, it can correctly pair its chromosomes during meiosis with those of other AA-type species. By definition, only *Oryza sativa* varieties and species with AA-type genomes fall under the purview of gene flow through traditional sexual hybridization.

2.4 Origin

The geographical site of the origin of rice domestication is not yet definitely known. The general consensus is that rice domestication occurred independently in China, India and Indonesia. Higher yields are usually obtained during the dry season when there is a greater solar energy than during the wet. Rice may be grown in many types of soils, from sandy loam and shallow literati soil to heavy clays, provided there is adequate water either from rain or irrigation. The optimum pH level for rice is 5.5-6.5 when the soil is dry but it usually becomes 7.0-7.2 on flooding. Rice is short day plant, but cultivar differs markedly in their response to photoperiod and temperature. In Bangladesh, rice is grown under different types and situation of land, varying from hill slopes to river beds. In some areas of the country rice crops is grown one time in a year, but there are some areas where rice is grown two times in the year. Some farmers are trying to produce rice even three times in the year using some fields. Rice is grown largely in rotation with jute, pulses, oil seeds, vegetables and wheat etc. (BBS 1995).

2.5 Diversity

The genus *Oryza* contains 25 species, which are found in subtropical parts of North and South America, Asia, Australia, and Africa. The two extensively farmed *Oryza sativa* Linn and *Oryza glaberrima* species stand out among the 25 species due to their plain, undivided panicle branches, shorter tunicate ligules (6 mm as opposed to 15 to 45 mm), and fertile lemma and plea. The most extensively grown rice plant, *Oryza sativa*, can be divided into three main subspecies (variant groupings): Indica, Japonica (Sinica) and Javonica, based on geographic distribution and morpho-physiological characteristics (Das and Khanda 2020). Japonica refers to short-grained, temperate-zone cultivars grown in China, Korea, and Japan. It also describes the long panicles of the Indonesian rice types gundil (awnless) and bulu (woned).

2.6 Chemical composition of rice

Range of the mean approximate analysis, including the percentage of rough rice's organic fraction and its milling fractions at 14% moisture content (Ambreen et al. 2006).

Table 1: Chemical composition of rice.

Nutrient	Rough[a]	Brown[b]	Milled[b,c]
Protein (Nx5.95)	5.8-7.7	7.1-8.3	6.3-7.1
Crude fat	1.5-2.3	1.6-2.8	0.3-0.5
Crude fiber	7.2-10.4	0.6-1.0	0.2-0.5
Crude ash	2.9-5.2	1.0-1.5	0.3-0.8
Available carbohydrates	63.6-73.2	72.9-75.9	76.7-78.4
Starch	53.4	66.4	77.6
Neutral detergent fiber	16.4	3.9	0.7-2.3
Pentosans	3.7-5.3	1.2-2.1	0.5-1.4
Hemicelluloses	-	-	0.1
1,3:1,4 B-glucans	-	0.11	0.11
Polyuronic acid	0.6	-	-
Free sugars	0.5-1.2	0.7-1.3	0.22-1.45
Lignin	3.4	-	0.1
Energy	15.8	15.2-16.1	14.6-15.6

2.7 Vitamin contents of rice

The following table lists the rough rice's mean vitamin content (µg/g) at 14% moisture content along with its milling fractions (Ghosh et al. 2019).

Table 2: Vitamin contents in rice.

Vitamin	Rough	Brown	Milled
Retinol (A)	0-0.08	0-0.11	0-tr[b]
Thiamin (B)	2.6-3.3	2.9-6.1	0.2-1.1
Riboflavin (B2)	0.6-1.1	0.4-1.4	0.2-0.6
Niacin (nicotinic acid)	29-56	35-53	13-24
Pyridoxine (B6)	4-7	5-9	0.4-1.2
Pantothenic acid	7-12	9-15	3-7
Biotin	0.04-0.08	0.04-0.10	0.01-0.06
Inositol	800	1000	90-110
Choline	760-980	950	390-880
p-Aminobenzoic acid	0.3	0.3	0.12-0.14
Folic acid	0.2-0.4	0.1-0.5	0.3-0.14
Cyanocobalamin (B12)	0-0.003	0-0.004	0-0.0014
Tocopherol (E)	9.20	9.258	Tr-3

2.8 Minerals

The availability of soil nutrients during crop growth and the analytical techniques employed by different researchers have a significant impact on the mineral composition of rice grains. Generally speaking, brown rice has more minerals than milled rice. Table 3 shows Brown, milled, and rough rice element range of mean content (Heinemann et al. 2005).

Table 3: Mineral content of rice.

Mineral	Element	Rough rice	Brown rice	Milled rice
Macro Element (mg/g at 14% moisture)	Calcium	0.1-0.8	0.1-0.5	0.1-0.3
	Magnesium	0.6-1.5	0.2-1.5	0.2-0.5
	Phosphorus	1.7-3.9	1.7-4.3	0.8-1.5
	Phytin phosphorus	1.8-2.1	1.3-2.7	0.3-0.7
	Potassium	1.5-3.7	0.6-2.8	0.7-1.3
	Silicon	10.8	0.6-104	0.1-0.4
	Sulphur	0.4-1.6	0.3-109	0.8
Micro Element (µ/g at 14% moisture)	Aluminum	26-540	0.3-26	0.1-2.2
	Bromine	-	-	0.9
	Codman	-	0.02-0.6	0.25
	Chlorine	500-800	210-560	200-300
	Cobalt	2-11	0.03-0.04	0.017
	Copper	-	1-6	2-3
	Fluorine	-	-	0.3

(Table 3 Contd...)

Mineral	Element	Rough rice	Brown rice	Milled rice
	Iodine	14-60	0.03	0.02
	Iron	17-94	2-52	2-28
	Manganese	-	2-36	6-17
	Mercury	-	-	0.05
	Molybdenum	-	0.3-1.0	1.4
	Nickel	-	0.2-0.5	0.14
	Rubidium	-	-	6
	Selenium	-	0.3	0.3
	Sodium	53-810	17-340	5-86
	Tin	-	-	1.1
	Zinc	1.7-3.1	6-28	6-23

2.9 Ecosystem of rice

Rice exhibits a high degree of flexibility and a greater tolerance for moist environments. It can tolerate temperatures between 45° and 45°S, as well as sea level or even lower when protected embankments are present, up to 3000 meters in the Himalayas. In monsoon areas, rice is also frequently grown, especially during the rainy season. Two more roots grow after the main root when there is enough water available. Next, adventitious roots emerge from the tillers and the parent stem's basal nodes. Ten phases are experienced by the rice plant during its growth cycle: (i) germination and emergence, (ii) seedling, (iii) tillering, (iv) stem

elongation, (v) panicle initiation, (vi) panicle development, (vii) flowering, (viii) milk grain, (ix) drought grain, and (x) mature grain stage. While current high yielding very early maturing varieties can be harvested in as little as 90 days after sowing, traditional types take around 150 days to reach the ripe grain stage.

2.10 Micropropagation

The rice crop is always multiplied via seed, which can be sown directly in the field, disseminated, or drilled. Alternatively, the seedlings can be raised in nurseries before being transplanted. Ratooning is a vegetative method of propagating rice.

2.11 Salinity problem of Bangladesh

In nations where rice is grown, salinity is the most prevalent soil issue (Datta et al. 1993). Around 100 million hectares of salt-affected land exist worldwide (Flowers et al. 2005). Bangladesh's southern sea is its source of salt. During cyclones and typhoons, the land (about 2.8 million hectares) in Bangladesh's coastal zones is exposed to sea water. In addition, the coastal zone's salinity is rising daily as a result of the extensive extraction of surface and ground water for agriculture and the Farrakha barrage. One of the main issues now is the salinity of Patuakhali, Noakhali, Barisal, and Khulna. When compared to water from other sources, river water is extremely salinized. During the dry season, the river water in Sonagazi may have a salt level of up to 20 dS/m (Raja 2015). According to reports, the salinity aquifer has reached a depth of 151 km and 46.3 km from the coasts of Khulna and Barisal, respectively. According to recent studies, the salinity zone in the southern and southeast regions of the country is continuing to grow as a result of the river Padma's gradually decreasing upstream flow. Researchers are finding indications

that salt, which has already harmed the environment in the coastal districts of Satkhira and Khulna, is moving upward and affecting neighboring areas like Magura and Narile.

2.12 Local varieties grown in coastal areas

Of the total 9 million hectares of farmed land, over one third is saltwater. In some of those areas, farmers typically grow a single wet season rice harvest. Even in the monsoon season, there aren't many high-yielding varieties suitable for cultivation in such areas. In the saline zone, where less than 20% of the arable ground is used for farming, people only grow low-yielding varieties like Binnatoa, Boilam, and Gunshee during the wet season.

2.13 Sensitivity of rice to salinity

Most people agree that rice has a modest sensitivity to salt. EC 3 dS/m is the threshold, or the highest soil salinity level at which yield is maintained. Beyond this point, the yield level drops. The yield is significantly decreased at EC 10 dS/m, and salt of varying grades negatively impacts 10% of rice. Producing rice in the extremely salinized region (12 dS/m and higher) is not recommended. Sensitivity, however, is dependent on a variety of plants and environmental elements. Major causes of salt injury in rice include osmotic effects, ion toxicity, and nutritional imbalances (Gregoria et al. 1997).

2.14 Effects on growth

The symptoms of salt injury in rice are stunted growth, rolling and withering of leaves, white blotch, browning and dying of older leaves. In general, all morphological characters are affected with increase of salinity in leaves. The effects of salinity on growth of rice is related to the stage of plant, development of salt

concentration, type of salt, duration of exposure to salt, soil pH, water regime temperature, humidity and solar radiation. Several study indicates that rice tolerant during germination, become very sensitive during early seedling stage (2-3 leaves stage), grains tolerance during vegetative growth becomes sensitive during pollination and fertilization and then become increasingly sensitive by more tolerant at maturity. Salinity at the reproductive stage decreases grain yield much more than at vegetative growth stage.

2.15 Effect of heat

It has been reported that thermal shock may influence induction and regeneration potential in both, anther and microspore culture. Keller and Armstrong (1979) applied an elevated temperature of 30-40°C for 1h in *B. napus* followed by 40^{o}C for 3h prior to another culture and found very effective results on embryoid yield and regeneration. Heat shock treatment along with starvation stress on rice and observed very efficient induction and regeneration. Ho et al. (1978) obtained high embryo yield and plantlets in rice anther culture following stress pretreatment at 30-40°C.

2.16 Aims and objectives

Bangladesh is mostly an agricultural nation, and this is the foundation of its economy. Bengal has a 14.4 mph speed territory. The area now planted to rice is 10.74 m. About 70% of Bangladeshi people's daily caloric intake comes from rice, which they eat every day. However, there has been a long-standing rice shortage throughout the nation. There is a lot of rice produced in northern Bangladesh. However, there aren't as many salt-tolerant rice cultivars available in the south currently. Furthermore, practically all of the farmers in our nation lack the necessary knowledge to cultivate rice. In light of the aforementioned information,

the current study has been conducted as a first step toward creating a variety that is suitable for growth on salt soil. Experiments were initially designed to accomplish the following objectives:

1. To screen the local Bangladeshi rice variety for them *in vitro* culture derived from mature or immature seeds and evaluation of their potentialities on somatic embryogenesis.

2. To establish an efficient regeneration system, optimization of media components, plant growth regulators (PGRs) using some recently released and local rice varieties for advance biotechnological work.

3. To screen the suitable indica rice cultivars for *in vitro* culture systems as well as for callus induction and plant regeneration for advanced biotechnological research.

4. To investigate the effect of various media and PGRs for the efficient callus induction and regeneration with stress pre-treatments factors.

CHAPTER-III

MATERIALS AND METHODS

3. MATERIALS AND METHODS

Three key methods, including *in vitro* somatic embryogenesis research, were applied in this work. Certain rice cultivars, medium, plant growth regulators (PGRs), and stress pre-treatments were selected, utilized, and altered for each of these operations.

3.1 Plant materials

Five rice varieties *viz*. BRRI dhan34, BRRI dhan51, BRRI dhan71, BRRI dhan79 and BRRI dhan95 were used under this study. Basic information of those rice varieties are mentioned below:

Table 4: Important characteristic of selected five rice varieties under this study.

Sl.	Variety Name	Plant height (cm)	Life days	Average yields/per hectors
1	BRRI 95	120	125	6.5
2	BRRI 79	112	135	5.5
3	BRRI 71	107-108	114-117	5
4	BRRI 51	90	140	5
5	BRRI 34	117	135	3.5

3.2 Methods

3.2.1 Preparation of stock solutions

First, five original stock solutions were developed employing a range of medium-sized ingredients. Because varied proportions of the components were required, different stock solutions for macro, micro, and vitamin nutrition were created in the following way:

Stock solution-I

Its production used all macronutrients, including (KNO_3, NH_4NO_3, KH_2PO_4 and $CaCl_2$, $Ca(NO_3)_2.H_2O$, $MgSO_4.7H_2O$, $(NH_4)_2SO_4$, NaH_2PO_4) etc. (20 times the strength needed for the medium). 400 milliliters of purified water were used to dissolve each macronutrient in a 1-liter glass beaker after each was carefully weighed using a 4-digit electric scale. For optimal mixing, the solution was quickly heated and agitated using a magnetic stirrer. After that, the mixture was moved to a 500 ml volumetric flask, and 500 ml more of distilled water was added. After being moved into a fresh reagent vial, the stock solution was cooled to 4°C.

Stock solution-II

The preparation of the medium involved the utilization of seven micronutrients, namely $MnSO_4.4H_2O$, H_3BO_3, $ZnSO_4.4H_2O$, KI, Na_2MoO_4, $CuSO_4.5H_2O$, $CoCl_2.6H_2O$, etc. at a concentration 500 times higher than the final strength required. As with stock A, the stock solution was made by fully mixing these ingredients with a glass of distilled water. The solution was collected in a reagent container and refrigerated to 4°C.

Stock solution-III

It was prepared using the two micronutrients *viz*. $FeSO_4.7H_2O$ and Na_2. EDTA at 100 times strength to that of the final strength needed for the medium.

Stock solution-IV

Stock D was prepared using the vitamins- Glycine, Nicotinic acid, Pyridoxine, Thiamine HCl at 500 times concentration to that of the final strength needed for the medium. The procedure was same as that followed in case of stock A. The solution was prepared in a refrigerator at 4°C.

Stock solution-V

It was made using 500 milliliters of distilled water at 20 times the final myo-inositol strength, as per the instructions for stock solution A, and refrigerated at 4°C.

3.2.2 pH of media

A digital pH meter and either 1M HCl or NaOH were used to bring the solution's pH down to 5.8.

3.2.3 Media preparation and sterilization

The macro- and micronutrients, vitamins, and Myo-inositol were extracted from the stock solutions based on the needs of the plant tissue culture medium. As the carbon source, sucrose (30 g/l) was added and properly mixed. With 0.1M NaOH or 0.1 HCl, the media's pH was raised to 5.6-5.8. The medium was autoclaved for 15 minutes at 121°C under 15 Ibs of pressure after being rendered solid by the addition of 0.8% agar. To guarantee sterilization, the culture vessels (test tubes, flasks, and bottles) holding medium were autoclaved for 15 minutes for the media and 20 minutes for any glassware at 121°C.

3.3 Culture media and maintenance of culture materials

MS (Murashige and Skoog 1962) and N6 (Chu et al. 1975) were used in this experiment (Table 5). Here 200 mg/l BAP, 300 mg/l L-proline, 500 mg/l L-glutamine, 2 mg/l 2,4-D, 500 mg/l casein hydrolysate, 3% sucrose, and 0.7% agar were used in the N6 and MS medium for callus induction. Only embryogenic calli were used in the investigation's callus induction media for multiplication. Prior to autoclaving, the pH was adjusted to 5.6 to 5.8. The working area of the laminar airflow cabinet was first surface sterilized with 70% ethanol. The petri dishes, together with other necessary instruments (forceps, scalpels, Whatman filter paper, glassware, etc.), were sterilized in an autoclave at 15 Ibs pressure at 121°C for 15 minutes before being used to inoculate the seeds. Prior to the inoculation, the surface was sanitized for 15 minutes using the UV lamp in the laminar air flow cabinet. The Petri plates were parafilm-sealed and kept at 25°C in the dark for three to four weeks. The vessels were then kept lit using 36-watt white fluorescent lights with a 16/8-hour light/dark cycle and a 5000 lux light intensity. The temperature in the growing chamber was maintained at 25°C. After being surface sterilized, the seeds were carefully put into various media. The germination percentage of various incubation techniques was calculated. Seed inoculation (%) is the total of the seeds that have been inoculated and have germinated.

3.4 Hardening of plants

In order to prevent soil contamination, the well-rooted plantlets were removed from the jar and had their root systems completely cleaned. The next step was to put sterilized, pre-soaked vermiculite with a diameter of 5 cm into a little plastic container. After then, pots were kept inside the damp chamber. To stop evapotranspiration from causing water loss, a plastic bag was placed over the plants

for one to two weeks. The plants were then moved to larger pots with a 20 cm diameter and filled with a 1:1:1 mixture of loamy soil, compost, and sand to harden them. To save the local ecologies, the field culture plants were kept in full sunshine.

3.5 Data recording and statistical analysis

Data were recorded as numbers and computed using the formulas mentioned below in order to ascertain the frequencies of callus induction and plant regeneration. Five replicates were used to calculate the means and average values. Using ANOVA in R 2021, a Tukey's HSD test was used to investigate significance variance in data at the $p<0.05$ level.

$$\text{Frequency of callus induction (\%)} = \frac{\text{No of explants inducted callus}}{\text{No of cultured explants}} \times 100$$

$$\text{Frequency of plant regeneration (\%)} = \frac{\text{No of callus regenerated shoot}}{\text{No of cultured callus}} \times 100$$

$$\text{Frequency of root induction (\%)} = \frac{\text{No of shoot inducted root}}{\text{No of cultured shoot}} \times 100$$

Table 5: Evaluation of MS and N6 basal media on callus inductions and plant regeneration of five rice varieties.

Nutrients/others	Composition/components constituents	Media (mg/l)	
		MS	**N6**
Macronutrients	KNO_3	1900.00	2830.00
	NH_4NO_3	1650.00	-
	KH_2PO_4	170.00	400.00
	$CaCl_2.2H_2O$	440.00	166.00
	$MgSO_4.7H_2O$	370.00	185.00
	$(NH_4)_2SO_4$	-	463.00
Macronutrients	$MnSO_4.4H_2O$	22.30	4.40
	$MnSO_4.H_2O$	-	3.30
	H_3BO_3	6.20	1.60
	$ZnSO_4.7H_2O$	8.60	1.50
	$ZnSO_4.4H_2O$	8.60	1.50
	KI	0.83	0.80
	Na_2MoO_4	0.25	-
	$Na_2MoO_4.\ 2H_2O$	0.25	-
	$CuSO_4.5H_2O$	0.025	-
	$CoCl_2.6H_2O$	0.025	-
	$AlCl_2$	-	0.03
	$NiCl_2$	-	0.03
Iron	$FeSO_4.7H_2O$	27.80	27.80
	Na_2EDTA	37.30	37.30
	$Na_2EDTA.2H_2O$	37.30	37.30
	EDTA Na ferric salt	-	-

(Table 5 Contd...)

Nutrients/others	**Composition/components constituents**	**Media (mg/l)**	
		MS	**N6**
Organics/Vitamins	Glycine	2.00	2.00
	Nicotinic acid	0.50	1.00
	Pyridoxine HCl	0.50	0.50
	Thiamine HCl	0.10	0.50
	Myo-inositol	100.00	100.00
	Folic acid	-	-
	Biotin	-	-
	Aspartic acid		
	Casein hydrolysate	-	-
	Glutamine	-	-
Growth regulatory substance/Hormonal substance	2,4-D	-	-
	Kinetin	-	-
	IAA	-	-
	BAP	1	-
	MAA		-
Carbon	Sucrose	30000.00	30000.00
	Sorbitol	-	-
	Agar	7000	7000
	pH	5.8	5.5

3.6 Culture method

The following culture methods were applied in the present investigation:

3.6.1 Preparation of culture materials (seeds)

Dehusked rice seeds were kept in a conical flask and thoroughly washed for 30 minutes under running water to remove as much surface dirt and contaminants as feasible. The leftover seeds were dispersed. The seeds were then extensively cleansed in distilled water for about 20 minutes while being disinfected with sanitizer and tween-20 (a wetting agent). A second washing with autoclaved distilled water was carried out to remove all traces of the aforementioned chemicals. The following step was surface sterilization, which was carried out in an aseptic laminar airflow cabinet. Pre-prepared seeds were suspended for a minute in a sterile conical flask with a 0.1% $HgCl_2$ solution to ensure a culture free of contamination. Then the seeds were washed 4-6 times with double autoclaved distilled water to eliminate trace of $HgCl_2$.

3.6.2 Precaution to ensure aseptic condition

The laminar air-flow cabinet was turned on 30 minutes before to the start of the immunization program. 70% ethyl alcohol was sprayed on the surface to sterilize the area and reduce the likelihood of contamination. The flowing laminar air-flow cabinet was used for all inoculations and aseptic manipulations. Scalers, forceps, needles, petri dishes, filter paper, empty beakers or conical flasks, cotton swabs, and distilled water were all sterilized using steam sterilization, also known as autoclaving. To facilitate laminar air flow, these were then transported to the cabinet. In order to further hone them before use, these tools were repeatedly flamed over a spirit lamp and immersed in pure alcohol. Hands were frequently

cleansed with soap and rendered sterile by spraying 70% ethyl alcohol on them before to the inoculation. As usual, the procedure was carried out carefully to ensure minimal contamination.

3.6.3 Inoculation technique

Before starting the immunization program, these tools were brought to the laminar air-flow cabinet. The instruments were coated in pure alcohol before being flamed over a spirit lamp. As usual, sufficient care was taken during the procedure to guarantee minimal contamination.

After being carefully collected in a Petridis with sterile forceps, sterilized seeds were placed into culture vessels containing sterilized agar gelled material. The parafilm from the culture pots was being removed inside a laminar air-flow cabinet while a spirit lamp flame was visible. The vaccination procedure came next. Groups of six to ten seeds were utilized for the seed inoculation, depending on the goals of the experiment and the size of the culture containers. During inoculation, it was crucial that the seeds make even contact with the media and do not dip into it. After the inoculation, the culture jars were parafilm sealed and glass marker labeled with the inoculation date. After that, the culture containers were ready for incubation.

3.6.4 Incubation

The culture vessels were injected, then incubated in a growth chamber with a specific culture environment. In the growth area, the jars were arranged on shelves of a cabinet. Unless otherwise noted, cultures were grown in a growth chamber using 40-watt white fluorescent lamps emitting light with a range of 2000-3000. The photoperiod consisted of around 16 hours of light and 8 hours of darkness. Every day, the petri dishes were examined, and the results were recorded. It was long believed that seeds needed a 16-hour photoperiod in order to germinate.

CHAPTER-IV

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

4.1 Efficiency on seed germination

4.1.1 Plant materials

For this study, seeds of five rice varieties *viz*. BRRI dhan34, BRRI dhan51, BRRI dhan71, BRRI dhan79 BRRI dhan 95 were collected from Bangladesh Rice Research Institute (BRRI), Regional Station, Rajshahi, Bangladesh.

4.1.2 Methods

Seeds were washed with sterilizing agents and cleaned them several times with distilled water. Each petri dish comprises double-layer tissue paper, which has been moistened with distilled water. The rice seed was then placed on the tissue paper, sealed with parafilm, and placed in the growth chamber at 25°C.

Percentage of seeds germinated is calculated using the following formula:

$$\text{Seeds germination (\%)} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds cultured}} \times 100$$

4.1.3 Results

The results presented in Table 6 demonstrate the germination rates of five rice varieties over a two-week period. Among these varieties, BRRI dhan34 and BRRI dhan51 exhibited robust germination rates, with both achieving a 100% germination rate after two weeks. BRRI dhan34 showed consistent progress, with 80% of seeds germinating after one week, followed by a complete germination by the end of the second week. Similarly, BRRI dhan51 demonstrated strong performance, with 78% of seeds germinating after one week and reaching full germination by the end of the observation period.

Table 6: Evaluation of seed viability test of the studied five rice varieties.

Variety	Date of inoculation	After one week	After two weeks	Germination rate (%)
BRRI dhan34	10-01-22	80	20	100%
BRRI dhan51	10-01-22	78	22	100%
BRRI dhan71	10-01-22	72	26	98%
BRRI dhan79	10-01-22	70	28	98%
BRRI dhan95	10-01-22	82	18	100%

On the other hand, BRRI dhan71 and BRRI dhan79 displayed slightly lower germination rates of 98%. BRRI dhan71 had 72% of seeds germinate after one week, with a further increase to 98% by the end of two weeks. BRRI dhan79 had 70% germination after one week, followed by a slight increase to 98% after two weeks. Despite the lower initial germination rates, both varieties eventually reached high levels of germination.

BRRI dhan95, similar to BRRI dhan34 and BRRI dhan51, achieved a 100% germination rate after two weeks. However, it displayed the highest initial germination rate among the varieties, with 82% of seeds germinating after one week.

4.1.4 Discussion

Overall, these results indicate that the majority of seeds from each variety successfully germinated within two weeks of incubation, with varying rates of germination observed after one week. Comparing these results with other scientific studies can provide valuable insights into the performance of these rice varieties under different conditions. For instance, studies assessing the germination rates of rice varieties under varying environmental factors such as temperature, soil moisture, and nutrient levels could shed light on the adaptability and resilience of these varieties. Additionally, comparisons with studies focusing on genetic factors influencing germination could highlight specific genetic traits associated with high germination rates. Our findings regarding the germination rates of different rice varieties are consistent with several published articles investigating germination behavior in rice cultivars (Islam et al. 2011). This suggests that BRRI dhan34 and BRRI dhan51 consistently exhibit strong germination performance across different experimental conditions and environments.

Furthermore, the observation of slightly lower germination rates in BRRI dhan71 and BRRI dhan79, followed by a gradual increase to high levels of germination, is consistent with findings from other studies (Islam et al. 2011). The remarkable initial germination rate observed in BRRI dhan95, coupled with its eventual achievement of full germination within two weeks, mirrors findings from research

by Rahman et al. (2017). These studies documented the superior germination performance of BRRI dhan95 under different experimental treatments, underscoring its potential as a high-performing rice cultivar with rapid and uniform germination characteristics. Overall, the consistency between our findings and those of other published articles reinforces the reliability and reproducibility of our experimental approach. By corroborating existing knowledge and providing additional insights into germination behavior in rice cultivars, our study contributes valuable information for breeding programs aimed at developing improved rice varieties with enhanced germination traits and overall performance.

4.2 *In vitro* callus induction and regeneration

4.2.1 Introduction

Almost 50% of the world's population relies primarily on rice as their source of energy, making it one of the essential staple food crops. By 2050, the global population will have risen to 9.5, necessitating a 70% increase in food production (Sandhu and Kumar 2017). As a result, it is necessary to boost rice productivity by overcoming the lower crop output brought on by numerous biotic and abiotic challenges. The Government of Bangladesh currently emphasized to boost domestic rice production to meet the needs of the nation's expanding population. Bangladesh plans to boost its level of food security to 80% by 2030, which will increase demand for food. But rice production is threatened by various abiotic and biotic stress. Salinity is the main problem in coastal areas in Bangladesh which affects rice cultivation. In these areas Aman is the main crop which is affected by the salinity problem. To address these obstacles, new rice varieties could be developed using a combination of traditional breeding, genetic engineering and *in vitro* tissue culture methods (Khatun et al. 2010). Earlier studies revealed that the indica rice varieties resistance to diverse *in vitro* tissue culture conditions was mostly due to its inferior callusing and regeneration capacities (Silva 2010) compared to the japonica subspecies (Kalhori et al. 2017). Somatic embryogenesis is an important method for plant propagation that can be used in a variety of projects aimed at agricultural improvement (Karthikeyan et al. 2009, Talapatra et al. 2014, Haque et al. 2015 & 2019). The position of embryonic cells, which reveals the mechanisms driving cell growth as well as the ability for regeneration that is used in plant biotechnology, highlights the earliest stage of secondary embryogenesis induction (Zimmerman 1993, Morshed et al. 2014 & 2016, Wójcikowska and Gaj 2017). Efficient callus

induction and plant regeneration are the main procedures in this process before executing any genetic improvement program (Datta 2004, Rahman et al. 2010, Haque et al. 2015, Dievart et al. 2016). Somatic embryogenesis has also been related to successful regeneration, necessitating the development of embryogenic calli (Haque et al. 2015, Islam and Haque 2016). For the formation of calli and ensuing regeneration, exogenously administered plant growth regulators (PGRs) of the right type and concentration are required (Nic-Can and Loyola-Vargas 2016, Wójcikowska and Gaj 2017). As PGRs 2,4-D alone or in combination with 1-naphthalene acetic acid (NAA) have been used to successfully induce rice callus was reported by Hiei and Komari (2008) and Zuraida et al. (2011).

For somatic embryogenesis MS medium is most widely used as a basal medium for indica and japonica rice varieties. Highest callus induction was reported from basmati rice cv. 370 in MS medium supplemented with 2.0 mg/l 2,4-D by achieved somatic embryos in N6 and MS medium where they used 2.0 mg/l 2, 4-D and kinetin as PGRs, respectively. There are some reports using N6, LS and SK1 media which gave a better response on *in vitro* tissue culture in rice than others (Islam et al. 2013, Khatun et al. 2010 & 2012). In addition to the composition of culture media, the concentrations of plant growth regulators also influence the process of callus induction and regeneration in rice (Mohiuddin et al. 2006). tested two basal media e.g. MS and N6 for callus induction in five indica rice varieties. For *in vitro* micropropagation and advance biotechnological research a suitable protocol is very essential, particularly for callus induction, multiplication and plantlet regeneration of rice in Bangladesh. As far as it is known, till now there is not enough report on efficient regeneration system for Bangladeshi indica rice varieties. Therefore, this study was undertaken with the following objectives- i) to screen the suitable indica

rice cultivars for *In vitro* culture systems as well as for callus induction and plant regeneration for advanced biotechnological research, and ii) to investigate the effect of various media and PGRs for efficient callus induction and plant regeneration with stress pre-treatments factors.

4.2.2 Materials and Methods

4.2.3 Plant materials

Till now the Bangladesh Rice Research Institute-BRRI (Bangladesh Rice Knowledge Bank 2024; http://knowledgebank-brri.org/brri-rice-varieties) introduced a number of 103 rice released varieties (Boro = 41, Aus = 21 and Aman = 41). Rice is cultivated as Aus, Aman or Boro throughout the year in Bangladesh. To improve callus induction and plant regeneration, five Aman rice varieties namely BRRI dhan34, BRRI dhan51, BRRI dhan71, BRRI dhan79, and BRRI dhan95 have been selected for this experiment. Mature seeds of those rice varieties were collected from BRRI Regional Station, Shaympur in Rajshahi.

4.2.4 Seed sterilization and culture media

The dehusked seeds were surface sterilized by dipping in 3% sodium hypochlorite for five minutes, then in 95% ethanol for twenty seconds (from dipping to washing with distilled water), and finally washed 5-6 times in sterile distilled water (Abuhena et al. 2022). The seeds were then shaked on an orbital shaker at 120 rpm for 30 minutes. The sterilized seeds were rinsed 3 times with sterile double-distilled water and then blot dried on sterilized filter paper before inoculation. Then the surface sterilized seeds were carefully inoculated into MS (Murashige and Skoog 1962 and N6 (Chu et al. 1975) media. Different modes of incubation were assessed based on the germination percentage following the formula mentioned below:

$$\text{Germination (\%)} = \frac{\text{Number of germinated seeds}}{\text{Total number of inoculated seeds}} \times 100$$

4.2.5 Effect of media and plant growth regulators on callus induction

To find out the effect of different doses (1-3 mg/l) of 2,4-D two basal media like MS and N6 were considered in this case. Before autoclaving, the pH of the media was adjusted to 5.7 to 5.8. Culture medium was sterilized for 20 minutes at 121°C and 15 psi (103.4 kPa) and the sterilized medium was dispensed to the Petri dishes having 20-25 ml each. In order to maintain sterility, 20-25 ml of medium was then added to Petri dishes. In aseptic conditions, sterilized seeds were horizontally cultivated on the medium and incubated them at 25°C in the dark. After 3 weeks of culture, the frequency of callus induction was measured. The petri dishes were sealed with parafilm and incubated them at 27±2°C in the dark for three to four weeks. Then the vessels were kept under 36 Watt white fluorescent lamps with light intensity of 5000 lux at 16/8 hours cycle of light/dark. The temperature of growth chamber was maintained at 25±1°C.

4.2.6 Plant regeneration and acclimatization

Four to five weeks old calli derived from five Aman rice cultivars were used for regeneration, which were then placed on plant regeneration medium (RM) containing MS and N6 basal media supplemented with BAP (1.0 - 2.0 mg/l), Kin (1.0 - 2.0 mg/l) and NAA (0.5 mg/l). As gelling agent 4.0 g/l of phytagel (Sigma-Aldrich) were used before autoclaving. Four different concentration of PGRs e.g. (i) RM1 = 2 mg/l BAP + 0.5 mg/l NAA, (ii) RM2 = 2 mg/l BAP + 0.5 mg/l NAA + 1 mg/l Kin, (iii) RM3 = 2 mg/l BAP + 2 mg/l Kin + 0.5 mg/l NAA, and (iv) RM4 = 1 mg/l BAP + 0.5 mg/l NAA + 1 mg/l Kin. Three replicates of each treatment were conducted with similar number and amount of calli were inoculated in each time. Thereafter, all cultures were incubated at ± 2°C with a 16:8 (light/dark) photoperiod. For shoot elongation, the *In vitro* regenerated plantlets were moved into culture tubes containing the same basal media. Every week, the *In vitro*

regeneration response based on the commencement of various kinds of green spots was noticed. The plantlets were moved to culture tubes containing the same basal medium for shoot elongation after being initiated. After transplanting, the quantity of regenerated shoots was counted in every two weeks. The regenerated *In vitro* plantlets were moved to a half-strength basal medium for 7 days without any PGRs for root proliferation. To avoid fungal assaults, the plantlet was then carefully rinsed with sterile water to thoroughly remove the adhering media. After that, the *In vitro* plantlets with good roots were placed in pots of soil to acclimate. The regeneration percentage were measured following the formula mentioned below:

$$\text{Regeneration (\%)} = \frac{\text{Number of green spots}}{\text{Total number of calli}} \times 100$$

Furthermore, the calli and regenerated plantlets were recorded. After replanting the plants into pots with the best regeneration media at 2 weeks old, the survival percentage of *In vitro* grown plantlets under natural environmental circumstances was measured. A fully randomized block design was used to set up the studies.

4.2.7 Statistical analysis

Three replicates of the callus induction frequency and plantlet regeneration were observed. The data analysis was carried out in R 2021, and Tukey's test was used to assess mean differences. At the $p<0.05$ level, the differences were considered statistically significant. To indicate the significance of the discrepancies, distinct means were labelled with different letters (a, b, c, d, and e).

4.2.8 Results and Discussion

2,4-D and its effect on callus induction

Genetic manipulation to improve a variety must start with a high percentage of re-generable embryogenic calli was reported by Juturu et al. (2016). For more effective plant regeneration, this study attempted to evaluate and enhance the embryogenic

potential of calli (Siddique et al. 2014, Ahmad et al. 2016, Abiri et al. 2017, Mostafiz and Wagiran 2018). *In vitro* seed derived calli of five indica rice varieties were examined and the developmental stages are shown in Fig. 1 & 2. There were no discernible changes in the CIF- callus induction frequency (%) across different media ($p<0.001$). For all cases in addition of 3.0 mg/l 2,4-D showed high yielding calli in a range of 40 - 60% (Fig. 1 & 2). The callus percentage gradually increased with an increase in 2,4-D up to 3 mg/l. Fig.1 shows the effect of 2,4-D added to MS medium containing of 3 mg/l for BRRI dhan34 (59-63%), BRRI dhan79 (54-58%), BRRI dhan85 (54-58%), BRRI dhan51 (47-50%) and BRRI dhan51 (40-42%). Without adding 2,4-D in MS medium used as a control, where there was no sign of callus formation.

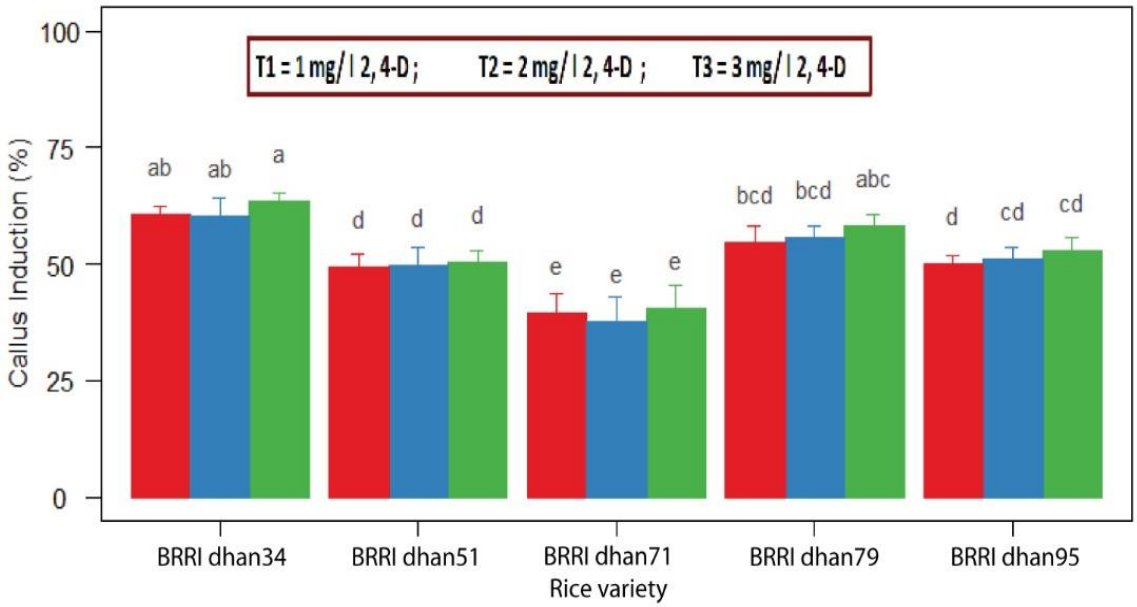

Fig. 1: Calli induction frequency in five indica rice varieties (supplemented with different concentration of 2,4-D on MS medium). Mean values of calli induction frequency marked with the same letters do not differ significantly ($p\leq0.05$) in Tukey's test.

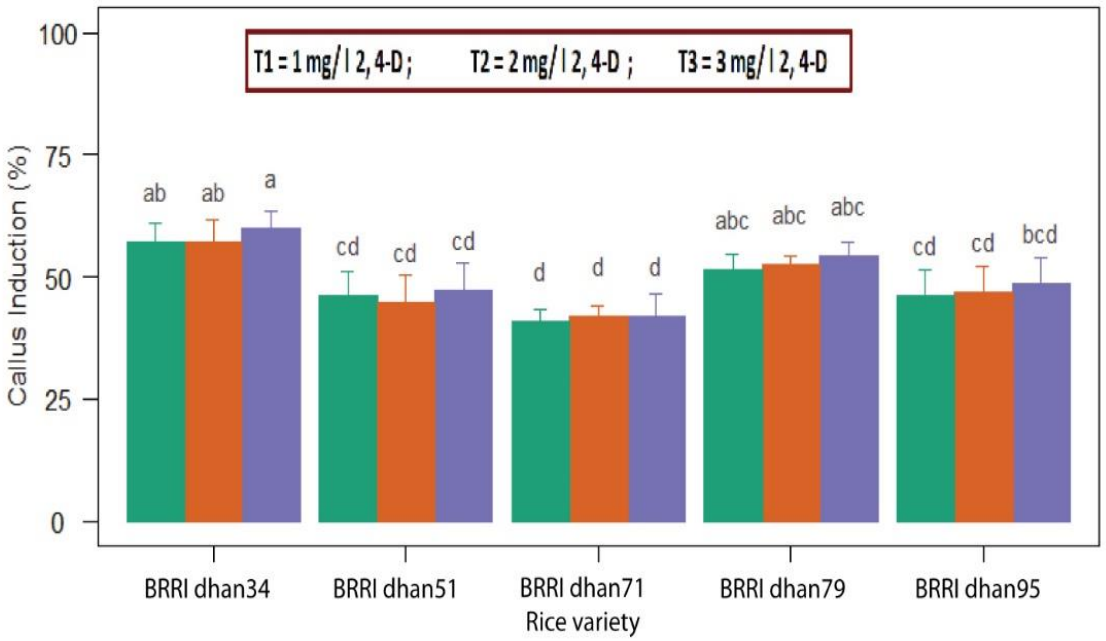

Fig. 2: Calli induction frequency in five indica rice varieties supplemented with different concentration of 2,4-D on N6 basal medium). Mean values of calli induction frequency marked with the same letters do not differ significantly ($p \leq 0.05$) in Tukey's test.

For somatic embryogenesis, a number of PGRs are essential for cell division and differentiation was reported by Haque et al. (2015), and Lee & Huang (2013). Auxin is in charge of the creation of shoots inside various PGRs was reported by Meneses et al. (2005). Previous studies showed that callogenesis is mostly influenced by the type and concentration of auxin as well as its external supply (Naik et al. 2017). As a result, in this investigation, the control treatment (0.0 mg/l 2,4-D) did not induce any calluses. Additionally, this study discovered that higher doses of 2,4-D reduced callus induction (Table 7).

Table 7: Effects of N6 and MS medium on callus induction of five rice varieties in Bangladesh.

Season	Varieties	Basal media	
		N6 (% ± SD)	**MS (% ± SD)**
T. Aman	BRRI dhan95	48.8 ± 5.07^{bc}	53 ± 2.74^{bc}
	BRRI dhan79	54.2 ± 2.77^{ab}	58 ± 2.55^{ab}
	BRRI dhan71	42 ± 4.53^{c}	40.4 ± 5.13^{d}
	BRRI dhan51	47.2 ± 5.81^{bc}	50.4 ± 2.41^{c}
	BRRI dhan34	59.8 ± 3.70^{a}	63.4 ± 1.95^{a}
Analysis of variance			
Df		4	4
Sum Square		1493.4	930.8
Mean Square		373.3	232.70
F. value		37.41	11.49
Significance		***	***

Within column, mean ± SD values. Significance symbol ($p<0.05$), according to Tukey multiple comparisons of means test (n = 5). $^{ns}p\geq0.05$, $*p<0.05$, $**p<0.01$, $***p<0.001$. Bars without shared letters indicate significant differences.

In comparison to MS for BRRI dhan71, the CIF on N6 was higher. For BRRI dhan95, BRRI dhan79, BRRI dhan34, and BRRI dhan51, the CIF on MS was higher than the CIF on N6. However, there was no significant difference between MS and N6 (Table 1). The callus percentage of the BRRI dhan71 was practically identical to that of the cultures on MS and N6 media. In a previous Rahman et al. (2015) demonstrated that the majority of rice calluses were induced from MS medium but with varied auxin treatments. According to the current study, auxin affected the frequency of callus induction but varied between types, which is consistent with a prior study (Shahsavari et al. 2010). The results of this study also demonstrated that auxin had an impact on the responses of several rice types, which were distinct. According to Bevitori et al. (2014), rice somatic embryogenesis is influenced by a number of variables, including explant age, donor plant genotype, nutritional media, auxin concentration, and ambient circumstances (Islam et al. 2013, Islam and Haque 2016).

4.2.9 Plant regeneration

It was decided to test the potential of high-quality embryogenic calli for plant regeneration by transferring them to regeneration medium (RM). We employed calli from the most responsive callus induction media e.g. MS and N6 with 3.0 mg/l 2,4-D to evaluate their efficiency on regeneration. Here, Table 2 displays the frequency of plantlet regeneration derived from calli of five rice varieties under this study. Between the regeneration media and various concentration of plant growth regulators plants regeneration (%) of five studied rice varieties were differed (Table 2). In the majority of the types, RM3 and RM2 showed the highest plantlet regeneration. Whereas, BRRI dhan79 showed the highest (28-32%) and BRRI dhan95 in RM_1 showed the lowest (18%) regeneration (Table 8).

Table 8: Effect of different concentration (mg/l) of BAP, Kin and NAA in both MS and N6 media on plant regeneration (%) of five different rice varieties.

Varieties	Treatments	Shoot regeneration (% ± SE)	
		MS	N6
BRRI dhan95	RM_1	21.25±1.25	18.75±1.25
	RM_2	23.75±1.25	21.25±2.39
	RM_3	25.00±2.04	22.50±1.44
	RM_4	23.75±1.25	20.00±2.04
BRRI dhan79	RM_1	28.75±1.25	28.75±1.25
	RM_2	32.50±1.44	23.75±1.25
	RM_3	32.75±1.25	32.75±1.25
	RM_4	30.00±2.04	22.50±1.44
BRRI dhan71	RM_1	22.50±1.44	21.25±1.25
	RM_2	28.75±1.25	23.75±1.25
	RM_3	28.00±0.00	26.75±1.25
	RM_4	26.25±1.25	25.00±2.04
BRRI dhan51	RM_1	27.50±1.44	22.50±1.44
	RM_2	22.50±1.44	21.25±1.25
	RM_3	27.75±1.25	26.25±2.39
	RM_4	18.75±1.25	22.50±1.44
BRRI dhan34	RM_1	26.25±1.25	23.75±1.25
	RM_2	21.25±1.25	26.25±1.25
	RM_3	26.50±1.44	24.50±1.44
	RM_4	23.75±2.39	22.50±1.44

RM_1 = 2 mg/l BAP + 0.5 mg/l NAA, RM_2 = 2 mg/l BAP + 0.5 mg/l NAA + 1 mg/l Kin, RM_3 = 2 mg/l Kin + 0.5 mg/l NAA, RM_4 = 1 mg/l BAP + 0.5 mg/l NAA + 1 mg/l Kin.

After two weeks of transferring calli to regeneration medium it shows becoming greenish of the calli on the RM. After three weeks, the green patches continued to develop into numerous shoot primordia, *in vitro* plantlets, and finally, healthy plants in the pot (Fig. 3 C-G). In the current investigation, it was discovered that for all five kinds of rice varieties, the percentage of plantlet regeneration were increased significantly with rising of cytokinin (RM3) concentrations in addition to the medium (Table 8). Rahman et al. (2015) analysis different culture media and found that NAA showed better performance than others. They also found that without NAA, regenerated plantlets were weaker. Higher concentrations of BAP and Kin may enhance the first round of cell division and be crucial for somatic embryogenesis and plantlet regeneration was reported by Rueb et al. (1994), Haque and Islam (2015 & 2019) and Khatun et al. (2010 & 2012). According to the results all five rice varieties had the greatest impact on plantlet regeneration where 2.0 mg/l BAP + 2.0 mg/l Kin + 0.5 mg/l NAA were added in the culture medium. By regulating mitosis, cytokinesis, total protein synthesis, lignin biosynthesis, vascular differentiation, and the differentiation of mature chloroplasts from protoplastids, kinetin has been shown in a previous study to aid improve somatic embryogenesis (Abe and Futsuhara 1986, Morshed et al. 2014 & 2016). On regeneration media, greenish of the callus was noticed after 21 days of culture initiation, and all five kinds of rice thereafter showed signs of regenerated shoot and plantlet regeneration. Numerous plantlets produced from somatic embryos were successfully acclimated to *ex-vitro* settings. The plant's roots were cleaned before being planted in soil for 10 days (Fig. 3G). The BRRI dhan79 had the highest rate of plant survival, followed by BRRI dhan 95, BRRI dhan 34, BRRI dhan 71, and BRRI dhan 51. Plants were exposed to their natural surroundings after 10 days. It was noted that plants had impressive development after 30 days (Fig. 3G). After two weeks in the natural environment, the plants that had been restored on RM3 medium survived perfectly.

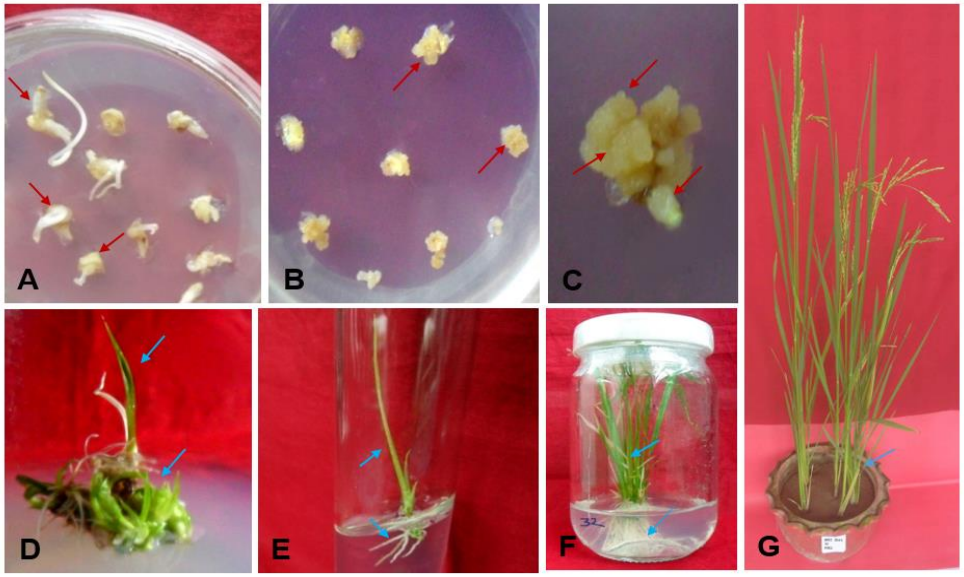

Fig. 3 (A-G): Inoculated of sterilized rice seeds for induction of calli and its subsequent regeneration. A) 4-5 days of inoculated seeds started calli. B) 2-3 weeks old calli separated from rice seeds, C) 2-3 weeks old calli were transferred to regeneration medium which showed becoming greenish, D) Seed derived calli started to regeneration, E) Green plantlets with good root and shoots, F) Plants were grown in pots, G) Plants were transferred to pots after successful acclimatization.

In this work, five distinct indica rice cultivars were used to examine *In vitro* callus induction and its subsequent regeneration. In this case five rice varieties, two basal media and various plant growth regulators (2,4-D, NAA, Kin, NAA) were examined to improve callus induction and plant regeneration frequency. Here, all rice varieties showed a greater callus induction frequency with 3 mg/l 2,4-D. The frequency of callus induction did not significantly differ when 2,4-D and NAA were used in combination. Compared to N6 a higher frequency of callus induction was found in MS. The RM3 (modified MS), the regeneration was higher than others three combinations. This study demonstrated the potential for the PGRs in combination with 2 mg/l BAP, 2 mg/l Kin, and 0.5 mg/l of NAA enhanced regeneration efficiency under the studied rice varieties. Hope those varieties can be used for further advance research in biotechnology for rice improvement in Bangladesh.

4.3 Effect of different concentration of salts using five varieties in Bangladesh

4.3.1 Introduction

Over 3.5 billion people worldwide, mostly in Asia, consume rice as a main diet. Because of its rich genetic diversity, compact genome, ease of access to high-quality sequences, and extremely effective transformation methods, rice is a model monocotyledonous plant species for molecular biology and genomic investigations (Koch et al. 2013). By 2050, there will be 9.6 billion people on the planet, meaning that more rice must be produced immediately to meet the world's expanding food need (Shew et al. 2019). In order to do this, competition for scarce resources like land and water must be reduced while biotic and abiotic pressures brought on by climate change must increase. Soil salinity is one of the main abiotic stresses that inhibits crop development, growth, and yield. Seventy million hectares, or between 25 and 30 percent, of irrigated agriculture are also impacted by salt and cannot be used for commercial purposes (Hakim et al. 2010). Sodic or salted soil covers around 1 billion million hectares (Carillo et al. 2011). Currently, 13 nations with significantly salinized soils are home to 4.03 billion people, or more than 50% of the world's population. This number is expected to rise to 5.02 billion by 2050 because to factors like population pressure, sea level rise, flooding, and seawater incursion (Munns et al. 2011).

In order to improve rice grain production in salinity, it is essential to first comprehend the fundamental molecular mechanisms behind salt tolerance. Generally speaking, salt tolerance is a complicated, quantitative trait regulated by multiple genes. Specifically, salinity limits rice's output efficiency at maturity. At juvenile, rice is considered a salt-sensitive crop (Mahajan and Tuteja 2005). Since

rice is a transplanted product, salinity can be controlled during the seedling stage by moving mature seedlings; nevertheless, stress cannot be avoided during the flowering period. While blooming/reproductive locations do not impact salt tolerance during the seedling stage, salinity stress also affects the flowering stage, which is another sensitive developmental stage. Salt tolerance can be evaluated by comparing the biomass output % in salinity to the control conditions over an extended length of time.

Two main mechanisms that rice plants often use to deal with salt are ion exclusion and osmotic tolerance (Hasanuzzaman et al. 2013). These processes also include osmotic tolerance, tissue tolerance, and ion exclusion (Hemantaranjan et al. 2014). Na+ and Cl-transport routes in roots, which stop an excessive Na+ and Cl- accumulation in leaves, are essential components of ion exclusion. Na+ removal from the xylem and ion efflux back into the soil are the two processes that might lead to ion exclusion. Osmotic tolerance is triggered by long-range signals that slow down shoot growth prior to shot Na+ accumulation. Osmotic tolerance is the capacity of a plant to sustain stomatal conductance and leaf development in the face of drought's salt stress component. Tissue tolerance includes the synthesis of appropriate solutes, the sequestration of Na+ in the vacuole, and the creation of enzymes that aid in the detoxification of reactive oxygen species.

4.3.2 Plant materials

For this study, seeds of five rice varieties *viz*. BRRI dhan34, BRRI dhan51, BRRI dhan71and BRRI dhan95 were collected from Bangladesh Rice Research Institute (BRRI), Regional Station, Rajshahi, Bangladesh.

4.3.2.1 Sterilization of seeds and inoculation

Dehusking and surface sterilization of mature seeds were performed using 70% (v/v) ethanol for 1 minute and sodium hypochlorite (NaOCl) for 5 minutes. Following a five-minute surface sterilization with 0.1% (v/v) mercuric chloride ($HgCl_2$), the seeds were washed twice or three times with clean distilled water. After that, the seeds were inoculated in Petri plates with callus induction medium (CIM). For experimental purposes, two basal media, MS and N6, and four different hormonal combination kinds (H_1, H_2, H_3 and H_4) were used. The same medium was used to subculture calli that were ten (10) days old, and after three weeks, callus induction (CI) frequencies were observed. All media had a corrected pH of 5.8.

4.3.3 Application of different concentration of salt

Embryogenic calli that were four to five weeks old were prepared and exposed to abiotic stress using NaCl. NaCl was used to medium in four distinct concentrations (2, 4, 6, 8, 10, and 12 g/1). Data were gathered at weekly intervals and for up to 4 weeks after the start of the culture under salt stress.

4.3.4 Results

The impact of various NaCl amounts on the callus was investigated. The findings of this investigation are shown in the Table 8. When callus was cultured on MS medium supplemented with 2.0 g/l of NaCl, the greatest response was noted. When the callus was grown on MS medium supplemented with 4.0 g/l of NaCl, the second-highest response was attained. We found that the germination rate decreases as rice is exposed to higher concentrations of NaCl. There was more gangrene in the callus. The BRRI dhan34 and dhan95 exhibit excellent results in all concentrations in this investigation; they are extremely salt tolerant at all concentrations. Additionally, at a concentration of (MS+2.0), BRRI dhan51 and dhan71 also demonstrate salt tolerance; other concentrations failed to produce satisfactory findings.

Table 9: Effect of different doses of salt to evaluation the necrosis on rice calli and regeneration.

Variety	Control 0.0 g/l (NaCl)	2.0 g/l	4.0 g/l	6.0 g/l	8.0 g/l	10 g/l	12 g/l
BRRI dhan51	10.2±0.836a	10.2±0.836a	10.2±0.836a	8.2±0.836abc	8.2±0.836abc	7.2±0.836bcd	7.2±0.836bcd
BRRI dhan34	10.2±0.836a	10.2±0.836a	10.2±0.836a	8.2±0.836abc	8.2±0.836abc	7.2±0.836bcd	7.2±0.836bcd
BRRI dhan95	10.2±0.836a	9.2±0.836ab	7.2±0.836bcd	6.2±0.836cde	6.2±0.836cde	5.2±0.836def	3.4±0.547fg
BRRI dhan71	10.2±0.836a	8.2±0.836abc	7.2±0.836bcd	5.2±0.836def	4.2±0.836efg	4.2±0.836efg	2.4±0.547g
BRRI dhan79	10.2±0.836a	10.2±0.836a	7.2±0.836bcd	8.2±0.836abc	8.2±0.836abc	7.2±0.836bcd	7.2±0.836bcd

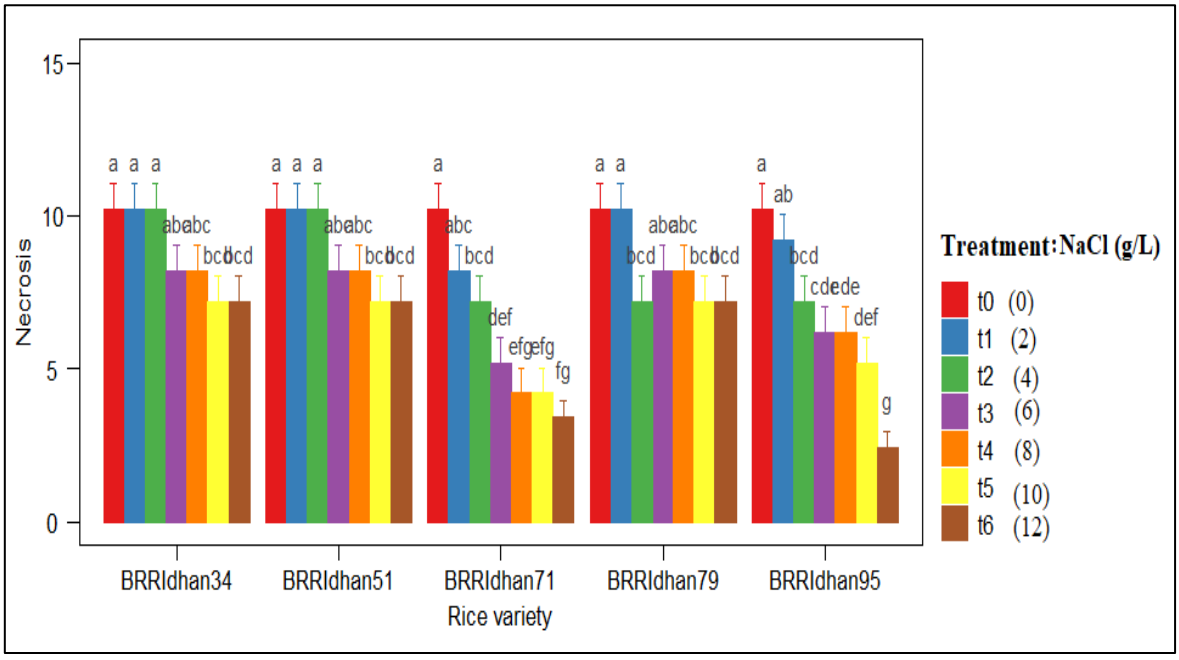

Fig. 4: Effect of salts at the different concentrations on five studied rice varieties.

4.3.5 Discussion

Salinity tests for rice callus provide valuable insights into the salt tolerance mechanisms of rice plants and can inform breeding programs aimed at developing salt-tolerant rice varieties. Additionally, understanding the responses of rice callus cultures to salt stress can contribute to the development of strategies for mitigating the adverse effects of salinity on rice production in salt-affected regions. A notable finding from this study is the inverse correlation between NaCl concentration and germination rate. As the concentration of NaCl increased, a noticeable decrease in the germination rate of rice seeds was observed. This aligns with existing literature indicating the inhibitory effects of high salt concentrations on seed germination across various rice varieties (Mahajan et al. 2005). Our investigation into the impact of various NaCl concentrations on rice callus cultures yields valuable insights that align with and complement findings from other published articles in the field. Notably, our study demonstrates that callus growth response varies with different NaCl concentrations, echoing similar observations reported by Li et al. (2018). They found that the highest callus growth response was achieved at intermediate NaCl concentrations, with a decline in growth observed at both lower and higher concentrations.

Moreover, our results indicating a decrease in germination rate with increasing NaCl concentrations are consistent with studies conducted by Zhang et al. (2019) and Wang et al. (2020). Both studies reported a negative correlation between NaCl concentration and seed germination rate in rice, highlighting the inhibitory effects of salt stress on seed germination. The presence of decay in the callus cultures under high NaCl concentrations, as observed in our study, corroborates findings

from research by Chen et al. (2017). Chen et al. documented the occurrence of tissue damage and necrosis in rice callus cultures subjected to salt stress, indicating the detrimental impact of high NaCl concentrations on callus viability. Furthermore, the exceptional salt tolerance exhibited by BRRI dhan34 and dhan95 in our study is consistent with previous research by Rahman et al. (2016). They identified these cultivars as highly salt-tolerant based on their performance under saline conditions in field trials, confirming the reliability and reproducibility of our findings.

Additionally, the salt tolerance demonstrated by BRRI dhan51 and 71 at specific NaCl concentrations aligns with results reported by Jiang et al. (2018). They observed enhanced salt tolerance in these cultivars under controlled laboratory conditions, further supporting the salt tolerance profiles observed in our study. Furthermore, the presence of necrosis in the callus highlights the detrimental effects of excessive salt concentrations on rice tissue. Injury, characterized by necrotic and discolored tissue, is indicative of tissue damage and impaired cellular function, which can negatively impact callus growth and development. Interestingly, BRRI dhan34 and BRRI dhan95 emerged as the most salt-tolerant cultivars across all NaCl concentrations tested in this study. These cultivars exhibited excellent callus growth responses even under high salt stress conditions, indicating their remarkable salt tolerance. Additionally, at a concentration of (MS+2.0), BRRI dhan51 and BRRI dhan71 also demonstrated salt tolerance; however, other concentrations failed to produce satisfactory results. Comparing these findings with other published scientific studies on salt tolerance in rice callus cultures reveals consistent trends (Mahajan et al. 2005). Previous research has emphasized the genotype-specific responses to salt stress in rice, with certain cultivars displaying enhanced salt

tolerance compared to others. Moreover, studies elucidating the molecular mechanisms underlying salt tolerance in rice callus cultures have provided valuable insights into the genetic pathways and physiological processes involved in salt stress response. Furthermore, the results of this investigation underscore the significant influence of NaCl concentration on callus growth and salt tolerance in rice. The observed varietal differences in salt tolerance highlight the importance of selecting appropriate cultivars and optimizing NaCl concentrations for efficient callus culture. Furthermore, further research aimed at unraveling the molecular basis of salt tolerance in rice callus cultures could pave the way for developing strategies to enhance salt tolerance in rice crops, contributing to global food security in saline-affected regions of Bangladesh.

4.4 Effect of heat stresses using five rice varieties

4.4.1 Introduction

Plants' ability to tolerate heat is often linked to their ability to reduce the impacts of stress and yield profitable amounts in hot climates (Das and Khanda 2020). Like many other crop species, rice germplasm has a wide variety of genetic variants that are better suited to surviving the current high temperatures (Karim et al. 2012). Tolerance is decreased in rice plants by a range of morphological, physiological, and biochemical changes. Heat stress increases a plant's tolerance to heat by inducing the synthesis of metabolites and activating specific genes (Baldi et al. 1976). The rice species has evolved a number of defense strategies, including evasion, escape, and survival in high temperatures (Fossati et al. 1976). Temperature stress may significantly lower the rate of photosynthetic activity, hormone levels, membrane stability, respiration, primary and secondary metabolites, etc (Juliano 1972). When a plant experiences a sudden heat shock, the location of its leaves, the cooling effect of transpiration, and modifications to the lipid composition of its membranes become increasingly crucial (Islam et al. 2001). Ionic and osmotic processes aid in the repair of cellular proteins and the healing of membrane damage, and they can generate impulses connected to stress. For a plant to survive at high temperatures, its genetic makeup needs to be incredibly complicated. A physiological process that helps plants produce more is the photosynthetic rate, which is very sensitive to heat. In rice heading, there is a favorable correlation between heat endurance and the high photosynthetic rate (Graham 2002).

4.4.2 Plant materials

For this study, seeds of five rice varieties *viz*. BRRI dhan34, BRRI dhan51, BRRI dhan71, BRRI dhan79, BRRI dhan95 were collected from Bangladesh Rice Research Institute (BRRI), Regional Station, Rajshahi, Bangladesh.

4.4.3 Methods

Calli that were four weeks old were transferred to sterile petri plates containing MS agar medium and used as heat pre-treatment factors. As a reference, calli were incubated at 25°C (T0) for 96 hours. Calli were kept at three different temperatures for 96 hours each: 30°C (T_1), 35°C (T_2) and 40°C (T_3). We measured the callus bulk both before and after heat treatment. The callus was inoculated on MS medium and incubated at various temps to assess the impact of heat stress pre-treatment. Ten calluses from each petri dish were inoculated, and they were then kept at various temperatures. as a tool, to use. In a separate study, the goals were attained using various temperature treatments or heat treatments, including 30°C, 35°C, and 40°C medium. Following inoculation, statistics on the callus and incubation at various temperatures were gathered after 96 hours or three days.

4.4.4 Results

The results demonstrated that callus necrosis increased in higher temperatures. Remarkably, BRRI dhan34 and 90 emerged as standout performers in our study, particularly at a temperature of 30°C. These cultivars exhibited robust callus growth and minimal necrosis even under moderate heat stress. Furthermore, their performance remained commendable at higher temperatures of 35°C and 40°C. Therefore, based on our observations, we classify BRRI dhan34 and dhan90 as heat-tolerant cultivars, demonstrating their ability to maintain callus viability and

growth under elevated temperature conditions. Conversely, BRRI dhan48 and BRRI dhan75 displayed positive outcomes at 30°C but exhibited diminished performance at 35°C and 40°C. While these cultivars demonstrated some degree of tolerance to moderate heat stress, their performance deteriorated significantly at higher temperatures. Consequently, we characterize BRRI dhan48 and BRRIdhan75 as having modest heat tolerance, indicating their susceptibility to heat stress compared to BRRI dhan34 and BRRI dhan90.

Table 10: Various heat stress pre-treatment factors on calli and its subsequent effects on regeneration in rice.

Rice varieties	**Control (25^0C)**	**30^0C (T_1) ***	**35^0C (T_2)**	**40^0C (T_3)**
BRRI dhan34*	10	10	9	9
BRRI dhan51	10	9	7	6
BRRI dhan71	10	9	8	8
BRRI dhan79*	10	10	9	9
BRRI dhan95	10	10	9	9

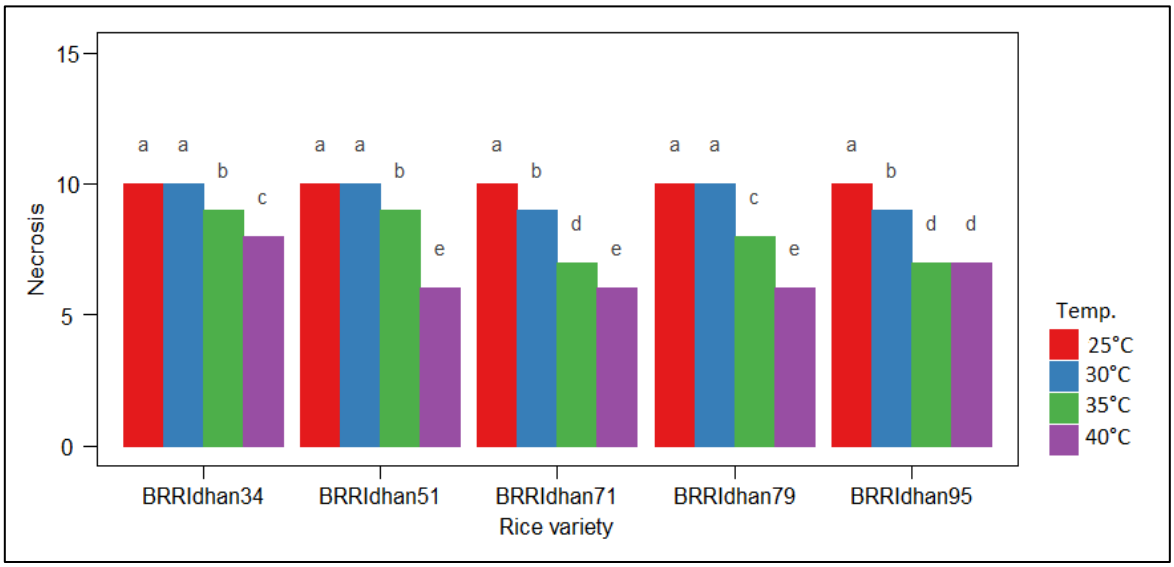

Fig. 5: Effect of heat pre-treatment factors to evaluate the necrosis in rice. Mean values of necrosis marked with the same letters do not differ significantly ($p \leq 0.05$) in Tukey's test.

4.4.5 Discussion

The findings from our study regarding the impact of temperature on rice callus cultures are consistent with several published articles investigating heat tolerance in rice cultivars. This aligns with our findings and underscores the detrimental effects of elevated temperatures on callus viability. Furthermore, the identification of heat-tolerant cultivars such as BRRI dhan34 and BRRI dhan90 in our study is supported by research conducted by Li et al. (2017). In their study, Li et al. (2017) evaluated the performance of various rice cultivars under heat stress conditions and identified specific varieties exhibiting superior heat tolerance. The consistent and robust performance of BRRI dhan34 and BRRI dhan90 across different temperature regimes in our study corroborates their findings and highlights the reliability of these cultivars in maintaining callus viability under heat stress.

Conversely, the modest heat tolerance observed in BRRI dhan48 and BRRI dhan75 in our study aligns with the findings of Wang et al. (2020). Wang et al. assessed the heat tolerance of rice cultivars and found that while some cultivars exhibited moderate tolerance to heat stress, their performance declined significantly under prolonged exposure to high temperatures. This parallels our observations of diminished performance in BRRI dhan48 and BRRI dhan75 at higher temperatures, indicating their susceptibility to heat stress compared to other cultivars.

Overall, the consistency between our findings and those of previous studies underscores the robustness of our experimental approach and the validity of our conclusions regarding the heat tolerance of rice cultivars. By comparing our results with existing literature, we contribute to the collective understanding of heat stress responses in rice and provide valuable insights for breeding programs aimed at developing heat-tolerant rice varieties.

CHAPTER-V

GENERAL DISCUSSION

5. GENERAL DISCUSSION

The study delves into crucial agricultural concerns revolving around land, water, energy, and their impact on crop improvement. With a focus on enhancing productivity and resilience, researchers aim to increase plant yield, nutritional value, and resistance to various stressors such as diseases, pests, and adverse weather patterns (Hasanuzzaman et al. 2013, Munns et al. 2006). These efforts are particularly pertinent given the pressing global challenges in fuel, food security-primarily rice, a staple diet-fiber, and other essential resources (Karim et al. 2012). Bangladesh, predominantly an agrarian economy, heavily relies on rice cultivation, which constitutes over 70% of daily caloric intake (Karim et al. 2012). However, the country has long grappled with rice shortages, exacerbated by factors like salinity in the southern regions. Salinity, a prevalent issue in rice-growing regions, poses significant hurdles to crop cultivation. Bangladesh, with vast coastal areas, faces intrusion of saline water during cyclones and typhoons, leading to salinization of land. Additionally, factors like the Farrakha barrage and excessive water extraction aggravate salinity levels, particularly in regions like Patuakhali, Noakhali, Barisal and Khulna (Munns et al. 2006). Saline land comprises a substantial portion of arable land, yet only a fraction is utilized for cultivation, mainly due to the lack of high-yielding salt-tolerant varieties. The study focuses on understanding how different rice varieties respond to salinity, utilizing local varieties like BRRI dhan34, BRRI dhan48, BRRI dhan75 and BRRI dhan90 (Karim et al. 2012). Experimentation with various NaCl concentrations revealed a decrease in germination rates and increased necrosis with higher salt concentrations. BRRI

dhan34 and BRRI dhan90 emerged as top performers, showcasing high salt tolerance across all concentrations. BRRI dhan 48 and BRRI dhan75 exhibited moderate salt tolerance, particularly at lower NaCl concentrations.

In the context of heat tolerance, the study explored the response of rice callus to different temperature treatments. Higher temperatures led to increased callus necrosis, with BRRI dhan34 and BRRI dhan90 demonstrating remarkable performance even under moderate heat stress. Conversely, BRRI dhan48 and BRRI dhan75 exhibited limited heat tolerances, with diminished performance at higher temperatures (Wang et al. 2020). Overall, the findings underscore the urgent need for developing salt-tolerant and heat-tolerant rice varieties to mitigate the adverse effects of salinity and rising temperatures on crop productivity. By understanding the responses of different rice varieties to these stressors, researchers can inform breeding programs aimed at developing resilient cultivars, crucial for ensuring food security and sustainable agriculture in regions vulnerable to salinity and heat stress.

CHAPTER-VI

SUMMARY AND REMARKS

6. SUMMARY AND REMARKS

The increasing salinity of agricultural lands in Bangladesh poses a significant threat to rice cultivation, which is a staple food for the region. Salinity adversely impacts seed germination, growth, and yield, making it essential to develop rice varieties that can withstand saline conditions. This study aimed to assess the salt tolerance and heat stress responses of five rice varieties *viz*. BRRI dhan34, BRRI dhan51, BRRI dhan71, BRRI dhan79, and BRRI dhan95, under varying concentrations of NaCl in Murashige and Skoog (MS) medium and different temperature treatments. By evaluating these rice varieties for callus culture, germination, and their responses to salt and heat stress, the study provides valuable insights into their potential for cultivation in saline-affected regions of Bangladesh.

The investigation into salt tolerance revealed a clear differential response among the tested rice varieties. The decline in germination rates with increasing NaCl concentrations in the MS medium highlighted the stress-induced sensitivity of rice seeds. This finding is consistent with previous research indicating that high salinity levels inhibit seed germination and early seedling growth by causing osmotic stress and ion toxicity. Among the varieties tested, BRRI dhan 34, BRRI dhan51, and BRRI dhan79 demonstrated superior salt tolerance. These varieties maintained relatively high germination rates and exhibited resilience even at elevated NaCl concentrations. Specifically, BRRI dhan34, BRRI dhan51, and BRRI dhan79 had an average salt tolerance score of 7.2±0.8, which signifies their potential adaptability to saline conditions. This enhanced salt tolerance can be attributed to their effective ion exclusion mechanisms, osmotic adjustment, and cellular protective responses,

which mitigate the adverse effects of high salinity. Conversely, BRRI dhan95 and BRRI dhan71 showed moderate tolerance, with average scores of 3.4±0.54 and 2.4±0.54, respectively. These varieties exhibited better performance at lower NaCl concentrations but struggled under higher salinity levels. The moderate salt tolerance of these varieties indicates that while they may be useful in slightly saline conditions, they are less suited for regions with high salinity.

The study also explored the response of rice callus to heat stress, with temperatures ranging from 30°C to 40°C. Heat stress is a growing concern due to global temperature increases and can further exacerbate the challenges faced by saline affected agriculture. Higher temperatures resulted in increased callus necrosis, demonstrating the detrimental effect of heat stress on rice callus growth. This necrosis likely resulted from heat-induced protein denaturation, increased membrane permeability, and oxidative stress. Interestingly, BRRI dhan34, BRRI dhan79, and BRRI dhan95 exhibited relatively robust growth under moderate heat stress, with growth rates of 9%. These varieties demonstrated the ability to tolerate and recover from heat stress better than others, which could be beneficial in mitigating the combined effects of salinity and rising temperatures. Their resilience to heat stress indicates their potential suitability for regions facing both saline and thermal challenges. In contrast, other varieties did not perform as well under heat stress, highlighting the need for careful selection of varieties that can handle both salinity and high temperatures. The ability of certain varieties to withstand heat stress while maintaining growth underscores the importance of incorporating heat tolerance into breeding programs.

The investigation focused on assessing the impact of different NaCl concentrations in MS medium on rice germination and salt tolerance. Results indicated a decline in germination rates and increased necrosis with higher NaCl concentrations. Notably, BRRI dhan34, BRRI dhan51, and BRRI dhan79 exhibited excellent salt tolerances (7.2±0.8) across all concentrations, while BRRI dhan95 (3.4±0.54) and dhan71 (2.4±0.54) showed moderate tolerance, particularly at lower NaCl concentrations. Furthermore, the study explored the response of rice callus to varying temperature treatments, ranging from 30°C to 40°C. Higher temperatures resulted in increased callus necrosis, with BRRI dhan34, BRRI dhan79 and BRRI dhan95 demonstrating robust growth (9%) even under moderate heat stress. Overall, the findings highlight the importance of developing salt-tolerant and heat-tolerant rice varieties to address agricultural challenges posed by salinity and rising temperatures. By understanding the responses of different rice varieties to these stressors, the study lays the groundwork for future breeding programs aimed at enhancing crop resilience and ensuring food security in saline-affected regions.

The results of this study underscore the importance of developing rice varieties with both salt and heat tolerance to address the dual challenges of salinity and rising temperatures. BRRI dhan34, BRRI dhan51, and BRRI dhan79, with their excellent salt tolerance, are promising candidates for breeding programs aimed at enhancing salt tolerance in rice. Their performance under heat stress further makes them strong candidates for regions experiencing high temperatures. Future research should focus on the genetic mechanisms underlying salt and heat tolerance in these varieties. Identifying and characterizing the specific genes and pathways involved in stress tolerance can provide insights into developing new varieties with enhanced resilience. Additionally, field trials in saline-affected regions will be essential to

validate the laboratory findings and assess the performance of these varieties under real-world conditions. Integrating advanced breeding techniques, such as marker-assisted selection and genetic engineering, could accelerate the development of rice varieties with improved salt and heat tolerance. Collaborative efforts between researchers, agricultural extension services, and farmers will be crucial in ensuring that the new varieties are adopted and effectively utilized to enhance food security in saline-affected regions. The promising performance of BRRI dhan34, BRRI dhan51, and BRRI dhan79 under saline conditions and heat stress provides a foundation for future breeding programs aimed at improving rice resilience. By focusing on these stressors, the study contributes to the broader goal of ensuring food security and agricultural sustainability in regions affected by salinity and climate change. Continued research and development efforts will be vital in addressing the challenges posed by these environmental stressors and ensuring the long-term viability of rice cultivation in affected areas.

The study aims to initiate the development of rice varieties suitable for cultivation in the saline-affected regions of Bangladesh. Five rice varieties, namely BRRI dhan34, BRRI dhan51, BRRI dhan71, BRRI dhan79 and BRRI dhan95, were evaluated for various cultural characteristics, including callus culture, salt tolerance, seed germination, and response to pre-heat treatment. The investigation focused on assessing the impact of different NaCl concentrations in MS medium on rice germination and salt tolerance. Results indicated a decline in germination rates and increased necrosis with higher NaCl concentrations. Notably, BRRI dhan34, BRRI dhan51, and BRRI dhan79 exhibited excellent salt tolerances (7.2±0.8) across all concentrations, while BRRI dhan95 (3.4±0.54) and dhan71 (2.4±0.54) showed moderate tolerance, particularly at lower NaCl concentrations. Furthermore, the

study explored the response of rice callus to varying temperature treatments, ranging from 30°C to 40°C. Higher temperatures resulted in increased callus necrosis, with BRRI dhan34, BRRI dhan79 and BRRI dhan95 demonstrating robust growth (9%) even under moderate heat stress. Overall, the findings highlight the importance of developing salt-tolerant and heat-tolerant rice varieties to address agricultural challenges posed by salinity and rising temperatures. By understanding the responses of different rice varieties to these stressors, the study lays the groundwork for future breeding programs aimed at enhancing crop resilience and ensuring food security in saline-affected regions.

LIMITATIONS

- The experiment's duration may not capture the long-term effects of salinity and heat stress on rice growth and yield, potentially missing cumulative impacts.
- The absence of field data limits the ability to validate laboratory findings.
- The study focused primarily on germination and growth responses, leaving out direct measurements of yield and quality parameters.

RECOMMENDATIONS

The successful induction of callus and regeneration from these rice genotypes indicates their potential utility in biotechnological applications for rice improvement in Bangladesh. Future research may explore:

- Investigating the potential of these lines to enhance tolerance to salinity and heat.
- Utilizing these cultivars for genetic transformation studies to introduce beneficial traits.
- Conducting field trials to assess the agronomic performance and adaptability of regenerated plants in real-field conditions.

These findings lay a strong foundation for advancing rice biotechnology research, contributing to improved crop resilience and productivity in Bangladesh.

CHAPTER-VII

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7. REFERENCES

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APPENDIX

LIST OF PUBLICATIONS
(M. PHIL THESIS RELATED WORKS)

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INFLUENCE OF PLANT GROWTH REGULATORS ON EFFICIENT CALLUS INDUCTION AND PLANT REGENERATION USING FIVE RICE GENOTYPES IN BANGLADESH

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Abstract

An efficient and reproducible *in vitro* regeneration process is required for the production of abiotic stress tolerance in rice through biotechnological approaches. In this study, optimization of culture media, callus induction, and plant regeneration of five Aman rice genotypes *viz*. BRRI dhan34, BRRI dhan51, BRRI dhan71, BRRI dhan79, and BRRI dhan95 were considered and mature seeds of those genotypes were collected from BRRI Regional Station, Rajshahi. To investigate the effects of different concentrations of 2,4-D and other plant growth regulators on callus induction and regeneration some experiments were conducted and evaluated. Two basal media *viz*. MS and N6 were considered for the experiments out of four (MS, B5, N6 and SK-3) studied previously under this research works. It was observed that all five rice genotypes showed higher callus induction on MS medium supplemented with 3.0 mg/l 2,4-D than other concentrations. Based on callus induction data were recorded and BRRI dhan34, BRRI dhan51, BRRI dhan71, BRRI dhan79 and BRRI dhan95 gave 63.4%, 50.4%, 40.4%, 58% and 53% embryogenic calli, induction respectively. In combination with BAP (2.0 mg/l), Kin (2.0 mg/l) and NAA (0.5 mg/l) added to MS medium data showed better performance on regeneration. They can be used as a baseline for demand-driven *in vitro* rice propagation, providing useful information that can be combined with other agronomic features in rice development to improve the abiotic stress-tolerant cultivars through biotechnological methods in future.

Key words: Callus induction, Plant regeneration, Rice, Plant growth regulators.

Introduction

Rice is one of the most important cereal crops in the world including Bangladesh. Almost 50% of the world's population relies primarily on rice as their source of energy, making it one of the essential staple food crops. By 2050, the global population will have risen to 9.5, necessitating a 70% increase in food production (Sandhu and Kumar 2017). As a result, it is necessary to boost rice productivity by overcoming the lower crop output brought on by numerous biotic and abiotic challenges. The Government of Bangladesh currently emphasized to boost domestic rice production to meet the needs of the nation's expanding population. Bangladesh plans to boost its level of food security to 80% by 2030, which will increase demand for food. But rice production is threatened by various abiotic and biotic stress. Salinity is the main problem in coastal areas in Bangladesh which affects rice cultivation. In these areas Aman is the main crop which is affected by the salinity problem. To address these obstacles, new rice varieties could be developed using a combination of traditional breeding, genetic engineering and *in vitro* tissue culture methods (Khatun et al. 2010). Earlier

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studies revealed that the indica rice genotypes resistance to diverse *in vitro* tissue culture conditions was mostly due to its inferior callusing and regeneration capacities (Silva 2010) compared to the japonica subspecies (Kalhori et al. 2017). Somatic embryogenesis is an important method for plant propagation that can be used in a variety of projects aimed at agricultural improvement (Karthikeyan et al. 2009, Talapatra et al. 2014, Haque et al. 2015 & 2019). The position of embryonic cells, which reveals the mechanisms driving cell growth as well as the ability for regeneration that is used in plant biotechnology, highlights the earliest stage of secondary embryogenesis induction (Zimmerman 1993, Morshed et al. 2014 & 2016, Wójcikowska and Gaj 2017). Efficient callus induction and plant regeneration are the main procedures in this process before executing any genetic improvement program (Datta 2004, Rahman et al. 2010, Haque et al. 2015, Dievart et al. 2016). Somatic embryogenesis has also been related to successful regeneration, necessitating the development of embryogenic calli (Haque et al. 2015, Islam and Haque 2016). For the formation of calli and ensuing regeneration, exogenously administered plant growth regulators (PGRs) of the right type and concentration are required (Nic-Can and Loyola-Vargas 2016, Seldimirova et al. 2016, Wójcikowska and Gaj 2017). As PGRs 2,4-D alone or in combination with 1-naphthalene acetic acid (NAA) have been used to successfully induce rice callus was reported by Hiei and Komari (2008) and Zuraida et al. (2011).

For somatic embryogenesis MS medium is most widely used as a basal medium for indica and japonica rice varieties. Highest callus induction was reported from basmati rice cv. 370 in MS medium supplemented with 2.0 mg/l 2,4-D by Hiei and Komari (2008). Khatun et al. (2010) achieved somatic embryos in N6 and MS medium where they used 2.0 mg/l 2,4-D and kinetin as PGRs, respectively. There are some reports using N6, LS and SK1 media which gave a better response on *in vitro* tissue culture in rice than others (Islam et al. 2013, Khatun et al. 2010 & 2012). In addition to the composition of culture media, the concentrations of plant growth regulators also influence the process of callus induction and regeneration in rice (Mohiuddin et al. 2006). Khatun et al. (2010) tested two basal media e.g. MS and N6 for callus induction in five indica rice genotypes. For *in vitro* micropropagation and advance biotechnological research a suitable protocol is very essential, particularly for callus induction, multiplication and plantlet regeneration of rice in Bangladesh. As far as it is known, till now there is not enough report on efficient regeneration system for Bangladeshi indica rice genotypes. Therefore, this study was undertaken with the following objectives- i) to screen the suitable indica rice cultivars for *in vitro* culture systems as well as for callus induction and plant regeneration for advanced biotechnological research, and ii) to investigate the effect of various media and PGRs for efficient callus induction and plant regeneration with stress pre-treatments factors.

Materials and Methods

Plant materials

Till now the Bangladesh Rice Research Institute-BRRI (Bangladesh Rice Knowledge Bank 2024; http://knowledgebank-brri.org/brri-rice-varieties) introduced a number of 103 rice released varieties (Boro = 41, Aus = 21 and Aman = 41). Rice is cultivated as Aus, Aman or Boro throughout the year in Bangladesh. To improve callus induction and plant regeneration, five Aman rice genotypes namely BRRI dhan34, BRRI dhan51, BRRI dhan71, BRRI dhan79, and BRRI dhan95 have been selected for this experiment. Mature seeds of those rice varieties were collected from BRRI Regional Station, Shaympur in Rajshahi.

Seed sterilization and culture media

The dehusked seeds were surface sterilized by dipping in 3% sodium hypochlorite for five minutes, then in 95% ethanol for twenty seconds (from dipping to washing with distilled water), and finally washed 5-6 times in sterile distilled water (Abuhena et al. 2022). The seeds were then shaked on an orbital shaker at 120 rpm for 30 minutes. The sterilized seeds were rinsed 3 times with sterile double-distilled water and then blot dried on sterilized filter paper before inoculation. Then the surface sterilized seeds were carefully inoculated into MS (Murashige and Skoog 1962 and N6 (Chu et al. 1975) media. Different modes of incubation were assessed based on the germination percentage following the formula mentioned below-

$$\text{Germination (\%)} = \frac{\text{Number of germinated seeds}}{\text{Total number of inoculated seeds}} \times 100$$

Effect of media and plant growth regulators on callus induction

To find out the effect of different doses (1-3 mg/l) of 2,4-D two basal media like MS and N6 were considered in this case. Before autoclaving, the pH of the media was adjusted to 5.7 to 5.8. Culture medium was sterilized for 20 minutes at 121°C and 15 psi (103.4 kPa) and the sterilized medium was dispensed to the Petri dishes having 20-25 ml each. In order to maintain sterility, 20-25 ml of medium was then added to Petri dishes. In aseptic conditions, sterilized seeds were horizontally cultivated on the medium and incubated them at 25°C in the dark. After 3 weeks of culture, the frequency of callus induction was measured. The petri dishes were sealed with parafilm and incubated them at 27±2°C in the dark for three to four weeks. Then the vessels were kept under 36 Watt white fluorescent lamps with light intensity of 5000 lux at 16/8 hours cycle of light/dark. The temperature of growth chamber was maintained at 25±1°C.

Plant regeneration and acclimatization

Four to five weeks old calli derived from five Aman rice cultivars were used for regeneration, which were then placed on plant regeneration medium (RM) containing MS and N6 basal media supplemented with BAP (1.0 - 2.0 mg/l), Kin (1.0 - 2.0 mg/l) and NAA (0.5 mg/l). As gelling agent 4.0 g/l of phytagel (Sigma-Aldrich) were used before autoclaving. Four different concentration of PGRs e.g. (i) RM1 = 2 mg/l BAP + 0.5 mg/l NAA, (ii) RM2 = 2 mg/l BAP + 0.5 mg/l NAA + 1 mg/l Kin, (iii) RM3 = 2 mg/l BAP + 2 mg/l Kin + 0.5 mg/l NAA, and (iv) RM4 = 1 mg/l BAP + 0.5 mg/l NAA + 1 mg/l Kin. Three replicates of each treatment were conducted with similar number and amount of calli were inoculated in each time. Thereafter, all cultures were incubated at ± 2°C with a 16:8 (light/dark) photoperiod. For shoot elongation, the *in vitro* regenerated plantlets were moved into culture tubes containing the same basal media. Every week, the *in vitro* regeneration response based on the commencement of various kinds of green spots was noticed. The plantlets were moved to culture tubes containing the same basal medium for shoot elongation after being initiated. After transplanting, the quantity of regenerated shoots was counted in every two weeks. The regenerated *in vitro* plantlets were moved to a half-strength basal medium for 7 days without any PGRs for root proliferation. To avoid fungal assaults, the plantlet was then carefully rinsed with sterile water to thoroughly remove the adhering media. After that, the

in vitro plantlets with good roots were placed in pots of soil to acclimate. The regeneration percentage were measured following the formula mentioned below:

$$\text{Regeneration (\%)} = \frac{\text{Number of green spots}}{\text{Total number of calli}} \times 100$$

Furthermore, the calli and regenerated plantlets were recorded. After replanting the plants into pots with the best regeneration media at 2 weeks old, the survival percentage of *in vitro* grown plantlets under natural environmental circumstances was measured. A fully randomized block design was used to set up the studies.

Statistical analysis

Three replicates of the callus induction frequency and plantlet regeneration were observed. The data analysis was carried out in R 2021, and Tukey's test was used to assess mean differences. At the $p<0.05$ level, the differences were considered statistically significant. To indicate the significance of the discrepancies, distinct means were labelled with different letters (a, b, c, d, and e).

Results and Discussion

2,4-D and its effect on callus induction

Genetic manipulation to improve a variety must start with a high percentage of re-generable embryogenic calli was reported by Juturu et al. (2016). For more effective plant regeneration, this study attempted to evaluate and enhance the embryogenic potential of calli (Siddique et al. 2014, Ahmad et al. 2016, Abiri et al. 2017, Mostafiz and Wagiran 2018). *In vitro* seed derived calli of five indica rice genotypes were examined and the developmental stages are shown in Fig. 1 & 2. There were no discernible changes in the CIF- callus induction frequency (%) across different media ($p<0.001$). For all cases in addition of 3.0 mg/l 2,4-D showed high yielding calli in a range of 40 - 60% (Fig. 1 & 2). The callus percentage gradually increased with an increase in 2,4-D up to 3 mg/l. Fig.1 shows the effect of 2,4-D added to MS medium containing of 3 mg/l for BRRI dhan34 (59-63%), BRRI dhan79 (54-58%), BRRI dhan85 (54-58%), BRRI dhan51 (47-50%) and BRRI dhan51 (40-42%). Without adding 2,4-D in MS medium used as a control, where there was no sign of callus formation.

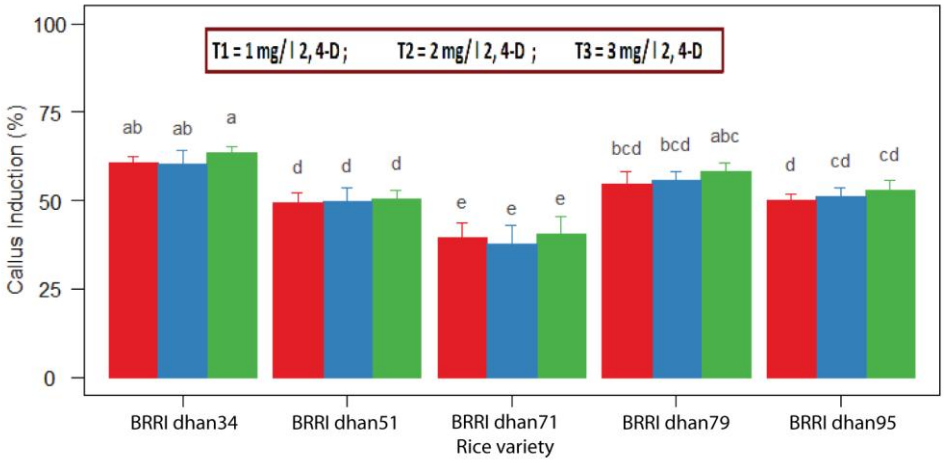

Fig. 1: Calli induction frequency in five rice genotypes supplemented with different concentration of 2,4-D in **MS** medium. Mean values of calli induction frequency marked with the same letters do not differ significantly ($p \leq 0.05$) in Tukey's test.

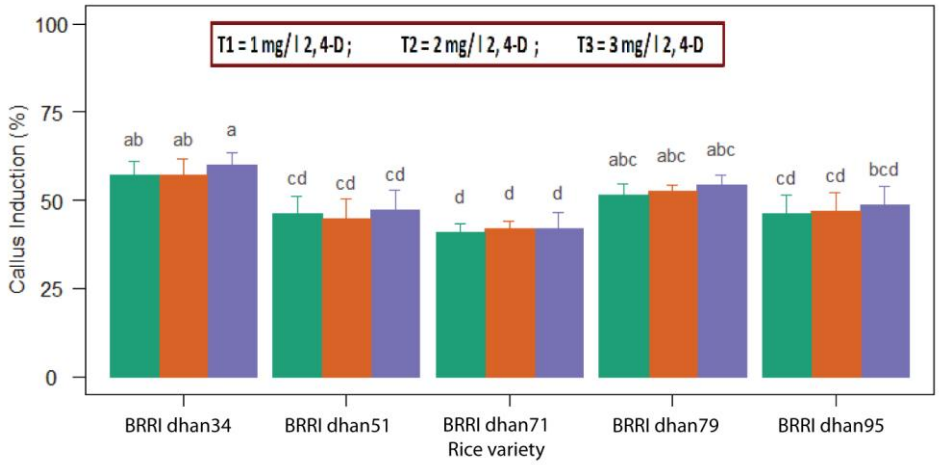

Fig. 2: Calli induction frequency in five indica rice genotypes (BRRI dhan34, BRRI dhan51, BRRI dhan71, BRRI dhan79 and BRRI dhan95) supplemented with different concentration of 2,4-D on **N6** basal medium. Mean values of calli induction frequency marked with the same letters do not differ significantly ($p \leq 0.05$) in Tukey's test.

For somatic embryogenesis, a number of PGRs are essential for cell division and differentiation was reported by Haque et al. (2015), and Lee & Huang (2013). Auxin is in charge of the creation of shoots inside various PGRs was reported by Meneses et al. (2005). Previous studies showed that callogenesis is mostly

influenced by the type and concentration of auxin as well as its external supply (Naik et al. 2017). As a result, in this investigation, the control treatment (0.0 mg/l 2,4-D) did not induce any calluses. Additionally, this study discovered that higher doses of 2,4-D reduced callus induction (Table 1).

Table 1: Effects of NS and MS medium on callus induction of five rice genotypes in Bangladesh.

Season	Genotypes	Basal media	
		N6 (% ± SD)	MS (% ± SD)
T. Aman	BRRI dhan95	48.8 ± 5.07^{bc}	53 ± 2.74^{bc}
	BRRI dhan79	54.2 ± 2.77^{ab}	58 ± 2.55^{ab}
	BRRI dhan71	42 ± 4.53^{c}	40.4 ± 5.13^{d}
	BRRI dhan51	47.2 ± 5.81^{bc}	50.4 ± 2.41^{c}
	BRRI dhan34	59.8 ± 3.70^{a}	63.4 ± 1.95^{a}
Analysis of variance			
Df		4	4
Sum Square		1493.4	930.8
Mean Square		373.3	232.70
F. value		37.41	11.49
Significance		***	***

Within column, mean ± SD values. Significance symbol ($p<0.05$), according to Tukey multiple comparisons of means test (n = 5). $^{ns}p \geq 0.05$, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. Bars without shared letters indicate significant differences.

In comparison to MS for BRRI dhan71, the CIF on N6 was higher. For BRRI dhan95, BRRI dhan79, BRRI dhan34, and BRRI dhan51, the CIF on MS was higher than the CIF on N6. However, there was no significant difference between MS and N6 (Table 1). The callus percentage of the BRRI dhan71 was practically identical to that of the cultures on MS and N6 media. In a previous Rahman et al. (2015) demonstrated that the majority of rice calluses were induced from MS medium but with varied auxin treatments. According to the current study, auxin affected the frequency of callus induction but varied between types, which is consistent with a prior study (Shahsavari et al. 2010). The results of this study also demonstrated that auxin had an impact on the responses of several rice types, which were distinct. According to Bevitori et al. (2014), rice somatic embryogenesis is influenced by a number of variables, including explant age, donor plant genotype, nutritional media, auxin concentration, and ambient circumstances (Islam et al. 2013, Islam and Haque 2016).

Plant regeneration

It was decided to test the potential of high-quality embryogenic calli for plant regeneration by transferring them to regeneration medium (RM). We employed calli from the most responsive callus induction media e.g. MS and N6 with 3.0 mg/l 2,4-D to evaluate their efficiency on regeneration. Here, Table 2 displays the frequency of plantlet regeneration derived from calli of five rice genotypes under this study. Between the regeneration media and various concentration of plant growth regulators plants regeneration (%) of five studied rice genotypes were differed (Table 2). In the majority of the types, RM3 and RM2 showed the highest plantlet regeneration. Whereas, BRRI dhan79 showed the highest (28-32%) and BRRI dhan95 in RM_1 showed the lowest (18%) regeneration (Table 2).

Table 2: The effect of different concentration (mg/l) of BAP + Kin + 0.5 NAA in MS and N6 media on plantlet regeneration (%) from the calli of five different rice genotypes.

Genotypes	Treatments	Shoot regeneration (% ± SE)	
		MS	N6
BRRI dhan95	RM_1	21.25±1.25	18.75±1.25
	RM_2	23.75±1.25	21.25±2.39
	RM_3	25.00±2.04	22.50±1.44
	RM_4	23.75±1.25	20.00±2.04
BRRI dhan79	RM_1	28.75±1.25	28.75±1.25
	RM_2	32.50±1.44	23.75±1.25
	RM_3	32.75±1.25	32.75±1.25
	RM_4	30.00±2.04	22.50±1.44
BRRI dhan71	RM_1	22.50±1.44	21.25±1.25
	RM_2	28.75±1.25	23.75±1.25
	RM_3	28.00±0.00	26.75±1.25
	RM_4	26.25±1.25	25.00±2.04

Contd. Table 2

BRRI dhan51	RM_1	27.50±1.44	22.50±1.44
	RM_2	22.50±1.44	21.25±1.25
	RM_3	27.75±1.25	26.25±2.39
	RM_4	18.75±1.25	22.50±1.44
BRRI dhan34	RM_1	26.25±1.25	23.75±1.25
	RM_2	21.25±1.25	26.25±1.25
	RM_3	26.50±1.44	24.50±1.44
	RM_4	23.75±2.39	22.50±1.44

RM_1 = 2 mg/l BAP + 0.5 mg/l NAA, RM_2 = 2 mg/l BAP + 0.5 mg/l NAA + 1 mg/l Kin, RM_3 = 2 mg/l Kin + 0.5 mg/l NAA, RM_4 = 1 mg/l BAP + 0.5 mg/l NAA + 1 mg/l Kin.

After two weeks of transferring calli to regeneration medium it shows becoming greenish of the calli on the RM. After three weeks, the green patches continued to develop into numerous shoot primordia, *in vitro* plantlets, and finally, healthy plants in the pot (Fig. 3 C-G). In the current investigation, it was discovered that for all five kinds of rice varieties, the percentage of plantlet regeneration were increased significantly with rising of cytokinin (RM3) concentrations in addition to the medium (Table 2). Rahman et al. (2015) analysis different culture media and found that NAA showed better performance than others. They also found that without NAA, regenerated plantlets were weaker. Higher concentrations of BAP and Kin may enhance the first round of cell division and be crucial for somatic embryogenesis and plantlet regeneration was reported by Rueb et al. (1994), Haque and Islam (2015 & 2019) and Khatun et al. (2010 & 2012). According to the results all five rice genotypes had the greatest impact on plantlet regeneration where 2.0 mg/l BAP + 2.0 mg/l Kin + 0.5 mg/l NAA were added in the culture medium. By regulating mitosis, cytokinesis, total protein synthesis, lignin biosynthesis, vascular differentiation, and the differentiation of mature chloroplasts from protoplastids, kinetin has been shown in a previous study to aid improve somatic embryogenesis (Abe and Futsuhara 1986, Morshed et al. 2014 & 2016). On regeneration media, greenish of the callus was noticed after 21 days of culture initiation, and all five kinds of rice thereafter showed signs of regenerated shoot and plantlet regeneration. Numerous plantlets produced from somatic embryos were successfully acclimated to *ex-vitro* settings. The plant's roots were cleaned before being planted in soil for 10 days (Fig. 3G). The BRRI dhan79 had the highest rate of plant survival, followed by BRRI dhan 95, BRRI dhan 34, BRRI dhan 71, and BRRI dhan 51. Plants were exposed to their natural surroundings after 10 days. It was noted that plants had impressive development after 30 days (Fig. 3G). After two weeks in the natural environment, the plants that had been restored on RM3 medium survived perfectly.

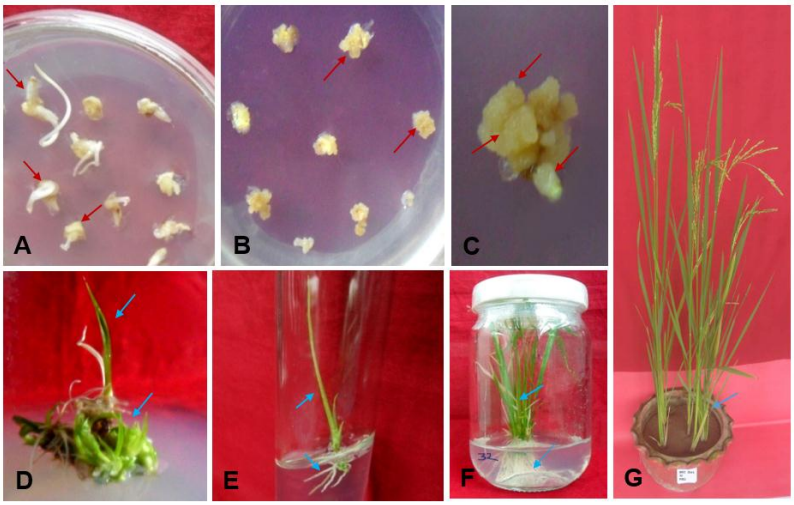

Fig. 3(A-G): Inoculated of mature rice seeds and several steps for development of calli and its subsequent regeneration. A) 4-5 days of inoculated seeds started calli. B) 2-3 weeks old calli separated from rice seeds, C) 2-3 weeks old calli were transferred to regeneration medium which showed becoming greenish, D) Seed derived calli started to regeneration, E) Green plantlets with good root and shoots, F) Plants were grown in pots, G) Plants were transferred to pots after successful acclimatization.

Conclusion

In this work, five distinct indica rice cultivars were used to examine *in vitro* callus induction and its subsequent regeneration. In this case five rice genotypes, two basal media and various plant growth regulators (2,4-D, NAA, Kin, NAA) were examined to improve callus induction and plant regeneration frequency. Here, all rice genotypes showed a greater callus induction frequency with 3 mg/l 2,4-D. The frequency of callus induction did not significantly differ when 2,4-D and NAA were used in combination. Compared to N6 a higher frequency of callus induction was found in MS. The RM3 (modified MS), the regeneration was higher than others three combinations. This study demonstrated the potential for the PGRs in combination with 2 mg/l BAP, 2 mg/l Kin, and 0.5 mg/l of NAA enhanced regeneration efficiency under the studied rice genotypes. Hope those genotypes can be used for further advance research in biotechnology for rice improvement in Bangladesh.

Conflict of interest

The authors declare no conflicts of interest.

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Conflict of interest: The authors hereby declare no conflict of interest regarding the publication of this article.

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Date of birth	:	28th October, 1989
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B. Educational background

Examination	Year	Board / University	G.P.A/C.G.P.A
S.S.C	2004	Rajshahi Board	3.75
H.S.C	2006	Rajshahi Board	4.10
B. Sc (Hons.) in Biotechnology and Genetic Engineering	2011	Islamic University	3.27
M. Sc in Biotechnology and Genetic Engineering	2012	Islamic University	3.73

C. List of Publications

A) M. Phil work related-01

1) **Toyfiquz Zaman** and S M Shahinul Islam (2024). Influence of plant growth regulators on efficient callus induction and plant regeneration using five rice genotypes in Bangladesh. Journal of Bio-Science,32(1): 83-94.

B) Others-07

1. Md. Ataur Rahman, Md. Rezanur Rahman, **Toyfiquz Zaman** (2020). Emerging potential of naturally occurring autophagy modulators against neurodegeneration. Current Pharmaceutical Design, 2(6): 1-8.
2. Md. Rezanur Rahman, Tania Islam, **Toyfiquz Zaman** (2020). Identification of molecular signatures and pathways to identify novel therapeutic targets in Alzheimer's disease: Insights from a systems biomedicine perspective. Genomics, 112(2): 1290-1299.
3. Md. Rezanur Rahman, Tania Islam, Md. Shahjaman, **Toyfiquz Zaman** (2019). Discovering biomarkers and pathways shared by Alzheimer's disease and Ischemic stroke to identify novel therapeutic targets. Medicina 55(5): 191.
4. Rezanur Rahman, Tania Islam, Beste Turanli, **Toyfiquz Zaman** (2019). Network-based approach to identify molecular signatures and therapeutic agents in Alzheimer's disease. Computational Biology and Chemistry 78: 431-439.
5. Md. Rezanur Rahman, Md. Abdullah Al-Mamun, **Toyfiquz Zaman** (2019). The influence of depression on ovarian cancer: discovering molecular pathways that identify novel biomarkers and therapeutic targets, Informatics in Medicine Unlocked**.**
6. Md. Foyzur Rahman, Md Rezanur Rahman, Tania Islam, **Toyfiquz Zaman** (2019). A bioinformatics approach to decode core genes and molecular pathways shared by breast cancer and endometrial cancer, Informatics in Medicine Unlocked.
7. Md. Rezaul Karim, Md. Tofazzal Hossain, Zinat Tamannaa, **Toyfiquz Zaman** (2017). Hepatoprotective efficacy of Oyster Mushroom (*Pleurotus ostreatus*). Journal of Khwaja Yunus Ali University. Vol: 02, Issue: 01