In vitro regeneration of selected rice (*Oryza sativa* L. *indica*) landraces of Manipur.

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By

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Certificate

This is to certify that the present thesis entitled "***In vitro* regeneration of selected rice (*Oryza sativa* L. *indica*) landraces of Manipur"** submitted by **Mr. Tongbram Punshi Singh**, M.Phil. Registration No: MZU/M.Phil/354 of dt. 26.5.2017 in partial fulfillment for the award of the Degree of Master of Philosophy in Biotechnology of Mizoram University has been carried out during 2016-2017 under my supervision. The thesis embodies original research and has not been submitted for any degree elsewhere.

The candidate has fulfilled all the requirements under M. Phil ordinance of the Mizoram University.

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Supervisor

Declaration of the Candidate

I, Tongbram Punshi Singh, hereby declare that the subject matter of this dissertation entitled **"*In vitro* regeneration of selected rice (*Oryza sativa* L. *indica*) landraces of Manipur"** is a record of work done by me, that the contents of this dissertation did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and the dissertation had not been submitted by me for any research degree in any other University/Institute.

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Date: *(TONGBRAM PUNSHI SINGH)*

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LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic Acid
SDS	Sodium Dodecyl Sulphate
CTAB	Cetyltrimethyl Ammonium Bromide
EDTA	Ethylene Diaminetetra Acetic Acid
NaCl	Sodium Chloride
PVP	Polyvinyl Pyrolidone
$MgCl_2$	Magnesium Chloride
dNTP	De-oxyribonucleotide Triphosphate
RNA	Ribonucleic Acid
UV	Ultra Violet
C	Cytosine
T	Thymine
PGR	Plant Growth regulator
2, 4-D	2, 4-Dichlorophenoxyacetic acid
NAA	Naphthalene acetic acid
BAP	6-Benzylaminopurine
K	Kinetin
AAC	Apparent Amylose content
PCR	Polymerase Chain Reaction

Chapter 1
Introduction

CHAPTER 1

INTRODUCTION

1.1 General introduction

Rice (*Oryza sativa* L.), is a member of the family Graminae. It is a herbaceous plant and one of the three major food crops of the world. The genus *Oryza* consists of 23 wild and weedy species and two cultivated species, viz., *O.sativa* and *O.glaberrima.*The domestication of *O. sativa* occured in Asia while *O.glaberrima* is mainly cultivated in the western tropical region of Africa (NBPGR, 2006). The primary centre of origin of *O. sativa* is reported to be in the foothills of Himalayas, North-east region of India, mountain ranges of South-east Asia and South-west China whereas *O.glaberrima*, the African rice, originated in the delta of river Niger in Africa (Maiti R *et al*., 2012). Rice is usually cultivated under the rain-fed or irrigated conditions with warm humid environment. Globally, the total area covered under rice farming is 164.12 million hectares with a production of 722.76 million tones and a standard productivity of 4037 kg per hectare (FAOSTAT, 2011). The application of biotechnology in rice has immensely contributed to the assembly of large number of improved varieties. Wide crossbreeding along with the technique of embryo rescue has contributed to the acquisition of helpful genes from the wild *Oryza* species to the elite cultivars. The utility of biotechnological tools driven wild crop relatives for the bio-prospecting of potentially useful genes for crop improvement has speed up the generation of new and improved novel varieties of crops.

Over the years, the potentiality of application of plant biotechnology tools and techniques for the development of useful and novel plant genotypes has been increasingly recognized. Continuous progress in developing techniques has been made for culturing and regeneration of plants using different explants of a large number of plant species (Thorpe, 1990). Tissue culture techniques coupled with advancement in molecular biology have opened a new vista for broadening crop gene pools, contributing to increasing the efficiency of conventional plant breeding methods. Several new rice cultivars have already been developed with the use of advanced techniques of biotechnology such as anther culture, embryo rescue and somaclonal variation (Brown and Thorpe, 1995; Zapata *et al*., 2004). Plant tissue culture, also cell, *in vitro* or sterile culture, is one of the very important tool in each basic and applied

studies, as well as for application in commercial purposes (Thorpe 1990). Skoog and Miller (1957) demonstrated that regulation and differentiation of roots and shoots (organogenesis) in tobacco pith culture relies on the relative concentrations of the plant growth regulator in the substance, therefore introducing the idea of hormonal regulation of organ formation. The phenomenon of somatic embryogenesis in suspension cultures of carrot was observed by Steward *et al*. (1958). By late 1970's, it became evident that significant contributions to agriculture and industries were brought by plant tissue culture technology. Black rice varieties are that rice with coloured cover (other than white and red). Black rice has high anthocyanin content situated within the cover layers, which provides it a dark purple colour. Additionally to ancient white or common rice, speciality rice have distinctive properties like distinctive flavour, aroma (unique aromas), colour (red, purple, black), nutrition (glossiness, viscousness), chemical composition, aesthetic, waxy (very low amylose content) and superior process quality and are increasing in demand and vastly grown in Thailand, Europe, India, United States, Pakistan and the Middle East (Chaudhary 2003; Choi *et al*. 2002; Yang et al. 2008; 2010).

1.2 Status of rice in India

In India, the total area under rice cultivation is about 43.86 million ha with a production level of 104.80 million tonnes and about 2390 kg/ha productivity (Agricultural Statistics at a glance- 2015). It is cultivated in a diverse soil and varied climatic conditions throughout the country with the productivity level being low as compared to many countries of the world. In India, marginal, small and medium farmers hold about 90 % of the lands under rice cultivation which is another problem in the effort to increase the rice productivity. China has the maximum productivity with 6582 kg per ha followed Vietnam (5228 kg /ha), Indonesia (4998 kg/ha), Bangladesh (4203 kg/ha) (National Food Security Mission, Feb-2014). Above all, to increase the rice productivity in the country, various new improved technologies and various interventions are available to be adapted. Hybrid rice has a high potential in increasing the productivity of rice and thus, the practice of implementation for hybrid rice cultivation to increase the productivity is an important move to be develop.

The rice growing regions in the country are broadly grouped in five regions as follows: (NBPGR, 2006):

i. North-Eastern Region: This region comprises of Assam and the North eastern states of India. In Assam, rice is mainly grown in the Basin of Brahmaputra River. As this region of the country receives heavy rainfall, cultivation of rice is done under rainfed condition.

ii. Eastern Region: The eastern region consist of the Indian states of Chhattisgarh, Orissa, Jharkhand, West Bengal, Madhya Pradesh, Bihar, and Eastern Uttar Pradesh. Rice is mainly grown in the basins of Ganga and Mahanadi rivers and mainly under rainfed condition.

iii. Northern Region: Uttarakhand, Haryana, Punjab, Jammu & Kashmir, Himachal Pradesh and Western Uttar Pradesh are group under this region. This region experiences low temperature during winter and rice is mainly cultivated during May-July to September-December.

iv. Western Region: Gujarat, Maharashtra and Rajasthan are grouped under this region. Rice is largely cultivated under rainfed condition during June-August to October - December.

v. Southern Region: The rice growing southern region comprises of Karnataka, Kerala, Tamil Nadu and Andhra Pradesh. The deltaic tracts of Godavari, Krishna and Cauvery rivers and the non-deltaic rainfed area of Tamil Nadu and Andhra Pradesh are the mostly under rice cultivation.

1.3 Scope of rice in Northeast India

The North-Eastern part of India comprises of Arunachal Pradesh, Assam, Manipur, Meghalaya, Nagaland and Tripura. This region accounts for 7.81 % of total area under rice and shares 6.07% of the total rice production in India. But, average yield per ha of rice in the region (1426 kg) is far below the national average (2390 kg) (Singh *et al.*, 2001). Rice germplasm collection in the form of local landraces and choice of the landraces for desirable traits represents an unprecedented rich heritage of rice germplasm within the region. There are several rice cultivars which contain coloured pigments and give rise to several varieties of black and red colour rice. The black scented rice (Chakhao) of Manipur, in NE India is widely known for its sweet aroma and dark purple colour and is used for special occasions as a delicacy. The literal meaning of "Chakhao" is delicious rice (Chak-rice: hao-delicious) (Somnath Roy *et al.,* 2014).The high anthocyanin content of the black rice within its pericarp layers is responsible for its dark purple colour (Abdel-Aal *et al.*, 2006). Till recently, the black rice is not cultivated commercially because the same cannot be used as staple food thereby

ensuing to restricted market access. The total area cultivated for black rice is relatively very low and even though the yield of"Chakhao" is very low as compared to different high yielding rice varieties, it is still more profitable in terms of economic returns as the cost is much higher. The black scented rice of Manipur "Chakhao amubi" are predominantly low in yield and is solely found in this state and is lesser known scientifically. As compared to the white rice, black rice has a higher mineral content which depend on the varieties and soil types of the planting area and are rich in minerals like Fe, Zn, Mn and P (Qiu *et al*. 1993; Liu *et al*. 1995; Zhang 2000). The pigment responsible for the black rice colour – anthocyanin with its antioxidant properties are recognized as a health promoting food ingredient (Philpott *et al*. 2004; Nam *et al*. 2006). "Taothabi" is one of the important rice landrace of Manipur which is known to grow in low lying plains and well known for their tolerance to submerged conditions. Taothabi is an indigenous red colour rice landrace which is reported to be tolerant to gall midge pest of rice (International Rice Research Newsletter, 1990). Sikder *et al*. (2006) reported that callus obtained from 2.0 mg/L of 2, 4-D showed the most effective results for the regeneration and also showed high shoot regeneration (40%) than higher concentration within the same regeneration media. Presence of auxin 2,4-D is considered a vital catapult factor in successful induction of rice callus (Lin and Zhang, 2005; Karthikeyan *et al*. 2009 ; Joyia and Khan, 2013) but there are also reports of 2,4-D in combination with BAP (Sahoo *et al*. 2011) or NAA (Bano *et al*. 2005). Moreover, for micro-propagation through calluses, the used of 2,4-D was determine as inevitable. Ramesh *et al*. (2009) reported that with the increase in concentration of 2,4-D, there is reduction in callus induction and facilitation solely in rooting in indica rice varieties.

Lee *et al*. (2002) reported that in terms of range, colour, size and morphology of callus developed, varied accordingly with the particular rice genotypes and also on the composition of media used, explants and interaction between these factors. Base on the morphology of the calli, there are four types of calli (Visarada *et al*. 2002) ; type I – compact creamy or whitish coloured organized callus, type II – organized yellow coloured callus, type III – unorganized brownish or yellowish callus, and type IV – rhizogenic callus.

One of the valuable techniques in tissue culture to exploit somaclonal variation is the dehusked rice seed culture. But its application is restricted by several factors that influence culture efficiency, like plant genotype (Liu *et al*. 1997), method of culture, the media and the conditions. Production of callus and its consequent regeneration are the most important steps in crop plants to be exploited using biotechnological techniques and to utilise somaclonal variation (Monirul Islam *et al*. 2005). Rashid *et al*. (2003) reported that the degree of callusing

varied according to the rice varieties. According to Rasheed *et al.* (2005) with respect to the incubation time and specific cultivar, there are wide ranges of variation in tissue culture. Tam and Lang, (2003); Shankhdhar *et al*. (2002); Jaseela *et al*. (2009) reported that callus formation was found with 60 – 100 percent in all the concentrations of 2,4- D used and is regarded the most efficient and therefore utilised in majority of embryogenic and tissue culture systems and also reported that 2 mg/L 2,4-D to be the foremost favourable for callus induction and callus proliferation. Although rice productivity in the NE region has made significant changes, particularly since the initiation of green revolution, there has been wide fluctuations in production over the years and also, in several states of the region. Thus, it is of important necessity to keep check and analyse the production and productivity trend of rice in North-East India where rice occupy about 89.46% of the total area under food grains and which contributes 92.32% of the total food grains production in the region. To keep pace with projected demand, rice production growth should be sustained with improved traits. Thus, development of an efficient *in vitro* regeneration system is important for the successful and productive utilization of biotechnology in rice crop improvement. Amylose is the most significant grain constituent that influences rice end-use quality and it is the foremost determinant used across the world to define rice market classes. The cooking and process quality of rice (*Oryza sativa*) is determine by the key factor i.e. amylose content (Juliano, 1985). Low amylose levels are typically related to soft, cohesive, glossy cooked rice, whereas higher amylose cultivars are usually dry when cooked, fluffy and separated (Juliano, 1971). The *waxy* gene located on the rice chromosome 6, encodes the enzyme – granule bound starch synthesis (GBSS) that plays a key role in apparent amylase synthesis (Smith *et al*., 1997). Two *waxy* gene alleles, Wx^a and Wx^b, have traditionally been related with the contents of GBSS and apparent amylase content (AAC) in rice endosperm, with the Wx^a allele synthesizing higher contents of GBSS, and thus AAC, than the Wx^b allele (Sano, 1984; Sano *et al*., 1985). The characteristics of the Wx^ballele is due to the single nucleotide change of G-T at the +1 position of the intron 1 consensus cleavage site: the presence of pre-mRNA (containing intron 1), low level of mature *Waxy* transcript, GBSS and AAC (Bligh *et al*., 1998; Cai *et al*., 1998; Hirano *et al*., 1998; Isshiki *et al*., 1998; Larkin and Park, 1999).

Bligh *et al*., (1995) reported that a polymorphic DNA microsatellite marker having a dinucleotide (cytosine–thymine) repeats (CT_n), located at exon 1 in the non-translated region of the rice *Waxy* gene. The CT_n alleles of this microsatellite, subsequently renamed RM190 (Temnykh *et al*., 2000), in a historical collection of 89 non-glutinous USA rice cultivars explained 82.9% of the variation in AAC (Ayres *et al*., 1997).

Consumer preference for rice around the world, when eaten as an intact grain, is largely dependent on a desire for its cooked texture to be either firm-and-not-sticky or soft and- sticky. Different useful properties such as freeze–thaw stability or flour thickening power are desirable in the industry of food processing base on their uses and application. Bergman *et al*., (2004) reported that the key factor of rice (*Oryza sativa*) cooking, sensory and processing characteristics of different cultivars are largely determine by its apparent amylose content(AAC). Consequently, rice breeders while preserving a targeted AAC that fits the desired end-use standard characteristics, can developed improved rice cultivars with enhanced agronomic traits.

The two rice landrace viz. “Chakhao amubi” and “Taothabi” of Manipur are poor in yield and are found only in this state and less aware throughout the Indian region. There is an enormous demand within the domestic market, having prospects for export. Keeping in view of this, the following objectives were designed with aimed in exploration and furthermore inclusion of these rice landraces in crop improvement program:

- Initiation and standardization of aseptic cultures
- To develop an efficient and reproducible *in vitro* plant regeneration system using mature seeds.
- Standardization of hardening and acclimatization of the *in vitro* regenerated plantlets.

Chapter 2
Materials and Methods

CHAPTER – 2

MATERIALS AND METHODS

2.1. Collection and maintenance of germplasm

For the present study, freshly harvested rice grains of two important rice landraces of Manipur namely – "Chakhao amubi" and "Taothabi", were collected during January 2016 from the low-lying farmer's fields of two districts of Manipur (Fig. 1 and Table 1), which are registered under the Agriculture Department, Government of Manipur and ICAR-NE region. The rice samples were germinated and maintained in pots for use in the study.

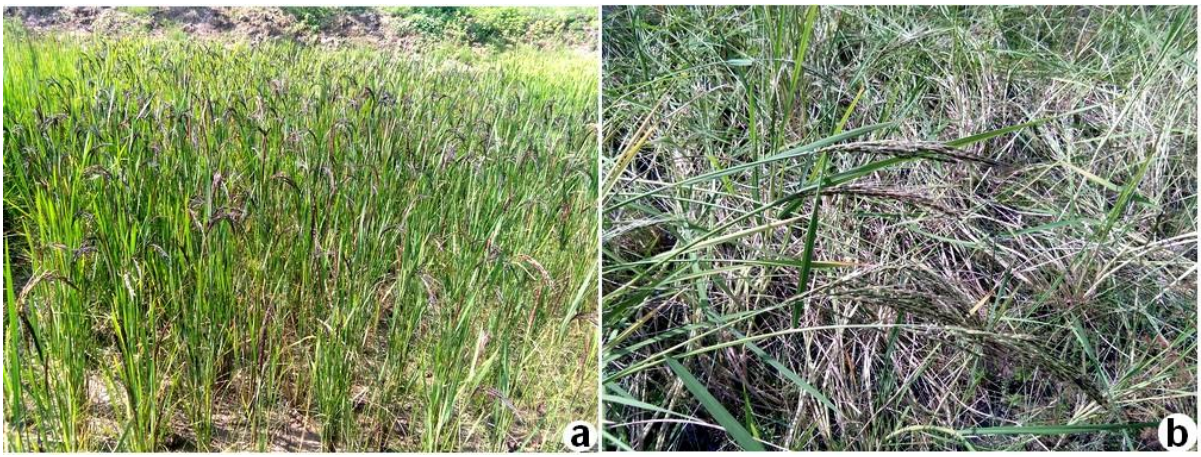

Figure 1: The two local rice landraces of Manipur in the field. a) Chakhao amubi; b) Taothabi

Table 1: Details of the collected rice landraces

Collector Number	Local name	Scientific name	Collection site
MR1	Chakhao amubi	*Oryza sativa* L.*indica*	Thoubal district
MR2	Taothabi	*Oryza sativa* L.*indica*	Bishnupur district

2.2 Grain Morphology

Classification and identification of the samples were carried out based on the morphological data as described by rice descriptors (Bioversity International, IRRI and WARDA, 2007). Determination of the grain dimensions and the shape of the two rice landraces viz. "Chakhao amubi" and "Taothabi" were carried out as follows:

Grain length (L), width (W) and L/W ratio were measured from 10 randomly selected grains. The distance from the base of the lowermost glume to the tip (apiculus) of the fertile palea or lemma is measured as the grain length (L). In case of awned varieties, length was measured to a point comparable to the tip of the apiculus. The distance across the fertile lemma and palea at the widest point was measured as the grain width (W). All the measurements were done using a digital calliper.

Length/ Width ratio was calculated by using the formula

$$\text{L/W ratio} = \frac{\text{Mean length of the kernel in mm}}{\text{Mean breadth of the kernel in mm}}$$

Based on the three morphological characters recorded, the rice landraces were classified using FAO scale for milled rice classification scheme (Table 2). The grain colour of the collected samples was studied by visual perception.

Table 2: Systematic Classification of rice grains (FAO scale for milled rice classification scheme, 1972)

Sl. No.	Grain length (L)	Size (Length)	Grain Length/Width ratio	Shape (L/W ratio)
1	7.00 mm and above	Extra long	3.00 mm and above	Slender/long
2	6.00-6.99 mm	Long	-	Medium
3	5.00-5.99 mm	Medium	2.00-3.00 mm	Bold
4	Less than 5.00 mm	Short	Less than 2.00 mm	Round/short

2.6. Determination of waxy or non-waxy character

Determination of the starchy nature of “Chakhao amubi”and “Taothabi” rice landraces from Manipur into waxy or glutinous and non-waxy or non-glutinous types was carried out by treating polished rice grains with iodine solution as described in IRRI Technical Bulletin (1965). Potassium iodide-iodine solution was prepared by dissolving 1 g of potassium iodide and 0.3 g of iodine in 100 ml water. The rice grains of the two samples were polished and 10 grains were selected randomly from each sample. The polished grains were treated with

iodine solution on a petridish and incubated for 10 s at room temperature. The grains were then observed for the colour stained developed and classified as follows:

a) Waxy or glutinous: brown colour staining when reaction with potassium iodide-iodine solution indicates the waxy nature of the rice grain.

b) Non-waxy or non-glutinous: dark blue staining when reaction with potassium iodide-iodine solution indicates the non-waxy nature of the rice grain.

2.7. *In vitro* regeneration studies

2.8. Media preparation

MS basal medium (Murashige & Skoog, 1962) was used for the explants regeneration in the study. MS media were supplemented with different concentrations of plant growth hormones (Table 3) and the pH was adjusted to 5.5 - 5.8 using 0.1N NaOH and 0.1N HCl. 0.8% (w/v). Agar was used for the solidification of the media and sterilized in an autoclave for 15 min at 121°C (15 lb psi pressure).

Table 3: Different concentrations of PGRs used in the study

Sl. No.	NAA (mg/L)	2,4-D (mg/L)	BAP (mg/L)	Kinetin (mg/L)
1	0	0	0	0
2	0	1	0	0
3	0	2	0	0
4	1	0	0	0
5	2	0	0	0
6	0	0	2	0
7	0	0	4	0
8	0	0	0	2
9	0	0	0	4

2.9. Preparation of explants

Matured seeds were used as explants for the *in vitro* regeneration of the rice samples. Various sterilization steps were carried out to avoid contamination. Sterilisation of the explants was done by treating the seeds with 1% NaOCl for a time period of 5 minutes followed by 3 times of surface rinsing with sterile distilled water. The seeds were again treated with 0.1% of HgCl

for another 5 minutes and rinsed 3 times with sterile distilled water. The sterilised seeds were then let off to dry on sterile tissue papers for another 5 minutes followed by inoculation on petriplates containing ~20 ml of MS solid basal medium.

2.1.0. Culture conditions for regeneration

Incubation of the cultures was carried out by maintaining the cultures in the culture room at 25±1°C, and kept 8:16 hours light and dark photoperiod with light intensity of 55 µmol $m^{-2}s^{-1}$ using cool white fluorescent light (Bajaj Ltd., India) and 50-60 % relative humidity.

2.1.1 Hardening

The effect on the *in vitro* regeneration ability of the selected rice in varied concentrations of PGRs was evaluated. The plantlets regenerated having fully developed leaves and roots were transplanted for primary hardening in the laboratory. The plantlets were removed and washed thoroughly to remove agar and transferred in pots containing sterilized soil: sand (1:1) and covered with polythene to maintain humidity.

2.1.2. Statistical Analysis

All the experiments were conducted in the plant tissue culture laboratory. Each experiment had three replicates per treatment. One petriplate with 5 explants represents as one replicate. The experiments were carried out at least thrice.The percentage data shown in the tables were arcsine transformed and analyzed for significance using analysis of variance (ANOVA; $P < 0.05$). All statistical analysis was carried out using SPSS statistical software package version 16.0 (SPSS, 2007).

2.1.3. Genotyping of waxy gene

2.1.4. Materials DNA extraction

For the genomic DNA isolation, the collected rice landraces *viz*. "Chakhao amubi" and "Taothabi" were grown and maintained in pots. DNA extraction was done by harvesting the young leaves from the 3-4 weeks old rice seedlings in the present study.

2.1.5. Chemicals and reagents used

1.0 M Tris-HCl pH 8.0 (himedia Pvt. Ltd.)

Glacial acetic acid

5.0 M NaCl

0.5 M Na_2EDTA pH 8.0

10 % SDS

Proteinase K (200 µg) (Bangalore Genei, India)

10 % CTAB

10 % PVP

Chloroform : Isoamyl alcohol (24:1) containing 5 % phenol

Absolute alcohol

Isopropanol

RNase (Sigma Aldrich Chemicals Pvt. Ltd., Baangalore, India)

10× Buffer (Bangalore Genei, India)

$MgCl_2$ (Bangalore Genei, India)

dNTP Mix (Bangalore Genei, India)

Taq DNA Polymerase (Bangalore Genei, India)

6× gel loading buffer (Bangalore Genei, India)

Ethidium bromide: 10 mg/ml (Bangalore Genei, India)

2.1.6.Extraction buffer

200 mM Tris-HCl (pH 8.0), 0.8 M NaCl, 25 mM Na_2EDTA (pH 8.0), 0.5 % SDS, 14 µg Proteinase K.

2.1.7. 2×CTAB solution

2 % CTAB, 100mM Tris-HCl (pH 8.0), 20 mM Na_2EDTA (pH 8.0), 1.4 M NaCl, 5 % PVP.

All the chemicals were purchased from Himedia Pvt. Ltd. India.

2.1.8. DNA extraction protocol

Extraction of genomic DNA was carried out following the modified CTAB method (Thangjam *et al*., 2003) with slight modifications as described below.

About 100 mg of young leaves from each rice landraces were collected, rinsed with tap water and blotted dry. The collected young leaves were then transferred in the micro-centrifuge tube (2 ml) containing 400 µl of extraction buffer. The leaves were then grinded in the buffer inside the tube with sterile glass rod. The ground leaves were incubated at 37°C for at least 90 minutes in a water bath followed by addition of 400 µl of 2% CTAB solution and incubation at 65°C for 120 minutes. It is then allowed to cool at room temperature and then

extracted by gentle addition of equal amount of chloroform: isoamyl alcohol (24:1) containing 5% phenol. Centrifugation was carried out at 10,000 rpm in a micro centrifuge at 4°C for 10 minutes. The upper aqueous layer was then carefully transferred to a new tube and the extraction process was repeated for 3 times to remove the cloudiness of the upper layer. It is then followed by addition of 2/3rd volume of ice cold isopropanol and mixed by gentle repeated inversions and incubate at room temperature for 30 minutes to precipitate DNA. It is then centrifuge at 10,000 rpm for 15 minutes at 4°C and the supernatant was removed. The pellet was washed with ice cold 70% ethanol. The supernatant was decanted and the pellet was air dried. The pellet is then resuspended in 50µl of TE buffer. RNA was removed by adding 1µl of RNase and incubating at 37°C for approximately 60 minutes.

2.1.9. Quantification and estimation of DNA quality

The amount of isolated DNA per milligram of leaf tissue was estimated by measuring absorbance at 260 nm and 280 nm using Bio-photometer plus (Eppendorf, Germany) according to the manufacturer's instruction. The quality of the extracted DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm. A 50 ng/µl DNA stock was prepared from the isolated DNA to be used for further experiments. The isolated DNA from each of the rice landraces was separated on 0.8% agarose gel in 1× TAEbuffer. For this, aliquot of2 µl (100 ng/µl) was loaded into the gel and then photographed using a gel documentation system (Bio-rad, Australia).

2.2.0. PCR amplification of intron 1 of the *waxy*gene

The G/T polymorphism in intron 1 of the *waxy* gene (In1G and In1T alleles) was genotyped by restriction enzyme digest of a polymerase chain reaction (PCR) fragment generated from this genomic region. Specifically, the polymorphic sequence of In1 SNP was amplified from genomic DNA using the forward primer RM-190F (5'-CTTTGTCTATCTCAAGACAC-3') and reverse primer GBSS-W2R (5'-TTTCCAGCCCAACACCTTAC-3') (Ayres *et al*., 1997).

The PCR amplification was carried out with 15 µl reaction mixture containing 10× PCR buffer, 50 ng of genomic DNA as template, 0.5 µM each of the primers, 1.5 mM of $MgCl_2$, 0.2 mM of dNTP (Himedia, India), 1 U of Taq Polymerase (Sigma-Aldrich Pvt. Ltd, Banglore). The PCR amplification was performed in a Thermal Cycler (Bio Rad, C1000™) with the following condition of 94 °C for 5 mins, 35 cycles of 94 °C for 45 s, 54 °C for 45 s, and 72°C for 1 min; followed by a final extension at 72°C for 7 min. The amplified fragments were resolved on a 2% agarose gel and the gels were stained with ethidium bromide and visualised under UV light with a 100 bp DNA ladder as a marker and then photograph using a gel documentation system (Biorad, Australia).

2.2.1. Polymorphism analysis

The G/T polymorphism in intron 1 of *waxy*gene was genotyped by restriction enzyme digest of the PCR fragment generated. 2µl of the PCR amplification product was digested with 2U of the restriction enzyme *Acc*I (New England Biolabs) in 10 µl total volume for 3 hrs and electrophorised on a 2.2 % agarose gel and then photograph using a gel documentation system (Biorad, Australia).

2.2.2. PCR amplification of RM190 microsatellite of the *waxy* gene

For genotyping the RM190 microsatellite CT_nalleles, two primers were used RM-190F (5'-CTTTGTCTATCTCAAGACAC-3') and RM-190R (5'-TTGCAGATGTTCTTCTGATG-3') (Ayres *et al*., 1997, Temnykh *et al*., 2000). The PCR amplification was carried out with 15 µl reaction mixture containing 10× PCR buffer, 50 ng genomic DNA as template, 0.5 µM each of the primers, 1.5 mM of $MgCl_2$, 0.2 mM of dNTP (Himedia, India), 1 U of Taq DNA polymerase (Sigma-Aldrich Pvt. Ltd, Bangalore). The PCR amplification was performed in a Thermal Cycler (Bio Rad, C1000™) with the following conditions of 94°C for 4 min., followed by 30 cycles of amplification at 94°C for 45s, 55°C for 45s and 72°C for 1 min. followed by a final extension at 72°C for 7 mins. The amplified fragments were resolved on a 2% agarose gel stained with ethidium bromide and visualized under UV light with a 100 bp DNA ladder as a marker and then photographed using a gel documentation system (Biorad, Australia).

2.2.3. Sequencing of the amplified product and analysis

For the sequencing of the amplified PCR products, the samples were sent to AgriGenome Labs Pvt Ltd. Kochi, Kerala, India. The sequence was analysed to determine the number of CT repeats using the online Microsatellite repeat finder (http://insilico.ehu.es/mini_tools/microsatellites/).

Chapter 3
Results

CHAPTER 3

RESULTS

3.1. Grain morphology characterization

Freshly harvested rice seeds of the two important landraces viz. “Chakhao amubi” and “Taothabi” were collected and analysed based on the morphological descriptors of Bioversity International, IRRI and WARDA (2007) from 10 randomly selected mature grains from each sample. Details of the morphological characters recorded from the two landraces are given in Fig. (2 and 3) and Table 4.

Table 4: Grain characteristics of the selected rice landraces based on FAO scale for milled rice (1972)

Sample	Grain colour	Grain length (mm)	Grain width (mm)	Length/ width ratio (mm)	Grain type	Aroma
Chakhao amubi	Dark purple	6.21±0.09	2.17±0.02	2.85	Long bold	Present
Taothabi	Light Red	6.42±0.07	2.75±0.04	2.33	Long bold	Absent

The grain colour of “Chakhao amubi” rice landrace was found to be dark purple colour while “Taothabi” rice landrace was of light red colour kernel. The mean grain length of “Chakhao amubi” was of 6.21 mm while the mean grain length of “Taothabi” was found to be slightly longer with 6.42 mm with a width of 2.75 mm. Based on the recorded length and shape of the rice grains, the two rice landraces were group with the grain type of long bold according to the grain characteristic classification described by “Descriptors of rice (*Oryza sativa* L.)”.

Based on the mean of the grain length and length/width ratio, the shape of “Chakhao amubi” and “Taothabi” were classified following the systematic classification of the grain characteristics as described by “Descriptors for rice (*Oryza sativa* L.)” (IBPGR-IRRI Rice Advisory Committee, 1980).

Figure 2: Grain length and colour variation of Chakhao amubi (*Oryza sativa* L. *indica)* landrace of Manipur; a) Grains with husk; b) Grains without husk.

Figure 3: Grain length and colour variation of Taothabi (*Oryza sativa* L. *indica*) landrace of Manipur; a) Grains with husk; b) Grains without husk.

3.2. Waxy or non-waxy character

To determine the starchy nature of “Chakhao amubi” and “Taothabi” rice landrace, 10 polished grains of each sample were treated with iodine solution. The polished rice grains were treated for 10s at room temperature and observed for colour stain development. Brownish colour stain development on the grains shows the waxy or glutinous nature while dark blue stain when reaction with the iodine solution shows the non waxy nature of the rice grains (IRRI Technical Bulletin,1965). The polished rice grains of “Chakhao amubi” when reaction with iodine solution was found to develop a brownish stain (Fig.4) on the grains while that of “Taothabi” showed a dark blue stain (Fig.5). Thus, from the study, it was found that “Chakhao amubi” rice landrace is a waxy rice while “Taothabi” is a non-waxy type of rice.

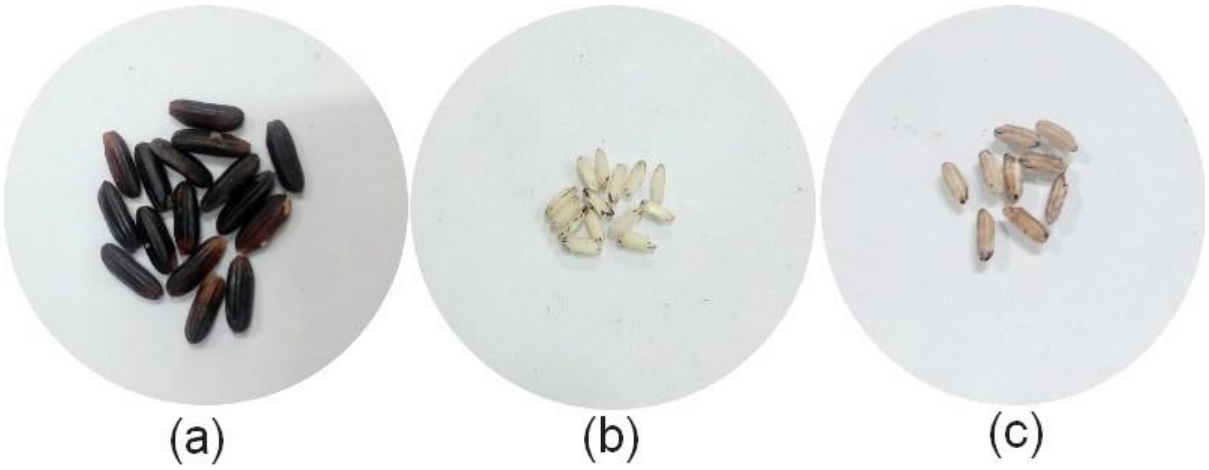

Figure 4: Detection of waxy/non-waxy character; a) Dehusked grains of Chakhao amubi; b) Polished rice grains; c) Brownish stain developed after treating with iodine solution.

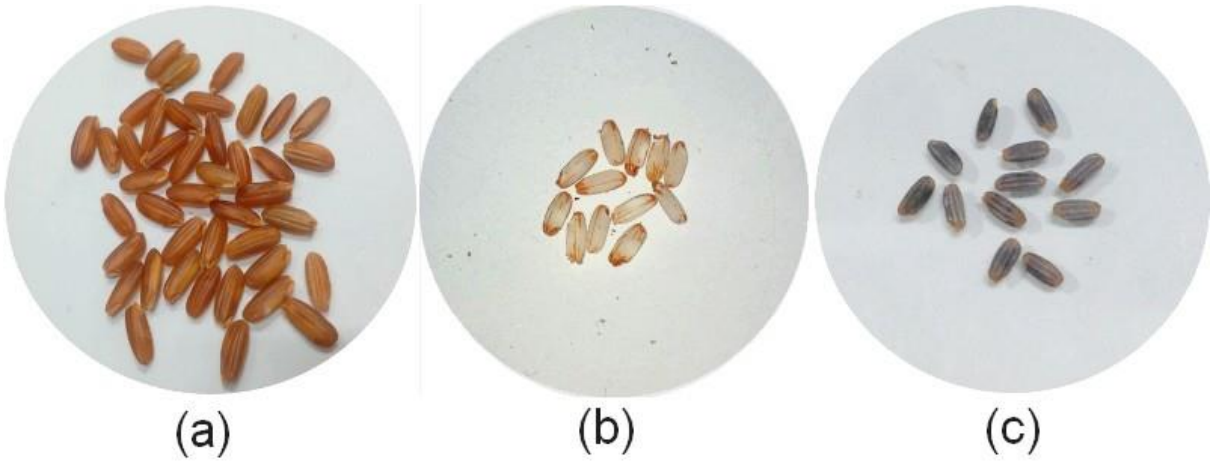

Figure 5: Detection of waxy/non-waxy character; a) Dehusked grains of Taothabi; b) Polished rice grains; c) Dark blue stain developed after treating with iodine solution.

3.3. *In vitro* regeneration studies

3.4. *In vitro* regeneration of "Chakhao amubi"

In vitro regeneration of "Chakhao amubi" was successfully established. The data recordedon the effect of MS basal medium supplemented with different concentrations of plant growth regulators (PGRs) viz. NAA, 2,4-D, BAP and Kinetin on the freshly harvested mature grains of "Chakhao amubi" is given in Table 5. All cultured rice explants showed different morphogenetic response in all the MS media supplemented with and without PGRs within the first 2 weeks of culture. The first morphogenetic change observed was the initiation of radical from the embryo of the mature grains cultured followed by the subsequent elongation of the radical. Germination of the rice seeds was observed as early on the 2nd day after culture. Germination percentage was high as observed in all the media sets with the maximum (93.33) observed in MS basal medium without any PGRs and the lowest percentage (82.00) was observed in MS medium with BAP (4 mg/L). The frequency and time taken for the root initiation varied with the different MS medium supplemented with PGRs. The root initiation of the cultured explants was recorded on the 6th day after culture. The highest percentage of root initiation (91.00) was observed in MS medium with 2,4-D (1 mg/L) and the least percentage (50.90) was observed in MS medium with BAP (2 mg/L). Shoot development of the germinated explants was observed from the 5th day after cultured and varied accordingly with the concentrations of the PGRs supplemented in the MS medium. Well developed shoot was observed in all of the media sets with different concentrations of PGRs used. The highest percentage of shoot formation (88.90) recorded on the 14th day after culture was found in MS medium without PGRs and in MS medium supplemented with NAA (2 mg/L). Root formation percentage was found maximum (91.00) in MS basal medium without any PGRs and in MS medium with NAA (2 mg/L). MS medium supplemented with BAP (4 mg/L) showed the least root formation. The regenerated individual explants were then sub cultured in a fresh media for further elongation. MS medium supplemented with 2,4-D hormone showed the formation of callus from the cultured explants with initiation of callus observed on the 7th day after culture. The frequency of callus formation varied with the concentrations of 2,4-D supplemented in MS medium. The highest percentage of callus formation as on the 30th day after culture was found highest (75.55) in MS medium with 2,4-D (2 mg/L) and least in MS medium with 2,4-D (1 mg/L). Fully regenerated plantlets of "Chakhao amubi" with well developed shoots and roots were observed on the 30th day after culture. The highest percentage of fully regenerated plantlets was observe in MS basal medium without any PGRs (91.00) and the lowest (75.60) was observed in MS medium with BAP (4 mg/L).

The regenerated plantlets were removed from the culture plates and thoroughly washed to remove any remains of medium and transferred in small plastic pots containing sterilized soil: sand (1:1). Plantlets were then covered with transparent polythene bags to sustain sufficient moisture for a week and transferred to the greenhouse.

Table 5: Effect of MS basal media supplemented with different PGRs on the *in vitro* regeneration of *Oryza sativa* L. landrace "Chakhao amubi" of Manipur.

Sl. No.	PGR (mg/L)				Percentage (%) of explants showing morohological changes (days)					
	NAA	2,4-D	BAP	Kinetin	Germination (3^{rd} day)	Root initiation (6^{th} day)	Shoot formation (14^{th} day)	Root formation (14^{th} day)	Callus formation (30^{th} day)	Fully regenerated plantlets (30^{th} day)
1	0	0	0	0	93.33±0.00a	88.90±2.15ab	88.90±2.15a	91.00±2.15a	-	91.00±2.15a
2	0	1	0	0	91.00±2.15a	91.00±2.15b	-	-	66.70±2.35a	-
3	0	2	0	0	91.00±2.15a	86.70±3.34ab	-	-	75.55±1.51b	-
4	1	0	0	0	88.90±2.15ab	86.70±3.34ab	82.22±1.71a	82.22±1.71b	-	82.22±1.71bc
5	2	0	0	0	93.33±0.00a	88.90±2.15ab	88.90±2.15a	91.00±2.15a	-	82.22±1.71c
6	0	0	2	0	88.90±2.15ab	50.90±1.40c	82.22±1.71a	55.60±1.28c	-	82.22±1.71bc
7	0	0	4	0	82.00±1.71b	53.33±0.00c	82.22±1.71a	48.91±1.27c	-	75.60±1.51b
8	0	0	0	2	91.00±2.15a	82.22±1.71a	88.90±2.15a	86.70±0.00ab	-	88.90±2.15a
9	0	0	0	4	91.00±2.15a	84.44±1.71ab	88.90±2.15a	88.90±2.15a	-	82.22±1.71bc

Mean (±) followed by the same letter(s) in each column were not significantly different at $P<0.05$ using Duncan's new multiple range test.

- absence of data

Figure 6: *In vitro* germination of Chakhao amubi seeds cultured on MS basal media with and without PGRs observed on the 3rd day; a) Without PGRs (Control); b) MS + NAA (2 mg/L); c) MS + BAP (4 mg/L).

Figure 7: Root initiation observed on *in vitro* germinated Chakhao amubi seeds cultured on MS basal media with and without PGRs on the 6th day; a) Without PGRs (Control); b) MS + 2,4-D (1 mg/L); c) MS + BAP (2 mg/L).

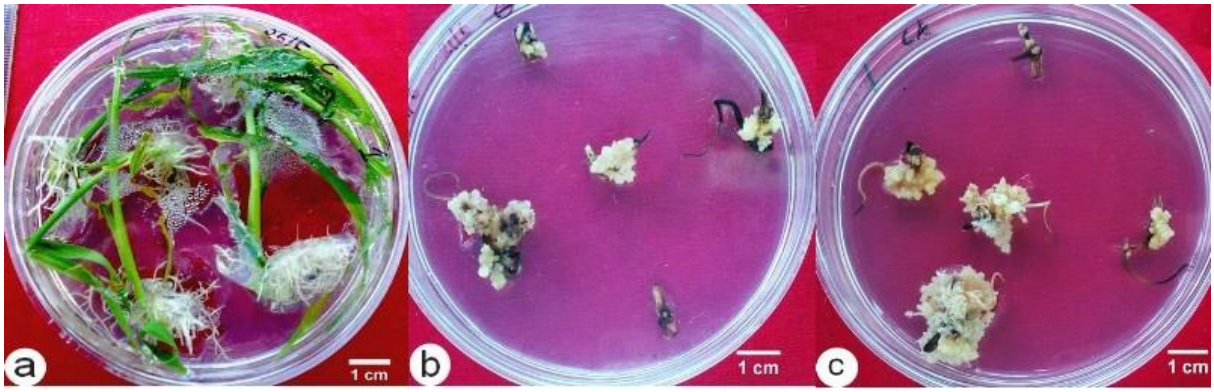

Figure 8: Callus formation observed on *in vitro* germinated Chakhao amubi seeds cultured on MS basal media with and without PGRs on the 30th day; a) Without PGRs (Control); b) MS + 2, 4-D (1 mg/L); c) MS + 2, 4-D (2 mg/L).

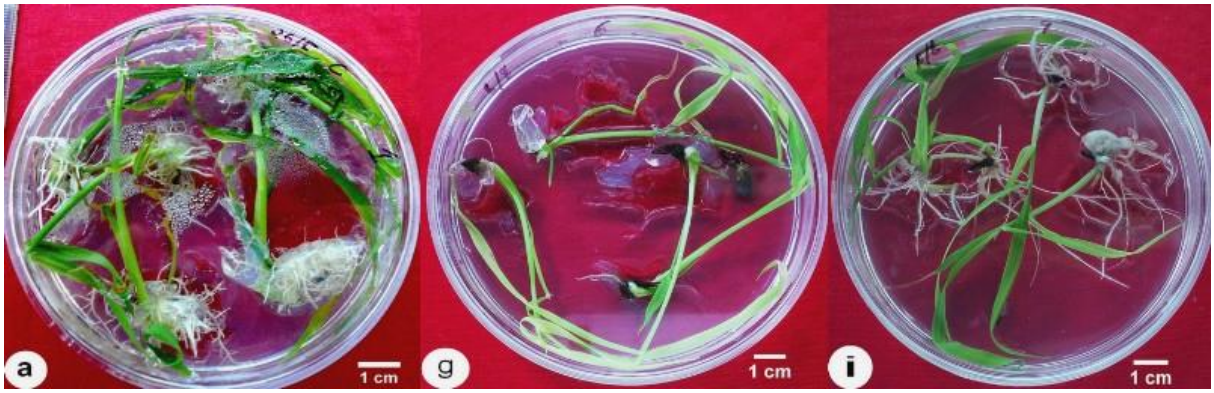

Figure 9: Plantlet regeneration and root formation observed on *in vitro* germinated Chakhao amubi seeds cultured on MS basal media with and without PGRs on the 30th day; a) Without PGRs (Control); b) MS + BAP (4 mg/L); c) MS + Kinetin (2 mg/L).

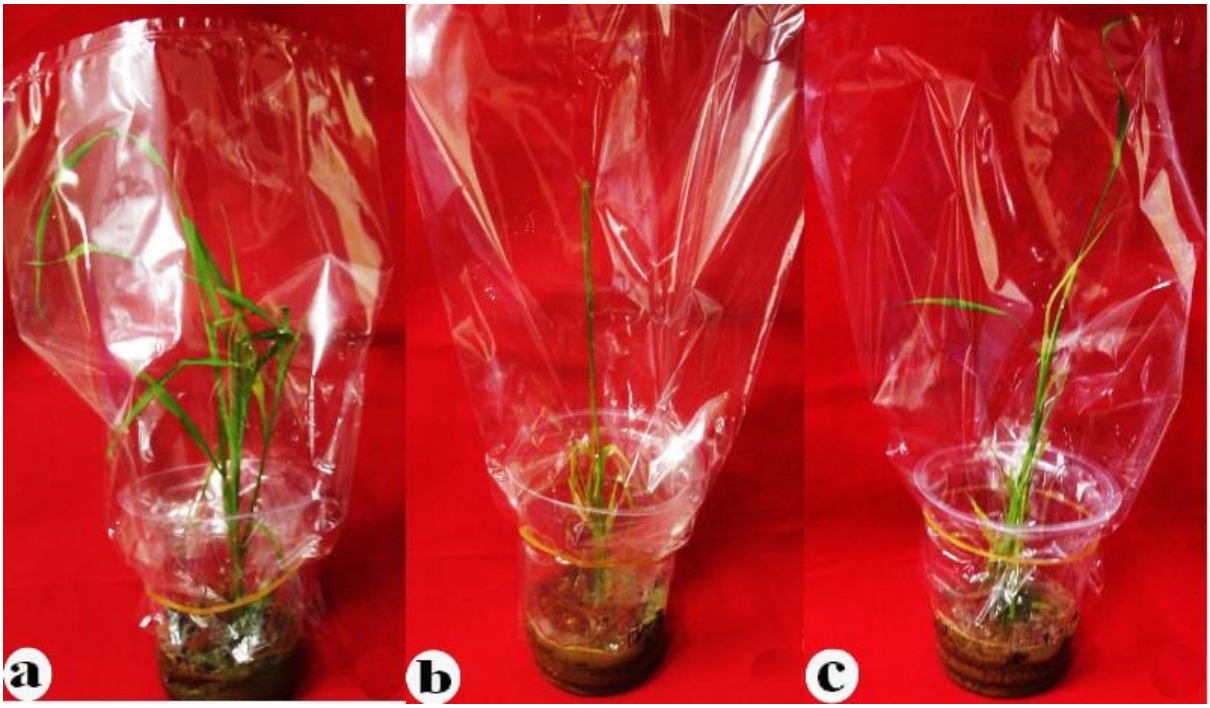

Figure 10: Hardened plantlets of *in vitro* germinated Chakhao amubi seeds cultured on MS basal media with and without PGRs. a) Without PGRs (Control); b) MS + BAP (4 mg/L); c) MS + Kinetin (2 mg/L).

3.5. *In vitro* regeneration of "Taothabi"

In vitro regeneration of Taothabi was successfully established using the mature seeds as explants. The data recorded on the effect of MS basal medium supplemented with different concentrations of plant growth regulators (PGRs) viz. NAA, 2,4-D, BAP and Kinetin on the freshly harvested mature grains of "Taothabi" is given in Table 6. All cultured rice explants showed different morphogenetic response in all the MS media supplemented with and without PGRs. The first morphogenetic change observed was the initiation of radical from the embryo of the mature grains cultured followed by the subsequent elongation of the radical. Cultured seeds germinated as early on the 2nd day after culture. Germination percentage was high as observed in all the media sets with the maximum (93.33) in MS medium without PGRs and MS medium with 2,4-D (1 mg/L) and NAA (1 mg/L). The frequency and time taken for the root initiation varied with the different concentrations of PGRs in the MS medium. The root initiation of the cultured explants was recorded on the 6th day after culture. The highest percentage of root initiation (91.00) was observed in MS medium supplemented with 2,4-D and in MS medium without any PGRs. Shoot development of the germinated mature grains was observed from the 5th day after cultured and varied with the different concentrations of the PGRs supplemented in the MS medium. Well developed shoot was observed in all MS medium with different concentrations of PGRs used. The highest percentage of shoot formation (91.00) recorded on the 14th day after culture was found in MS medium without any PGRs. Maximum percentage of root formation (91.00) was observed in MS basal medium without any PGRs. The regenerated individual explants were then sub cultured in a fresh media for further elongation. MS medium supplemented with 2,4-D hormone showed the formation of callus from the cultured explants with initiation of callus observed on the 7th day after culture. The frequency of callus formation varied with the concentrations of 2,4-D supplemented in MS medium. The highest percentage of callus formation as on the 30th day after culture was found highest (68.91) in MS medium with 2,4-D (2 mg/L) and lowest percentage (55.55) in MS medium with 2,4-D (1 mg/L). Root formation of the cultured explants were observed from the 14th day after culture with highest percentage of root formation (91.00) observed in MS medium without PGRs and the lowest percentage (51.12) in MS medium supplemented with BAP (4 mg/L). Fully regenerated plantlets of "Taothabi" were observed on the 30th day after culture. The highest percentage (91.00) of fully regenerated plantlets was observe in MS basal medium without any PGRs.

The regenerated plantlets were removed from the culture plates and thoroughly washed to remove any remains of medium and transferred in small plastic pots containing sterilized soil: sand (1:1). Plantlets were then covered with a transparent polythene bags to sustain sufficient moisture for a week and transferred to the greenhouse.

Table 6: Effect of MS basal media supplemented with different PGRs on the *in vitro* regeneration of *Oryza sativa* L. landrace "Taothabi" of Manipur

Sl. No.	PGR (mg/L)				Percentage (%) of explants showing morohological changes (days)					
	NAA	2,4-D	BAP	Kinetin	Germination (3^{rd} day)	Root initiation (6^{th} day)	Shoot formation (14^{th} day)	Root formation (14^{th} day)	Callus formation (30^{th} day)	Fully regenerated plantlets (30^{th} day)
1	0	0	0	0	93.33±0.00a	91.00±2.15a	91.00±2.15a	91.00±2.15a	-	91.00±2.15a
2	0	1	0	0	93.33±0.00a	91.00±2.15a	-	-	55.55±1.28a	-
3	0	2	0	0	91.00±2.15a	91.00±2.09a	-	-	68.91±1.39b	-
4	1	0	0	0	93.33±0.00a	73.33±2.51bc	88.91±2.15a	88.91±2.15a	-	88.91±2.15a
5	2	0	0	0	88.91±2.15a	77.77±3.22cd	86.67±3.35a	84.46±1.71a	-	84.46±1.71b
6	0	0	2	0	88.91±2.15a	71.12±1.39bc	84.46±1.71a	60.01±2.26b	-	82.23±1.71ab
7	0	0	4	0	88.91±2.15a	72.24±2.61b	84.46±1.71a	51.12±1.27b	-	82.23±1.71b
8	0	0	0	2	88.91±2.15a	84.46±1.71ad	86.67±3.35a	86.67±3.35a	-	82.23±1.71b
9	0	0	0	4	91.10±2.15a	88.91±2.15a	86.67±3.35a	84.44±3.86a	-	84.44±3.35ab

Mean (±) followed by the same letter(s) in each column were not significantly different at $P<0.05$ using Duncan's new multiple range test

-absence of data

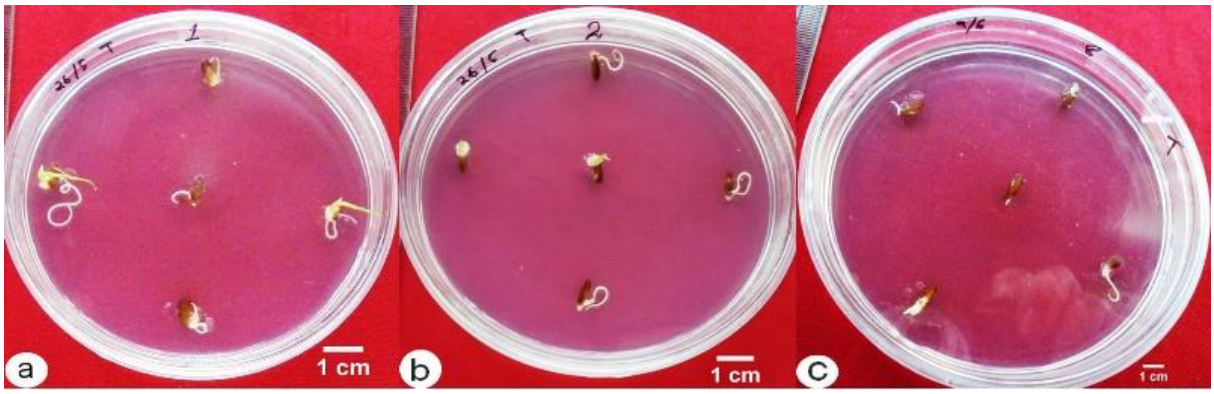

Figure 11:*In vitro* germination of Taothabi seeds cultured on MS basal media with and without PGRs observed on the 3rd day; a) Without PGRs (Control); b) MS + NAA (2 mg/L); c) MS + BAP (2 mg/L).

Figure 12: Root initiation observed on *in vitro* germinated Taothabi seeds cultured on MS basal media with and without PGRs on the 6th day; a) Without PGRs (Control); b) MS + 2,4-D (2 mg/L); c) MS + (BAP 2 mg/L).

Figure 13: Callus formation observed on *in vitro* germinated Taothabi seeds cultured on MS basal media with and without PGRs on the 30th day; a) Without PGRs (Control); b) MS + 2,4-D (1 mg/L); c) MS + 2,4-D (2 mg/L).

Figure 14: Plantlet regeneration and root formation observed on *in vitro* germinated Taothabi seeds cultured on MS basal media with and without PGRs on the 30th day; a) Without PGRs (Control); b) MS + NAA (2 mg/L); c) MS + BAP (4 mg/L).

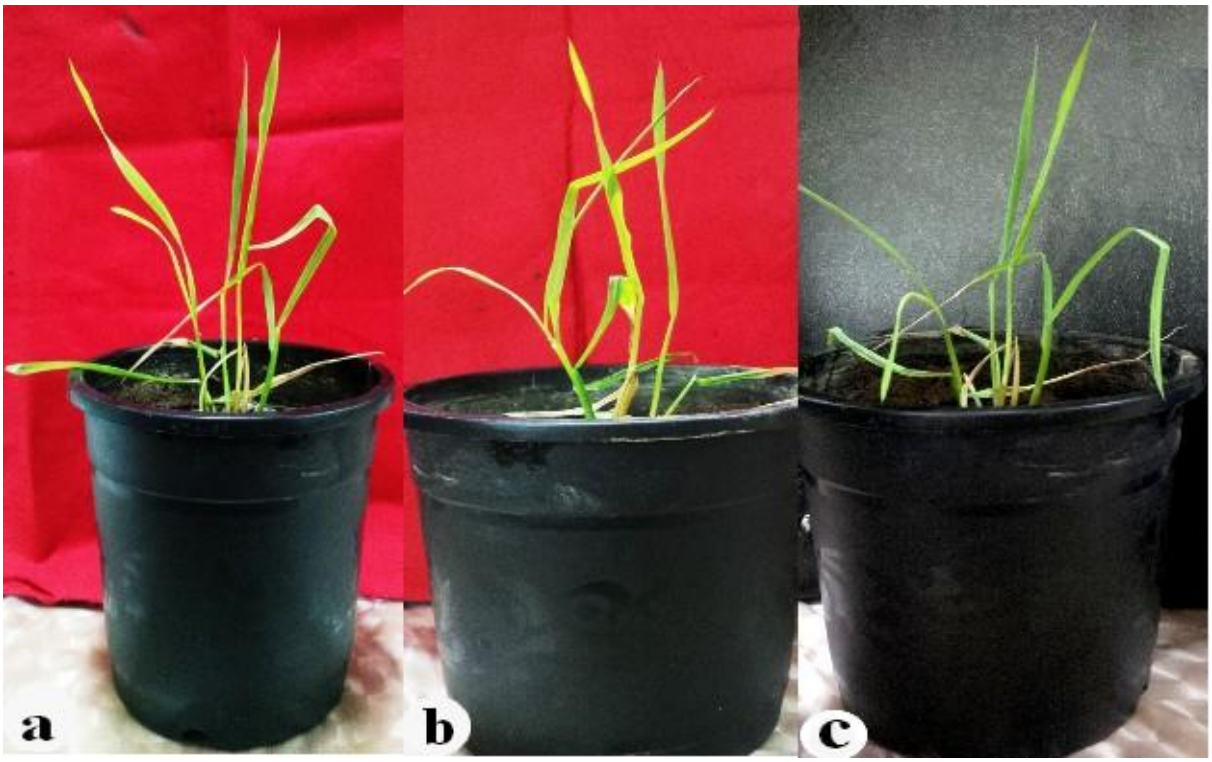

Figure 15: Hardened plantlets of *in vitro* germinated Taothabi seeds cultured on MS basal media with and without PGRs;a) Without PGRs (Control); b) MS + BAP (4 mg/L); c) MS + NAA (2 mg/L).

3.6. Genotyping of the *waxy* gene

3.7. Quality of the extracted genomic DNA

The quality of the extracted genomic DNA from the two rice landraces viz. “Chakhao amubi” and “Taothabi” rice landraces were evaluated by loading 2 µl (100 ng/µl) of the DNA and electrophorised on 0.8 % agarose gel and then photographed using a gel documentation system (Bio-rad, Australia). The electrophoregram showed a distinct sharp band of high molecular weight DNA.

3.8. PCR amplification of the intron 1 of the *waxy* gene

Using the optimised PCR conditions, the amplification of the intron 1 of the *waxy* gene of the two rice landraces viz. “Chakhao amubi”and “Taothabi” resulted in the generation of a single sharp fragment of around 250 bp (Fig.16).

3.9. Polymorphism analysis

Restriction digestion of the PCR products of the two rice samples was carried out with enzyme *Acc*I (New England Biolabs) using the optimised conditions. Restriction digestion of the PCR amplified product was carried out in a 10µl total volume for 3 hrs. The digestion product was run on a 2.2 % agarose gel and viewed using a gel documentation system. The digestion product of the PCR product shows that “Taothabi” rice landrace (MR2) was found to be cleaved by the enzyme *Acc*I while “Chakhao amubi” rice landrace (MR1) was not cleaved (Fig. 17). This confirms the presence of the sequence AGGTATA in the amplified fragments of “Taothabi” landrace, while the corresponding amplified fragments from “Chakhao amubi” rice landrace having the sequence AGTTATA at this position was not cleaved. Thus, this key G-T polymorphism in the intron 1 of the *waxy* gene was assayed.

3.1.0. PCR amplification of RM190 microsatellite of the *waxy* gene

Using the optimised PCR conditions, the amplification of the RM190 microsatellite of the *waxy* gene of “Chakhao amubi”and “Taaothabi” rice landraces resulted in the generation of a single sharp fragment of around 105 bp (Fig. 18).

3.1.2. Sequencing of the amplified product and analysis

The PCR amplified product of the RM190 microsatellite for the two rice landraces was sent for sequencing and the sequence were analysed to determine the number of CT repeats in the sequence using the online microsatellite repeat finder. Two CT_n alleles of the *waxy* microsatellite RM190 was identified from the two rice landrace. “Chakhao amubi” (MR1) rice landrace was found to have 14 CT repeats while “Taothabi” (MR2) rice landrace was found to have 10 CT repeats in the RM190 microsatellite sequence of the *waxy* gene.For the non-glutinous, CT_8, CT_{10} and CT_{11} are associated with high apparent amylose content (AAC $\geq$ 23 %) and CT_{14} and CT_{16} are intermediate AAC–types (AAC 18 – 23 %) (M.H. Chen *et al*., 2007).

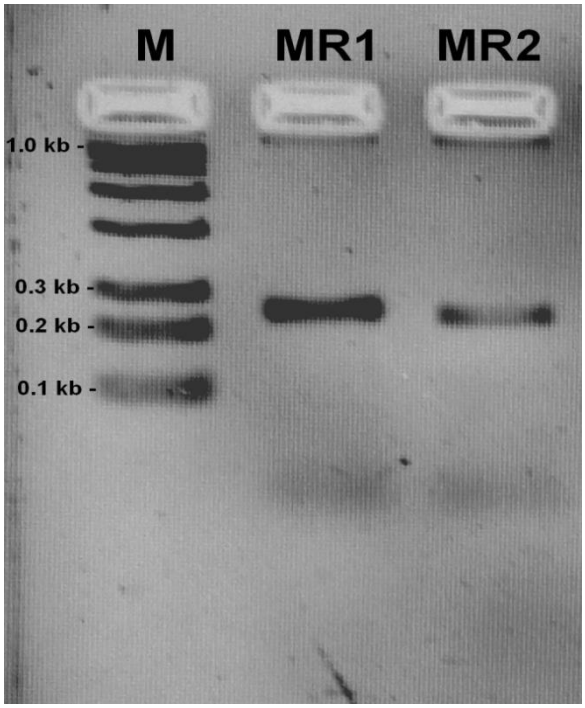

Figure 16: PCR amplification profile of intron 1 of *waxy* gene (M= 100 bp marker; MR1= Chakhao amubi; MR2= Taothabi).

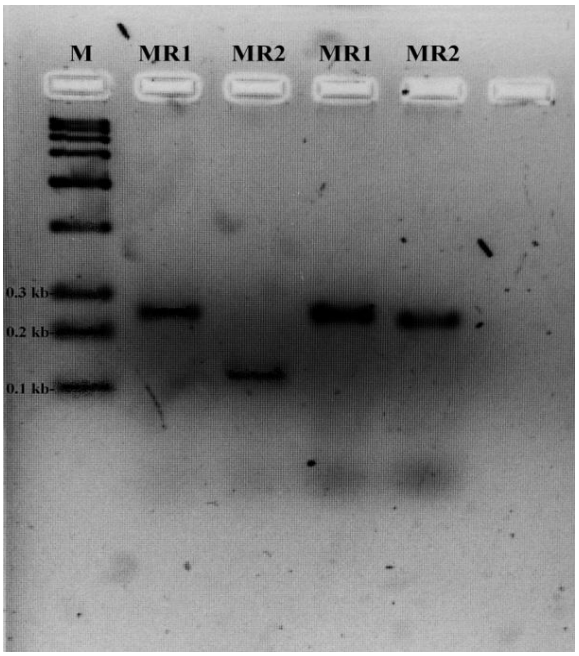

Figure 17: Electrophoregram of the restriction digestion by the enzyme *Acc*I of the PCR amplified product on a 2.2 % agarose gel (M= 100 bp marker; MR1= "Chakhao amubi"; MR2= Taothabi).

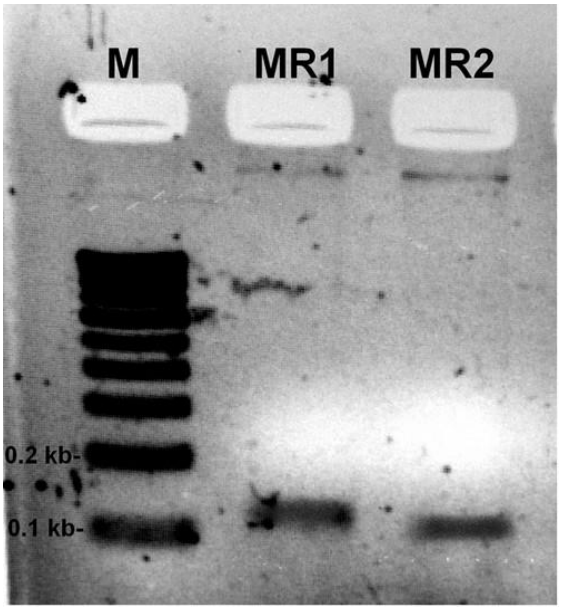

Figure 18: PCR amplified product of the RM190 microsatellite of the *waxy* gene(M= 100 bp marker; MR1= Chakhao amubi; MR2= "Taothabi.

Table 7: Details of the RM190 microsatellite sequences (underlined) of the two rice landraces obtained

Sample	RM190 sequence	CT_n allele	Apparent amylase class (AAC %) based on Ayres *et al.* (1997)
Chakhao amubi	GCAGCATAATGT<u>CTCTCTCTCTCTCTCTCTCTCTCTCTCT</u>GTGATACACATCTATGAGTGTGTTGGTCTGTTGTTGTCCGGAAGATATCTGAAAA (95 bp)	14	Intermediate (AAC 18-23%)
Taaothabi	AAGGCATAA<u>CTCTCTCTCTCTCTCTCTCTCT</u>TCACTTCTCTGCTTGTGTTGTTCTGTTGTTCATCACGAAAACATCTGCAAA (80 bp)	10	High (AAC ≥23%)

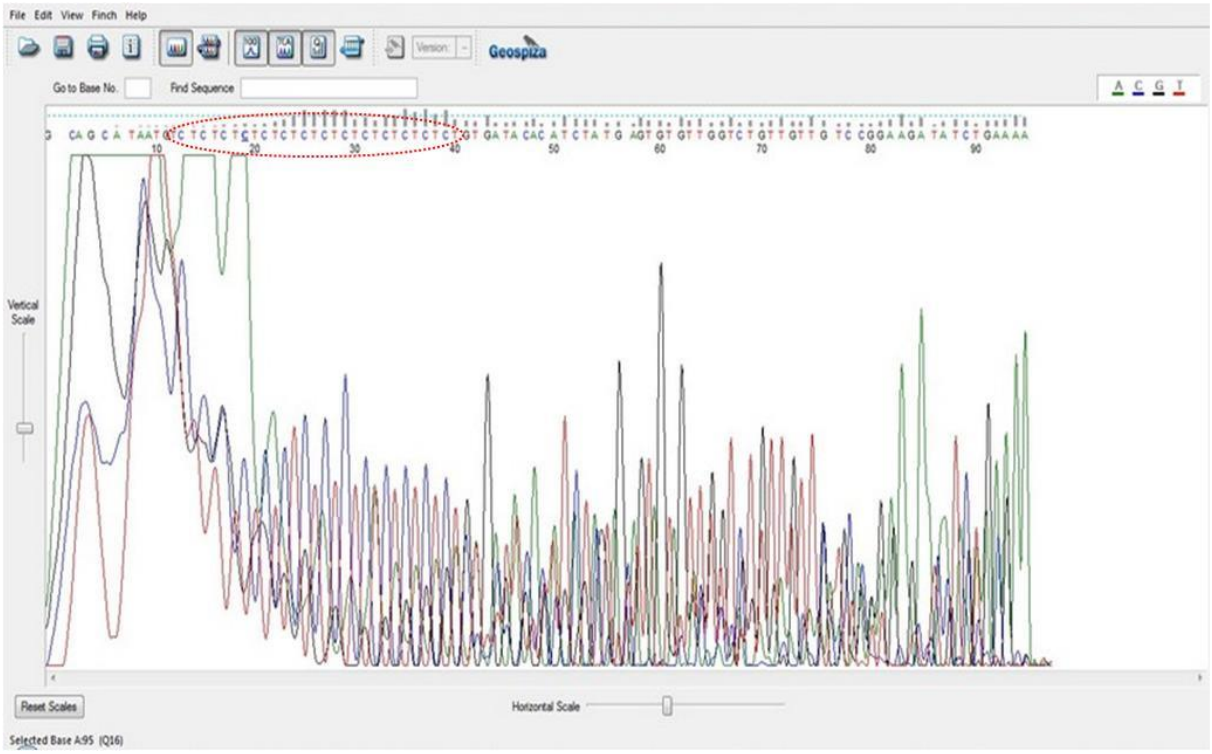

Figure 19: Chromatogram of the RM190 microsatellite sequence of Chakhao amubi.

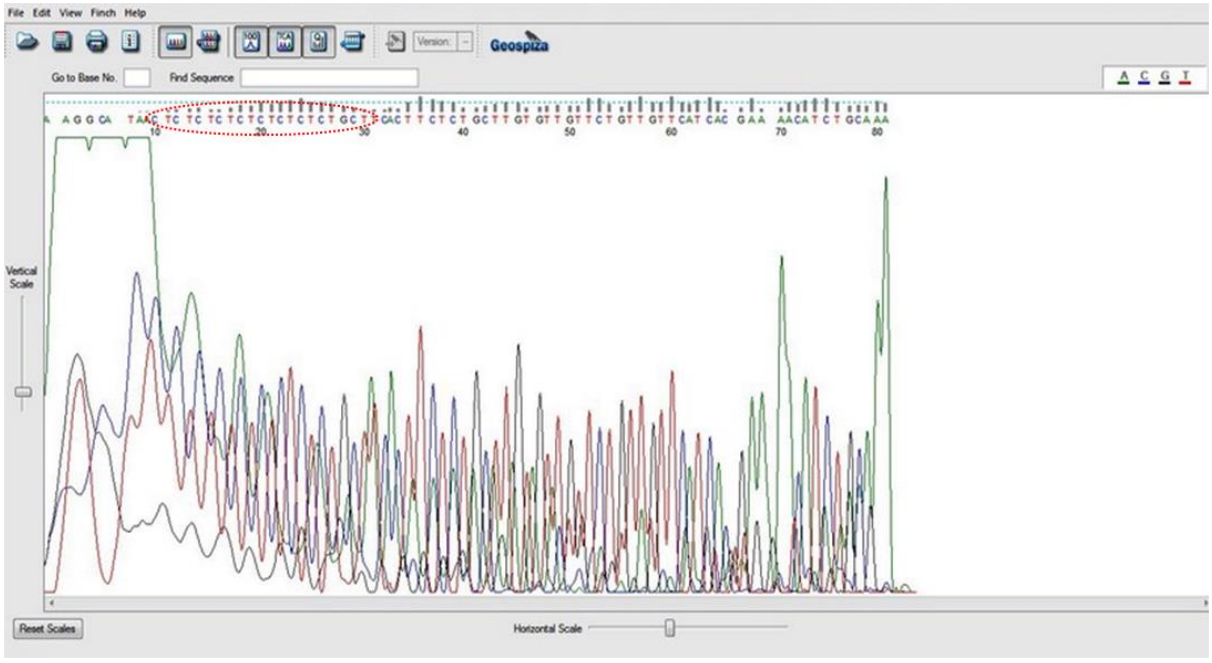

Figure 20: Chromatogram of the RM190 microsatellite sequence of Taothabi

Chapter 4
Discussion

CHAPTER 4

DISCUSSION

In the present study two local rice landraces of Manipur namely - "Chakhao amubi" and "Taothabi" were studied. The evaluation of the grain characteristics, classification of starchy nature, *in vitro* regeneration and genotyping of *waxy* gene were carried out. The grain colours of the two rice landraces studied varied from light red of "Taothabi" to dark purple colour of "Chakhao amubi". The grain length of "Taothabi" was found to be slightly longer and wider than that of "Chakhao amubi". As per the FAO scale for milled rice both the rice grains were found to be of long bold grain type. "Chakhao amubi" with a distinctive aroma was found to be waxy or glutinous type of rice with development of brownish stain when reaction with iodine solution while "Taothabi" was found to be of non-waxy or non-glutinous rice with darkish blue stain. The brownish stain development is due to the reaction with amylopectin as the starch component while darkish blue stain is due to the reaction of iodine with starch amylose (IRRI Technical Bulletin, 1965).

In the present study, successful *in vitro* regeneration of the two rice landraces "Chakhao amubi" and "Taothabi" was achieved using dehusked mature grains. One of the valuable techniques in tissue culture to exploit somaclonal variation is the dehusked rice seed culture (Liu *et al*. 1997). In the study, callus formation was found in MS medium supplemented with 2, 4-D. The percentage of callus development was found higher in "Chakhao amubi" than "Taothabi" and varied with the concentration of 2, 4-D used in the medium. Many studies have revealed that the presence of 2,4-D as an important catapult factor in rice callus induction (Lin and Zhang, 2005; Karthikeyan *et al*. 2009 ; Joyia and Khan, 2013). Rashid *et al*. (2003) also reported that the degree of callusing in rice varies accordingly with the rice varieties. Rooting was found maximum in MS basal media without any PGR supplements.

In order to carry out a successful PCR-amplification of isolated DNAs, it is essential to optimise the PCR conditions specific for the particular species (Pandey *et al*., 2012). The presence of unwanted bands or smearing of the PCR products may be observed due to the use of inappropriate PCR conditions such as concentrations of template DNA, primer, $MgCl_2$, Taq DNA polymerase and dNTPs. In the present study, several parameters such as concentration of template DNA, $MgCl_2$, dNTPs, primer, Taq DNA polymerase have been tested to standardize the efficiency of amplification. The PCR amplification of the *waxy* gene was optimised for a 15 µl reaction mixture containing 10× PCR buffer, 50 ng of genomic DNA as template, 0.5 µM each of the primers, 1.5 mM of $MgCl_2$, 0.2 mM of dNTP

(Himedia, India), 1 U of Taq Polymerase (Sigma-Aldrich Pvt. Ltd, Bangalore).The need for standardization of PCR conditions for specific analysis and their reasons have been widely reported. Excessive concentrations of genomic DNA and chemicals ($MgCl_2$, dNTP, Taq DNA polymerase and primers) in the PCR amplification reduced the amplification efficiency thereby leading to smearing and non-specific yield of the products (Padmalatha and Prasad, 2006; McPherson and Moller, 2006).

The rice end-use quality attributes is determine by its apparent amylose content (AAC). The *waxy* gene, located on rice chromosome 6, encodes the enzyme-granule bound starch synthase (GBSS), which plays a key role in apparent amylase synthesis (Smith *et al*., 1997). Genotyping of intron1 of the *waxy* gene (In1G and In1T alleles) showed that "Taothabi" rice landrace was cleaved by the enzyme*Acc*I but not in "Chakhao amubi". This confirms the presence of AGGTATA sequence in the intron 1 region of *Waxy* gene of "Taothabi" rice landrace. Li *et al*. (1995) and Wang *et al*. (1995) also reported that high amylose cultivars have the sequence AGGTATA in the putative 5′-leader intron splice site. Ayres *et al*., (1997) also reported that the sequence AGTTATA at the putative leader intron 5′ splice site was found in cultivars with 18% or less amylose whereas cultivars with a higher proportion of amylose had AGGTATA. The RM190 microsatellite CT_n alleles in the *waxy* gene were first identified by Bligh *et al.* (1995). Ayres *et al.* (1997) reported eight RM190 CT_n alleles and one in addition by Bergman *et al.* (2000). In the study, two RM190 microsatellite allele viz. CT_{10} and CT_{14} was found. "Taothabi" rice landrace was found to have CT_{10} allele which is associated with a higher apparent amylose content of ≥23%. While "Chakhao amubi" was found with CT_{14} allele which is associated with intermediate apparent amylose content with 18-23% (Ayres *et al.* 1997). The existing level of genetic diversity among the crop populations is fundamental and serves as a backbone of the genetic resources to conserve for both their local survival as well as for future development which forms the basis for advanced breeding and improvement programs (Thangjam, 2014).

Thus, in the present study, grain characteristics, starchy nature and the polymorphism of *waxy* gene along with standardization of an efficient *in vitro* regeneration protocol of the two local rice landraces of Manipur namely - "Chakhao amubi" and "Taothabi" were established. These findings will be useful for understanding the scientific basic of the two rice landraces studied for any breeding or improvement programs.

SUMMARY

CHAPTER 5

SUMMARY

Rice, *Oryza sativa* L. (2n =24) is an annual herbaceous plant of the family Graminae. The genus *Oryza* comprises of 23 wild and weedy species and two cultivated species, viz., O. *sativa* and O. *glaberrima* and rest are wild species which include both diploid and tetraploid forms.

The major findings of the present study are summarized as follows:

- ✓ Two economically important rice landrace viz. "Chakhao amubi" and "Taothabi" of Manipur was studied for its grain characteristics and variation. Both the rice landraces studied was found to be of long bold grain type with "Taothabi" grains slightly longer in length as compared to "Chakhao amubi".
- ✓ Grain colour of the two selected rice landraces varied from dark purple colour of "Chakhao amubi" to light red colour of "Taothabi" rice landrace.
- ✓ Grains of the two rice landrace was studied to determine it waxy or non-waxy character with "Chakhao amubi" found to be of waxy type while "Taothabi" rice landrace was found to be non-waxy type of rice grains.
- ✓ An efficient *in vitro* regeneration protocol was established for "Chakhao amubi" rice landrace using mature grains as the explants. Germination percentage was found to be high in all the media sets used in the study. MS medium with 2, 4-D (1 mg/L) showed the maximum percentage of root initiation from the explants. MS medium supplemented with 2, 4-D (2 mg/L) showed the highest percentage of callus formation. Highest percentage of root formation and plantlet regeneration was observed in MS medium without PGRs.
- ✓ An efficient *in vitro* regeneration protocol was established for "Taothabi" rice landrace using mature grains as explants. Germination percentage was found high in all the MS medium with different concentrations of PGRs used in the study. Thehighest response for shoot and root regeneration in the *in vitro* cultured explants was found in MS medium without any PGRs.
- ✓ In the genotyping of intron 1 of the *waxy* gene, the G/T polymorphism was successfully assayed by enzyme digestion with *Acc*I. "Taothabi" rice landrace was found to be cleaved by *Acc*I which showed the presence of the sequence AGGTATA.

While “Chakhao amubi” was not cleaved, this showed the presence of AGTTATA in the amplified fragments of the intron 1 of the *waxy* gene.

- ✓ For the genotyping of the RM190 microsatellite CT_n alleles of the two rice landraces, the amplified fragments were sequence and analysed for the determination of CT repeats. “Chakhao amubi” rice landrace was found to have CT_{14} allele and “Taothabi” rice landrace was found with CT_{10} allele.

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ANNEXURES

Bio-data

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EDUCATIONAL QUALIFICATION

Sl. No.	Examination passed	Year	Board/University	Percentage
1	HSLC	2007	BOSE, Manipur	74
2	HSSLC	2009	COHSE, Manipur	67.6
3	B. Sc (Agriculture)	2013	Annamalai University	82.8
4	M. Sc (Agriculture)	2015	SHIATS, Allahabad	72.6

Papers presentation/participation in conference/symposiums:

1. Presented a poster entitled "*In vitro* regeneration of Chakhao amubi, indigenous black aromatic rice (*Oryza sativa* L. *indica*) of Manipur" in the "International Conference on Natural Resources Management for Sustainable Development and Rural Livelihoods" held during 26-28 October, 2017 at Mizoram University.
2. Presented an oral presentation entitled "*In vitro* regeneration of Taothabi (*Oryza sativa* L. *indica*), an indigenous cultivated rice landrace of Manipur" in the "National Conference on Recent Advances in Biotechnology" held during 9-10 November, 2017 at Mizoram University.

INTERNATIONAL CONFERENCE ON NATURAL RESOURCES MANAGEMENT FOR SUSTAINABLE DEVELOPMENT AND RURAL LIVELIHOODS

26-28 October, 2017

Certificate of Presentation

This is to certify that Prof./Dr./Mr./Ms. Punshi Singh has delivered a key note/presented a lead paper/ paper/poster/chaired an academic session entitled In Vitro Regeneration of Chakhao Amubi, Indigenous Black Aromatic Rice (Oryza Sativa L. Indica) of Manipur in the International conference hosted by the Department of Geography and Resource Management, School of Earth Sciences and Natural Resources Management, Mizoram University (a Central University), Aizawl, Mizoram, India on 26th-28th October, 2017.

Prof. Vishwambhar Prasad Sati
Convener and Head

Dr. K. C. Lalmalsawmzauva
Organizing Secretary

In Vitro Regeneration of Chakhao Amubi, Indigenous Black Aromatic Rice (*Oryza sativa* L. *indica*) of Manipur

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Abstract

Northeast India, located in the Indo-Burma mega-biodiversity hotspot region, in known for its huge reserve biological resources. Rice is the main staple food in the region and more than 30,000 cultivars are planted including more than 2,000 local landraces. Among the landraces, an indigenous black aromatic rice (*Oryza sativa* L. *indica*) of Manipur known as chakhao amubi is considered as one of the most important owing to its anthocyanin content and scented qualities. The major issues related with this local rice are low yield and restricted planting areas. Biotechnology provides a viable option for the genetic improvement of crop plants including rice. For any genetic improvement programs through genetic engineering, an efficient *in vitro* regeneration protocol is needed. In the present study *in vitro* regeneration of chakhao rice was successfully established. Seeds cultured on basal MS media supplemented. High germination and regeneration potential was observed with NAA and 2,4-D supplemented media. *In vitro* germination was recorded within 2 days of culture. Subsequent regeneration and shoot multiplication were observed by the 7th day and well developed roots were observed in 14 days. Successfully regenerated plantlets were hardened and transplanted into soil condition.

Keywords: Chakhoa, black aromatic rice, Manipur, *in vitro* regeneration

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Mizoram University (A Central University), Aizawl – 796004

Certificate

Certified that Mr./Ms./Dr./Prof. J. Premlata Singh *of* Department of Biotechnology, Mizoram University *participated and presented a paper/poster entitled* "In vitro regeneration of Tadhabi (Oryza sativa L. indica), an indigenous cultivated rice landrace of Manipur." *in the National Conference on "Recent Advances in Biotechnology" organized by Department of Biotechnology, School of Life Sciences, Mizoram University, Aizawl – 796004 during 9th & 10th November, 2017.*

(Dr. Thangjam Robert Singh)
Convenor

(Dr. J. Bhattacharya)
Head, Department of Biotechnology
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Lianzela
(Prof. Lianzela)
Vice Chancellor
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studied and it could be utilized for future conservation and sustainable utilization of the genetic resources.

PB4(Oral): *In vitro* regeneration of "Taothabi" (*Oryza sativa* L. *indica*), an indigenous cultivated rice land race of Manipur.

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Abstract

As the state of Manipur is located in the Indo-China region, which is considered to be the primary centre of origin of rice, diverse forms of indigenous rice land races are found here. Rice is the main staple food in the region and around 300 local land races including two wild rice have been reported in the region. Among the land races, an indigenous red coloured local rice (*Oryza sativa L. indica*) taothabi is known for its tolerance to water lodging and resistance to gall midge pest. Biotechnology provides a viable option for the genetic improvement of crop plants including rice. For any genetic improvement programs through genetic engineering, an efficient and reproducible *in vitro* plant regeneration protocol is needed. In the present study, *in vitro* plant regeneration of taothabi was successfully established. Seeds were cultured on MS media supplemented with different concentrations of 2,4-D, NAA, BAP and kinetin hormones. Callus induction was found in MS media supplemented with 1 mg/L, 2 mg/L 2,4-D and 2 mg/L NAA. Highest callus induction frequency was observed with 2 mg/L 2,4-D supplemented medium. *In vitro* germination of mature

seed was recorded within 2 days of culture. Subsequent regeneration and shoot initiation were observed by the 7th day and well developed roots were observed in 2 weeks after culture. Successfully regenerated plantlets were hardened and transplanted in soil condition.

PB5(Oral): Characterization of grain morphology and *in vitro* regeneration of Mizo indigenous rice (*Oryza sativa* L.) landraces

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Abstract

Rice is one of the most important crop species in the world. It is the main food source for nearly half of human population and 23% of global caloric consumption comes from this plant. Rice is cultivated on an estimated 3% of the world's agricultural land, and serves as a primary source of calories for over half the world's population. In the present study, in vitro germination and regeneration studies and characterization of grain morphology was carried out on 5 rice landraces of Mizoram viz. MZR-01, MZR-02, MZR-03, MZR-04 and MZR-05. Mature rice grain embryos were used as sources of primary explants and were surface sterilized with 50% of Sodium hypochloride and 0.1% Mercury Chloride. The explants were inoculated on MS media supplemented with different concentration of Plant Growth Regulators (PGRs). Characterization of grain morphology was done

In vitro regeneration of selected rice (*Oryza sativa* L. *indica*) landrace of Manipur.

Dissertation submitted in partial fulfilment
of the requirements for the
Degree of Master of Philosophy in Biotechnology

By

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2017

Abstract

Abstract

Rice (*Oryza sativa* L.) is a plant belonging to the family of grasses, Gramineae (Poaceae). It is one of the three major food crops of the world and forms the staple diet of about half of the world's population. The genus *Oryza* consists of 23 wild species and two cultivated species viz. *O. sativa* and *O. glaberrima*. *O. sativa* is an Asian cultivated rice while O. *glaberrima* is grown only in limited areas of West Africa. *O. sativa* is recognised into three sub species namely *indica, japonica* and *javanica*. India has a long history of rice cultivation. Globally, it stands first in rice area and second in rice production after China. India is also known for its quality rice, like basmati and other fine grain aromatic types grown in different regions of the country. The North eastern region of India is considered to be one of the hot pockets of rice genetic resources in the world and a potential rice-growing region with extremely diverse rice growing conditions as compared to other parts of the country. Manipur, a north eastern state located in the Indo-China region, which is considered to be the primary centre of origin of rice, has a diverse forms of indigenous rice land races. Rice is the main staple food in the region and around 300 local land races including two wild rice have been reported in the region. Among the landraces, "Chakhao amubi"- black scented rice is popular for its flavour, aroma and anthocyanin content. It is mostly confined to serving only as delicacy in local feast and festivals. Till very late, the black rice is not cultivated commercially due to its low yield and also as the same cannot be used as staple food thereby resulting to limited market access. "Taothabi" is also an indigenous rice landrace of the region well known for its tolerance to water lodging and resistance to gall midge pest. The type of starch in the rice grain determines the preference by different populations. Amylose is the most important grain constituent that influences rice end-use quality and it is the major determinant used across the world to define rice market classes.

Biotechnology provides a viable option for the genetic improvement of crop plants including rice. For any genetic improvement programs through genetic engineering, an efficient and reproducible *in vitro* plant regeneration protocol is needed. The present study was carried out for the characterization of the grain, starchy nature, initiation and standardization of aseptic cultures to develop an efficient and reproducible *in vitro* plant regeneration system and to understand the *waxy* gene polymorphism in the two rice landraces of Manipur viz. "Chakhao amubi" and "Taothabi".

In the grain morphology study, “Chakhao amubi” grains were found to be of dark purple colour and “Taothabi” with light red colour. The grains of “Chakhao amubi” and “Taothabi” were found to have long bold type. The starchy nature of the rice grain was also studied. Polished rice grains of “Chakhao amubi” was found with brownish stain while “Taothabi” showed darkish blue stain when reaction with potassium iodide-iodine solution. The dark blue stain in “Taothabi” grains was due to the presence of high amylose which showed the non-waxy character of the rice grains. While “Chakhao amubi” grains was found to be of waxy character with brownish stain whe reaction with potassium iodide-iodine solution. *In vitro* regeneration of the two rice landraces was successfully established during the study. High germination percentage was observed in all the media sets used with germination observed from the 2^{nd} day after culture in both “Chakhao amubi” and “Taothabi”. Callus induction was found in MS media supplemented with 1 mg/L and 2 mg/L 2,4-D. Highest callus induction frequency was observed with 2 mg/L 2,4-D supplemented medium in both the rice landraces. Subsequent regeneration and shoot initiation were observed by the 7^{th} day and well developed roots were observed in 2 weeks after culture. Successfully regenerated plantlets were hardened and transplanted in soil condition. During the study, intron 1 of the *waxy* gene was successfully genotype and the G/T polymorphism was also detected using the enzyme *Acc*I. “Chakhao amubi” was found to have the AGTTATA sequence in the intron 1 of the *waxy* gene and was not cleaved by the cleaved by the enzyme *Acc*I. While “Taothabi” was cleaved this showed the presence of the sequence AGGTATA in the intron 1 of the *waxy* gene. In the genotyping of the RM190 microsatellite of the *waxy* gene, the CT allele repeats were analysed from the sequenced product. “Chakhao amubi” was found with CT_{14} allele while “Taothabi” was found to have CT_{10} allele. As reported, CT_{14} alleles was found to be associated with intermediate apparent amylose content (AAC 18-23 %) while CT_{10} allele was associated with high AAc content of more than 23 %. Thus, “Chakhao amubi” was found to be of intermediate AAC type whereas “Taothabi” was of high AAC type.

The present study revealed the grain characteristics, starchy nature and the polymorphism of *waxy* gene of the two local rice landraces of Manipur namely along with the standardization of an efficient *in vitro* regeneration protocol. These findings will be useful for understanding the scientific basic of the two rice landraces studied for any breeding or improvement programs.