"*IN VITRO* PROPAGATION OF THERMOSENSITIVE GENIC MALE STERILE RICE (*Oryza sativa* L.)"

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By

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Thesis

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2016

DECLARATION

I hereby declare that this thesis entitled "***In vitro*** **propagation of thermosensitive genic male sterile rice (*Oryza sativa* L.)"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associate ship, fellowship or other similar title, of any other university or society.

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Nisthar E.

Dedicated

To my family

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LIST OF ABBREVIATIONS AND SYMBOLS USED

%	Per cent
±	Plus or minus
µg	Microgram
µl	Micro litre
µM	Micro Molar
2 ip	2-isopentenyladenine
2, 4-D	2, 4-dichlorophenoxyacetic acid
BA	N^6- benzyl adenine
$Cacl_2$	Calcium Chloride
CD (0.05)	Critical Difference at 5% level
cm	Centi Metre
CMS	Cytoplasmic male sterile system
EGMS	Environmental genic male sterile system
et al.	And other co workers
etc	Etcetera
g	Gram
g^{-1}	Per gram
GA	Gibberellic acid
HCl	Hydrochloric acid
$HgCl_2$	Mercuric Chloride
hrs	Hours

IAA	Indole-3-acetic acid
IBA	Indole-3-buteric acid
Kn	Kinetin (6-furfurylaminopurine)
M	Molar
mg	Milligram
min	Minutes
ml	Millilitre
mM	millimolar
MS	Murash and Skoog (1962)
NAA	α-Naphthalene acetic acid
NaCl	Sodium Chloride
NaOH	Sodium hydroxide
ºC	Degree Celsius
PGMS	Photosensitive genic male sterile
sec	Seconds
sp.	Species
spp.	Species (plural)
TGMS	Thermosensitive genic male sterile
viz	Namely

Introduction

1. INTRODUCTION

The crop improvement and development in major food crops to tackle the needs of the world's growing population requires implementation of new and conventional hybridization techniques. Other constrains like shrinking area of land, water scarcity, evolution of newer biotypes of pests, diseases and threats from climate change figure serious challenges to rice breeders. Thus high yielding varieties in rice are needed to overcome these problems. Rice (*Oryza sativa* L.) belongs to Poaceae family and is one of important cereal staple food after wheat and maize. It is cultivated largely in humid tropical and subtropical regions. Being self-pollinated crop, rice can be extensively used in crop improvement and hybridization techniques. The use of male sterile hybrid system in rice has revolutionized the hybrid seed production technology. It is widely used as a simple, efficient and inexpensive system for development of new hybrids. To overcome the tedious procedure of emasculation, rice breeders extensively use male sterile systems.

The three line hybrid system (Cytoplasmic male sterile system - CMS) is the largely used method among male sterile systems. It is maternally inherited and male sterility is induced by cytoplasmic gene. But it has major drawback such as it requires specific maintainer lines and restorer lines To overcome these constraints a novel male sterile system is required. Environmental genic male sterile system (EGMS) or two line hybrid system, the male sterility induced by an environmental factor (temperature or photoperiod). The photoperiod sensitive genic male sterile system (PGMS) is widely utilized in China. But in India, temperature sensitive genic male sterile system (TGMS) is highly recommended due to small or marginal variation in photoperiodic differences. The TGMS genes are heritable in nature and pollen abortion is controlled by nuclear genes. In TGMS system, the production of hybrid seeds depend upon the high temperature (>30°C) and at low temperature (<23°C) it turns fertile. These characteristic features of TGMS enables the hybrid seed production and it was

observed as more effective and efficient to increase the grain yield (Yuan, 1990). The main advantages of TGMS system include the ability to use wide range of genotypes as male parents, absence of maintainer lines and restorer lines. Thermosensitive genic male sterility (TGMS) in rice was first reported in China (Yuan, 1987), Japan (Maruyama *et al.*, 1990), and IRRI (Virmani and Voc, 1991). In tropical region TGMS lines are multiplied through selfing at specific temperature after 5-15 days of panicle initiation (Maruyama *et al*, 1990; Borkakati and Virmani, 1996). The International Rice Research Institute (IRRI), Philippines developed most of the TGMS donor lines for hybrid seed production.

Due to lack of male sterile red rice line, currently there is no red rice hybrid available in Kerala. Thus TGMS system is one of the best possible system to develop a male sterile red rice hybrid. The red rice pericarp is devoid of flavonoid group (Proanthocyanididne) a condensed form of tannin (Oki *et al.,* 2002). It is reported to have some medicinal property and antioxidant property. Also it is reported to decrease the formation of antherosclerotic plaque formation in cardiovascular diseases (Ling *et al.,* 2001).

During the development of thermosensitive genic male sterile plants in red rice background, the problem pose among the breeder that the male sterile plants can be identified only at flowering stage by assessing pollen and spikelet sterility during summer. Hence, these sterile plants will not set seeds, but multiplication of thermosensitive genic rice plants is necessary for further breeding. So, *in vitro* propagation is the only alternative to multiply the developed male sterile lines. The successful implementation of *in vitro* procedures depends on the age, genotype of explant, nature of explant, culture media and culture condition.

Hence, the main objective of present study was to standardize the method for *in vitro* propagation of thermosensitive genic male sterile rice.

Review of literature

2. REVIEW OF LITERATURE

The world population is doubling very quickly and the problems like hunger, malnutrition, famine in food crops have affected most of the developing countries. To ensure food security to next millennium a better scientific development in the field of crop science is needed. As a result of green revolution, high yielding varieties (HYVs) of major food crops were developed (especially in rice and wheat). And there was a tremendous increase in rice 2.8 fold and wheat 2.6 fold in global production.

Rice, (*Oryza sativa* L.) belongs from Poaceae family (old Graminae). The genus includes 26 species (Khush, 1997). Only two species are cultivated (*Oryza sativa* L. and *O. glaberrima Steud*) and others are wild varieties. According to Datta *et al*. (2003), rice (*Oryza sativa* L.) is further divided into 3 subspecies - *Indica, Japonica* and *Javanica* and out of which both *Indica* and *Japonica* are cultivated largely in most of the Asian countries. Among the rice cultivation, 80 per cent composed of *Indica* rice variety (Ramesh *et al*., 2009 and Tie *et al*., 2012). It is the basic food for over half of the human population and one of the most widely cultivated food crop in south Asia. The consumption of rice is increasing with respect to production at a rate of 1.6-1.8 per cent (Shobarani *et al*., 2010). In India, rice carries major role in the development of annual GDP at 15 per cent and support required nutrient with 43 per cent calorie to over 70 per cent of the total population. Thus high yielding rice varieties are required to satisfy the future needs.

The crop improvement and development in rice breeding utilize various strategies since, it is a highly self -pollinated crop. The introgression of superior traits in to cultivated varieties is possible in both conventional and non-conventional breeding methods. The hybrid rice development technology uses different methods and systems to ensure its applicability. Among them male sterile system, cytoplasmic genic male sterile system (CMS) and environmental genic male sterile system (EGMS) have been widely used by rice breeders. The first hybrid development in rice was initiated

in china (Yuan, 1977). From 1976 to 1991, hybrid technology boosted the production of rice in China (around 200 million tons) (Yuan, 1994). Later the International Rice Research Institute (IRRI) started research on hybrid rice in 1979.

In cytoplasmic male sterile system (CMS), male sterility is determined by cytoplasmic and nuclear genes. Currently, the three line system (CMS) is the largely exploited system which is used by rice breeders to create progenies. Although it is widely commercialized, it pose some difficulties such as it requires a maintainer line and high production cost of hybrid progenies. To overcome these constrains the two line system was adopted in China (Yuan, 1997). The major advantage of two line hybrids over three line hybrid includes possibility to use different genotype as donor parent, not requiring specific maintainer lines and minimizing the negative effect of cytoplasmic sterility factor. (Arasakesary *et al*., 2015). Hence it enables hybrid seed production much simpler and cost effective. In two line hybrid system (EGMS), sterility factor is determined by nuclear genes and by the effect of environmental factors such as temperature and photoperiod. The first report of male sterility system was recorded by Martin and Crawford (1951) in pepper. Later Shi (1981, 1985), Zhou *et al.* (1988), Sun *et al.* (1989), Maruyama *et al.* (1991), Virmani and Voc (1991) also studied about male sterile system in rice. Among the EGMS system, temperature sensitive genic male sterile system (TGMS) is more preferred above the photoperiod sensitive genic male sterility systems (PGMS) under tropical conditions in which day length differences are marginal (Virmani *et al.,* 1996).

The thermo sensitive genic male sterile system (TGMS) was first identified in China (Yuan, 1987), later in Japan (Maruyama *et al*., 1990), and followed by IRRI (Virmani and Voc, 1991). The male sterile character is governed by recessive single nuclear gene and sterility-fertility behavior is restored with varying temperature (Maruyama *et al.*, 1990; Borkakati & Virmani, 1996). During hybrid development TGMS lines with any fertile line can be used as parental lines. The trait of male sterility

in TGMS is heritable in nature. At a higher temperature it shows sterility (>30^0C) and fertility is restored at lower temperature (<23^0C). Thus, TGMS promises higher yield and seed developmental efficiency among hybrid seed production methods (Yuan, 1990). The TGMS lines provided by IRRI had greater heterotic frequency than CMS lines (Lopez and Virmani, 2000).

There is no sufficient information about the genetic background of TGMS genes. Currently, 12 TGMS genes have been identified by the investigators. The mapped *tgms* genes includes *tms1* on chromosome 8 (Wang *et al*., 2003), *tms2* on chromosome 7 (Lopez *et al*., 2003*), tms3* on chromosome 6 (Subudhi *et al*., 1997)*, tms4-1* on chromosome 2 (Dong *et al*., 2000)*, tms5* on chromosome 2 (Wang *et al*., 2003)*, rtms1* on chromosome 10 (Jia *et al*., 2001)*, ms-h* on chromosome 9 (Koh *et al*., 1999) *tms6* on chromosomes 5 *(*Lee *et al*., 2005) , *tms8* on chromosome 11 (Appibhai *et al*., 2012), *tms9* on chromosome 5 (Sheng *et al*., 2013), *tmsX* on chromosome 10 (Peng *et al*., 2006) and *ptgms2-1* on chromosome 2 (Xu *et al*., 2011). TGMS system can be well recommended in tropical country like India where temperature variation is huge between Rabi and Kharif seasons (Singh *et al*., 2011).

In Kerala, The TGMS system is the best suitable system to develop hybrid rice and it satisfy the increasing needs of consumption of rice. The state under tropical zone shows marginal changes in temperature between seasons. Red rice is most preferred by consumers among the other rices due to its medicinal values and wide acceptability in Kerala. Uma and Jyothi are the two ruling verities released by Kerala Agricultural University.

Kerala has no red rice hybrid yet and so the TGMS system can be used to develop the hybrids in red rice background. But during the developmental procedure through conventional breeding technique, the developed TGMS rice plants do not set seeds due to sterile nature of lines. Which could be identified only at a flowering stage. In order to multiply the developed male sterile lines at summer, *in vitro* procedure is

the only possible alternative. Hence the present investigation aim at checking the possibilities of multiplication of TGMS plants with different explants as source material.

The present study on "*in vitro* propagation of thermosensitive genic male sterile rice (*Oriza sativa*. L.)" is aimed to standardize the method for *in vitro* propagation of TGMS rice plants.

2.1 *IN VITRO* PROPAGATION OF MALE STERILE RICE

Biotechnological tools promises new frontiers to tackle the increasing demand of rice production with respect to growing population. *In vitro* regeneration of rice depends upon the genotypes of the explants used. It is used as a method for production of genetically identical progenies.

The first successful report on *in vitro* propagation of rice by Kawata and Ishihara (1968) on root as explants source. Later Nishi *et al*. (1968) cultured from seed and Tamura (1968) from embryos. Rueb *et al*. (1994) reported that explant genotype, nature, age, energy source, plant growth substances, chemicals used in culture medium, culture condition determine the result of *in vitro* propagation.

2.1.1 Type of explants

Efficient callus induction and green plant development from different type explants such as anther, young panicle, young embryo, and mature embryo of 19 rice varieties were reported by Xiu-hong *et al*. (2005). Anthers resulted in low callus induction with only 3.97 per cent compared to other explants 69.17 per cent (young panicle), 69.21 per cent (young embryo) and 62.75 per cent (mature embryo) respectively. According to Carsono and Yoshida (2006) interaction of different genotype of rice as seed explants resulted higher regeneration potential (cultured on MS medium).

The seeds of Basmati-370 cultured on MS media supplemented with 2, 4-D (0.5 mg) and 2iP (0.1 mg) resulted in highest callus induction frequency and plantlet regenerated on BA (1.5 mg) (Marassi *et al*., 1996). Rashid *et al*. (2001) cultured super basmati on MS and N6 medium with higher percentage of callus induction obtained on 2, 4-D treatment which followed regeneration on BAP and NAA. Mannan *et al.* (2013) reported that the rate of callus induction increased with treatment of 2-4D (2 mg L^{-1}) alone in two traditional varieties Kalijira and Chinigura in Bangladesh. The callus initiation rate decreased in combination of 2, 4-D with BAP and kinetin.

Li-hui *et al.* (2000) studied on micropropagation of young panicle of wild rice with different genotypes. They confirmed that regeneration percentage of wild rice varied with its genotype. Zafar *et al*. (1992) used mature embryo and immature inflorescence as explant source for Basmati 370 in MS medium with 2, 4-D as growth hormone. Deepti *et al*. (2001) experimented on culturability of mature embryos of Pusa Basmati1 in MS medium with lower concentration of 2, 4-D (2 mg L^{-1}), which helped to obtain better callus initiation and higher regeneration potential on BA (0.5 mg L^{-1})

Bhuiyan *et al.* (2014) experimented on in vitro callus induction and regeneration frequency of two submergence tolerant *Indica* varieties (BRRI dhan 52 and FR13A). Efficient callus induction was obtained on MS media supplemented with 2, 4-D (3.0 mg L^{-1}) and highest shoots regeneration was recorded in the MS media supplemented with kinetin (2 mg L^{-1}), BA (2 mg L^{-1}) and NAA (1 mg L^{-1}). Efficient callus induction and regeneration of plantlets from verities of Sita, Rupali and Swarna Masuri was reported by Upadhyaya *et al*. (2015) on MS medium with varying concentration of 2, 4-D and in which effective callus induction obtained on 2, 4-D (2 mg L^{-1}).

2.1.1.1 Anther as Explants

Androgenic potential of plants was first revealed by Guha and Maheshwari in 1964 from *Datura* species. Later Bourgin and Nitsch (1967) developed haploids from *Nicotiana tobaccum.* Androgenic induction on rice was first reported by Niizeki and Oono (1968) in Japan, followed by Guha *et al.* (1970) in India.

The fate of regeneration from anthers involves either by callus formation or via. direct embryogenesis. The problems like lower rate of green plant regeneration, high degree of albino regeneration and lower culture response of anthers leads to inefficient regeneration from anther culture in rice. According to Hartke and Lorz (1989) *Indica* varieties respond very low to *in vitro* techniques, due to constraint like poor callus proliferation, early anther necrosis and higher rate albino plant generation (Chen *et al*., 1991). Explant genotype, culture medium, physiology of donor plant and pre-treatment determines the successful regeneration protocols.

According to Hu (1985), and Raina (1997) *Japonica* rice verities show higher androgenic induction than the *Indica* cultivars. Lentini *et al*. (1995) exhibited that out of 35 *Indica* rice varieties tried on N6 medium only one *Indica* variety regenerated from callus. Raina and Zapata (1997) reported that efficient androgenic induction was observed in IR43 rice plants grown under long days (>12 h), high solar radiation (>18Mj m^{-2}), sunshine (>8 h) and day/night temperature (34ºC/24ºC).

Efficient androgenic callus induction involves different pre-treatment methods for induction stress resulted in higher regeneration potential. The pretreatment includes sugar starvation, heat treatment, cold treatment and osmotic stress (Bhojwani and Razdan, 1996). Type of treatment and time duration depend up on the explant genotype (Datta, 2001).

2.1.1.1.1. **Low temperature treatment**

It is the widely used pretreatment method to induce cold stress. Rate and duration cold of cold pretreatment depended up on explant genotype and nature. Efficient androgenic calli were obtained when panicle treated with 5^0C for 5 days compare to untreated ones (Chaleff and Stolarz, 1981).

Khatun *et al.* (2012) reported that cold treatment at 4^0C for 3-7 days could induce best frequency of regeneration by studying 20 different type anthers cultured on five induction media. Among 5 genotypes IR 43 resulted in high response on SK3 medium. Lower temperature pre-treatment of *Japonica* cv. Nipponbare at 10^0C for 10-30 days enhanced sporophyte development by Matsushima *et al.* (1988). Pande (1997) reported that efficient androgenic induction depend up on lower temperature treatment at 10^0C for 10 days and after 11 days resulted in albino regeneration in cultivated varieties of IR43.

2.1.1.2 Node as explant

Furuhashi and Yatazawa (1964) reported the effective plantlets of rice derived from the nodes of in vitro grown callus on Heller's medium with vitamins and 2, 4-D (2 mg L^{-1}). The calli for regeneration was obtained from *in vitro* grown seedlings.

Efficient callus induction and green plants were obtained with shoot nodes and roots of *in vitro* germinated seedlings on MS basal medium supplemented with 2,4-D and Kinetin as growth regulators (Yamada *et al*., 1967).

The potential of rice nodes in the clonal propagation technique was described by Kumar (1981). He also found that first node found below the panicle had very low regeneration potential compared to other nodes.

Reddy (1981) investigated on efficient regeneration of haploid plants via callus formation from internode as explant source in rice. Effective callus could be induced on Linsmaier medium (LS) supplemented with 2, 4-D.

Rashid *et al.* (2000) demonstrated efficient protocol for shoot regeneration from *in vitro* germinated callus culture of Basmati rice. Maximum shoot regeneration occurred on MS medium supplemented with sorbitol 3 per cent, sucrose 3 per cent, amino acids (2 g L^{-1}), BAP (5mg L^{-1}), NAA (1 mg L^{-1}) and gelrite (4 g L^{-1}). Raghavendra *et al.* (2010) developed multiple shoots from basal part of *Indica* cultivar Rasi on MS medium provided with thidiazuron (TDZ) and BAP.

2.1.1.3 Leaf as explant

Yan and Zhao *et al.* (1981) regenerated callus from young leaf blades of rice, *Indica* variety of Quang-Lu-Aion on MS medium provided with 2, 4-D (4 mg L^{-1}), NAA (1 mg L^{-1}) and IAA (1 mg L^{-1}). The obtained callus were transferred to regeneration medium supplemented on MS medium with BAP (1.5 mg L^{-1}), IAA (0.2 mg L^{-1}) and GA3 (4 mg L^{-1}).

Ramesh *et al*. (2009) Obtained callus induction in 4 day old *in vitro* developed leaf segments of *Indica* rice variety Rasi, on Linsmaier Skoog medium (LS) with 2, 4-D (11.3 µM) as growth regulator. Efficient plant regeneration was recorded on MS medium supplemented with BAP (2.2 µM) and NAA (0.27 µM).

Priya *et al.* (2011) developed effective procedure for regeneration plantlets through callus from leaf bases of abiotic stress sensitive IR 64 *Indica* rice obtained from *in vitro* grown seedlings on MS media provided with 2, 4-D and kinetin. Which resulted in higher frequency of callus regeneration (87.5 per cent) from 2, 4-D (13.5 µM) and kinetin (1.3 µM).

2.1.3 Surface sterilization of explants

The collected explants for *in vitro* regeneration usually colonized by many microorganisms. To overcome this problem adequate level of surface sterilizing agent such as sodium or calcium hypochlorite(5-10 per cent),ethyl alcohol (50-95 per cent)

and mercuric chloride (0.01-0.1 per cent) followed by rinsing with sterile water were carried out.

Sha *et al.* (2008) gave the treatment of rice anthers with ethanol (70 per cent) for few seconds and followed by treatment with mercuric chloride (0.1 per cent) for 20 min.

Surface sterilization of leaf base samples of *Indica* rice variety was conducted using 70 per cent ethanol for 60 sec and dipped in 1 per cent sodium hypochlorite for 15 min (Ramesh *et al.,* 2009).

The seeds of Ranbir and Basmati cultivars were given a treatment of detergent tween 20 followed by ethanol (70 per cent) and 0.1 per cent mercuric chloride (3min) in laminar air flow cabinet for surface sterilization (Mahajan *et al.,* 2013).

2.1.4 Culture medium

Effective *in vitro* regeneration of plantlets depends on culture medium and its adequate chemical composition. Chu (1975) reported that effective rice androgenic induction varied with culture medium supplemented with ammonium nitrogen level. Raina (1989, 1997) suggested that N6 medium as the best medium for japonica rice anther culture. Reddy *et al.* (1985) recommended He2 medium over N6 medium for *Indica* verities with lower NH_4+ions and which was modified from N6 medium with twice concentration of KH_2PO_4. Raina and Zapata (1997) studied with *Indica* variety IR43 and formulated M-019 medium. This medium was derived from SK-1 medium with replacement CH by a little amount of $(NH_4)_2SO_4$ and the level of KH_2PO_4 was increased from 170 mg L^{-1} to 540 mg L^{-1}. Ogawa *et al*. (1995) reported on effect of nitrogen source on androgenic callus induction in *Indica* rice variety IR24 cultured on R-2 medium. The medium supplemented with 40 mM KNO_3 and 2.5 mM $(NH_4)_2SO_4$ along with treatments of glutamine or alanine, among them best frequency of callus induction with maximum development of green plants were observed in alanine.

Benefits of maltose over sucrose is due to low degradation rate into glucose and sucrose is degraded into fructose which act as growth inhibitor in callus induction. (Last and Brettel, 1990).

Islam *et al.* (2004) studied with anthers of a hybrid rice line Pakhal 9690 on N6 medium supplemented with 2, 4-D (1.0 mg L^{-1}) and α-NAA (2.0 mg L^{-1}) was resulted most efficient callus induction (35.5 per cent). Followed by regeneration medium with α- NAA (2.0 mg L^{-1}) and kinetin (1.0 mg L^{-1}) treatment. Sah (2008) demonstrated the effect of culture medium with rice anthers of different genotype and reported that N6 medium provided with myoinositol (100 mg L^{-1}), 2, 4-D (2.5 mg L^{-1}), kinetin (0.5 mg L^{-1}), silver nitrate (10 mg L^{-1}) and maltose (50 g L^{-1}) resulted high callus induction frequency (17.65 per cent).

Thuan *et al.* (2001) studied the anthers of F_1 plants obtained from four crosses of aromatic and improved rice cultivars and subsequently regenerated on N6 and MS media supplemented with 2,4-D (0.5 mg L^{-1}), NAA (1.0 mg L^{-1}) and BAP (0.5 mg L^{-1}) for callus initiation. Regeneration of these plantlet were carried on MS and N6 media supplemented with BAP (1.0 mg L^{-1}) and NAA (1.0 mg L^{-1}) and higher frequency of callus induction was obtained in N6 medium with respect to MS medium (11.9 per cent and 7.95 per cent). This study revealed that percentage of green plant regeneration in N6 medium was eight times higher than MS medium.

Gioi *et al.* (2002) experimented on interaction of genotype and culture media in anther culture of F_1 plants obtained from four different crosses. Among N6, LS and MS with 2, 4-D (0.5 mg L^{-1}), NAA (2.0 mg L^{-1}) used for callus induction N6 medium exhibited high frequency of callus initiation (3.5 per cent) followed by (2.37 per cent), (2.63 per cent). The induced calli were sub cultured in regeneration medium N6 provided with NAA (0.5 mg L^{-1}), BAP (2 mg L^{-1}). The induced calli of F_1 progeny IR64/IR68530 resulted in highest plant regeneration percentage of 1.12 and no sufficient green plants could be regenerated from cross of IR64/IR70441.

Lutts *et al.* (1999) studied the effect of different concentrations of ABA, PEG, IAA, proline, tryptophan and NaCl in basal medium of two *Japonica* (Ilong Poa and Aiwu) and two *Indica* (IR 2153 and Nano Bolera) cultivars of rice. The experiment revealed that ABA, PEG, proline exhibited lower regeneration frequency and treatment with IAA increased the root regeneration but decreased shoot regeneration. Tryptophan promoted the regeneration and treatment of NaCl reduced the regeneration frequency.

Effect of 4 per cent sucrose along with sorbitol and 2, 4-D (0.5 mg L^{-1}) increased callus induction and regeneration in US rice genotypes Al-Khayri *et al.* (1996). According to Valdez *et a1*. (1996), MS medium was found as the highest callus induction medium and efficiency of regeneration was depending on the genotype of explants. Highest percentage of callus induction was obtained with 20 mM L-lysine on MS medium with 2, 4-D (2 mg L^{-1}), L-proline (l g L^{-1}) and casein hydrolysate (300 mg L^{-1}) in the Thai rice c.v. KDML105 (Pongtongkam *et al.,* 2004)

N6 media supplemented with organic adjuvants such as yeast extract, coconut water (5 and 10 per cent) and casein hydrolysate at different levels induced androgenic calli with sufficient growth regulators in five *Indica* rice genotypes (IR 72, Mansarovar, Taraori Basmati, Pusa Basmati and Karnal local 95). Yeast extract at concentration of 100 mg L^{-1} and coconut water (5 and 10 per cent) enhanced androgenic callus induction and green plant regeneration in N6 medium. The dosage of casein hydrolysate also had influence on enhancing the callus induction due to its organic nitrogen composition (Roy and Mandal, 2005).

Niroula *et al.* (2009) reported that efficient callus induction and regeneration from anthers depend on composition of medium and plant growth regulators. Highest callus induction frequency was achieved (14.1 per cent) on N6 medium fortified with 2, 4-D (2.5 mg L^{-1}), Kinetin (0.5 mg L^{-1}), myo-inositol (100 mg L^{-1}), silver nitrate (10 mg L^{-1}) and maltose 50 (g L^{-1}).

Silva and Ratnayake (2009) investigated anther culture potential of two traditional *Indica* cultivars *Kurulu Thuda* and *BG 250* on basal medium of N6 and SK-I. They reported that androgenic callus induction was nearly three times higher on N6 medium compared to SK-I medium in *Kurulu Thuda c*ultivar *with* 17.2 per cent. Only 1.4 per cent callus induction was reported from BG 250 and there was no plant regenerated from induced calli.

Pazuki *et al.* (2015) worked on the effect of various organic nitrogen sources and antibiotics in MS medium provided with 2, 4-D (4 mg L^{-1}), benzyl-aminopurine (0.4 mg L^{-1}), sucrose(30 g L^{-1}), Agar-agar (8 g L^{-1}) for *in vitro* callus induction from scutellum of four indigenous Iranian *Indica* rice (*Oryza sativa* L.). They identified that nitrogen source such as proline (2.8 g L^{-1}), promoted the callus induction but asparagine (0.75 g L^{-1}) inhibited the regeneration of calli.

Herath and Bandara (2011) studied androgenic potential of F_1 and F_2 progenies of *Indica* rice (Bg 90-2, Bg 379-2, Bg 94-1) crossed with *Japonica* rice variety, Hu lo tao on N6 medium supplemented with 5 per cent (w/v) sucrose, 2, 4-D (2.0 mg L^{-1}) and Kinetin (0.5 mg L^{-1}). The regeneration frequency of F_1 and F_2 parents were high compared to the parents. Addition of sugar alcohol such as mannitol (100 mg L^{-1}) promoted callus induction and regeneration in culture medium to about 1.5 per cent in several experimental genotypes of several rice cultivars. (Kaushal *et al*., 2014).

Certain compounds in culture medium such as presence of auxins, sucrose and calcium may cause production of ethylene gas in closed culture bottles which inhibit the successful callus induction and regeneration. (Yang *et al*., 1984), (Zhou *et al*., 1991), (Ghamemi *et al*., 1994) and (Hepler *et al*., 2005). Silva *et al.* (2009) reported the effect of silver nitrate in culture medium in reducing the ethylene gas and promoting the androgenic potential in *Indica* cultivars.

Addition of sucrose or maltose as carbohydrate source in media could enhance the callus induction and subsequent regeneration of plantlet in most cases.

Sucrose is widely used in most of the *in vitro* studies. Park *et al.* (2013) demonstrated the effect of maltose concentration on androgenic callus induction and regeneration in five genotypes of rice (*Oryza sativa*). They observed that low concentrations (0.04 g L^{-1}) of maltose leads to high green plant regeneration with reduced callus induction when N6 medium was supplemented with NAA (2.0 mg L^{-1}) and kinetin (0.5 mg L^{-1}).

The composition of maltose on culture media was reported to induce the androgenic callus induction in cereals (Jane and Corz, 1995; Raina, 1997). Javed *et al*. (2007) reported that maltose concentration in culture medium enhanced the anther culture potential in Pokkali and Nona Bokra supplemented with N6 medium.

According to Sengsai *et al.* (2007) one step plantlet regeneration was obtained when maltose and NAA were supplemented together in the N6 induction media of hybrid BC_1F_1 (KDML 105/IRBB5/KDML105). The rate of green plant regeneration increased (1.29 per cent) when regeneration medium MS was provided with BAP (2 mg L^{-1}), NAA (0.2 mg L^{-1}), casein hydrolysate (300 mg L^{-1}), 15 per cent coconut water, and sucrose (30 g L^{-1}).

Presence of activated charcoal on culture medium could enhance the culture effect and enable the darkening the medium and absorption of inhibitory compounds produced by the medium or explant (Pan and Staden, 1999)

2.1.5 Growth regulators

Effective callus induction and regeneration of plantlets depend up on the optimization of plant growth regulators in culture medium (Ge *et al.,* 2006). Xie *et al*. (1995) reported that *Japonica* rice cultivar can be used as model plant to optimize the dosage of plant growth enhancer.

Among the different growth regulators, 2, 4-D is the most extensively used growth regulator for *in vitro* regeneration of cereals. 2, 4-D along with other auxins

and cytokinins enhance callus induction and regeneration in rice (Iyre and Raina, 1972). Kaushal (2015) reported a significant effect of 2, 4-D in callus induction and regeneration of anther culture of F_1 hybrid rice (IR58025eB x Dular). They obtained highest regeneration potential (19.33-40.46 per cent) with 2, 4-D (2 mg L^{-1}) in He2 medium.

2.1.6 Culture conditions

A suitable culture environment is highly important in successful *in vitro* multiplication procedure. Incubation under dark condition compare to light condition with temperature 25±2°C could enhance callus induction and development in rice reported by Summart *et al.* (2008). The induced calli could be then regenerated in the regeneration medium under temperature 25±2°C with 16 hour light photoperiod (Perez *et al*., 2003).

2.1.7 *In vitro* rooting

The *in vitro* regenerated shoots are then transferred to rooting medium with adequate level of auxins which enhance root induction. Highest root induction could be obtained on MS media provided with IBA (1 mg L^{-1}) and 3 per cent sucrose in shoots of *Indica* rice Jaya obtained from seeds (Alam *et al*., 2012).

Maximum rooting of regenerated shoot via. callus on MS with hormone free media was observed in BRRI dhan 52 and FR13A rice varieties (Bhuiyan *et al.,* 2014).

2.1.8 Hardening and planting out

The complete success of micro propagation depend on effective transfer of *in vitro* raised plantlets to *ex vitro* condition. Plants grown under *in vitro* condition exhibit different morphology, anatomy and physiology (Pospilova *et al.,* 1999). Hardening and acclimatization is essential to withstand transplanting shock and survival of regenerated plantlets.

Kshyap and Dhiman (2011) reported maximum plant height, number of leaves and survival per cent (79.02 per cent) in the medium provided with cocopeat: perlite (3:1) compared to sand: soil (45.16 percent) in micropropagation of Gloxinia (*Sinningia speciose* Hierm) and Saintpaulia (*Saintpaulia ionantha* Wendl).

Puhan and Siddiq (2013) used sand, soil and farmyard manure to acclimatize both direct and indirect *in vitro* regenerated plantlets in several *Indica, Japonica*, aromatic and wild varieties of rice. Plantlets regenerated from mesocotyl segments exhibited 100 per cent survival rate compare to callus derived plants (30-40 per cent).

Materials and methods

3. MATERIALS AND METHODS

The present investigation entitled "*In vitro* propagation of thermosensitive genic male sterile rice (*Oryza sativa L.*)" was carried out at the Department of Plant Physiology, College of Agriculture, Vellayani, Kerala Agricultural University during the year 2015-2016. The main objective of study was to standardize method for *in vitro* propagation of thermosensitive genic male sterile rice.

The study was carried out in two phases *viz.,* generation and screening of F_2 population and standardization of protocol for *in vitro* multiplication of thermosensitive genic rice plants. The stable TGMS rice line IR75589-31-27-8-33 was collected from The International Rice Research Institute (IRRI), Philippines by Standard Materials Transfer Agreement (SMTA) through National Bureau for Plant Genetic Resources (NBPGR), New Delhi. The imported TGMS line (female) along with two popular red varieties of Kerala state, Uma and Jyothi (pollen donors) were used as parents for hybridization program to develop F_2 population.

The details of materials and method adopted for the study are given below.

3.1 PHASE 1: GENERATION OF F_2 POPULATION

The TGMS rice line IR75589-31-27-8-33 (EC 720903) imported from IRRI Philipines and two red rice varieties Uma, Jyothi were crossed to develop F_1. The method adopted for hybridization programme was proximal hybridization method. In proximal hybridization method, the panicles of two parents were placed together by covering with butter paper and allowed the pollination happen to naturally. The developed F_1 plants were then selfed to produce F_2 plants. The developed F_2 population screened for viability and sterility studies were done using I-KI solution at flowering stage.

3.1.1 SCREENING FOR F_2 STERLE PLANTS

During the time of anthesis, spikelets collected from each plant were sampled and anthers were stained in one per cent Iodine Pottasium Iodide (I-KI) solution for screening the fertility and sterility status of pollen grains. The collected anthers were crushed using a needle and debris was removed. Stained anthers were placed in a sterile glass slide and observed under stereo zoom microscope (LEICA EZ4D). The sterile pollen grains appeared as irregularly shaped and lightly stained but round and darkly stained pollen grains taken from fertile plants. Pollen sterility was determined by staining pollen grains with one per cent Iodine Potassium Iodide (I-KI) solution, while spikelet fertility was determined by counting the filled spikelet's in proportion to the total number of spikelet's of randomly selected bagged panicles of a plant.

The screened sterile plants were subjected as explants for *in vitro* multiplication procedure.

3.2 PHASE 2: STANDARDIZATION OF PROTOCOL FOR *IN VITRO* MULTIPLICATION OF TGMS RICE PLANTS

3.2.1 Explants Used

Three explants viz. anther, leaves and nodes were collected from sterile rice plants in the morning. For anther sampling, panicles of the F_2 plants were collected when the auricle distance of the flag leaf to that of the next leaf was around 5-10 cm. Collected panicles were wrapped in moistened paper and stored at 5^0C for 10 days. All the explants were wiped with 70 per cent ethyl alcohol before surface sterilization

3.2.2 Surface Sterilization

The explants were washed thoroughly under running tap water for 30 minutes and dipped in water with a few drops of detergent (Tween 20) for 30 minutes, followed by washing with distilled water for about 4-5 times. The explants were then immersed

in systemic fungicide bavistin (0.1 per cent) for 30 min followed by rinsing several times with distilled water. Then the explants were aseptically sterilized with a dip for 2-3 seconds in absolute alcohol followed by sterilization with disinfectant mercuric chloride under LAF. Different levels of mercuric chloride (0.05 per cent, 0.08 per cent, 0.1per cent, 0.15 per cent and 0.2 per cent) were tried with exposure period of 10 min for obtaining contamination free cultures. After the treatment with mercuric chloride the explants were rinsed thoroughly with sterile distilled water for 4-5 times to remove the remaining mercuric chloride.

3.2.3 Culture Media

The basal medium used for the study were N6 (Chu, 1978), MS (Murashige and Skoog, 1962) and B5 (Gamborg). The chemicals used for preparation of culture media were of analytical grade from Merck (Mumbai) and HiMedia Laboratories Pvt. Ltd. (Mumbai).

Standard procedures were followed for the preparation of basal medium (Thorpe, 1980). Stock solutions of major and minor nutrients, organic supplements and plant growth hormones were prepared by dissolving the required quantity of chemicals in specific volume of distilled water/ 1 N NaOH/ 1N HCl/ Ethyl Alcohol depending up on the nature of chemical and were stored under refrigerated conditions (4^0C).

The glassware's used for the investigation were washed with dilute liquid detergent (Labolene or Cleansol) and rinsed with single distilled water followed by air drying with hot air oven. Adequate quantities of the stock solutions were pipetted out in to 1L beaker containing 600 ml distilled water. Heat stable growth substance were added in required amount in the media. Sucrose and inositol were added in required quantity and dissolved by using glass rod with constant stirring. The initial pH of the medium is noted and final pH is adjusted to 5.7 by using 1N HCL/1N NaOH with an electronic pH meter (EUTECH). Final volume was made up to 1000 ml by clean volumetric flask. Required amount of agar was added to medium and melted by using

a microwave oven (LG). The melted medium was poured in to pre sterilized culture bottles (300 ml), test tubes (25 x 150 mm) and petri plates. About 40 ml of medium were dispensed to each jam bottles and 20 ml to the test tubes respectively. Test tube containing medium were then cotton plugged with pre sterilized non-absorbent cotton wool and culture bottles were tightly sealed with plastic lid. They were then autoclaved at 121^0C and 1.06 kg cm^3 pressure for 45 minutes using a STERI horizontal cylindrical autoclave (Yorko, India). The prepared media were incubated under controlled condition about a week to monitor media contamination.

3.2.4 Inoculation

The materials and glassware's required for inoculation were washed thoroughly with tap water, rinsed with distilled water, ethanol wiped (70 per cent) and covered with polypropylene cover and autoclaved at 121^0C and 15 1.06 kg cm^3 pressure for 45 minutes using a STERI horizontal cylindrical autoclave (Yorko, India).

The inoculation procedures were carried out in aseptic condition under Laminar Air Flow cabinet by using sterile forceps and surgical blades. Anthers were aseptically excised without causing injury. The surface sterilized nodes were excised about (1-2 cm long) by sterile surgical blades in a sterile petri plate.

3.2.5 Culture Initiation and Multiplication

Sterile anthers and leaves were inoculated on to callus induction media for further initiation and regeneration. N6, B5 and MS media supplemented with different combination of auxins 2, 4-D, NAA, and IBA were used for callus induction and regeneration. Nodal segments (1-2 cm) were inoculated on to MS media supplemented with cytokinins (BA and Kinetin) and auxins (IAA, NAA, and IBA) for shoot induction and further regeneration. Treatments involved different levels of 2, 4-D (0.5-3mg L^{-1}), IBA (0.5-3mg L^{-1}), NAA (0.5-3mg L^{-1}), BA (0.5-3mg L^{-1}), kinetin (0.5-3mg L^{-1}) (Table 1 and 2). The treatments were replicated six times.

Observations recorded in callus induction media were number of explant plated, number of explant converted to callus, callus induction (per cent), growth score, callus index and nature of callus after six weeks culture.

Observation recorded on shoot induction media were number of days for shoot initiation, shoot/culture and shoot length after three weeks of culture.

3.2.5.1 ***Multiple Shoot induction***

The nodal cultures were then sub cultured on MS media provided with BA (4 mg L^{-1}) and NAA (0.5 mg L^{-1}) for induction of multiple shoots.

3.2.6 Incubation

The nodal cultures were then incubated in a controlled conditioned room at light intensity 40 µmol $m^{-2}s^{-1}$ using white florescent tubes for 15 h. Callus induction cultures were maintained in dark condition. The temperature of the room was regulated using air conditioner at 25±2^{0}C at a relative humidity of 60 per cent.

3.2.7 ***In vitro*** **Rooting**

Regenerated shoots having 5-9 cm length were separated and subjected to different rooting treatments. The shoots were inoculated in to MS medium with different combinations of NAA (0.5-1.5mg L^{-1}), IBA (0.5-1.5mg L^{-1}) and IAA (0.5-1.5 mg L^{-1}) (Table 3). Each treatment were replicated six times.

Observations were taken as per cent culture initiating root, number of days for root initiation, root/culture and length of root after six weeks of culture.

Table 1. Plant growth regulators tried for callus induction.

Treatment No.	Plant growth regulators (mg L^{-1})		
	2,4D	IBA	NAA
T1	0.5	-	-
T2	1	-	-
T3	1.5	-	-
T4	2	-	-
T5	3	-	-
T6	-	0.5	-
T7	-	1	-
T8	-	1.5	-
T9	-	2	-
T10	-	3	-
T11	-	-	0.5
T12	-	-	1
T13	-	-	1.5
T14	-	-	2
T15	-	-	3
Control	-	-	-

Table 2. Plant growth regulators (Auxins and Cytokinins) tried for shoot induction.

Treatment No.	Plant growth regulators (mg L^{-1})				
	BA	IAA	IBA	NAA	Kn
T1	0.5	-	-	-	-
T2	1	-	-	-	-
T3	2	-	-	-	-
T4	3	-	-	-	-
T5	0.5	0.5	-	-	-
T6	1	0.5	-	-	-
T7	2	0.5	-	-	-
T8	3	0.5	-	-	-
T9	0.5	-	0.5	-	-
T10	1	-	0.5	-	-
T11	2	-	0.5	0.5	-
T12	3	-	0.5	0.5	-
T13	0.5	-	-	0.5	-
T14	1	-	-	0.5	-
T15	2	-	-	-	-
T16	3	-	-	-	-
T17	0.5	-	-	-	0.5
T18	1	-	-	-	0.5
T19	2	-	-	-	0.5
T20	3	-	-	-	0.5
Control	-	-	-	-	-

Table 3. Plant growth regulators tried for *in vitro* rooting

Treatment No.	Plant growth regulators (mg L^{-1})		
	NAA	IBA	IAA
R1	0.5	-	-
R2	1	-	-
R3	1.5	-	-
R4	-	0.5	-
R5	-	1	-
R6	-	1.5	-
R7	-	-	0.5
R8	-	-	1
R9	-	-	1.5
Control	-	-	-

3.2.8 Hardening of the Regenerated Plantlets

The *in vitro* regenerated plantlets having length 6-10 cm with 5-10 roots were removed from the test tubes by the help of forceps. The agar adhering to the roots was completely removed by washing in running tap water. The plants were treated with 0.1 per cent anti-fungal agent (Bavistin) for 1h before planting out. The plants were then transferred to paper cups filled with potting medium consisting of coirpith: vermicompost (1:1), sand: soil: cowdung (1:1:1), sand: soil: vermicompost (1:1:1), sand: soil (1:1) and 10g VAM (Vesicular Arbuscular Mycorrizha). The transferred plants were kept under 25 per cent shade net house and irrigated twice a day with spraying bottles. Observations recorded were survival percent and days to emergence of new leaves after 4 weeks of transfer.

3.2.9 Statistical Analysis

The experimental design was completely randomized with six replicates. The data were subjected to analysis of variance (ANNOVA) and significant difference between treatments were determined by Duncan Multiple Range Test (DMRT).

Results

4. RESULTS

The present study was carried out on "*In vitro* propagation of thermosensitive genic male sterile rice (*Oryza sativa* L.)" at the Department of Plant Physiology, College of Agriculture, Vellayani during 2015-2016. The results obtained from the study are described here.

4.1 PHASE 1: GENERATION OF F_2 POPULATION

The TGMS line IR75589-31-27-8-33 (EC 720903) imported from IRRI were field grown and crossed with two promising red rice varieties Uma and Jyothi to develop F_1 generation through proximal hybridization technique. The subsequent F_1 progenies of Uma and Jyothi have undergone self-pollination and the F_2 generation was developed. The parents and obtained progenies are figured in Plates-1, 2, 3, 4 and 5.

4.1.1 Screening of F_2 Population

The spikelet's of field grown F_2 plants were collected and stained under zoom stereo microscope (LEICA EZ4D) with 1 per cent I-KI solution for the identification of TGMS F_2 plants. The obtained status of spikelet sterility and pollen sterility of parents, F_1 and F_2 are expressed on Table 4. After staining, the fertile anthers appeared as darkly stained with round shaped pollen grains while sterile anthers appeared as lightly stained with irregular shape. Maximum (100 per cent) pollen and spikelet sterility was observed on TGMS parent plant and F_2 sterile plants, whereas fertile parents and fertile progenies exhibited pollen sterility ranging from 2 per cent to 3.9 per cent and spikelet sterility from 25.2 per cent to 30 per cent. The identified TGMS plants from screening were used as source material for *in vitro* propagation techniques. The figures of F_2 sterile and fertile anthers are illustrated on Plates 6, 7 and 8.


TGMS (EC 720903)


Uma


Jyothi

Plate 1. Rice parental lines used for hybridization


TGMS (EC 720903) x Jyothi


TGMS (EC 720903) x Uma

Plate 2. Proximal hybridization technique of rice employed in the present study

F_1 (Uma x TGMS)

F_1 (Jyothi X TGMS)

Plate 3. F_1 progenies

Sterile

Fertile

Plate 4. F_2 progenies (Uma x TGMS)

Sterile

Fertile

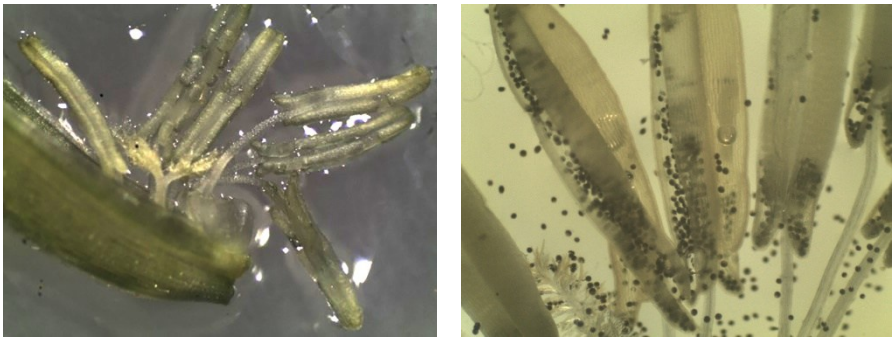
Plate 5. F_2 progenies (Jyothi x TGMS)

Sterile Fertile

Plate 6. F_2 anthers (Jyothi x TGMS) before stained with I-KI (1 per cent) solution

Sterile Fertile

Plate 7. F_2 anthers (Jyothi x TGMS) after stained with I-KI (1 per cent) solution



Sterile Fertile

Plate 8. F_2 pollen grains (Uma x TGMS) after stained with I-KI (1 per cent) solution

Table 4. Details of pollen sterility and spikelet sterility

Plants	Pollen Sterility (%)	Spikelet Sterility (%)
Jyothi	F (2.00)	28.00
Uma	F (2.30)	25.20
TGMS 03	S (100)	100
F_1 (Jyothi x TGMS)	F(2.55)	26.00
F_1 (Uma x TGMS)	F(2.86)	25.50
F_2 (Jyothi x TGMS) Fertile	F(4.26)	30.00
F_2 (Jyothi x TGMS) Sterile	S(100)	100
F_2 (Uma x TGMS) Fertile	F(3.90)	28.89
F_1 (Jyothi x TGMS) Sterile	S(100)	100

4.2 PHASE 2: STANDARDIZATION OF PROTOCOL FOR *IN VITRO* MULTIPLICATION OF TGMS RICE PLANTS

4.2.1 Surface Sterilization

The explants (anthers, leaves and nodes) were collected from screened TGMS rice plants at anthesis stage. They were subjected to several surface sterilization treatments. Among the treatments tried, the highest survival (75 per cent) was obtained in T3 (0.1 per cent $HgCl_2$ for 10 min) with low level of contamination (Plate 10). In T4 (0.15 per cent) $HgCl_2$ for 10 min., T5 (0.2 per cent) $HgCl_2$ for 10 min gave the contamination free cultures but lower culture regeneration (25 per cent and 10 per cent respectively). No explants could survived in T1 (0.05 per cent) and T2 (0.08 per cent)

A. Fungal contamination

B. Bacterial contamination

Plate 9. Contamination in the establishment medium

Plate 10. Explants after surface sterilization with 0.1 per cent mercuric chloride

$HgCl_2$ 10 min treatments due to contamination. The cultures showed predominantly fungal over bacterial contamination. The pictures of contaminated cultures from bacteria and fungus are showed in Plate 9.

4.2.2 Callus induction

Anthers and leaf samples from field grown TGMS rice plants after anthesis were collected and used as explants for callus induction and multiplication studies.

Sixteen treatments involving different combination of plant growth regulators (2, 4-D, NAA and IBA) were supplemented in MS, N6 and B5 media to study their effect on callus induction potential from the explants. Result obtained from the treatments are given in Table 5. *In vitro* response from anther and leaf explants are represented in Plate 11 and 12.

No callus induction and subsequent regeneration was obtained from any treatment tried with the both explants. Only clumpy appearance of anthers were observed on (T4) N6 medium supplemented with 2, 4-D (2 mg L^{-1}). Anther response in T4 treatment are figured in Plate 13. Most of the anthers turned brownish after one week of culture and became unresponsive.

Plate 11. TGMS anther culture after 6 week

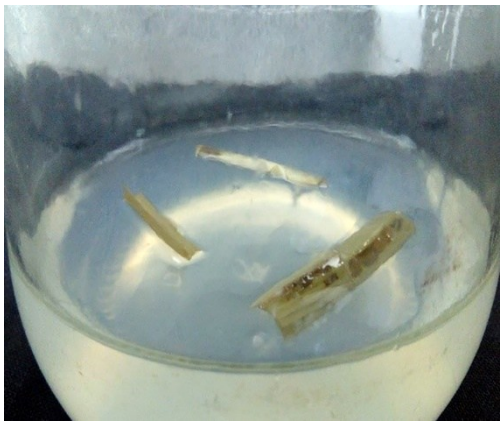


Plate 12. TGMS leaves culture after 6 week

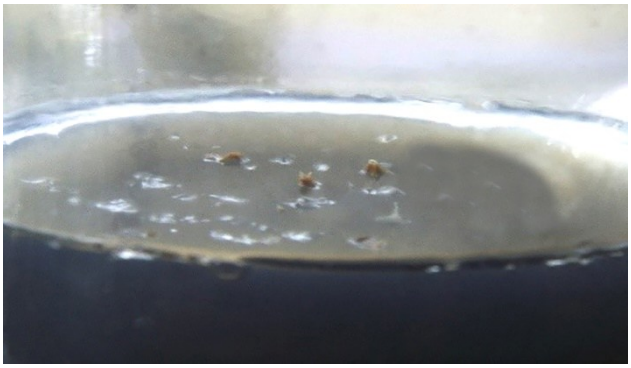


Plate 13. *In vitro* anther culture and growth condition in T4 (N6 +2, 4-D (2 mg L^{-1}) + 3 per cent maltose)

Table 5. Standardization of callus induction media for TGMS anthers and leaves

Treatments	Plant growth regulators (mg l^{-1})			Media	No. of anthers plated	No. of leaves plated	No. of explant converted in to	Callus Induction (%)	Growth Score	Callus Index	Nature of Callus
	2,4 D	IBA	NAA								
T1	0.5	-	-	N6 B5 MS	250	50	Nil	-	-	-	-
T2	1	-	-	N6 B5 MS	250	50	Nil	-	-	-	-
T3	1.5	-	-	N6 B5 MS	250	50	Nil	-	-	-	-
T4	2	-	-	N6 B5 MS	250	50	Nil	-	-	-	-
T5	3	-	-	N6 B5 MS	250	50	Nil	-	-	-	-
T6	-	0.5	-	N6 B5 MS	250	50	Nil	-	-	-	-
T7	-	1	-	N6 B5 MS	250	50	Nil	-	-	-	-
T8	-	1.5		N6 B5 MS	250	50	Nil	-	-	-	-

Table 5. Continued……

Treatments	Plant growth regulators (mg l^{-1})			Media	No. of anthers plated	No. of leaves plated	No. of explant converted in to callus	Callus Induction (%)	Growth Score	Callus Index	Nature of Callus
	2,4 D	IBA	NAA								
T9	-	2	-	N6 B5 MS	250	50	Nil	-	-	-	-
T10	-	3	-	N6 B5 MS	250	50	Nil	-	-	-	-
T11	-	-	0.5	N6 B5 MS	250	50	Nil	-	-	-	-
T12	-	-	1	N6 B5 MS	250	50	Nil	-	-	-	-
T13	-	-	1.5	N6 B5 MS	250	50	Nil	-	-	-	-
T14	-	-	2	N6 B5 MS	250	50	Nil	-	-	-	-
T15	-	-	3	N6 B5 MS	250	50	Nil	-	-	-	-
Control	-	-	-	N6 B5 MS	250	50	Nil	-	-	-	-

4.2.3 Shoot induction

Nodal segments from TGMS parent plant, sterile F_2 progenies (Uma x TGMS) and sterile F_2 progenies (Jyothi x TGMS) were collected and surface sterilized. These excised nodes (1-2cm) were used as explants for shoot induction and regeneration.

Twenty one different treatments with combination of different plant growth regulators (BA, IAA, NAA, IBA, and Kinetin) in MS medium were tried for regeneration. The results obtained from the treatments are furnished in Table 6, 7 and 8 respectively.

Hundred per cent shoot initiation was obtained with all the treatments tried. The shoot initiation was obtained from nodal segments (Plate 14). The photographs of regenerated shoots from TGMS parent plant, sterile F_2 progeny (Jyothi x TGMS) and sterile F_2 progeny (Uma x TGMS) are represented in Plate 15.

The different treatments showed remarkable variation with regard to the number of days taken for shoot initiation, shoot/culture and shoot length in all the genotype tried (TGMS parent plant, sterile F_2 progeny (Jyothi x TGMS) and of sterile F_2 progeny (Uma x TGMS)).

The earliest shoot initiation (6.83, 6.50 and 7.66 days) on TGMS parent plant, F_2 of sterile (Jyothi x TGMS) and (Uma x TGMS) respectively was recorded in T15 (MS + BA 2 mg L^{-1}+ NAA 0.5 mg L^{-1}) which was on par with T3, T7, T11, and T16. The shoot initiation in TGMS parent, F_2 progenies (Jyothi x TGMS) and (Uma x TGMS) was late (14.34, 14.66 and 15.56 days respectively) in the control treatment. Among the genotype tried, Uma exhibited late shoot initiation of (11.66 days) in T9 treatment.

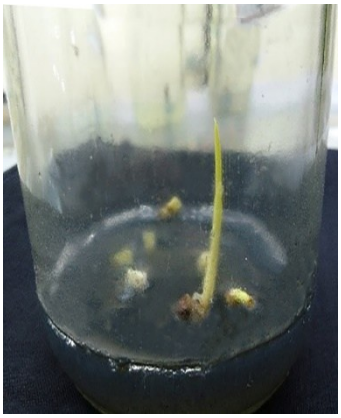
With respect to the number of shoot/culture, the TGMS parent (3.33), F_2 progenies (Jyothi x TGMS) 3.33 and (Uma x TGMS) 2.50 were showed maximum number of shoots/ culture in T15 (MS + BA 2 mg L^{-1} + NAA 0.5 mg L^{-1}). This treatment

Plate 14. Enlarged view of shoot initiation from nodal segments of TGMS rice



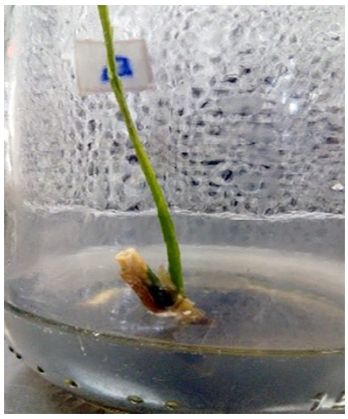
TGMS Parent (EC 720903) | Sterile F_2 progeny (Jyothi x TGMS) | Sterile F_2 progeny (Uma x TGMS)

A. Shoot induction after 10 days of culture



TGMS Parent (EC 720903) | Sterile F_2 progeny (Jyothi x TGMS) | Sterile F_2 progeny (Uma x TGMS)

B. Shoot induction after 2 weeks of culture


TGMS Parent (EC 720903) | Sterile F_2 progeny (Jyothi x TGMS) | Sterile F_2 progeny (Uma x TGMS)

C. Shoot induction after 4 weeks of culture

Plate 15. Shoot initiation from nodal explants of TGMS rice on MS+BA (2 mg L^{-1}) + NAA (0.5 mg L^{-1})

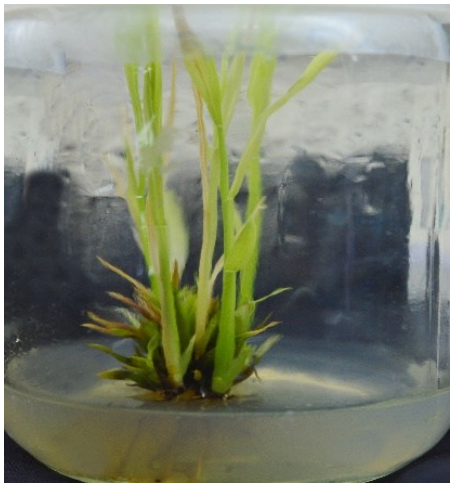


Plate 16. Multiple shoot induction of TGMS rice on MS+BA (4 mg L^{-1}) + NAA (0.5 mg L^{-1})

showed significant variation with respect to all other treatments. The least number of shoot/culture were obtained in control (hormone free medium) which was on par with T1, T8, T9, T12 treatments in all genotypes.

Maximum shoot length (7.51 cm for TGMS parent, 7.83 cm for sterile F_2 progeny (Jyothi x TGMS) and 6.18 cm for sterile F_2 progeny (Uma x TGMS)) were observed in T15 (MS + BA 2 mg L^{-1} + NAA 0.5 mg L^{-1}) which were on par with T7, T11 and T19. The minimum shoot length was observed in control with hormone free MS media in all genotypes.

The earliest shoot initiation, maximum shoot/culture and shoot length were observed in T15 (MS + BA 2 mg L^{-1} + NAA0.5 mg L^{-1}) with all genotypes.

4.2.4 *In vitro* Rooting

Ten treatments with different levels of auxins (IAA, IBA, and NAA) were tried for *in vitro* rooting. Result obtained from the investigation are presented in Table 9,10 and 11 for TGMS parent plant, sterile F_2 progeny (Jyothi x TGMS) and sterile F_2 progeny (Uma x TGMS) respectively.

All the treatment with different growth hormone resulted in cent per cent root initiation (Plate 17). But late rooting and lower root/culture was observed in control treatment with hormone free media.

The earliest (5.33 days) root initiation was observed in TGMS parent plant in R8 (NAA 1 mg $L^{-1)}$ followed by sterile F_2 progeny (Jyothi x TGMS) (5.83 days) and sterile F_2 progeny (Uma x TGMS) (6.7 days).

Late root initiation (9.50 days) was recorded in R3 (IAA 1.5 mg L^{-1}) for sterile F_2 progeny of Uma which was on par with R1, R4 and R5 treatments. Highest number of roots obtained (12.50 root/culture) on TGMS parent in R8 (NAA 1 mg L^{-1}) treatment followed by Sterile F_2 progeny (Jyothi x TGMS) (12.33 root/culture) and sterile F_2 progeny (Uma x TGMS) (11.83 root/culture) in same treatment. The least number of

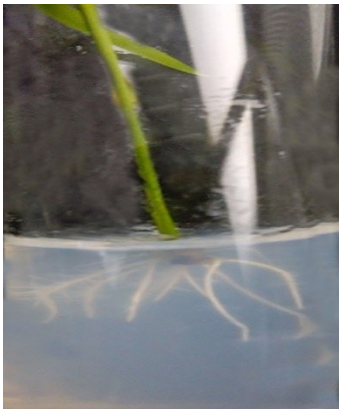
TGMS Parent
(EC 720903)

Sterile F_2 progeny
(Jyothi x TGMS)

Sterile F_2 progeny
(Uma x TGMS)

A.Root induction after 10 days of culture

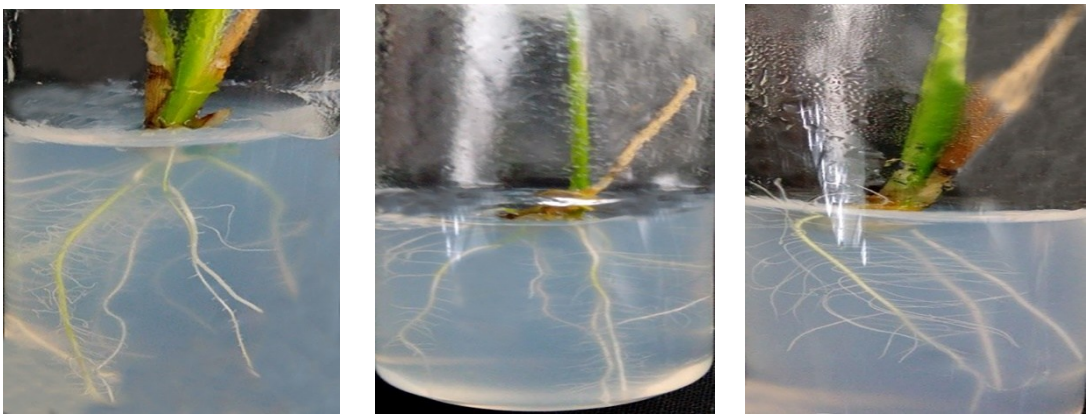
TGMS Parent
(EC 720903)



Sterile F_2 progeny
(Jyothi x TGMS)

Sterile F_2 progeny
(Uma x TGMS)

B. Root induction after 4 weeks of culture



TGMS Parent (EC 720903) | Sterile F_2 progeny (Jyothi x TGMS) | Sterile F_2 progeny (Uma x TGMS)

C. Root induction after 5 weeks of transfer

Plate 17. Root initiation from regenerated shoots of TGMS rice plants in MS with NAA (1 mg L^{-1})

root observed in control (5.33 root/culture) by sterile F_2 progeny (Uma x TGMS) and was on par with R1, R2, R4 and R6 in all genotypes. The maximum root length was showed by sterile F_2 progeny (Jyothi x TGMS) (3.88 cm) in R8 treatment, TGMS parent with 3.78 cm followed by sterile F_2 progeny of (Uma x TGMS) 3.77 cm in the same treatment. The minimum root length was recorded in control of sterile F_2 progeny (Uma x TGMS) 1.7 cm which was on par with R1, R4 and R9 treatments of all genotypes.

Table 6. Effect of different plant growth regulators on *in vitro* shoot induction of TGMS parent plant

Treatment No.	Plant growth regulators (mg L^{-1})								
	BA	IAA	IBA	NAA	Kn	Survival (%)	Days for shoot initiation	Shoot length	Shoot/culture
T1	0.5	-	-	-	-	100%	10.18±0.56	4.06±0.10	1.50±0.30
T2	1	-	-	-	-	100%	8.67±0.25	4.75±0.11	1.66±0.26
T3	2	-	-	-	-	100%	7.83±0.54^{bcd}	5.42±0.19	2.33±0.66
T4	3	-	-	-	-	100%	9.34±0.67	3.83±0.10	1.83±0.56
T5	0.5	0.5	-	-	-	100%	9.84±0.56	5.33±0.03	1.66±0.26
T6	1	0.5	-	-	-	100%	8.66±0.27	5.96±0.08	1.50±0.30
T7	2	0.5	-	-	-	100%	8.17±0.57	6.61±0.29^{cd}	2.1±0.56^{bcd}
T8	3	0.5	-	-	-	100%	10.83±0.54	5.46±0.18	1.33±0.26
T9	0.5	-	0.5	-	-	100%	9.67±1.4	4.35±0.07	1.50±0.09
T10	1	-	0.5	-	-	100%	8.50±1.6	5.61±0.11	1.33±0.26
T11	2	-	0.5	-	-	100%	7.17±0.97^{ab}	6.26±0.10	2.66±0.12^{b}
T12	3	-	0.5	-	-	100%	10.34±0.06	5.36±0.04	1.16±0.09
T13	0.5	-	-	0.5	-	100%	8.00±0.56^{bcde}	5.49±0.09	1.33±0.26
T14	1	-	-	0.5	-	100%	7.17±0.56^{ab}	6.03±0.37	2.50±0.30^{b}
T15	2	-	-	0.5	-	100%	6.83±0.50^{a}	7.51±0.27^{a}	3.33±0.16^{a}
T16	3	-	-	0.5	-	100%	7.33±0.66^{abc}	7.41±0.30^{a}	1.83±0.56
T17	0.5	-	-	-	0.5	100%	8.50±0.30	6.23±0.19	2.33±0.26^{bc}
T18	1	-	-	-	0.5	100%	8.34±0.67	6.73±0.07^{bc}	1.16±0.16
T19	2	-	-	-	0.5	100%	8.16±0.16	7.116±0.16^{ab}	1.66±0.26
T20	3	-	-	-	0.5	100%	8.84±0.56	6.60±0.11	1.33±0.11
Control	-	-	-	-	-	100%	14.34±0.66	2.78±0.97	1.07±0.16
CD (5%)	-	-	-	-	-		0.94	0.47	0.647

Means denoted by the same letters are not significantly different (P = 0.05) using Dungan Multiple Range Test

Table 7. Effect of different plant growth regulators on *in vitro* shoot induction of sterile F_2 progeny (Jyothi x TGMS)

Treatment No.	Plant growth regulators (mg L^{-1})								
	BA	IAA	IBA	NAA	Kn	Survival (%)	Days for shoot initiation	Shoot length	Shoot/culture
T1	0.5	-	-	-	-	100%	10.89±0.56	4.01±0.06	1.33±0.26
T2	1	-	-	-	-	100%	8.84±0.57	4.35±0.08	1.66±0.23
T3	2	-	-	-	-	100%	8.17±1.34	5.63±0.06	1.83±0.56
T4	3	-	-	-	-	100%	10.33±0.66	3.93±0.22	1.50±0.30
T5	0.5	0.5	-	-	-	100%	10.66±0.67	5.45±0.05	1.66±0.26
T6	1	0.5	-	-	-	100%	8.50±0.36	6.01±0.05	1.83±0.16
T7	2	0.5	-	-	-	100%	7.33±0.26^{bc}	7.11±0.03^{bc}	2.33±0.26^{bc}
T8	3	0.5	-	-	-	100%	8.17±0.54	5.46±0.08	1.33±.016
T9	0.5	-	0.5	-	-	100%	9.16±0.26	4.43±0.07	1.16±0.16
T10	1	-	0.5	-	-	100%	8.33±0.16	5.96±0.16	2.33±0.66^{bc}
T11	2	-	0.5	-	-	100%	6.84±0.26^{ab}	7.35±0.13^{b}	2.50±0.30b
T12	3	-	0.5	-	-	100%	8.66±0.17	5.41±0.01	1.33±0.26
T13	0.5	-	-	0.5	-	100%	8.88±0.27	5.46±0.04	1.66±0.22^{bc}
T14	1	-	-	0.5	-	100%	7.33±0.30^{bc}	6.78±0.06^{c}	2.33±0.26
T15	2	-	-	0.5	-	100%	6.50±0.56^{a}	7.83±0.07^{a}	3.33±0.66^{a}
T16	3	-	-	0.5	-	100%	7.83±0.54^{cd}	6.85±0.12^{c}	1.66±0.53
T17	0.5	-	-	-	0.5	100%	9.16±0.26	5.83±0.17	1.83±0.56
T18	1	-	-	-	0.5	100%	8.33±0.16	5.91±0.31	2.33±0.26^{bc}
T19	2	-	-	-	0.5	100%	8.16±0.34	6.36±0.04^{d}	1.66±0.20
T20	3	-	-	-	0.5	100%	9.16±0.16	5.95±0.13	1.83±0.16
Control	-	-	-	-	-	100%	14.66±0.56	3.03±0.16	1.16±0.16
CD (5%)	-	-	-	-	-		0.75	0.37	0.65

Means denoted by the same letters are not significantly different (P = 0.05) using Dungan Multiple Range Test

Table 8. Effect of different plant growth regulators on *in vitro* shoot induction of sterile F_2 progeny (Uma x TGMS)

Treatment No.	Plant growth regulators (mg L^{-1})								
	BA	IAA	IBA	NAA	Kn	Survival (%)	Days for shoot initiation	Shoot length	Shoot/culture
T1	0.5	-	-	-	-	100%	11.16±0.56	3.61±0.14	1.16±0.16
T2	1	-	-	-	-	100%	10.50±1.3	3.83±0.22	1.33±0.26^{bcd}
T3	2	-	-	-	-	100%	10.33±1.26	4.21±0.25	2.16±0.56
T4	3	-	-	-	-	100%	10.66±1.46	3.66±0.17	1.16±0.16
T5	0.5	0.5	-	-	-	100%	9.83±0.96	4.78±0.06	1.33±0.26
T6	1	0.5	-	-	-	100%	8.33±0.66	5.36±0.04	1.16±0.16
T7	2	0.5	-	-	-	100%	8.66±0.64^{abc}	5.86±0.12^{ab}	2.33±0.66^{abc}
T8	3	0.5	-	-	-	100%	10.50±1.1	5.31±0.07	1.11±0.16
T9	0.5	-	0.5	-	-	100%	10.83±1.16	4.28±0.01	1.83±0.12
T10	1	-	0.5	-	-	100%	9.66±0.26	4.95±0.22	2.00±0.40^{bcde}
T11	2	-	0.5	-	-	100%	8.83±0.56	5.75±0.11^{bc}	2.83±0.16^{a}
T12	3	-	0.5	-	-	100%	10.33±1.16	5.18±0.04	1.33±0.26
T13	0.5	-	-	0.5	-	100%	8.33±0.96^{bcd}	5.33±0.08	1.50±0.30
T14	1	-	-	0.5	-	100%	8.16±0.56^{ab}	5.78±0.15^{b}	1.83±0.16
T15	2	-	-	0.5	-	100%	7.66±0.66^{a}	6.18±0.125^{a}	2.50±0.30^{ab}
T16	3	-	-	0.5	-	100%	8.33±0.68^{ab}	5.31±0.21	1.33±0.26
T17	0.5	-	-	-	0.5	100%	10.16±0.56	5.2±0.02	1.83.±0.16
T18	1	-	-	-	0.5	100%	10.84±0.96	5.21±0.17	2.00±0.40
T19	2	-	-	-	0.5	100%	9.67±0.60	5.56±0.06^{bcd}	1.66±0.26
T20	3	-	-	-	0.5	100%	11.66±1.06	5.06±0.08	1.33±0.23
Control	-	-	-	-	-	100%	15.50±0.70	2.93±0.12	1.17±0.116
CD (5%)	-	-	-	-	-	-	1.088	0.39	0.57

Means denoted by the same letters are not significantly different (P =0.05) using Dungan Multiple Range Test

Table 9. Effect of different plant growth regulators on *in vitro* root induction of TGMS parent plant

Treatment No.	Plant growth regulators (mg L^{-1})						
	IAA	IBA	NAA	Survival (%)	Days for root initiation	Root length	Root/culture
R1	0.5	-	-	100%	8.83±0.17	3.15±.08	10.17±0.17
R2	1	-	-	100%	7.83±0.57	$3.83±0.19^{bc}$	$10.83±0.97^{cde}$
R3	1.5	-	-	100%	8.67±0.27	$3.33±0.04^{bc}$	10.67±0.67
R4	-	0.5	-	100%	$7.33±0.27^{cd}$	3.17±0.22	10.17±0.97
R5	-	1	-	100%	$6.50±0.30^{b}$	$3.58±0.08^{ab}$	$11.83±0.17^{ab}$
R6	-	0.5	-	100%	7.67±0.27	3.20±0.08	10.67±0.67
R7	-	-	0.5	100%	$6.17±0.17^{b}$	$3.50±0.04^{bc}$	$11.67±0.27^{abc}$
R8	-	-	1	100%	$5.33±0.27^{a}$	$3.78±0.06^{a}$	$12.50±0.30^{a}$
R9	-		1.5	100%	$6.83±0.17^{bc}$	3.25±0.04	$11.50±0.30^{bd}$
Control	-	-	-	100%	14.17±0.97	1.85±0.12	6.33±0.67
CD (5%)	-	-	-	-	0.67	0.34	0.86

Means denoted by the same letters are not significantly different (P = 0.05) using Dungan Multiple Range Test

Table 10. Effect of different plant growth regulators on *in vitro* root induction of sterile F_2 progeny (Jyothi x TGMS)

Treatment No.	Plant growth regulators (mg L^{-1})						
	IAA	IBA	NAA	Survival (%)	Days for root initiation	Root length	Root/culture
R1	0.5	-	-	100%	9.166±0.56	3.18±0.04	9.83±0.16
R2	1	-	-	100%	8.66±0.26	3.36±0.02^{bc}	10.5±0.70
R3	1.5	-	-	100%	8.50±0.30	3.16±0.03	10.66±0.26
R4	-	0.5	-	100%	7.33±0.66	3.43±0.01^{bc}	10.83±0.96
R5	-	1	-	100%	6.50±0.30^{ab}	3.48±0.17^{b}	11.50±0.30
R6	-	0.5	-	100%	7.16±0.56^{bc}	3.28±0.01	10.16±0.56
R7	-	-	0.5	100%	6.84±0.16^{bc}	3.45±0.03^{bc}	11.66±0.66^{b}
R8	-	-	1	100%	5.83±0.26^{a}	3.88±0.07^{a}	12.33±0.66^{a}
R9	-		1.5	100%	7.33±0.26	3.23±0.01	11.16±0.56^{c}
Control	-	-	-	100%	15.33±0.27	1.88±0.20	5.83±0.16
CD (5%)	-	-	-	-	0.72	0.29	0.82

Means denoted by the same letters are not significantly different (P = 0.05) using Dungan Multiple Range Test

Table 11. Effect of different plant growth regulators on *in vitro* root induction of sterile F_2 progeny (Uma X TGMS)

Treatment No.	Plant growth regulators (mg L^{-1})						
	IAA	IBA	NAA	Survival (%)	Days for root initiation	Root length	Root/culture
R1	0.5	-	-	100%	9.33±0.27	3.42±0.03	9.50±0.30
R2	1	-	-	100%	8.50±0.30	3.45±0.02^{bc}	11.0±0.80^{abc}
R3	1.5	-	-	100%	9.50±0.30	3.23±0.01	11.16±0.57^{ab}
R4	-	0.5	-	100%	8.33±0.27^{de}	3.52±0.01^{b}	10.16±0.97
R5	-	1	-	100%	7.17±0.57^{bc}	3.42±.03b^{c}	11.0±0.80^{abc}
R6	-	0.5	-	100%	8.67±0.27	3.33±0.02	9.83±0.57
R7	-	-	0.5	100%	7.67±0.67^{cd}	3.50±0.01^{b}	10.83±0.57
R8	-	-	1	100%	6.17±0.17^{a}	3.77±0.23^{a}	11.83±0.57^{a}
R9	-		1.5	100%	6.83±0.17^{ab}	3.32±0.01	11.16±0.97^{ab}
Control	-	-	-	100%	16.17±0.57	1.7±0.06	5.33±0.67
CD (5%)	-	-	-	-	0.68	0.25	0.95

Means denoted by the same letters are not significantly different (P = 0.05) using Dungan Multiple Range Test

4.2.5 Hardening of the Regenerated Plantlets

The *in vitro* regenerated plants with well-developed root system were carefully removed from the culture bottles and transplanted in four different media viz. coirpith: vermicompost (1:1), sand: soil: cowdung (1:1:1), sand: soil: vermicompost (1:1:1), sand: soil (1:1) and 10g VAM (Vesicular Arbuscular Mycorrizha) to study the effect of *ex vitro* establishment after 4 weeks of transfer. Cent per cent survival rate was observed on coirpith: vermicompost (1:1) media with emergence of new leaves after 14 days of transfer. The plants showed a survival rate of 75 per cent, 80 per cent, and 72 per cent in the treatments of sand: soil: cowdung (1:1:1), sand: soil: vermicompost (1:1:1), sand: soil (1:1) and 10g VAM respectively. Plate 18 demonstrates the *ex vitro* establishment of TGMS plants.

A. Coirpith: vermicompost (1:1)

B. Sand: soil: cowdung (1:1:1)

C. Sand: soil: vermicompost (1:1:1)

D. Sand: soil (1:1) and 10g VAM)

E. Transplanted plantlets in

Plate 18. *Ex vitro* establishment of TGMS plants.

Discussion

5. DISCUSSION

The present investigation entitled "*In vitro* propagation of thermosensitive genic male sterile rice (*Oryza sativa* L.)" was carried out during 2015-2016 at the Department of Plant Physiology, College of Agriculture, Vellayani. The result obtained from the study are discussed in this chapter.

Hybrid development in rice utilizes male sterile system to avoid tedious emasculation procedure. In hybrid rice programme, environmental male sterile system (EGMS) is highly preferred over cytoplasmic male sterile system. The EGMS system involves thermosensitive and photosensitive genic male sterile lines (TGMS and PGMS). The TGMS system is widely used in tropical climate due to marginal effect of day length differences. (Virmani *et al*., 1996). So, these study utilizes TGMS line (EC720903) as female parent for hybridization and two ruling red rice varieties (Uma and Jyothi) used as pollen donors. The male sterility trait is recessive and monogenically controlled and also can be introgressed to any rice varieties (Ali and Khan., 1997). The successful evaluation of flowering, morphological and agronomical traits are important tool for identification of commercially used TGMS lines. (Virmani *et al.*, 1997; Kalaiyarasi and Vaidyanathan, 2002).

The study of pollen and spikelet sterility status of parents and progenies revealed that pollen sterility ranged from 2.00 to 4.26 per cent in fertile plants but TGMS plants showed 100 per cent pollen sterility. The spikelet sterility ranged from 25.20 per cent to 28.89 per cent in fertile plants while TGMS plants exhibited 100 per cent spikelet sterility. The results indicated that absence of pollen grains in TGMS plants leads to complete male sterility and spikelet sterility status. The observation are in agreement with earlier report that the TGMS lines with maximum pollen sterility at high temperature and self-seed set at lower temperature are preferred in commercial utilization (Lu *et al*., 1994). The morphological study of TGMS lines IR 68301-11-6-4-4-3-6-6 and IR 76753-41-6-34-13 exhibited complete male sterility at temperature

greater than 30^{o}C and fertility restored at temperature between 19^{o}C to 28^{o}C (Akhter *et al.*, 2008). The TGMS lines with broad pollen grains would have very less fertility chances under suitable conditions (Virmani *et al.,* 2003). The low seed production ability of the three TGMS (TNAU60S, TNAU18S and TS29-150-GY) lines decided by pollen sterility status (Arasakesary *et al.,* 2015). The results showed that male sterile and fertile status were different in each genotypes and depend up on climatic condition.

In the developed TGMS plants the male sterility can be identified only at flowering stage and it will not set seeds at above critical sterility period. But the TGMS lines are needed for hybrid development programme. So, *in vitro* technique were adopted to multiply the developed TGMS plants. Rongbai *et al*. (1998) reported that ovary culture was the only option for *in vitro* multiplication in male sterile systems. They cultured unpollinated young ovaries from F_1plants of cross between UPRI95-140 a TGMS donor with UPRI9S-117 on N6 medium supplemented with 2,4-D (4 mg L^{1}), NAA 2 (mg L^{-1}) and BA (1.0 mg L^{-1}). The study resulted with only 0.5 per cent callus induction with low regeneration of green plants.

In the present investigation, the *in vitro* regeneration potential of TGMS plants were tried with different explants (Anther, Leaves and Nodal segments) available only after confirming male sterility at flowering stage.

The major constraints in the successful anther culture technique were low frequency of callus induction and poor percentage of green plant regeneration. Most of the *Indica* rice varieties exhibit recalcitrant to anther culture. (Roy and Mandal, 2005). In the present study sterile anthers were attempted to regenerate on three different media (MS, N6 and B5) supplemented with different auxins (2, 4-D, NAA and IBA). In all the treatment tried only N6 provided with 2, 4-D (2 mg L^{-1}) responded with clumpy appearance on medium. But no calli induced from the responded anthers. Hence this result indicated that negative response of male sterile anthers in the *in vitro* multiplication process when compared with study of androgenic callus induction of

Indica rice hybrid of Chakao amubi and Basmati 370 (Medhabati *et al.,* 2014). They reported that highest androgenic callus induction observed on N6 medium supplemented with 2, 4-D 2 (mg L^{-1}) with 18 anthers turned callus. The media composition were same as in both experiments but no calli regeneration was obtained from the present investigation. The negative effect of sterile anthers on *in vitro* procedure may be due to absence pollen grains in the anthers of this specific TGMS lines. Hence the only possibility for regeneration is from anther wall. But poor regeneration ability limits its application. In TGMS system, sterility caused by premature PCD (Programmed Cell Death) of tapetum layer (Ku *et al*., 2003). Hence anther wall also functionally not active. The TGMS anthers exhibited complete pollen sterility confirmed with 1 per cent I-KI solution. So, haploid plants regeneration is not possible from the sterile anthers. The present investigation confirming that it's difficult to regenerate plantlet from the anthers of TGMS plants.

Attempt was also made on regeneration of callus from the leaves as explant source. There is no sufficient literature on potential of rice leaves from mature plants for micro propagation studies. No callus was induced from the treatment tried in this experiment with different combination of plant growth regulators. This result was in agreement with the previous reports. Henke *et al*. (1978) reported that leaf bases of 3 to 4 days old seedlings were most suitable for the callus induction. Age of seedlings and genotype of providing explant have reported detrimental effect in successful regeneration procedure. Young (4 days old) seedling derived leaf bases showed highest callus induction whereas no calli was induced from 10 days old leaf base in LS medium supplemented with 2, 4-D 11.3µM.

Efficient plant regeneration from young *in vitro* regenerated stem node was reported earlier in callus induction media. (Furuhashi *et al.,* 1964). The regeneration potential of *in vitro* raised nodes in culture was further confirmed MS basal medium supplemented with 2, 4-D (Yamada *et al*., 1967). Hence the present study utilized nodal segments of rice inflorescence for *in vitro* multiplication. There is no sufficient

literature on direct *in vitro* plantlet regeneration from rice inflorescence nodes. The inter nodal segments of TGMS rice plants were used as source material for *in vitro* propagation studies. The present investigation reveals that *in vitro* multiplication of TGMS rice plants possible through nodal explants.

Direct multiplication of TGMS plants achieved through inter nodal segments of rice plant provided with MS media. Direct shoot regeneration was observed in all treatments provided with combination of BA with slight auxins concentration. Among twenty one treatment tried all the treatment showed excellent shoot initiation. Highest shoot induction was obtained when MS media was supplemented with BA 2 mg L^{-1} along with NAA 0.5 mg L^{-1}. Earliest shoot initiation (6.83 days), maximum shoot length (7.41cm) and shoot/culture (3.33) were observed in treatment MS with BA (2 mg L^{-1}) and NAA (0.5 mg L^{-1}). The control treatment with hormone free medium produced least number of shoot/ culture. These results indicated the synergic effect of BA and IAA on shoot proliferation. The effect of BA in shoot induction media were in agreement with the findings of Ragavendra *et al.* (2010) in rice Rasi. On MS medium supplemented with BA alone gave better shoot regeneration. In the present investigation shoot induction rate varied with each genotypes. Efficient regeneration protocol in rice depends up on genotype of explant (Visrada and Sarma, 2002). Significant variation in culture response of *Indica* rice varieties earlier reported by Qinam and Kothari (1993), Kahana and Raina (1998) and Serej *et al.* (1997).

In vitro induction of roots from actively growing shoots has been achieved in basal medium containing auxin or absence of auxins depending on genotype of explant. (Rout *et al.,* 1999). In the present study 10 treatments with different combination of auxin such as IAA (0.5 to 1.5 mg L^{-1}), IBA (0.5 to 1.5 mg L^{-1}) and NAA (0.5 to 1.5 mg L^{-1}) were tried for *in vitro* root formation. Earliest root initiation (5.33 days), maximum number of root and maximum root length was recorded in MS medium supplemented with NAA (1.0 mg L^{-1}). Roots were regenerated in control treatment (hormone free medium) also. Bhuiyan *et al.* (2014) reported that highest root induction of rice variety

BRRI dhan 52 and FR13A on hormone free MS medium. Effect of IBA in root induction of rice was observed by Alam *et al.* (2012). Highest root induction obtained in MS media with IBA (1.0 mg L^{-1}).

The successful completion of *in vitro* propagation marked only after effective hardening of regenerated plantlets. Suitable planting out medium and *ex vitro* condition are crucial for any *in vitro* procedure.

In the present investigation *in vitro* regenerated plants were acclimatized in four different potting media such as coirpith: vermicompost (1:1), sand: soil: cowdung (1:1:1), sand: soil: vermicompost (1:1:1) and sand: soil (1:1) and 10g VAM. The plants showed a maximum survival rate in coirpith: vermicompost (1:1) media while other three treatment showed 75 per cent, 80 per cent, 72 per cent respectively and there was no significant difference between them. Highest survival per cent of survival rate in coirpith: vermicompost medium may be due to the water holding capacity of the medium.

Kshyap and Dhiman (2011) observed that more than 79 per cent plantlet survived on hardening of Gloxinia and Saintpaulia in cocopeat: perlite (3:1) compare to sand: soil (45 per cent). Puhan *et al*. (2013) reported that 100 per cent rice plantlets were survived in sand, soil and farmyard manure on *ex vitro* establishment of mesocotyl derived plants of *Indica, Japonica* and aromatic varieties.

Summary

6. SUMMARY

The study entitled "*In vitro* propagation of thermosensitive genic male sterile rice (*Oryza sativa* L.)" was conducted at the Department of Plant Physiology, College of Agriculture, Vellayani. The main objective of the study was to standardize the method for *in vitro* propagation of thermosensitive genic male sterile rice.

The thermossensitive genic male sterile system has great potential in rice hybrid development which emerge as alternative for tedious emasculation procedure. The developed male sterile hybrids will not set seeds due to their specific physiological and morphological characters. The present investigation is based on the possibility of *in vitro* multiplication of TGMS plants. The TGMS plant were developed from the cross between female parent (TGMS-EC720903) and male parents (Uma and Jyothi) *via.* proximal hybridization method. The F_1 generation obtained from the crosses were allowed to self-pollinate to produce F_2 population. The developed F_2 population were further screened for male sterility using the pollen grain with I-KI solution. These sterile TGMS plants were used as source material for *in vitro* propagation.

The present study utilized three explants from TGMS plants for *in vitro* studies. The explants of anther, leaves and nodal segments were collected and surface sterilized with fungicide (bavistin 0.3 per cent) and mercuric chloride (0.1 per cent). Anther and leaf samples were inoculated on three different callus induction media (N6, MS and B5) supplemented with different combinations of auxins (2, 4-D, NAA and IBA) ranging from 0.5 to 3.0 mg L^{-1}. Nodal segments of TGMS plants were excised (1-2 cm long) and inoculated in to shoot induction media of MS provided with plant growth regulators (BA, NAA, IAA and Kinetin).

The present study standardized the *in vitro* regeneration protocols for TGMS multiplication through nodal segments as explant source. Hence, this is the first report in male sterile system that utilized nodes as explant for *in vitro* multiplication. Efficient TGMS plantlet were regenerated from the nodal parts of mature plants. Highest shoot

induction, shoot length and shoot per culture obtained on MS media supplemented with BA (2mg L^{-1}) + NAA (0.5 mg L^{-1}). Significant regeneration was obtained from all the treatments tried. The regenerated shoots were then subjected to root induction medium using MS with auxins (NAA, IBA and IAA) at 0.5 to 1.5 mg L^{-1}. Among ten different treatment tried efficient root induction were recorded in all the treatments. Maximum root length, root induction and root per culture culture were observed on MS medium provided with NAA (1.0 mg L^{-1}). The *in vitro* regenerated TGMS plants were further transferred to *ex vitro* conditions provided with four different potting media viz. coirpith: vermicompost (1:1), sand: soil: cowdung (1:1:1), sand: soil: vermicompost (1:1:1), sand: soil (1:1) and 10g VAM (Vesicular Arbuscular Mycorrizha). Among the four different media used, maximum (100 per cent) survival percentage was obtained in coirpith: vermicompost (1:1) media. No significant difference was observed in other three media with survival percent of 75 per cent, 80 per cent, and 72 per cent respectively. All the data were subjected to statistical analysis with ANNOVA. The statistical analysis of obtained data showed that all the treatments were significant and it is efficient for further multiplication studies.

The present study also revealed the negative effect of selecting sterile anther and leaves from mature rice plant as explant towards *in vitro* procedures. These results point out the efficient *in vitro* regeneration protocol was highly influenced by culture media and culture condition, genotype of explant and age of explant.

Future line of work:

The identified potential of nodal explants of TGMS rice plants can be used for *in vitro* multiplication procedure. Induction of multiple shoot from the regenerated shoots of TGMS plants can be used as mass multiplication process with specific growth regulators in basal medium. Thus it can be used for multiplication of TGMS lines within a short time duration and without waiting the male fertility inducing condition for multiplication TGMS hybrids.

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Appendices

APPENDIX I

Murashige and Skoog medium (1962)

Components	mg L^{-1}
NH_4NO_3	1650
KNO_3	1900
$CaCl_2$. $7H_2O$	440
$MgSO_4$. $7H_2O$	370
KH_2PO_4. $3H_2O$	170
KI	0.83
H_3BO_3	6.2
$MnSO_4$. $4H_2O$	22.3
$ZnSO_4$. $7H_2O$	8.6
Na_2MoO_4. $2H_2O$	0.25
$CuSO_4$. $5H_2O$	0.025
$CoCl_2$. $6H_20$	0.025
$FeSO_4$. $7H_2O$	27.8
Na_2EDTA $2H_2O$	37.3
$AgNO_3$	8.0
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thymine HCl	0.1
Glycine	2.0
Inositol	100
Sucrose	3%
Agar	0.7%
pH-5.7	

APPENDIX II

CHU (N6) Medium

Components	**mg L^{-1}**
KNO_3	2830
$MgSO_4$. $7H_2O$	9037
KH_2PO_4. $3H_2O$	400
$(NH_4)_2$ SO_4	463
$CaCl_2$. $2H_2O$	166
KI	80
$MnSO_4$. H_2O	3.33
H_3BO_3	1.6
$ZnSO_4$	1.5
$FeSO_4$. $4H_2O$	27.85
Na_2EDTA. $2H_2O$	37.25
$AgNO_3$	8.0
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thymine HCl	0.1
Glycine	2.0
Inositol	100
Sucrose	3%
Agar	0.7%
pH-5.7	

APPENDIX III

Gamborg B5 Medium

Components	mg L^{-1}
KNO_3	2500
$CaCl_2$. $7H_2O$	150
$MgSO_4$. $7H_2O$	250
KH_2PO_4. $3H_2O$	150
$(NH_4)_2$ SO_4	134
KI	0.75
H_3BO_3	3.0
$MnSO_4$. $4H_2O$	0.6
$ZnSO_4$. $7H_2O$	2.0
Na_2MoO_4. $2H_2O$	0.25
$CuSO_4$. $5H_2O$	0.025
$CoCl_2$. $6H_2O$	0.025
$FeSO_4$ $7H_2O$	27.8
Na_2EDTA $2H_2O$	37.2
$AgNO_3$	8.0
Nicotinic acid	1.0
Pyridoxine HCl	1.0
Thymine HCl	10
Glycine	2.0
Inositol	100
Sucrose	3%
Agar	0.7%
pH-5.7	

"*IN VITRO* PROPAGATION OF THERMOSENSITIVE GENIC MALE STERILE RICE (*Oryza sativa* L.)"

By

NISTHAR E.

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Abstract of Thesis

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ABSTRACT

The project entitled "*In vitro* propagation of thermosensitive genic male rice (*Oryza sativa* L.)" was conducted in the Department of Plant Physiology, College of Agriculture, Vellayani during 2015-2016. The main objective of the study was to standardize the method for *in vitro* propagation of thermosensitive genic male sterile rice. The plant material used for this study were the TGMS line (EC720903) imported from IRRI, Philippines as female parent and two red varieties viz. Uma and Jyothi were selected as pollen donors.

F_1 progenies were generated from the cross between TGMS line (EC720903) with Uma and Jyothi through proximal hybridization technique. The obtained F_1 population have subjected to self-pollination to produce F_2 population. The screened sterile plants were used as source material for *in vitro* multiplication.

In this present study, three explants (anthers, leaves and node) from TGMS rice were used for *in vitro* multiplication. Sterile anthers and leaf explants were inoculated on callus induction media (N6, B5 and MS) supplemented with different combination of auxins (2, 4-D, NAA, IBA) and nodal segments were inoculated in to shoot induction media (MS) with different concentration of growth regulators.

In vitro regeneration protocol of TGMS rice was optimized from nodal segments through various steps of shoot regeneration, *in vitro* rooting and acclimatization and planting out.

Shoot regeneration from nodal segments with maximum shoot induction was obtained in the medium MS supplemented with BA (2 mg L^{-1})+ NAA (0.5 mg L^{-1}). Highest root induction was obtained in MS with NAA (1 mg L^{-1}). The *ex vitro* establishment of *in vitro* regenerated plantlets showed maximum survival rate in coirpith: vermicompost (1:1) media. No callus regeneration was observed from any of the treatment tried in sterile anthers and mature leaves.