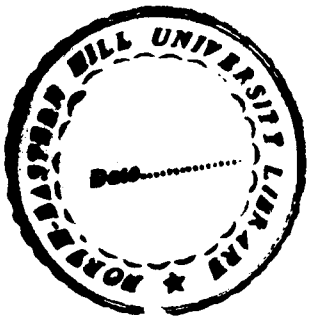
PLANT REGENERATION FROM PROTOPLASTS AND FUSION-MEDIATED PRODUCTION OF CYBRIDS IN RICE

By

BIJOYA BHATTACHARJEE



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DECLARATION

I, Miss Bijoya Bhattacharjee, hereby declare that the subject matter of the thesis entitled "Plant regeneration from protoplasts and fusion-mediated production of cybrids in rice" is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/ Institute.

This is being submitted to the North-Eastern Hill University for the degree of Doctor of Philosophy in Botany.

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ABBREVIATIONS

atp6	gene for subunit 6 of F0-ATPase complex
bp	base pair(s)
°0	degrees Celsius
cm	centimetre
coxII	cytochrome oxidase subunit II gene
Co.	company
Cont.	continued
Corp.	corporation
CPW	cell and protoplast washing solution
CTAB	Cetyl-trimethylammonium bromide
cv(s)	cultivar(s)
DC	Direct current
2,4-D	2,4-dichlorophenoxyacetic acid
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetra acetic acid
e.g.	for example
et al.	*et alia* (Latin; and others)
EtBr	Ethidium Bromide
Fig.	figure
F1	first generation
F2	second generation
γ-ray	Gamma ray
gm	gramme
g.f.wt.	gramme fresh weight
EDTA	ethylene diamine tetraacetic acid
HCl	hydrochloric acid
IOA	iodoacetamide
Kao	Kao (1977) basal medium
kb	kilobase (s)
KD	kiloDalton
krad	kilorad
λ	lambda
l	litre (s)
Ltd.	limited
m	metre
M	molar
mg	milligramme (s)
mg l^{-1}	milligramme (s) per litre
ml	millilitre (s)
mm	millimetre (s)

mRNA	messenger ribonucleic acid
mM	milimolar
µm	micron
µl	microlitre (s)
mW	milliwalt
°N	degrees North
No.	number
O.D.	optical density (absorbance units)
PCV	packed cell volume
pH	hydrogen potential; logarithm of the reciprocal of hydrogen ion concentration i.e. $\log_{10}(1/[H^+])$ or $-\log_{10}[H^+]$
pp	page
RNAase	ribonulease
RNA	ribonucleic acid
rpm	revolutions per minute
°S	degrees South
SD	standard deviation
S.E.	standard error
Tris	Tris (hydroxymethyl) aminoethane
UK	United Kingdom
USA	United States of America
USSR	Union of Soviet Socialist Republics
UV	ultraviolet light
v/v	volume to volume
V	volt
w/v	weight to volume
x g	x gravity (relative centrifugal force)
%	percentage
x	magnification
<	less than
>	more than

CHAPTER 1

INTRODUCTION

CHAPTER 1 : INTRODUCTION

Cereals are the most important group of food plants, which constitute the centrepiece of world agriculture by providing 52% of the total food calories. Of the more than 250,000 species of angiosperms, a mere 29 provides most of our calories. The most important of these are the eight cereal grains, viz. wheat, rice, maize, barley, oats, rye, sorghum and pearl millet of the family Gramineae (Poaceae). Therefore, cereals are the major targets for the application of cellular and molecular genetic manipulations with particular focus on recovering fertile transgenic plants. Among the major cereal crops the only one that is grown almost exclusively as human food is rice which accounts 27% of the world's food production and contributes substantially to global food security (Paroda, 1998).

1. Introduction to rice crop

Rice is the world's single most important food crop and a source of calories for more than one third of the world's population. More than 90% of the total rice is produced and consumed in Asia where 60% of the world's population live. By 2025 AD, the global population is likely to touch 8.5 billion from the current level of 5.7 billion, of which more than 3 billion will be primarily rice consumers (Khush, 1997; Paroda, 1998). With increasing population, global rice production needs to be increased from the 1995 level of 460 million tonnes to 560 million tonnes by 2000 AD and to 980 million tonnes by 2020 AD (Swaminathan, 1998). The task of producing the additional rice to meet the demand of the early 21st century poses major challenges. The world's rice area, however, has changed little since the early 1980s. A significant increase in

rice area is unlikely in the future. In fact, rice area is going down in several countries due to the pressure of urbanisation, industrialisation and other economic factors. Therefore, in order to meet this required increase in rice production, varieties must developed which have higher yield potential, superior grain quality, multiple resistance to diseases and pests and tolerance to abiotic stresses. Recent breakthrough in molecular and cell biology have provided tools which will increasingly be used as aids to conventional plant breeding for developing rice varieties for the 21st century (Khush, 1997).

The advances in biotechnology offer enormous potential in enhancing the efficiency of breeding methods for rice improvement. Integration of the technology with conventional breeding has enabled the breeder to achieve several targets more quickly and efficiently, than was conceivable in the past. Transformation, somatic hybridization and cytoplasmic hybridization have already been employed in rice improvement programs. Transformation enables the integration of agronomically important genes into the plant genome, whilst somatic and cytoplasmic hybridization allows the transfer of genetic material between plants parasexually, thereby overcoming sexual incompatibility which may occur between desirable species combinations. However, for all these genetic manipulations, tissue culture is a prerequisite that allows recovery of plants from the manipulated cells.

1.1. Economic importance of rice

1.1.1. Cultivation of rice

The cultivation of rice dates to the earliest age of man and long before the era of which mankind have historical evidence. Rice was probably the staple food and the first cultivated crop in Asia. According to the Archaeological survey of India, four terraces of rice cultivation on the banks of the river Ravi in south-west Kashmir dates back to the Pleistocene or ice age (Grist 1986). Rice grows in diverse environments almost unparalleled in the plant kingdom. Natural dispersal and human selection have extended the cultivation of rice from the 55° north latitude (on the border between the USSR and China) to 43° south latitude (Central Argentina). It is also cultivated from sea level at the deltas of the great rivers to an altitude of 3,000 m (10,000 ft) elevation in the Himalayas (Swaminathan, 1984; Grist, 1986; Khush, 1997). Rice is cultivated in the cool climate of Nepal and India and in the hot desert of Pakistan, Iran and Egypt. Although typically grown on irrigated or rainfed, puddled, lowland soil, rice is the only crop that grows in the river deltas where water may rise as high as 4 m. Rice also grows without standing water, as an upland crop, particularly on rolling land.

1.1.2. Utilisation of rice

Rice is of vital importance to the economy of developing countries and nutritionally, produces more carbohydrate per hectare than any other cereal crop (De Datta, 1981). Rice accounts for 35 to 60% of the calories consumed by 3 billion Asians. The importance of rice in the diet accounts for over 70% of the daily calorie intake in

countries such as Bangladesh, Cambodia, Laos and Myanmar but drops to about 40% in countries such as China and India where wheat is also included in the diet. Rice is also an important staple food in Latin America, Africa and Middle East. Rice accounts for 8-9% of the protein for human consumption whereas wheat constitute 11-12% (Khush, 1997). Rice is planted on about 148 million hectares annually, or on 11% of the world's cultivated land. It is the only major cereal crop, which is consumed exclusively by human. In 1996, the world rice production was 553 million tons. The largest producer was China (187 million tons) followed by India (122 million tons), Indonesia (50 million tons), Bangladesh (27 million tons), Vietnam (24 million tons), Thailand (21 million tons) and Mianmar (20 million tons) (Khush, 1997). Thailand is world's leading rice exporter, selling about 4-6 million tons annually followed by USA which ranks 11th in production (produces 6 million tons) and exports about 40% of it.

1.2. The genus *Oryza* : origin, classification, distribution, morphology and brief genetics

1.2.1. The genus *Oryza*

The genus *Oryza* is a member of the grass family *Gramineae*, order *Glumifloreae*, class *Monocotyledoneae* and division *Angiospermeae*. *Oryza* consists of two cultivated and twenty one wild species. The two cultivated *Oryza* species are *Oryza sativa*, the Asian cultivated rice grown all over the world and *Oryza glaberrima* that is grown on a small scale in West Africa. Twenty one wild species of *Oryza* are scattered in Asia, Africa, Australia and Central and South America. It is estimated that 4,205,000 rice accessions

are conserved in various gene banks worldwide and of this, 10% are wild accessions. Over 80,640 cultivated accessions are preserved in Genetic Resources Center of International Rice Research Institute and of this, 15,000 are from India. The size of the national collection ranges from about 40,000 in China to 25,000 in India, 7,000 in U.S. and other countries have smaller collections (Swaminathan, 1984; Khush, 1997; Paroda, 1998). These germplasm are not only utilized as a source of genetic variability in plant breeding programs but also possess important genetic material for resistance to some insect pests (Swaminathan, 1984).

1.2.2. Origin of rice

The archaeological evidences suggest that the genus *Oryza* to which the cultivated rice belongs, probably originated at least 130 million years ago. The ancient super continent of Gondwana is believed to be the original habitat of this genus. When Gondwana broke up and became Africa, Antarctica, Australia, Malagasy, South America and Southeast Asia, *Oryza* species drifted into different geographic habitats. Today's species of this genus are distributed in all of these continents except Antarctica (Chang, 1976; Swaminathan, 1984; Khush, 1997). W G. Solheim II in 1966 discovered the earliest and most convincing archaeological evidence for domestication of rice in Southeast Asia (Solheim, 1966). Ancient India is undoubtedly one of the oldest regions where cultivation of *O. sativa* began (Khush, 1997). The excavation from Hastinapura revealed that rice had already existed by 1000 BC in Northern India. The oldest carbonized grains found in India date back to about 6750 BC. The second oldest

carbonized rough rice grain excavated in 1973 in Hemudu, a village near Ningpo, Central China, is estimated to be 6,000 to 7,000 years old (Matsuo *et al.*, 1997; Khush, 1997). The primary center of diversity for *O. glaberrima*, is probably the swampy basin of the upper Niger river, formed around 1500 BC and two secondary centers to the South-West near Guinean Coast which were formed 500 years later (Khush, 1997). The evolutionary pathways of two cultivated rice species are presented in Fig. 1.

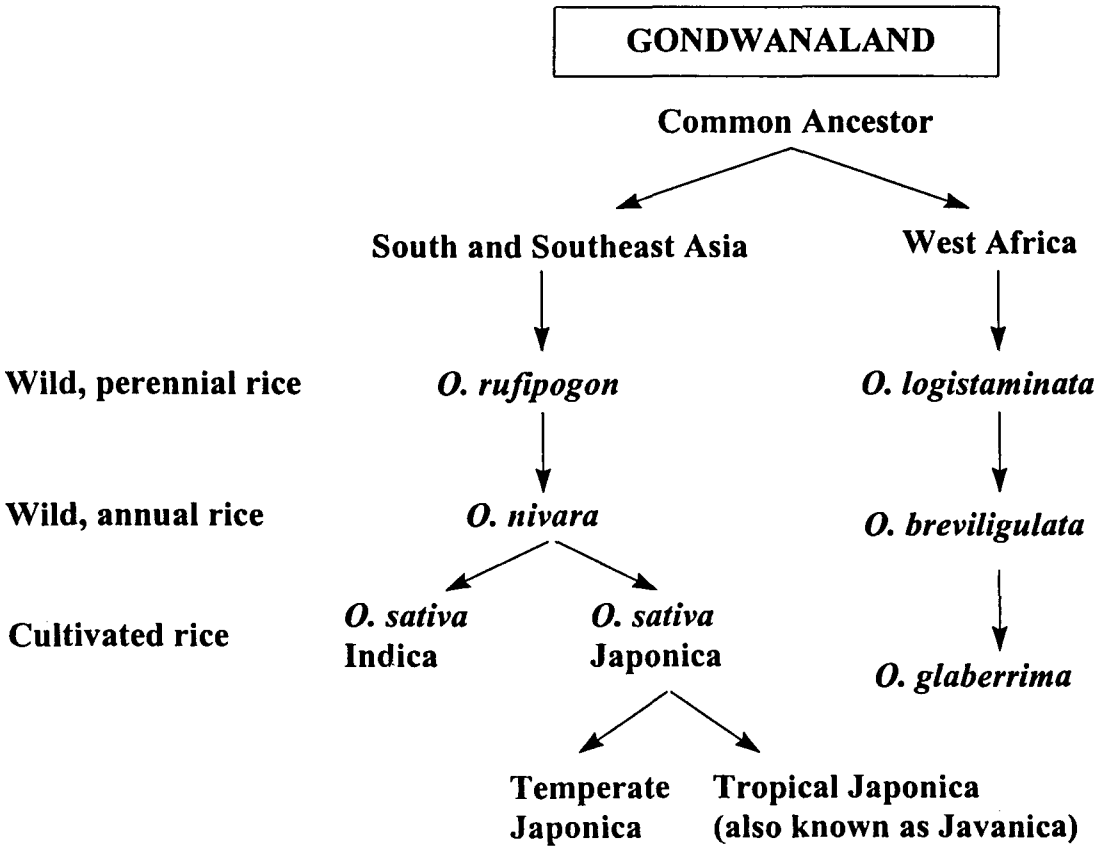


Fig. 1. Evolutionary pathways of two cultivated species of rice[1]

[1]Adapted from Khush (1997)

The cultivated rice, *O. sativa* and the African rice *O. glaberrima* are thought to be an example of parallel evolution in crop plants (Fig 1.). *Oryza sativa* probably derived from *O. nivara*, the wild annual rice, via the perennial rice, known as *O. rufipogon*. In another parallel evolutionary path, *O. glabarrima* was domesticated for annual *O. breviligulata,* which in turn evolved from perennial *O. longistaminata* (Chang, 1976; Khush, 1997).

1.2.3. Classification of rice

Since the third century BC, rice varieties in China have been clustered under three groups: 'hsien', 'keng' and glutinous. Kato *et al.* in 1928 divided cultivated rice *O. sativa* into two subspecies, Indica and Japonica, on basis of geographical distribution, plant and grain morphology, hybrid sterility and serological reaction. The Indica group is the old 'hsien' and the Japonica, the 'keng' (Grist, 1986). Moringa and Kuriyama (1958) added a third group Javanica to designate the bulu and gundil varieties of Indonesia.

Glaszmann classified the cultivated rice of the Asian countries into six groups on basis of genetic affinity using isozyme analysis (Glaszmann, 1987). This classification involves no morphological characters. When the six groups were compared with the varietal groups classified on the basis of morphological characters, Group I corresponded to the Indica and Group VI to the Japonicas. Javanica rices also belong to Group VI and designated as tropical Japonicas and the so-called Japonicas are referred to as temperate Japonicas (Khush, 1997).

The three ecospecies, Indica, Japonica and Javanica differ in their agronomic characters such as low temperature sensitivity, drought resistance, awn length, first internode length, grain shape and size and phoperiodic response (Oka, 1988). Indica rices are a large, diverse group of short day cultivars grown commercially in the tropics and sub-tropics. They generally are tall, heavy tillering, with pendant leaves and characterized by long, slender and non-glutinous grains. The Japonicas have darker green, erect leaves with moderate tillering, shorter, round and glutinous grains and are cultivated mainly in temperate zones and sub-tropics. The Javanica rices are found mostly in Indonesia, Java and to some extent, elsewhere. They are tall, insensitive to photoperiod and have a long vegetative phase, low tillering, long, broad and thick grains borne on long panicles (Grist, 1986; Oka, 1988; Khush, 1997).

1.2.4. Distribution of rice

According to most investigators, the cultivated species of *O. sativa*, spread from the Himalayan foot hills to Western and Northern India, to Afganistan and Iran and South India to Sri Lanka (Swaminathan, 1984; Khush, 1997). As early as 1000 BC, rice was a major crop in Northern India and Sri Lanka (Khush, 1997). Rice might have traveled from India to Madagascar and East Africa and then to countries of West Africa. The rice crop is believed to have been introduced to Greece and neighboring countries of Mediterranean. Rice grown in the Mediterranean regions, are Japonica while the rice grown in the Indian sub-continent is Indica. The Indica rice spread eastward to Southeast Asia and north to China. The Japonica rice was most likely domesticated

somewhere in northern parts of Southeast Asia or South China. It moved north to became a temperate Japonica. Around the beginning of 1st century, the temperate Japonicas were introduced in Korea and from Korea to Japan and to China. The Indica and Japonica rice was introduced from the mainland of Southeast Asia into Malaysia, Philippines, and Indonesia and from Philippines to Taiwan. The Portuguese introduced the tropical Japonica rice from Indonesia into Guinea-Bissau from where they spread to other West African countries. Lowland Indica rice was brought to Brazil and then to other Latin American countries by Spanish people. However, in all probability rice did not become an established crop in Europe much later perhaps in 15th or 16th century. The first record of rice in U.S.A dates from 1685, and it is believed to be introduced from Madagascar, South Asia and East Asia (Swaminathan, 1984; Khush, 1997).

The primary center of diversity for *O. glaberrima* is, from the swampy basin of the Niger southwest into two areas, near the Guinean coast. In West Africa *O. glaberrima* is a dominant crop grown in flooded areas of the Niger and Sokoto basins (Swaminathan, 1984).

1.2.5. Morphology of cultivated rice

Rice (*O. sativa* L) is an annual grass species with a more or less erect, cylindrical, smooth and hollow jointed stem or culm, flat sessile leaf blades and a terminal panicle (Grist 1986). A hull tightly encloses rice grain (caryopsis). Hull is composed of empty glumes, lemma and palea. The grain mainly consists of endosperm and embryo. Embryo is composed of an embryogenic axis, plumule and radicle. The plumule is

bound at the inner side by the scutellum or cotyledon, which lies next to the endosperm and is attached to the hypocotyl. The epiblast is seen as a protruding structure, which extends towards the upper end of the tip of the plumule and overlaps with the upper end of the scutellum. The radicle is enclosed in the coleorrhyza. When the grain germinates, the coleoptile emerges before the coleorrhyza. If grown in soil, the radicle protrudes first, but if the seed is submerged in water, the coleoptile emerges before the radicle. The radicle develops into root system and the young leaves emerge from the coleoptile.

The main stem or culm is differentiated from the growing point of the embryo, enclosed at first by the coleoptile. Stem has many nodes and covered by the leaf sheath. The leaves are alternate and borne in two ranks along the stem. The first leaf of the plant is the coleoptile. The second leaf, emerging through the lateral slit of the coleoptile, is reduced in size and has practically no blade. The remaining leaves are normal, except the uppermost or 'flag' leaf just below the panicle is slightly modified and plays an important role in assimilation of plant nutrients and therefore influences grain yield. The normal leaf has sheath, ligule, auricle and blade.

The inflorescence is a panicle, more or less lax, much branched and bearing spikelets. The spikelets are laterally compressed, oval, oblong or lanceolate, with or without awn. They are borne on a short pedicel, the rachilla dis-articulating below the lower floret and not produced beyond the uppermost floret. There are three florets in each spikelet but the two lower florets are reduced to sterile, scale-like lemmas while the terminal florat is fertile and forms the easily recognised 'grain'. The awn, when present, may be a mere

tip of about 1-2 mm or may be as much as 10 cm long. Inside the boat-shaped lemma and palea of the fertile floret the two lodicules, six stamens with slender filaments bearing versatile anthers and the pistil which consists of a one-celled ovary with a single ovule are the main organs. The style bears two plumose stigmas. At the time of flowering, the lemma and palea separates and the stigma protrudes followed rapidly by the anther bursting. Soon after pollination, the ovary gradually develops into the caryopsis and leads into mature seeds. (Grist, 1986).

Height of the plant is an important trait, related to the harvest index, growth duration, nitrogen response and lodging resistance. Apart from deep-water or so called floating varieties, the height of the rice plant is from 1 to 2 m but some deep water cultivars grow up to a height of more than 7 m. The dwarf varieties and some mutants grow to a height of little more than 0.5 m (Grist, 1986; Khush, 1997). Maturation period of rice plant depends on whether the variety is season-fixed or date-fixed. Some rice plants mature in less than 80 days from seed to seed while others like photo-period sensitive rice Rayada have a growth cycle of about 280 days (Grist, 1986; Khush, 1997).

1.2.6. Brief genetics of rice

The haploid chromosome numbers of *Oryza sativa* L. was first reported and presented by Kuwada in 1910, who demonstrated n = 12 (2n = 24). Studies by Audulov in 1931 provide strong evidence that the basic number of chromosome of the genus *Oryza* is 12. (Matsuo *et al.*, 1997). The two cultivated species of the genus *Oryza*, *O. sativa* and *O. glaberrima* are diploid 2n = 24 and posses 12 pairs of chromosomes. They are

numbered according to the decreasing order of length at pachytene stage of sexual cell division. Thus, the longest chromosome is number 1, second longest number 2 and the shortest is number 12. Chromosomes of both the cultivated species and closely related wild species are similar and their genomes are designated as AA genomes.

The chromosomes of other wild species, however, differ from those of cultivated rice and they belong to genomes designated as BB, CC, DD, EE, FF and GG. A few of the tetraploid species have BBCC, CCDD and HHJJ genomes (Brar and Khush, 1997; Khush, 1997; Khush *et al.*, 1998). Somatic chromosome number, genomic composition and potentially useful traits of *Oryza* species are given in the Table 1.

Table 1. Somatic chromosome number, genomic composition and potentially useful traits of *Oryza* species (adapted from Khush, 1997)

Species	2n	Genome type	Distribution	Useful or potentially useful traits[1]
O. sativa* complex				
O. sativa L.	24	AA	World wide	Cultivated rice
O.nivara Sharma et Shastry	24	AA	Tropical and sub-tropical Asia	Resistance to grassy stunt virus, blast, drought tolerance
Table 1.Cont.				
O. rufipogon Griff.	24	AA	Tropical and sub-tropical Asia	Elongation ability, resistance to BB, source of CMS
O.breviligulata A. Chev. Et Roehr.	24	A^gA^g	Africa	Resistance to GLH, BB, drought tolerance
O. glaberrima Steud.	24	A^gA^g	West Africa	Cultivated rice
O.longistaminata A. Chev. Et Roehr.	24	A^gA^g	Africa	Resistance to BB, drought tolerance

Table 1 Cont.

O. meridionalis Ng.	24	A^mA^m	Tropical Australia	Elongation ability, drought tolerance
O. glumaepatula Steud.	24	$A^{gp}A^{gp}$	South and Central America	Elongation ability, source of CMS
***O. officinalis* complex**				
O.punctata Kotschy ex Steud.	24 48	BB BBCC	Africa	Resistance to BPH, Zigzag leafhopper
O.minuta J.S. Pesl.ex C.B. Presl.		BBCC	*Philippines and* Papua New Guinea	*Resistance to sheath* blight, BB, BPH, GLH
O.officinalis Walls ex Watt	24	CC	Tropical and sub-tropical Asia, tropical Astralia	Resistance to thrips, BPH,GLH, WBPH
O. rhizomatis Vaughan	24	CC	Sri Lanka	Drought tolerance, rhizomatous
O. eichingeri A. Peter	24	CC	South Asia and East Africa	Resistance to yellow mottle virus, BPH, WBPH, GLH
O. latifolia Desv.	48	CCDD	South and Central America	Resistance to BPH, high biomass production
O. alta Swallen	48	CCDD	South and Central America	Resistance to striped stemborer, high biomass production
O.grandiglumis (Doell) Prod.	48	CCDD	South and Central America	High biomass production
O. australiensis Domin.	24	EE	Tropical Australia	Drought tolerance, resistance to BPH
O.brachyantha A. Chev. et Roehr.	24	FF	Africa	Resistance to yellow stemborer, leaf-folder, whorl maggot, tolerance to laterite soil
***O. meyeriana* complex**				
O.granulata Nees et Arn.	24	GG	South and South East	Shade tolerance,

Table 1 Cont.			Asia	adaptation to aerobic soil
Ex Watt				
O. meyeriana (Zoll. Et Mor. Ex Steud.) Baill.	24	GG	South East Asia	Shade tolerance, adaptation to aerobic soil
***O. ridleyi* complex**				
O. longiglumis Jansen	48	HHJJ	Irian Jaya, Indonesia and Papua New Guinea	Resistance to blast, BB
O. ridleyi Hook. f.	48	HHJJ	South Asia	Resistance to stemborer, whorl maggot, blast, BB
Unknown genome				
O. schlechteri Pilger	48	unknown	Papua New Guinea	Increased tillering

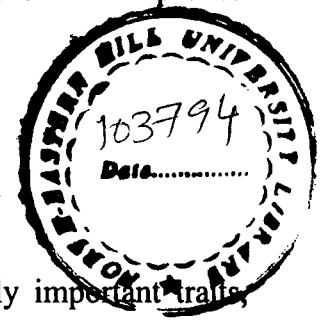
[1]BPH = brown planthropper; GLH = green leafhopper; WBPH = white-backed planthopper; BB = bacterial blight; CMS = cytoplasmic male sterility.

***All species of *O. sativa* complex have the same genome, AA. However, genomic differences have been observed in samples from different continents. These differences are indicated by adding a superscript to the AA designation, e.g. A^EA^E for *O. glaberrima*.**

1.3. Rice improvement through conventional breeding

Rice is a self-pollinated crop with less than 5% cross-pollination. Commercial varieties consist of homozygous, pure lines, which breed true-to-type. Natural crossing between commonly existing rice population produces new and mixed plant types in the field. The selection of special types from the mixed population was usually, achieved in the past by pure line selection and in a few instances by hybridization. This was done to not only suit various environmental conditions but also for the better yield. The rice breeding programme using hybridization was first initiated by Kano in 1904 in Japan (Grist, 1986). The International Rice Research institute in the Philippines that was set up in the mid-1960s undertook extensive rice breeding programs. Since then, rice

breeders of different countries of the world have developed new varieties by crossing carefully selected parents and eventually created many new cultivars with improved qualities.



1.3.1. Objectives of rice breeding

Initially, rice breeding programs were undertaken for agronomically important traits such as tolerance to drought, flood, salinity and soil alkalinity mainly through selection from mixed natural population and through introduction. Now selection is used for the isolation of superior genotypes from segregating population following hybridisation (Poehlman and Sleper, 1995). One of the most important objectives of rice breeding is to develop varieties, which will assure the stable and maximum production. To attain this, objective of breeding programs are different in different countries and even within the country. However, the overall objectives include improvement in productivity coupled with disease and pest resistance, tolerance to abiotic stresses, grain quality and nutritional value.

1.3.2. Improvement in productivity

1.3.2.1. High yield technology

A major achievement of Green Revolution is the tremendous increase in the yields of cereal grain production including rice through conventional breeding and selection methods. This revolution in agriculture, started in 1960s, kept pace with population growth and assured food security to half of the world's population for over 3 decades

(Vasil, 1995). One single factor that brought about the yield breakthrough in tropical rice, as in case of wheat is the dwarfing gene Dee-Geo-Woo-Gen (DGWG), a spontaneous mutant isolated in China. Extensive exploitation of this unique dwarfing gene source in crosses with tall standard native varieties led to the evolution of the first high yielding dwarf variety Taichung (Native 1) followed by the miracle rice IR8 in the year 1966. Since then, several hundred high yielding varieties were released for general cultivation all over tropical Asia, Africa and Latin America (Paroda, 1998). This achievement increased the production at the rate of 3% per year, which was adequate to meet the demand of the rapidly growing world population. Since then, the world's rice production has been, doubled from 257 million tons in 1966 to 520 million tons in 1990. In the last 25 year nearly all of the rice growing countries in Asia including the most populous China and India attained self-sufficiency in rice production (Khush, 1997; Paroda, 1998). Recently, through the development of molecular genetic maps, through which, gene blocks governing component traits of quantitative characters known by quantitative trait loci (QTL) can be located and exploited in direct breeding, for improvement of complex characters like grain yield. Using molecular markers, probably it is for the first time that two valuable yield-related QTLs (*Yld 1-1* and *Yld 2-1*) could be found in the wild species, *O. rufipogon.* They were then, transferred to a hybrid background resulting in as much as 17% higher yield (Xiao *et al.*, 1996; Tanksley and MaCouch, 1997). The demand of rice is still increasing and yield is plateuing. In order to meet this ever-increasing demand, hybrid rice technology has been employed.

1.3.2.2. Hybrid rice technology

One of the major break-through of conventional breeding, is exploitation of heterosis using hybrid varieties. The successful development and utilization of maize hybrids beginning around 1930 was a landmark in crop breeding. It provided inpetus to plant breeders to explore commercial exploitation of hybrid vigor or heterosis phenomenon in other crops. Rice is one of the most important crops in the world and is currently the focus of intensive efforts to increase yield through heterosis. Successful exploitation of hybrid vigor on commercial scale for last 20 years by Chinese has convinced many countries in and beyond Asia of hybrid technology as the potential strategy to step up the yield level. A farm-level study showed 15-16% yield advantage of hybrid rice over inbred rice in China. Comparison of average yield of hybrid rice and conventional rice varieties grown in China during 1981-1990 (Yuan *et al.*, 1994) showed a 29% to 45% yield advantage of hybrids. During the year 1976 to 1995, hybrid rice technology had helped China to increase rice production by nearly 300 million tons (Yuan, 1998).

In 1991, about 17.6 million hectares of land was cultivated with hybrid rice in China, which is about 55% of the total area of rice (Yuan, 1994). The success of hybrid seed development and use in China led to the establishment of hybrid rice programs at IRRI in 1979 and at least 20 other countries; majority of which are developing ones including India during 1996. In addition to China, hybrid rice was planted in 102,000 hectare in Vietnam and about 65,000 hectare in India. Limited commercial cultivation of hybrid rice has also been reported in Bangladesh, Korea, Myanmar and Philippines (Pulver, 1998). Rice, being a self-pollinated crop, must involve use of an effective, male sterility

system to develop and produce hybrids. Cytoplasmic male sterile lines are normally hybridized with restorer lines for the production of hybrid seeds. Cytoplasmic male sterility (CMS) system or three line method of hybrid production involving CMS lines, maintainer lines and restore lines has been found to be the most effective and practical for developing rice hybrids. However, Two line method or PGMS (photoperiod *sensitive genetic male sterility)* and TGMS (*thermo sensitive genetic male sterility)* systems also hold promise to increase efficiency of breeding rice hybrids and reduce cost of seed production. Genetic male sterility (GMS) can also be used in hybrid seed production but due to its recessive nature, it entails rejection of 50% of the plants and thus CMS is preferred over GMS.

Among four different types of CMS systems, S1 (Chinsura Boro II or BT), S2 (Wild Abortive or WA), S3 (Gambiaca) and S4 (Not designated earlier) only two systems are currently used for hybrids seed production of Indica and Japonica rices. The level of heterosis in Japonica hybrids is not as good as in Indica hybrids. About 90% CMS line (A line) for the commercial production of the hybrids in China are based on a single source of wild abortive (WA) cytoplasmic male sterility. Records have shown that these varieties are significantly out-yielded over pure line cultivars (Yuan, 1994). During the last several years, this WA system of CMS has also been used extensively in hybrid rice programs of other countries to produce CMS lines in various genetic backgrounds (Virmani, 1994, Virmani, 1996).

1.3.3. Resistance to disease and pest

Rice productivity is effected by several biotic stresses. Rice is susceptible to many diseases and more than 200 insect-pests are known to herbivore on rice. Some of the major pests affecting rice production include bacterial blight, blast, and sheath blight, tungro, brown planthopper and stem-borers. Changes in insect's biotypes and disease races are becoming a continuing threat to increased rice production (Khush and Brar, 1991). Approximately 55% of the rice yield is lost by disease annually, whilst 10-30% is damaged by insects.

Although, pests and diseases can be controlled by using insecticides, pesticides and fungicides, the optimal and safe use of these chemicals is a growing concern. Exposure to these chemicals seriously affects farmer's health, productivity and environment. Development of new plant types with enhanced resistance to specific diseases and pests can overcome these problems. Whilst such a resistant variety can combat potential damage by pests and diseases for several years, new, emerging variants or biotypes of pests and diseases will still be able to break this resistance down (Diehl and Bush, 1984). Therefore, discovery of new sources of resistance and their introgression in the elite breeding lines is required for successful breeding programs in rice (Bennet *et al.*, 1993). The introgression of a gene for grassy stunt virus resistance from the wild species *O. nivara* to cultivated rice varieties, was the first example of useful gene transfer to rice. The resistant gene for brown planthopper (BPH) from *O. officinalis* into cultivated rice was also successfully transferred (Jena and Khush, 1990). In order to develop IR36, thirteen rice cultivars from six different nations and a wild species *O. nivara* were

utilized by the breeders (Innes, 1992). This rice variety was released as a result of international co-operation among the plant breeders, plant pathologists, entomologists, agronomists and plant physiologists in early 1980s. IR36 is resistant to many diseases and pests including green leaf hopper, bacterial blight, blast, stem borer, tungro, brown plant hopper and grassy stunt viruses. Furthermore, IR36 is tolerant to zinc and iron deficiency in soils and also to drought. Interestingly, wild rices are the important reservoir of useful genes for improvement. *Xa21* was originally identified in the wild species *Oryza longistaminata*, and subsequently transferred to the cultivated rice IR24, generating the near-isogenic line IRBB21 (Ikeda *et al.*, 1990; Khush *et al.*, 1990).

1.3.4. Tolerance to abiotic stress

The tremendous success of plant breeding programs in the 1960s enhanced rice production significantly. However, most of the high yielding rice varieties released in the 1960s were inadequately adapted to stress conditions. The enormous yield losses attributable to abiotic stresses, such as drought, cold, salinity, alkalinity and flooding, on the rice crop have been well documented (Anonymous, 1986). In such cases, new type of varieties that are tolerant to adverse soil condition, including salinity, drought, cold and alkalinity are required. For instance, *P. coarctata* has been identified as a source of salt tolerance. Production of intergeneric hybrids between IR28 or IR36 (salt-sensitive) rice cultivars and *P. coarctata* through sexual hybridization was reported (Jena, 1994). However, transfer of genes from sexually incompatible species to *O. sativa* and from wild species having genome other than AA to *O. sativa* through conventional breeding,

is difficult to achieve due to the low crossability and limited recombination between chromosomes of the different partners. Alternatively, somatic hybridization may provide a means of overcoming sexual incompatibility between the cultivars and wild species (Jelodar, 1996).

1.3.5. Enhancement in grain quality and nutritional value

Nutritive quality plays an important role where rice is principle source of dietary protein (Juliano and Villareel, 1993; Swaminathan, 1998). The nutritional aspect of rice becomes an important consideration in the context of improvement, because it supplies most of the energy and protein for the dependent population (Bajaj, 1991). Rice provides about 40% of the protein in the Asian diet. Among the cereal proteins, rice protein is considered the richest biologically by virtue of its high digestibility (80%), high lysine content and relatively better net protein utilisation. Yet, it is nutritionally handicapped due to its inherently low protein content (6-8%) and inevitable milling loss of as much as 15-20% (Swaminathan, 1998). More importantly, milling removes essential amino acids such as cysteine, methionine, lysine, also vitamin B1, and minerals (Fe, Ca) which are largely located on the outer layer of the endosperm. Consequently, there is an urgent need for improving quality of the grain protein so that the people consuming rice are supplied with adequate amount of protein. Breeding for high protein content undertaken at IRRI, India and other countries has so far met with limited success (Swaminathan, 1998). Complex mode of inheritance, non-linear relationship between yield and protein content and proneness to profound environmental conditions is

responsible for this slow progress. There may be two ways to minimise the losses in milled rice viz., (i) exploiting genetic variation in the distribution pattern of protein bodies, and (ii) differential resistance of outer layers to scrapping. Among several factors, thickness of aleurone layer seems to determine the level of milling loss (Pehu and Siddiq, 1986). Once their genetics and response to selection are established, breeding for 'Low protein loss' may become feasible (Swaminathan, 1998).

1.3.6. Limitations of conventional breeding methods

Achieving high yields had been the research priority in the sixties and seventies, whereas stability of yield performance became the thrust in seventies. Nearly all the high yielding varieties available then were vulnerable to a host of insect-pests and diseases and none was adapted to abiotic stresses of all kinds (Paroda, 1998). As we march into the next millennium, the world would face the most challenging task of sustaining even the current level of rice availability. Success in achieving the goals would depend on exploitable genetic variability for the desired traits that directly or indirectly contribute to the enhancement of yield and its stability. Primary and secondary gene pools have been the major sources of variability for breeders to make use off. Induced mutation is yet another potential but least exploited source of variability. However, there is a lack of genetic variability in cultivated rice and, in many cases, variability in the cultivated species for important economic traits is limited (Khush and Brar, 1991). However, at the same time, the wild rice germplasm has been found to constitute a rich reservoir of useful genes for varietal improvement. In this

case, related wild species can be used as a source of useful genes and offer great potential to incorporate such genes into commercial rice cultivars for resistance to major diseases, insects and tolerance to various abiotic stresses. However, crossability barriers limit the gene transfer from wild species to cultivated species. One of the most important barriers is the limited recombination among homologous chromosomes of cultivated rices and wild species and the introgression is poorly understood (Brar and Khush, 1997). The major problems in conventional rice breeding are summarized as follows:

i) The rice breeders have to conduct breeding programs with the primary gene pool of the species.

ii) The breeders are always confined to naturally occurring genetic variability

iii) There is a very limited variability in the cultivated species.

iv) Although wild species are an important reservoir of useful genes but transfer of these genes from wild species to cultivated species is very limited. It is due to several incompatibility barriers such as crossability and limited recombination between chromosomes of wild and cultivated species.

v) The time taken to develop a new variety by conventional method is always long and time consuming. For instance, in a successful hybrid seed production programme, approximately 6-8 repeated back-crossings are necessary which requires 5-6 years for developing a new male sterile line.

vi) Development of improved rice cultivar, which can withstand diseases, insects and abiotic stresses for the adverse environmental conditions still, remains a major challenge to rice breeders.

vii) The extent of land required for conventional breeding methods is also very large.

These limitations of the conventional breeding method necessitate the use of biotechnology and plant molecular biology in rice improvement.

1.3.7. Biotechnological approaches to rice improvement

In recent years, much attention has been directed to the newly emerging and invents of novel technologies in molecular and cell biology known as biotechnology. Advances in plant cell culture research, especially of major crop species, have played an increasingly critical role in the development of modern plant biotechnology (Vasil, 1990). In comparison to conventional methods, which aim to expand genetic variation through gene recombination with sexual reproduction, this technology enables to use much wider variation by overcoming the inter-specific barrier. More specifically, this method can transfer a character (or a gene) from an entirely different species or genus, thereby reveling out serious difficulties existing in sexual reproduction. The application of tissue culture and genetic engineering in rice crop improvement will be discussed in this section with special emphasis on mitochondrial gene transfer, for the production of cytoplasmic hybrids in rice.

1.4. Development of tissue culture systems for rice

1.4.1. General status of cereal tissue culture

Gottlieb Haberlandt in 1902 developed the idea of totipotency in plant cells (Haberlandt, 1902). According to his idea, plant cells are automatic in function, and in principle, are capable of regenerating to produce a complete plant. This scientific proposition has since become a basic principle in studies pertaining to plant tissue culture. The successful regeneration of plants from single cell and tissues cultured *in vitro* caused considerable upsurge of interest and activity in the possible combined uses of plant cell culture and modern molecular genetic techniques in the improvement of crop plant. The first successful tissue culture among cereals was reported from maize endosperm by La Rue in 1949 (La Rue, 1949). Fertile, diploid maize plants were first regenerated by Green and Phillips in 1975 from callus derived from the scutellum of immature embryos (Green and Phillips, 1975). Norstog succeeded with barley embryo culture in 1961 (Norstog, 1961). In 1973, the first barley plants were regenerated *in vitro* from anther cultures (Clapham, 1973). The importance of corrects nutritional and hormonal conditions were recognized by Skoog and Miller in 1957 and have influenced the techniques used since then (Skoog and Miller, 1957).

The modern work on tissue culture of cereals is based on, some important discoveries made in 1980-81 (Haydu and Vasil, 1981; Lu and Vasil, 1981; Vasil and Vasil, 1980). They were, the use of (i) meristemetic tissues (mature embryos, young inflorescence, immature leaf bases (at defined stages of development) as explants and (ii) nutrient media supplemented with a strong auxin, usually 2,4-dichlorophenoxy acetic acid.

These findings, led to the (i) establishment of long term callus cultures in which plant regeneration takes place via somatic embryo formation and (ii) establishment of cell suspension culture derived from embryogenic calli that yield totipotent protoplasts. These pioneering works show the ability to control organogenesis from various cereal callus, laid a foundation for subsequent advances in cereal cell and tissue culture and application of these techniques to plant breeding and genetic engineering of plants. Since then, an established plant regeneration systems has been reported for all major crops including wheat, rice, maize, barley, sorghum, sugarcane, oats, millet and few grass species under the family Gramineae (Srinivasan and Vasil, 1986; Chen *et al.*, 1988; Harris *et al.*, 1988; Prioli and Sondhall, 1989; Yamada, 1989; Roest and Gilssen, 1989; Hartke and Lörz, 1989; Lynch *et al.*, 1991; Yan *et al.*, 1990; Ghosh- Biswas *et al.*, 1994; Aftab *et al.*, 1996; Ahmed *et al.*, 1997; Southgate, 1996; Singh *et al.*, 1997; Elkonin *et al.*1995; Bekele *et al.*, 1995; Khanna and Raina, 1997, Azhakanandam, 1999).

1.4.2. General status of rice tissue culture

The tissue culture of rice was initiated in the mid-1950s with culture of excised rice roots (Fujiwara and Ojima, 1955) and immature rice embryos (Amemiya *et al.*, 1956). Regeneration of rice plants was subsequently reported from root-derived callus (Kawata and Ishihara, 1968). In the same year, shoots were successfully obtained from callus initiated from rice embryos (Tamura, 1968). Since the early report of production of haploid rice plants from anthers (Niizeki and Oono, 1968), anther culture has been

widely employed in rice breeding. Since then, different useful technologies such as plant regeneration from suspension cultures (Lieb *et al.*, 1973; Abe and Futsuhara, 1986) and plant regeneration from protoplast culture has been developed for rice (Fujimura *et al.*, 1985; Yamada, 1989; Abdulla *et al.*, 1986; Kyozuka *et al.*, 1987; Toriyama *et al.*, 1986; Masuda *et al.*, 1989; Jenes and Pauk, 1989; Li and Murai, 1990; Meijer *et al.*, 1991b; Jain *et al.*, 1995; Bhattacharjee and Gupta, 1995, Bhattacharjee *et al.*, 1998).

1.4.2.1. Callus induction and plant regeneration of rice

1.4.2.1.1. Embryo culture

Embryo culture is a technique in which scutella of immature and mature embryos excised from the ovules are aseptically raised in a liquid or semi-solid medium. Embryo culture was not applied to rice until the beginning of the 1950s. Amemiya *et al.* (1956) initiated studies pertaining to the immature embryo culture of rice plants. However, the use of the scutella of mature and immature embryos as explant sources was a breakthrough in cereal tissue culture, and is widely used even today to initiate callus cultures and plant regeneration from many rice varieties and other cereals. Cultured cells differ in properties among themselves. Heyser *et al.* (1983) classified the calli into two types: embryogenic and non-embryogenic. Embryogenic calli can regenerate a completely fertile plant, while non-embryogenic calli occasionally bear buds but can not bear any shoots. These two types of calli could be distinguished morphologically from each other. Embryogenic calli consist of small meristemetic cells and produce many plants through somatic embryogenesis while the non-embryogenic calli consists of long

tubular and sometimes vacuolated cells which produce few or no plants (Siriwardana and Nabors, 1983). Embryogenic calli maintain their competence for long periods of time and give rise to genetically uniform and normal plant population. In rice, there are several reports on callus induction from immature embryos (Lai and Liu, 1976; Lai and Liu, 1982; Peng and Hodges, 1989; Wu and Zapata, 1992; Bhattacharjee and Gupta, 1995, Bhattacharjee *et al.*, 1998) and mature embryos (Maeda, 1967; Bajaj, 1991; Kavi Kishor and Reddy, 1986a; Yasuda *et al.*, 1990; Peterson and Smith, 1991; Datta *et al.*, 1992; Torrizo and Zapata, 1992; Chowdhry *et al.*, 1993; Rance *et al.*, 1994; Bhattacharjee and Gupta, 1995; Bhattacharjee *et al.*, 1998; Azhakanandam, 1999. Bhattacharjee *et al.*, 1999). Scutellum cells of immature embryos were found most responsive explants for tissue culture in rice. However, mature embryos are also suitable for tissue culture due to their availability throughout the year (Peterson and Smith, 1991; Datta *et al.*, 1992; Torrizo and Zapata, 1992; Chowdhry *et al.*, 1993; Rance *et al.*, 1994; Bhattacharjee and Gupta, 1995) and provided that a high frequency of regeneration can be achieved (Rueb *et al.*, 1994). The immature organs and meristemetic tissues, which contain undifferentiated cells, are known to be more suitable for plant regeneration (Morrish *et al.*, 1987).

1.4.2.1.4. Plant regeneration through somatic embryogenesis and organogenesis

Somatic embryogenesis is the predominant mode of regeneration in cereals (Vasil, 1994). Both Japonica and Indica rice varieties can be regenerated from callus initiated from various parts of the plant through somatic embryogenesis (Rueb *et al.*, 1994; Rout

and Lucas, 1996) and organogenesis (Suzuki *et al.*, 1993). Plantlet regeneration through somatic embryogenesis has been reported in many rice varieties (Heyser *et al.*, 1983; Siriwardana and Nabors, 1983; Abe and Futsuhara, 1986; Wang *et al.*, 1987; Hartke and Lorz, 1989; Poonsapaya *et al.*1989; Choudhry *et al.*, 1993; Rance *et al.*, 1994; Rueb *et al.*, 1994; Adkins *et al.*, 1995; Bajaj and Rajam, 1995; Khanna and Raina, 1998; Azhakanandam, 1999). Efficient plant regeneration, via somatic embryos, in rice depends on various factors including, genotype (Abe and Futsuhara, 1986; Hartke and Lorz, 1989; Choudhry *et al.*, 1993; Khanna and Raina, 1998; Azhakanandam, 1999), manipulation of medium (Siriwardana and Nabors, 1983; Bajaj and Rajam, 1995; Azhakanandam, 1999), carbohydrate source (Ghosh Biswas and Zapata, 1993), partial desiccation of callus (Rance *et al.*, 1994) and dehydration of callus using high concentration of gelling agent in the regeneration media (Azhakanandam, 1999). The addition of organic additives such as L-tryptophan, L-proline (Siriwardana and Nabors, 1983; Chowdhry *et al.*, 1993), abscisic acid (Peterson and Smith, 1991; Xu *et al.*, 1995), spermidine (Bajaj and Rajam, 1995), cytokinins such as 6-benzylaminopurine (BAP) and kinetin (Lee *et al.*, 1989; Datta *et al.*, 1990; 1992) to regeneration medium - all potentially influence plant regeneration from rice callus. In rice, the interaction of plant phyto-hormones, such as 2,4-D and kinetin, is considered a key to induction of embryogenic callus and plant regeneration from such tissues (Peterson and Smith, 1991; Mitsuoka *et al.*, 1994). Somatic embryogenesis is useful, because the somatic embryos, like their zygotic counterparts, are directly or indirectly derived from single cells and therefore are non-chimaric in nature and posses a root-shoot meristem which is vital for

survival in soil. Plants derived from somatic embryos maintain uniformity and genetic fidelity (Vasil, 1995).

Improved plant regeneration through organogenesis has also been reported from rice callus tissues (Kawata and Ishihara, 1968; Nakano and Maeda, 1979). Osmotic stress, caused by a simple treatment like partial desiccation of calli, promotes organogenesis in calli and leads to a higher regeneration ability in calli (Tsukahara and Hirosawa, 1992; Rance *et al.*, 1994).

1.4.2.1.5. Somaclonal variation in tissue culture-derived plants

Somaclonal variation is an important phenomenon observed in plants regenerated from tissue culture and can be utilized as a tool by plant breeders. Regeneration of plants through tissue culture should normally result in clones that are phenotypically and genetically identical to the progenitor plants. In many cases, however, regenerants exhibit deviation from the parental type (Brown *et al.*, 1991). This phenomenon is widespread in a range of plant species and defined as somaclonal variation (Larkin and Scowcroft, 1981). This topic has been extensively reviewed by several workers (Sun and Zheng, 1990; Peschke and Phillips, 1992; Ahmed and Sagi, 1993; Phillips *et al.*, 1994; Skirvin *et al.*, 1994; Karp, 1995). This variability (somaclonal variation) can however, be utilized for the development of new varieties Somaclonal variation has been observed in many cereal species such as wheat (Karp and Maddock, 1984; Cooper *et al.*, 1986), maize (Brettel *et al.*, 1986), sorgham (Bhaskaran *et al.*, 1987) and rice (Adkins *et al.*, 1990; 1995).

Nishi *et al.* in 1968 first reported the phenotypically variant plants of rice originating from seed-derived callus, including dwarf and twisted plants. Various factors such as cytoplasmic or nuclear mutation (Evans and Sharp, 1983), polyploidy and other chromosomal abnormalities (Ahloowalia, 1882; Orton, 1983), may be the reason for the origin of somaclonal variation. The variation in plants regenerated from haploid tissue is comparatively high (Lynch *et al.*, 1991). Male sterile somaclones have been identified from the Indica varieties IR24 and IR54 (Ling *et al.*, 1988). Zhang *et al.* (1989) analyzed plants regenerated from seed-derived callus of four Chinese varieties. Rice calli have also been screened for resistance to phytotoxin (Ling *et al.*, 1986). More recently, Azhakanandam (1999) has reported the characterization of somatic embryo- and seed-derived plants of the aromatic Indica rice, Pusa Basmati 1. Commonly observed variations in tissue culture-derived plants includes the number of tillers per plant, plant height, flag-leaf length, heading date, panicle length, fertility, and a number of seeds produced (Oono, 1978, 1981, 1985; Sun *et al.*, 1981, 1983; Schaeffer *et al.*, 1984; Murai and Kinoshita, 1986; Azhakanandam, 1999).

1.4.2.1.6. Cell suspension culture

Lieb *et al.* (1973) were the first to report suspension cultures of rice from root callus using modified Heller's medium (Heller, 1953). Since then, several basal media, including R2 (Ohira *et al.*, 1973), MS (Murashige and Skoog, 1962), LS (Linsmaier and Skoog, 1965), B5 (Gamborg *et al.*, 1968), CC (Potrykus *et al.*, 1977), N6 (Chu *et al.*, 1975) and AA (Muller and Grafe, 1978) have been used to initiate suspension cultures

from various calli derived from different explants of many varieties of rice. R2 medium, has more often been used for Indica rices (Torrizo and Zapata, 1992; Ghosh Biswas and Zapata, 1993; Jain *et al.*, 1995) while AA medium, which contains four different amino acids as source of carbon, has been used for the initiation of Japonica rice suspension cultures (Abdulla *et al.*, 1986; Guiderdoni and Chair, 1992; Su *et al.*, 1992). The nitrogen source of the medium also plays an important role in the initiation and establishment of suspension culture in rice (Toriyama and Hinata, 1985; Thompson, 1986). However, the establishment of embryogenic cell suspension cultures seems to be under the control of the plant genotype.

To establish suspension culture of rice, different explants such as root, anther, leaf base, immature panicle, stem base, immature and mature embryos are used (Abe and Futsuhara, 1991). Friable embryogenic calli are commonly used as source for the initiation of suspension cultures in rice. Suspension cultures are initiated with a small amount of embryogenic calli in a suitable liquid medium. They are maintained on a shaker in the dark and subcultured with regular interval to increase highly dispersed fine and rapidly proliferating cells rich in cytoplasmic substances. A fine embryogenic cell suspension consists of small clumps composed of 10-20 cells, each actively proliferating, densely cytoplasmic with minimum number of highly vacuolated cells (Abe and Futsuhara, 1991). To maintain the embryogenicity of the cells they are sub-cultured at 3 to 7 days, with regular interval. Once established, these cultures exhibit a rapid growth with cell doubling time of 27 to 32 hours (Karlson and Vasil, 1986). A

significant feature of an established rice suspension culture is that it appears to posses a higher percentage of totipotent cells than other explants or callus (Hodges *et al.*, 1991). Plantlets regeneration, directly from suspension cells, have been reported in Japonica as well as Indica rice (Abe and Futsuhara, 1991; Binh *et al*, 1992; Ghosh Biswas and Zapata, 1992; Tsukahara *et al.* 1992). Plant regeneration has been achieved from long term suspension cultures initiated from root-derived (Zimny and Lörz, 1986; Abe and Futsuhara, 1986; and seed-derived (Ye, 1984; Honda *et al.*, 1992; Kobayashi *et al.*, 1992) embryogenic calli of Japonica rice. Abe and Futsuhara (1991) reported that the regeneration capacity of embryogenic cell suspensions of Japonica varieties, in culture, was higher than for Indica, Indica-Japonica hybrids and Javanica types. Ozawa and Komamine (1989) established a system of high-frequency embryogenesis from long-term cell suspension cultures initiated from excised immature embryos of rice.

In rice, embryogenic cell suspension cultures have been used as the main source material for the enzymatic isolation of protoplasts (cells without cell wall). The suspension cells are also amenable to *Agrobacterium*- mediated transformation (Hiei *et al.*, 1994). However, the establishment and maintenance of embryogenic suspension cultures particularly of Indica rice varieties, presents difficulties. It has been observed that the morphogenic competence of suspensions usually declines with successive subculture over prolonged period of time (Abe and Futsuhara, 1991). Suspension cultures of cereals lose their morphogenic potential in about 6 months (Jahne *et al.*, 1991) to 12 months (Shillito *et al.*, 1989) and tend to accumulate genetic changes (Chang *et al.*, 1991; Wang *et al.*, 1992; Karp, 1993) during prolonged culture. To overcome this

problem, competent cultures can be preserved over liquid nitrogen (cryopreservation) for ensuring a constant supply of suspension cells over the most responsive state later (Krautwig and Lörz, 1995).

1.4.2.1.7. Protoplast culture

A fundamental requirement for nearly all applications in biotechnology is the regeneration of whole plants from cells/tissues or protoplasts cultured *in vitro*. Due to the fact that protoplast represents a true single cell system, they offer a valuable baseline system for plant genetic manipulation. They are also used for various fundamental studies, such as membrane transport, metabolic regulation and studies on gene expression after gene transfer by direct DNA delivery into protoplasts (Krautwig and Lörz, 1995). Protoplasts are the versatile tool for genetic manipulation of plant cells. Reproducible plant regeneration from protoplasts is therefore, an essential prerequisite for practical application of the techniques like direct DNA uptake, somatic hybridization and cytoplasmic hybridization.

The first report of enzymatic isolation of protoplasts (Cocking, 1960) offered a novel and promising technique to the plant cell and tissue culture. It open a new area of research and protoplasts can now be obtained from nearly all plants tissues and cultured cells. Many studies were carried out on rice protoplasts (Deka and Sen, 1976; Lai and Liu, 1982, Wakasa *et al.*, 1984). Although formation of callus from protoplasts of rice has reported in some of these studies, but none of them succeed in regenerating plants from the calli derived from rice protoplasts. The first successful plant regeneration from

protoplasts was reported from Japonica rice (Fujimura *et al.*, 1985) and since then, a reproducible system has been developed from protoplasts of Japonica, Indica and Javanica rices.

In contrast to most dicotyledonous species, where the protoplasts isolated even from mature tissues have the ability to dedifferentiate and to re-enter the cell cycle, the majority of monocotyledonous plants, which include the most important crop species seem to be recalcitrant to tissue culture, especially protoplast culture. Later, it was reported that cultured cells provides the best source of protoplasts with division capacity in the family Gramineae (Thompson, 1986; Rueb *et al.*, 1994). However, due to the difficulties involved in the culture of protoplasts isolated directly from the plant tissues, embryogenic calli derived from mature and immature scutella and leaf base-derived calli were used as a source material for initiation of cell suspension.

Since 1985, substantial progress has been made in plant regeneration from protoplasts of Japonica, Indica and Javanica rices where mostly embryogenic cell suspensions have been utilized as a source material for the isolation of large number of totipotent protoplasts. Plant regeneration from the protoplasts of Japonica rice has been successfully accomplished for a range of varieties (Fujimura *et al.*, 1985; Yamada *et al.*, 1986; Abdulla *et al.*, 1986; Kyozuka *et al.*, 1987; Toriyama *et al.*, 1986; Masuda *et al.*, 1989; Jenes and Pauk, 1989; Li and Murai, 1990; Meijer *et al.*, 1991b; Jain *et al.*, 1995; Bhattacharjee and Gupta, 1995). Indica rice varieties are the most widely cultivated in the tropical regions of the world, and are the most important food source for half of the world's population. Hence, attention has also been focused on the improvement of these

varieties using biotechnology including protoplast to plant regeneration systems. In general, it is difficult to culture and regenerate plants from the protoplasts of Indica rices. Since Kyozuka *et al* (1989) established a protocol for regeneration of Indica rices using nurse culture methods, a number of researchers have reported plant regeneration from Indica rices. Most of them have utilized embryogenic cell suspensions derived from embryogenic callus obtained from scutellum of immature embryos (Lee *et al.*, 1989; Lee and Kim, 1991; Su *et al.*, 1992; Yin *et al.*, 1993), mature seeds (Kyozuka *et al.*, 1988; Wang *et al.*, 1989; Torrizo and Zapata, 1992; Datta *et al.*, 1992b; Ghosh Biswas and Zapata, 1993; Timothy and Rangaswamy, 1993; Jain *et al.*, 1995; Ramaswamy *et al.*, 1995) and anthers (Datta *et al.*, 1990a; Ghosh Biswas and Zapata, 1991). There have been few reports on culture and plant regeneration of Javanica rice protoplasts. Plantlets have been regenerated from protoplasts of Javanica rices using embryogenic cell suspensions, initiated from calli obtained from scutellum of mature seed (Wang *et al.*, 1989; Suh *et al.*, 1992) and immature embryos (Suh *et al.*, 1992) and also from immature panicles (Li *et al.*, 1992; Utomo *et al.*, 1995).

In order to avoid difficulties, time and labour involved in the establishment of cell suspensions and variations due to prolonged *in vitro* culture in the regenerated plants; protoplasts derived from materials other than cell suspensions is clearly of considerable interest. Successful plant regeneration has been achieved from protoplasts isolated directly from rice immature embryo-derived callus (Wu and Zapata, 1992), scutellar tissues of immature zygotic embryos of rice (Ghosh Biswas *et al.*, 1994) and messophyll tissues of leaf base and sheath explants (Gupta and Pattanayak, 1993). However, the

fact that protoplast yield from intact tissues and plating efficiency were comparatively low or that protoplasts could not be reproducibly induced to regenerate plants (Ghosh Biswas *et al.*, 1994a), has prevented these options from being adopted as routine source material for protoplasts isolation and culture.

Success in rice protoplast culture depends on many variables. This includes genotype, explant source, age of cell suspension, medium composition, culture conditions, use of nurse culture and selection of carbon source in plant regeneration media (Abdulla *et al.*, 1986; Kyozuka *et al.*, 1987; Li and Murai, 1990; Datta *et al.*, 1990; Hodges *et al.*, 1990; 1991; Su *et al.*, 1992; Torrizo and Zapata, 1992; Ghosh Biswas and Zapata, 1993).

The effect of pre-culture treatment on rice protoplasts, in the term of heat shock (at 45°C, 5 min followed by 20 sec on ice or 45°C for 8 min followed by 10 sec in ice water), to improve plating efficiency has been reported by Thompson *et al.*, (1987) and Lin M-Yu *et al.*, (1991) respectively.

The use of feeder cells has greatly influenced protoplast culture in cereals (Rhodes *et al.*, 1988; Shillito *et al.*, 1989; Prioli and Sondahl, 1989; Funatsuki *et al.*, 1992; Krautwig and Lörz, 1995). Feeder cells from several species have been used to support cell division from cultured protoplasts of Indica and Japonica rices. This includes, cultivated rice species (Lee and Kim, 1991; Wen *et al.*, 1991; Su *et al.*, 1992; Guiderdoni and Chair, 1992; Timothy and Sree Rangasamy, 1993, Bhattacharjee and Gupta, 1995), wild rice species, *O. ridleyi* (Torrizo and Zapata, 1992; Jain *et al.*, 1995) and allied graminaceous plants e.g *Panicum maximum*, *Lolium multiflorum*, *Panicum serobiculatum* (Timothy and Sree Rangasamy, 1993; Jain *et al.*, 1995). Two different

types of nurse culture methods have been established using feeder cell system and adopted by several researchers. In one method, protoplasts are suspended in agarose-solidified protoplast culture medium inside 'donut' holes surrounded by nurse as established by Kyozuka *et al.*, (1987; 1988). In the second method, protoplasts are suspended in agarose-solidified protoplast culture medium and plated on the surface of a filter membrane positioned on the top of agarose-solidified feeder cells embedded as nurse as reported by Lee *et al.*, (1989). In the second method, membrane filters (0.8 μm) were used as a partition between the nurse cells and protoplasts (Wen *et al.*, 1991; Su *et al.*, 1992; Guiderdoni and Chair, 1992; Torrizo and Zapata, 1992; Ghosh Biswas and Zapata, 1993; Timothy and Rangasamy, 1993; Bhattacharjee and Gupta, 1995, Jain *et al.*, 1995).

The presence of feeder cells in the protoplast culture of Japonica rice varieties improved protoplast to plant regeneration system by increasing plating efficiency and plant regeneration frequency. It was demonstrated that in some Indica varieties feeder cells were necessary to induce protoplast-derived colony formation and plant regeneration (Bhattacharjee and Gupta, 1995; Jain *et al.*, 1995).

Several workers (Kyozuka *et al.*, 1988) have done extensive work on the effect of different sources of carbohydrate on plant regeneration of rice. The use of maltose instead of sucrose, as a carbon source, is an important modification in plant regeneration medium that enhances frequency of plant regeneration (Torrizo and Zapata, 1992; Ghosh Biswas and Zapata, 1994; Jain *et al.*, 1995).

It has been reported that the water content of rice callus tissue is an important factor in relation to plant regeneration. A poorly regenerating callus can be changed to a vigorously regenerating one by exposure to water stress (Liu and Lai, 1991; Binh *et al.*, 1992; Tsukahara and Hirosawa, 1992).

1.4.2.1.8. Protoclonal variation

The development of a protoplast-to-plant regeneration system in rice has led to the generation of protoclonal variation in protoplast-derived plants (protoclones) and their seed progeny (Lynch *et al.*, 1991). Variations in agronomic characteristics were observed among protoclones of rice. In rice protoclones, changes in agronomic characters, such as heading time, plant height, number of tillers per plant, number of fertile tillers, days to maturity, panicle length, grain size (Abdullah *et al.*, 1989; Sukekiyo and Kimura, 1991; Mezencev *et al.*, 1995), chlorophyll content of plants (Guiderdoni and Chaïr, 1992) and ploidy level have been reported (Guiderdoni and Chaïr, 1992; Kawata *et al.*, 1992).

Among protoplast derived rice plants, variation was also found for nuclear DNA content (Brown *et al*, 1990). The degree of variation was lower in many lines of protoplasts regenerants than in callus-derived plants (Müller *et al.*, 1990). Somaclonal, protoclonal and molecular variations have been reported to exist in the progeny of plants regenerated from both callus and protoplast-derived tissues of rice (Abdullah *et al.*, 1989; Zheng *et al.*, 1989; Saleh *et al.*, 1990; Müller *et al.*, 1990; Davey *et al.*, 1991).

1.4.3. Utilization of protoplast technology

1.4.3.1. Transformation: Direct gene transfer to rice protoplasts

Genetic transformation is one of the most versatile tools in modern biology. In higher plant, genetic transformation was first attained by the use of a natural gene transfer system possessed by *Agrobacterium tumefaciens* (Schell, 1987). Unfortunately, Graminaceous species including rice, wheat, maize and barley were thought to be outside the host range for *Agrobacterium* infection and therefore, alternative methods like direct DNA transfer had to be devised for transformation of these plant species. Because of lack of rigid cell wall, protoplasts are excellent recipients of a variety of macromolecules including foreign DNA. It was established that DNA taken up by protoplasts could be stably integrated into the host chromosome. Gene transfer by direct delivery of DNA into protoplasts has been successful either through chemical treatment with polyethelene glycol (PEG) or through electroporation.

1.4.3.1.1. Polyethelene glycol induced gene transfer

Incubation of protoplasts with long chain cations, such as poly-L-arginine, poly-D-lysine and polyethelene glycol (PEG) can induce protoplasts to take up foreign DNA from the medium. Since the first reported use of PEG to introduce plastids into tobacco protoplasts (Krens *et al.*, 1982), this agent has been extensively used to mediate plasmid uptake into a number of protoplast systems, including rice (Davey *et al.*, 1989). Since then many rice varieties have been transformed using this method (Datta *et al.*, 1990b;

Zhang *et al.*, 1991; Peng *et al.*, 1992; Datta *et al.*, 1992a; Xu *et al.*, 1993; Ayres and Park, 1994; Ghosh Biswas *et al.*, 1994a; Cornejo *et al.*, 1995; Lee, 1996).

1.4.3.1.2. Direct gene transfer via electroporation

Electroporation-mediated gene transfer has been widely utilized for transformation of plant protoplasts although it was developed for animal cell transformation (Neumann *et al.*, 1982). Electroporation-mediated gene transfer into protoplasts has been reported in rice by several researchers throughout world (Shimamoto *et al.*, 1989; Yang *et al.*, 1988; Zhang *et al.*, 1988; Battraw and Hall, 1990; Tada *et al.*, 1990; Schuh *et al.*, 1993; Ayres and Park, 1994; Chaudhury *et al.*, 1994; Lee, 1996; Bhattacharjee *et al.*, 1997).

1.4.3.1.3. Limitations of protoplast -mediated transformation

Establishment and maintenance of embryogenic suspensions is technically difficult with many Indica rices as the morphogenic competence decline during prolonged sub-culture (Abe and Futsuhara, 1991). Moreover, direct DNA uptake methods often result in multiple copies of genes incorporating into the genome, rearrangement and fragmentation of genes, high frequency of sterile plants (Flavell, 1994) and non-Mendelian inheritance of transgenes (Peng *et al.*, 1995; Aldemita and Hodges, 1996). With the recent development of *Agrobacterium tumefaciens* - mediated transformation in rice these problems in direct DNA transfer can be avoided (Chan *et al.*1992; Chan *et al.*1993; Hiei *et al.*, 1994; Rashid *et al.*, 1996; Aldemita and Hodges, 1996; Dong *et al.*, 1996; Cheng *et al.*, 1998; Azhakanandam, 1999, Khanna and Raina, 1999).

1.4.3.2. Protoplasts fusion

Sexual hybridization between closely related species has been used for years to improve cultivated crops. Introduction of new traits has been based mainly on sexual crosses between different genotypes within or between closely related species. However, due to the presence of various reproductive barriers, gene transfer has been restricted to sexually compatible species or at best to a few wild species closely related to cultivated crop and thus limiting the possibilities of modifying and improving crop plants (Waara and Glimelius, 1995). In most of the cases, many desirable and agronomically interesting traits were found only in distantly related species or even in unrelated species. Since they constitute a genetic resource potential, considerable efforts have been made to identify and isolate these genes and transfer them into crops. Through the rapid development of somatic cell genetics, methods now exists for transferring genes across sexual barriers and over taxonomic distances. Wherever interesting genes have been identified and isolated, they can be transferred through transformation, but for most traits the genes have not yet been identified. Somatic cell fusion might be useful tool in overcoming the limitation of sexual hybridization because cluster of genes can be transformed through this method.

Fusion of somatic cell in an original form is impossible in plant species because plant cells have a cell wall consisting of cellulose and are jointed to each other with pectin. However, when cell wall is removed from the cell (mechanically or enzymatically), fusion of these somatic cells (known as protoplasts) permits hybridization of both sexually compatible and incompatible species (somatic hybridisation). This is

accompanied with fusion of cytoplasmic organelles like mitochondria and chloroplasts between species (cytoplasmic hybridization). Kuster (1909) was the first to point out the possibility of producing somatic hybrids through protoplast fusion and made the early attempts in this direction. However, at that time no methods were available to provide large number of protoplasts for the fusion experiments. Therefore, the experiments to establish a technique for the production of hybrids from somatic cells remained unsuccessful. With development of the method of enzymatic isolation of protoplasts (Cocking, 1960; Takabe *et al.*, 1968) and their subsequent regeneration into plants (Vasil and Vasil, 1980), made protoplast system available for somatic hybridization. Since the first report on protoplast fusion-derived somatic hybrid between *Nicotiana tabacum* x *N. langsdorfi* (Carlson *et al.*, 1972), several successful experiments were made on (i) somatic hybridization (to produce different combination of somatic hybrids) and (ii) cytoplasmic hybridization (to transfer cytoplasmic traits like cytoplasmic male sterility) in several plant families through protoplast fusion.

1.4.3.2.1. Somatic hybridization

One of the most important practical uses of protoplast culture is the somatic hybridization, which has opened up new possibilities for the parasexual hybridization of plants. Somatic hybridization permits nuclear and organeller genomes to be inherited biparentally whereas sexual hybridization allows only the nuclear genomes to be inherited biparentally and organelle genomes are inherited uniparentally. When the trait of interest is present in a species that is sexually incompatible with the other species,

transfer of this trait is achieved by somatic hybridization through fusion of protoplasts. Technically, there are no limitations in the fusion of protoplasts of different species. The absence of cell wall enables the isolated protoplasts to be fused chemically, electrically or by combination of these two processes. Therefore, somatic hybridization is a unique tool for the transfer of useful genes and traits between plants to create a novel source of germplasm.

In rice, sexual crossing between Indica and Japonica varieties frequently results in sterility in the F1 (Ikehashi and Araki, 1986), and weak plants in the F2 (Oka, 1988). Due to non-availability of reproducible protoplast-to-plant regeneration system and the low frequency of plant recovery from protoplasts, successes in the production of somatic hybrids in rice has been limited for quite sometime. The first somatic hybrid plants of rice and barnyard grass (*Echinichloa oryzicola* Vasing) were reported by Terada *et al.* (1981). Earlier somatic hybrid callus formation of *Oryza sativa* x *Glycine max* and *Oryza sativa* x *Daucus carota* was reported by Niizeki *et al.* (1985) and Sala *et al.* (1985) respectively. Following the establishment of successful plant regeneration protocol from rice protoplasts interspecific somatic hybrids between cultivated rice and wild species was reported by Hayashi *et al.* (1988). Later, the use of electrofusion in obtaining somatic hybrids in rice was demonstrated by several workers, e.g. diploid somatic hybrid plants between two *O. sativa* cvs. Murasakidaikoku and Yamahoushi (Toriyama and Hinata, 1988); putative hybrid plants between two *O. sativa* varieties (Lee and Kameya, 1989); heterokarayons betweeen *O. sativa* and *Porteresia coarcta* (Finch *et al.*, 1990); hybrid plants between *O. sativa* and *Lotus corniculatus* L. (Nakajo

et al., 1994); assymetric hybrid plant between *O. sativa* and *Daucus carota* (Kisaka *et al.*, 1994) and somatic hybrid plant between *O. sativa* and *Porteresia coarctata* (Jelodar, 1996).

1.4.3.2.2. Cytoplasmic hybridization

Another important application of protoplast fusion technology is cytoplasmic hybridization that is used when the DNA of the cytoplasmic organelles encodes the trait of interest. Higher plant cell contains apart from others, two cytoplasmic organelles, viz. plastids and mitochondria. These organelle possess relatively small genomes compared to the nucleus. This DNA codes for approximately 10% of the polypeptides necessary to ensure organelle functioning and the remaining 90% are encoded by nuclear genes and imported into cytoplasm through the membrane of the organelle. Cytoplasmically encoded traits are usually inherited maternally through female parent in higher plants. A few traits encoded by cytoplasmic organelles have been reported, such as herbicides resistance, non-chromosomal stripe mutants of maize, T-toxin sensitivity of T (Texas) male sterile cytoplasm in maize and cytoplasmic male sterility (Vasil I.K., 1982; Leaver and Gray, 1982; Levings, 1983; EcKenrode and Levings, 1986; Newton and Coe, 1986; Londsdale, 1987).

CMS is a characteristics which can be used to produce stable male sterile lines for a successful hybrid rice breeding programme. Mitochondrial DNA determination of CMS is well-documented (Srivastava and Gupta, 1990; Leaver and Gray, 1982). In conventional breeding method, an intensive and laborious backcrossing is needed to

introduce CMS in the desired species to produce a new CMS line. To bypass the backcrossing, direct transfer of isolated mitochondria or mitochondrial genomes encoding CMS trait in the fertile plant would be of great significance. Cytoplasmic hybridization or hybridization, involving a donor recipient protoplast fusion system has been developed in several dicotyledonous species viz *Nicotiana* (Zelcer *et al.*, 1978), *Brassica* (Menczel *et al.*, 1987), *Daucus* (Tanno-Suenaga *et al.*, 1988) and *Petunia* (Izhar *et al.*, 1983) to accomplish the transfer of CMS in one step.

Donor-recipient protoplast fusion has been proved a successful tool in achieving transfer of cytoplasmic genomes from one fusion partner to the other. In this method, protoplast of irradiated donor parent is usually fused with iodoacetamide-inactivated recipient parent. Irradiation of CMS donor protoplasts with a lethal dose of X-ray or Gamma-ray can successfully eliminate the nucleus of donor protoplasts (Dudits *et al.*, 1980; Ichikawa *et al.*, 1987; Sidrov *et al.*, 1987) whereas, iodoacetamide treatment of the protoplast prevents cell division (Nehls, 1978) of the recipient protoplast. As a result, fused product contains cytoplasm from the donor parent and nucleus from the recipient parent. In effect, in the cytoplasmic hybrid, CMS traits encoded in the mitochondrial genome of a sterile line is transferred into a desired fertile line in a single step.

1.4.3.2.2.1. General status of cytoplasmic hybridization in plants

Extensive work has been done on cybridization, which has far-reaching implications in plant breeding. Transfer of CMS via protoplast fusion has been successfully demonstrated in several dicotyledons, viz. *Daucus carota* (Tanno-Suenaga *et al.*, 1988),

Brassica napus (Yarrow *et al.*, 1986; Barsby *et al.*, 1987a; 1987b; Menczel *et al.*, 1987; Cardi and Earle. 1997), *Nicotiana* (Aviv and Galun, 1980; Menczel *et al.*, 1983) and *Petunia* (Izhar *et al.*, 1983). In monocots, there are relatively few reports on transfer of CMS through protoplast fusion mainly due to the lack of reproducible protoplast-to-plant regeneration system. Among the important cereal crops, rice is the most important one on which a lot of work has been done to transfer the CMS trait through protoplast fusion.

1.4.3.2.2.2. Current status of cytoplasmic hybridization in rice

Yang *et al.* (1988) produced cybrid calli between CMS line A-58, which carries CMS cytoplasm of Chinsura Boro II and fertile Japanese cultivar Fujiminori. After electrofusion between irradiated protoplasts of A-58 and iodoacetamide-treated protoplasts of Fujiminori; 14 calli were regenerated, some of which were cybrid as shown by isozyme patterns of peroxidase as markers of nuclear DNA and plasmid-like DNA as marker of mitochondria. Regenerated plantlets were also obtained from one of the five cybrid calli (Yang *et al*, 1989). Morphological features, isozyme patterns chromosome numbers and restriction patterns of the mitochondrial DNA indicated that these plants were cybrids. However, the nature of the sterility of these cybrid plants was unclear. CMS cytoplasm was successfully transferred to a fertile Japanese cultivar directly from Chinsurah Boro II through asymmetric protoplast fusion (Kyozuka *et al.*, 1989). Sixteen plants with diploid chromosomes were grown to maturity. Only one of these plants was male sterile and the progeny inherited male sterility. The restoration of

gametophytic fertility by the *Rf-1* gene suggested that the CMS trait of this cybrid plant were identical to those of Chinsurah Boro II. Production of cybrid plants also have been reported by Akagi *et al.* (1989, 1995a, 1995b, 1995c) using donor-recipient protoplast fusion method. More than 80% of the cybrid plants did not set seeds upon selfing. (Akagi *et al.*, 1989, 1995a, 1995b). It was further shown that male sterile cybrid plants were female fertile. In rice cybrids, occasional modification of mtDNA has been observed (Akagi *et al*, 1989, 1995b) and about 20% of the cybrid plants between Indica and Japonica subspecies have been reported to be fertile (Akagi *et al.*, 1989; 1995a; 1995b).

1.5. Male sterility and plant breeding

Male sterility in flowering plants is of tremendous importance not only in molecular and developmental studies of stamen / pollen grains and evolutionary studies on the origin of dioecy but also in its commercial application in hybrid seed production programme in plant breeding. The existence of male sterility in a particular species eliminates the laborious and often difficult task of hand emasculation, which is otherwise required for the production of hybrid seed. Mail sterility enables the combination of traits from two parental lines, with the ultimate goal to suppress negative traits from either parent with the genes from the other parent and to superimpose the positive traits carried by both parents. This results in a vigor that equals or exceeds either parental line. Several natural sources of genetic and cytoplasmic male sterility have been used for many years. The occurrence of male sterility has been exploited in many crop species such as corn,

sorghum, sugar beet and rice in order to exploit heterosis (Yuan, 1977; Virmani and Shinjyo, 1988). However, in crops where natural sources of male sterility are not available or not suitable, e.g. in tomato, hand emasculation and manual hybridization is still being carried out.

1.5.1. Male sterility in higher plant

In higher plants, structural and functional disturbances in pollen development can cause gametophytic abortion or production of non-viable pollens. These aberrations in pollen development constitute male sterile phenotype, which either can be induced or can occur naturally due to nuclear-cytoplasmic incompatibility or other intrinsic biological factors. Male sterility, although generally defined as the condition when viable pollen is not produced, is variable in expression and can range from the complete absence of stamens to the failure of anther dehiscence and release of normal viable pollen (Gabelman, 1956; Frankel and Galun, 1977). The conversion of stamens to different types of floral organs also represents as male sterile condition. Thus, one or more aberrations in stamen development and microsporogenesis can result in male sterility (Sawhney and Shukla, 1994).

1.5.2. Types of male sterility

Several systems for classifying male sterility have been proposed by various researchers. Summarizing these proposals, Kaul (1988), classified male sterility into three types on a genotypic basis. These are, (i) genetic male sterility (GMS) - controlled by nuclear

genes, (ii) cytoplasmic male sterility (CMS) - controlled by male sterility inducing cytoplasm and (iii) genic- cytoplasmic male sterility (GCMS) - resulting from a nuclear cytoplasmic interaction. In practice, however, differentiation of later two groups is difficult, since a certain degree of interaction between the cytoplasm and the nucleus does occur in most cases of CMS plants. Therefore, the later two types are considered under the same heading 'cytoplasmic male sterility'.

1.5.2.1. Genetic male sterility

Genetic male sterility also known as nuclear or genetic or Mendelian male sterility, originating through spontaneous mutation, is a common phenomenon in nature. Generally, a single recessive gene can control this trait. Mutant nuclear genes affecting male gamete and organ development are designated as male sterile genes. These male sterile genes affect male fertility and thus, create complete or partial male sterility. They usually are recessive but there are a few that are dominant (Ms), and both are typically expressed in specific sporophytic tissues at different stages. (Horner and Palmer, 1995). Dominant male sterility can be used for hybrid seed formation, if propagation of the female line is possible (e.g. via *in vitro* clonal propagation). On the other hand, recessive sterility can be used if sterile and fertile plants are easily discriminated. Commercial utility of genetic male sterility system is limited however by the high expenses involved in clonal propagation and rouging out the female rows of self-fertile plants. Mutations resulting in genetic male sterility have also been induced in several important crop plants. Both ionizing radiation and chemical mutagens such as ethyl

methyl suphonate (EMS) and ethylene imine (EI) have been used (Kaul, 1988) for this purpose.

1.5.2.2. Cytoplasmic male sterility

Cytoplasmic male sterility is a well-characterized valuable exception in nature that prevents production of functional pollen grains but does not affect female fertility. CMS occurs widely in higher plants and is due to incompatibility between nuclear and cytoplasmic gene products which results in a failure to produce mature pollen grains (Newton, 1988). This male sterile system relies on cytoplasmic factors/organelle genes that affect adversely the development of one or more types of cells in the anther during microsporogenesis. This type of sterility is maternally inherited, since all cytoplasmic organelles are inherited from the egg cell only and generally leads to complete sterility under normal environmental conditions.

In general, CMS results either from inter-specific crosses or from intra-specific crosses. Histological studies have demonstrated that tapetal abnormality occurs in about 35% of recorded cases of CMS in different species (Kaul, 1988, Laser and Lersten, 1972) where tapetal abnormalities were observed at different stages of microsporogenesis. Although CMS plants are unable to self-fertilize, their ovules are fertile and normal seed set can be obtained when they are fertilized with pollen from normal plants. Since CMS eliminates the possibilities of self-pollination, it has commercial application in the production of hybrid seed for economically important plants (Newton, 1988).

Specific nuclear genes (restorer genes) allow pollen formation in plants with CMS cytoplasm. In a CMS system, when the restorer gene is dominant, pollen fertility is restored, but when it is recessive, the plant remains sterile. Hybrid rice breeding has so far adopted this mechanism, which is called the 'three line method'. The seed parent (sterile line) has cytoplasm, which is effective in preventing pollen formation in the plant. The pollen parent (fertile) must have a specific nuclear gene that is adaptive to the cytoplasm of the seed parent, so the F1 variety can develop pollens and become fertile. This is known as restorer line. Since the seed parent is unable to produce self-fertilized seeds, it is necessary to have a cultivar that has the same nucleus as of the CMS lines, with the exception that the cytoplasm is normal and produces fertile pollens, to maintain CMS lines. Such a cultivar is called maintainer line. The maintainer and its corresponding CMS are normally isogenic but differ in their cytoplasmic background.

1.5.3. Cytoplasmic male sterility in rice

Sampath and Mohanty (1954) and Weeraratne (1954) first reported the role of cytoplasm in causing male sterility in rice. Subsequently, Katsuo and Mizushima (1958) also observed a similar phenomenon in a progeny of the first backcross *O. sativa* f. spontanea/*O. sativa* cv. Fujisaka 5. The first CMS line in cultivated rice was developed from the cytoplasmic source of Chinsurah Boro II variety designated as BT. Following this, many CMS lines were developed in rice (Virmani and Edwards, 1983). However, at that time, researchers did not deploy the CMS lines for hybrid seed production. In order to develop commercial F1 rice hybrids of rice, the first cytoplasmic male sterile

line was developed in China in 1973 from a male sterile plant occurring naturally in a population of wild rice (*Oryza sativa* f. *spontanea*) growing on the Hainan island in 1970 (Yuan, 1977). This plant was designated as wild rice with aborted pollen (WA). Since then, a number of CMS lines have been developed from various wild and cultivated rice accessions (Lin and Yuan, 1980). CMS in rice has been classified into four groups, (i) S1 (CMS-boro or CMS-bo or BT), (ii) S2 (Wild abortive), (iii) S3 (Gambiaca) and (iv) S4 (not designated earlier) (Young *et al.*, 1983, Virmani *et al.*, 1986). However, only one source (viz., WA type) has been extensively used in hybrid seed production. The practical use of CMS in developing hybrid varieties in grain crop is possible only when the effective restorer lines are identified and/or developed. Therefore, the understanding and knowledge of CMS and fertility restoration system is crucial in using these genetic tools more effectively in F1 hybrid seed production.

1.5.4. Fertility restoration in F1 hybrid in rice

In order to deploy CMS system to develop fertile F1 hybrid seeds on commercial basis, availability of an effective fertility restorer system is very essential. The fertility restorer, generally designated as 'restorer line', carries dominant nuclear gene(s) for fertility restoration (*Rf*) which is (are) able to override the sterility factor present in the cytoplasm of the CMS plant. Two types of interactions are observed between fertility restorer gene and cytoplasm. One occurs at diploid level, known as sporophytic and other one at haploid level, known as gametophytic. Fertility restoration in the CMS-BT (S1) male sterile lines of rice is controlled by a single dominant gene (*Rf*) which has

gametophytic effect and cause pollen fertility, in F1 hybrids (Shinjyo, 1969). This *Rf* gene of CMS-BT (S1) cytoplasm is located on chromosome C (Shinjyo, 1975), now designated as chromosome 10 using trisomic analysis (Bharaj *et al.*, 1995; Kinosita, 1995; Virmani, 1996).

On the other hand, the effect of two restorer genes for CMS-WA (S2) cytoplasm is sporophytic and gives normal pollen and spikelet fertility in F1 hybrids. Govinda Raj and Virmani (1988) found that mode of action of the two restorer genes for CMS-WA cytoplasm varied with the cross; certain crosses show dominant epistatis, while others showed recessive epistatis or epiststis with incomplete dominance. Further studies showed that, one of the two fertility restorer genes for CMS-WA cytoplasm was stronger in action than other or one is dominant and the other is incompletely dominant (Virmani, 1996). Bharaj *et al.*, (1995) located the two restorer gene of CMS-WA cytoplasm using trisomic analysis. The stronger restore gene (*Rf*-WA-1) was located on chromosome 7 and the weaker restorer gene (*Rf*-WA-2) was located on chromosome 10. Due to the fact that, the success of hybrid rice technology is dependent on the heterotic potential of the CMS and fertility restoration system, it is very important to develop restorer lines in Indica rices and more specially for Japonica rices which are mostly non-restorers and therefore requires restorer genes to be incorporated into them for hybrid seed production programme.

1.5.5. Molecular basis of cytoplasmic male sterility

Mitochondrial genomes of plants have been implicated in specifying the trait cytoplasmic male sterility, a defect, which causes pollen abortion, but give no reduction

in female fertility. CMS is due to incompatibility between nuclear and cytoplasmic gene products, which results in a failure to develop mature pollen grains. Numerous studies have shown that mutations in the mitochondrial genome are associated with CMS and that the CMS phenotype can be alleviated by the presence of appropriate (usually dominant) restorer genes in the nucleus. A wide range of mitochondrial mutations can cause CMS and thus there must be a correspondingly diverse spectrum of functions among fertility restorer genes. A significant advance in the understanding of fertility restoration has come with the realisation that mitochondrial gene expression may be controlled in a tissue specific manner.

1.5.5.1. Molecular biology of cytoplasmic male sterility in higher plant

In most cases of CMS, it has been shown that the mitochondrial genome is involved in the male sterile trait (Londsdale, 1987; Newton, 1988; Levings and Brown, 1989; Hanson, 1991, Srivastava and Gupta, 1991). Earlier research on CMS in crop plants generally focused on the molecular organization of the heterogenous population of mtDNA molecules.

Plasmid like molecules of the main mitochondrial genome have been described in several fertile and CMS lines of *Zea mays* (Kemble and Bedbrook, 1980; Dale *et al.*, 1981; Ludwig *et al.*, 1985); sorghum (Pring *et al.*, 1982; Chase and Pring, 1985;), wheat (Henda *et al.*, 1984), *Brassica* (Palmer *et al.*, 1983), sugar beet (Powling, 1981), *Vicia faba* (Boutry and Briquet, 1982, Negruk *et al.*, 1982) and rice (Yamaguchi and Kakiuchi, 1983; Kadowaki *et al.*, 1986; Mignouna *et al.*, 1987; Shikanai *et al.*, 1987,

Saleh *et al.*, 1989). In maize, CMS has been associated with alteration in the sequence organization of the mitochondrial genome (Levings *et al.*, 1979). Molecular analysis of mtDNA has been used to distinguish three CMS lines of maize from one another and from a normal male sterile line. In maize, CMS-C, rearrangement involving portions of the mitochondrial gene *atp9*, *atp6* and *coxII*, and a region of mtDNA homologous to cpDNA, may be associated with the CMS trait. On the other hand, a novel mitochondrial gene T-*urf13* is responsible for CMS and susceptibility traits born by CMS-T maize (Levings and Dewey, 1988). The 13 KDa polypeptide encoded by the T-*urf13* gene is a component of inner mitochondrial membrane. However, its effect is reduced in the presence of the fertility restorer genes, *Rf1* and *Rf2* (Forde and Leaver, 1980). Evidences from transgenic experiments indicate that *urf13* is toxic to cell viability in bacteria as well as insect cell culture. CMS in petunia and the mt-locus that encodes it (*pcf*) is believed to be associated with CMS. *pcf*, like T-*urf13* is a fusion gene. *pcf* contains 5' flanking and coding region of *atp9*, part of each exon of *coxII* and carboxyl terminous and 3' flanking sequence of an unidentified open reading frames, *urfS* (Young and Hanson, 1987). Two mitochondrial genes, *nad3* and *rps12*, are located closely downstream of the petunia *pcf* gene. In petunia, however, the *pcf* gene encodes a 25 KDa polypeptide (Nivision and Hanson, 1989) which is completely absent in fertile line and much reduced in presence of fertility restorer gene. Thus, in CMS-T maize and petunia, restored genes reduced the abundance of unique polypeptides that associated with the CMS. In sorghum, mutation of the *coxI* gene may play an important role in causing CMS.

1.5.5.2. Molecular biology of cytoplasmic male sterility in rice

Existence of a heterogenous population of linear (1 - 150 kb) and circular (1 - 96 kb) molecules in rice mtDNA was established by electron microscopic study of the mtDNA (Wang *et al.*, 1989a). Size of the rice mtDNA was thought to be greater than 150 kb (Yamaguchi *et al.*, 1986), and on the basis of DNA restriction profile, perhaps up to 300 kb in size (Wang *et al.*, 1989a). However, in 1990, Hirai *et al* reported the construction of a 350 kb rice mtDNA physical and genetic map. Later a genetic map of rice mitochondrial DNA was created by "walking" along the genome in a lambda phase library constructed from the mtDNA from green leaves. It was reported that rice mtDNA was organized as five basic circular DNAs, each of which shares homologous sequences with others. A master circle was created from the results of recombination across repeated sequences and its size was estimated to be 492 kb (Iwahashi *et al.*, 1992). In 1993, it was reported that the rice mt genome has a genetic complexity of about 300 kb (Narayanan *et al*, 1993) and this is smaller than that of several others cereals like wheat (more than 400 kb) or maize (more than 500 kb). Further studies showed that the rice mitochondrial genome is organized as several circular, subgenomic molecules or mitochondrial chromosomes (Narayanan *et al.*, 1995).

Plasmid like DNA molecules also have been observed in both fertile and CMS lines of many rice varieties (Yamaguchi and Kakiuchi, 1983; Kadowaki *et al.*, 1986, 1988; Mignouna *et al.*, 1987; Shikanai *et al.*, 1987; Seleh *et al.*, 1989). These plasmids exist in linear or circular forms and can apparently repplicate independently of the main mitochondrial genome.

The presence of double stranded RNA (dsRNA) has also been documented in rice, particularly in those varieties carrying BT cytoplasm and a nuclear male sterile line, Nongken 58S. However, dsRNA was not detected either in rice carrying WA cytoplasm or in the fertile maintainer of BT CMS (Wang *et al.*, 1990).

Among the four different types of CMS in rice (S1, S2, S3 and S4), intensive work has been done on the molecular mechanism of BT (S1) and WA (S2) type CMS. In case of BT type of CMS, the *atp6* gene has been implicated in sterility on the basis of (i) expression of a chimaric *atp6* gene in the CMS line, (ii) incomplete processing or editing of *atp6* transcripts in the male sterile line and (iii) expression of antisense transcripts from an open reading frame downstream from *atp6* gene. However, in WA CMS (S2), when *atp6* gene was studied, results were not the same as in case of BT CMS.

A simple amplified mitochondrial DNA fragment $R_{2\text{-}630}$ WA was obtained from wild WA male sterile cytoplasm by AP-PCR technique. Polymorphism between mtDNAs of male sterile and normal cytoplasm was detected by Southern hybridization with the probe of $R_{2\text{-}630}$ WA fragment. This fragment was sequenced by the didoxy chain-termination method. It is 609 bp in length. Its base components of A+T are 54.1%. A comparison of $R_{2\text{-}630}$ WA fragment and 1236 plant genes (including sixteen mitochondrial genes of rice) from Gene Bank reveals low homology (<50%). The nucleotide sequence of this DNA fragment contains a small inverted repeated sequence 5′- ACCATATGGT-3′, which is located at the position from 262-272. In addition, it has a coding region of 20 amino acids residues, which is located at the position from 279-439. The results in this study imply that this specific DNA may associate with WA type

cytoplasmic male sterility in rice. The small inverted repeated sequence probably plays a role in the generation of male sterility (Xu *et al.*, 1995).

Differences in transcription of *orf155* mitochondrial gene between WA CMS and fertile lines have been reported. All the fertile maintainer and restorer lines exhibit a single transcript of 0.7 kb in size. Equivalent steady state levels of this transcript were also present in the sterile line. However, an additional 1.1 kb transcript (of lower intensity) was found in the WA CMS cytoplasm (Seth *et al.* 1995). Later it was confirmed that, this gene encodes a membrane bound, 155 amino acids long polypeptide. In contrast, in wheat the same protein is 156 amino acids long (Srivastava *et al.*, 1997).

1.6. Biotechnological manipulation and CMS transfer

Traditional method of transfer of CMS between different varieties through sexual crossing is accomplished by 5-6 recurrent backcrossings, which require 5-6 years if one crop is grown every year. This process, however, can be hastened through biotechnological manipulations using (i) protoplast fusion between CMS line and the variety which is to be sterilized and (ii) through transformation of the variety with foreign gene having capability to inhibit or hamper pollen development. Both the methods will be briefly discussed here.

1.6.1. Transfer of CMS through Protoplast fusion

Because of far-reaching implications of cell fusion for somatic and cytoplasmic hybridization, much effort in the past has centered on the development of efficient and

reproducible techniques for cell fusion. In an asymmetric protoplast fusion system, where the DNA of cytoplasmic organelles, such as chloroplasts or mitochondria encodes the trait of interest, only the organelles have to be transferred from one parent to the other. To attain such a transfer, a donor-recipient protoplast fusion techniques was developed by Zelcer *et al.*, (1978); and further modified by Sidorov *et al.*, (1987). This technique provides an opportunity to study nucleo-organellar and organellar-organellar genomic interaction in the heterokaryotic cell and in any cybrid plant (Galun and Aviv, 1988). The scheme for donor-recipient cytoplasmic gene transfer involves inactivation of the protoplasts of one parent, which is used as donor by a physical mutagen to get rid of the nucleus. Cytoplasm of the other parent, the recipient, is inactivated by chemical treatment viz. iodoacetamide.

X or Gamma irradiation of protoplasts causes practical elimination of chromosomes or nuclei (Dudits *et al.*, 1980; Sidrov *et al.*, 1987; Ichikawa *et al.*, 1987) in somatic hybrid plants. This treatment has however, no deleterious or mutagenic effects on organelle genomes recovered in the progeny. Effect of various dosages of X-rays on protoplasts of CMS lines has been investigated. It has been reported that the gamma ray or X-ray dose was the most important factor in cybrid production. At X-ray doses greater then 120 krad (2 krad / min), no colony formation was observed, although most protoplasts were still alive based on a microscopic inspection and limited cell division was apparent (Yang *et al.*, 1988, Fujimura *et al.*, 1996). A dose of 50 to 60 krad gamma rays (source Cobalt-60) was required for virtually complete division-arrest of citrus protoplasts (Vardi *et al.*, 1987).

Metabolic inhibition method using anti-metabolite was first described in mammalian cell genetics (Wright, 1978) as a method to recover somatic hybrid. Chemicals such as iodoacetate or iodoacetamide are used for this purpose. Iodoacetamide is known to inhibit the division of protoplast (Nehls, 1978). Different doses of iodoacetamide work for different species without any deleterious effect on the progeny. Concentration of iodoacetamide is also an important factor in the production of cybrid plants.

With the development of an active cell fusion system for the production of somatic hybrid and cytoplasmic hybrids in a vast range of plants, a number of different fusion techniques have been reported. Protoplast fusion can be mediated by either chemical or electrical method. In case of chemical fusion, Carlson *et al.* (1972) used sodium nitrate as the fusogen. Since then, several other fusogens have been used for this purpose. Later the development of electrofusion gave a boost to the technology of somatic hybridization for *en masse* fusion of protoplasts. Electrofusion of protoplasts was first reported by Senda *et al.* (1979). Various modifications in the electrofusion system have been employed in order to use di-electrophoresis for protoplast alignment (Zimmermann and Schleurich, 1981; Zimmermann and Vienken, 1982; Watts and King, 1984; Jones *et al.*, 1996).

The electrofusion method has many advantages over the chemical fusion. This technique eliminates the use of potentially toxic chemicals. It is extremely rapid and, under appropriate conditions, can produce fusion rates in excess of 50% (Zimmermann and Scheurich, 1981; Waara and Glimelius, 1995). The fusion frequency in electrofusion is however, affected by several factors. Different populations of

protoplasts with different sizes exhibit different degrees of fusibility. Electrical parameters and the fusion medium also affect the fusion frequency (Pillai, 1990).

Considerable attention has been attracted by demonstrations of cell and protoplast fusion using electric field. Electrically induced protoplast fusion is a two step process. First, intimate membrane contact is established then localized bilayer disruption that initiate fusion. In this method, cell-to-cell contact is established by application of a high frequency AC (Alternate Current) electrical field. This creates charge on the surface of the protoplast, which results in the protoplasts being arranged in chains on the electrode. With cell contact established, fusion is initiated by the application of one or more short DC (Direct Current) pulses of sufficient magnitude to cause reversible membrane breakdown. Following electrofusion, protoplasts are successfully cultured in appropriate protoplast culture medium. In proper culture conditions these protoplasts divide and give rise to protocolonies which, in turn, can be regenerated into putative cybrid or hybrid plant.

1.6.2. Induction of male sterility through genetic engineering

Use of molecular biology for induction of male sterility in tobacco and oilseed rape was reported for the first time in 1990. Plants were engineered through transformation using chimaeric ribonuclease gene fused to a fragment of 1.5 kb TA-29 promoter having tapetum-specific expression. With the expression of these chimaeric genes, tapetum degenerated and pollen production was prevented. The plants ultimately showed male sterility (Mariani *et al.*, 1990). This male sterility system was a dominant genetic male

sterility and the ribonuclease gene was further linked to bialophos-resistant gene for use in hybrid seed production of oil seed rape. Later, Worrall *et al.* (1992) reported induction of male sterility through premature dissolution of the microsporocyte callose wall in transgenic tobacco. Another report was published on antisense inhibition of flavonoid biosynthesis that resulted in male sterility in Petunia anthers (Van der Meer *et al.*, 1992).

1.6.3. Usefulness of the cybridisation in rice breeding

1.6.3.1. Development of new CMS lines

The cytoplasmic traits of Chinsura Boro II were transferred to 40 Japanese cultivars by asymmetric protoplast fusion in order to convert fertile cultivars to CMS. More than 80% of the diploid cybrid plants were sterile and did not set seeds on selfing except for the cybrids that had the nucleus from Hoshiyutaka (Akagi *et al.*, 1995a;). The restorer gene for Chinsura Boro II are widely distributed in the tropics where Indica varieties are grown (Shinjyo, 1972). Since, Hoshiyutaka was bred by crossing Japonica and Indica rice, it might have had the restorer gene. Cybrid plants were analyzed using PCR-based method for specifically detecting the mitochondrial gene region *orf79* which is a chimera of another mitochondrial gene (Akagi *et al.*, 1994) and all the sterile plants showed to have this region. Inter-parental recombination between two mitochondrial genomes occurs in rice cybrids and the recombinant genomes as well as the parental genomes segregate during protoplast culture (Akagi *et al.*, 1995c).

A novel CMS line, called 'Bio-Mother 1', was bred using the cybridization method from the cultivar 'Yukigesyo' during 1991 to 1993 (Nakamura *et al.*, 1995). To date, 40

Japanese cultivars have been converted to CMS lines as candidates for used in hybrid rice seed production. CMS cybrid plants were also produced using WA cytoplasm derived from 'wild abortive' type which expressed CMS in a sporophytic manner and is widely used for Indica subspecies.

1.6.3. 2. Application of new CMS lines in hybrid rice breeding

One of the most important features of hybrid rice technology lies in its yield advantage. In order to evaluate the yield performance, many F1 lines are necessary. For this purpose, candidates for female parents were converted to CMS through hybridization method and using these new CMS lines, F1 seeds of several candidate varieties were produced in Japan. These F1 seeds, showed yields that were 30 to 40% higher than those of the leading representative varieties. In addition, their quality was good enough for Japanese consumers (Oka *et al.*, 1995). In Japan, many hybrid rice varieties that are acceptable in the competitive Japanese market were developed using cybrid-derived CMS lines.

It takes about 8 months to produce new CMS lines by the hybridization method and two additional backcrossing is necessary for elimination of somaclonal mutation. Therefore, it takes about 2 years for establish the new CMS lines, while conventional recurrent backcrossing method requires 5-6 years. Thus, the hybridization method will be useful, especially, for conversion a large number of fertile cultivars into CMS, which is the most important, step in hybrid seed production.

1.7. Specific objectives of the thesis

The present investigation was undertaken with the following specific objectives:

i) Callus induction, maintenance of embryogenicity and plant regeneration from different explants of CMS, maintainer and restorer lines of rice.

ii) Initiation, establishment and maintenance of embryogenic cell suspensions from CMS, maintainer and restorer lines of rice.

iii) Isolation, culture, induction of division and plant regeneration from suspension-derived protoplasts and

iv) Transfer of cytoplasmic male sterility through protoplast fusion.

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2 : MATERIALS AND METHODS

2.1. Source of plant material

Seeds of *Oryza sativa* L. cvs. RCPL 1-3C, RCPL 1-2C, Nami (Japonica rices); IR36, IR64, IR65 and IR74 (Group 1 Indica rices), V20A, IR62829A, IR54752A (cytoplasmic male sterile lines), V20B, IR62829B, and IR54752B (respective maintainers of male sterile lines) were used in this study. The RCPL lines are advanced breeding lines from a cross, Pusa 33 x Khonorullo. RCPL lines belong to Group VI of Glassman's (Glaszmann, 1987) isozyme classification and thus are Japonica rice. Seeds of IR varieties, cytoplasmic male sterile lines and their respective maintainers were obtained from the International Rice Research Institute (IRRI), Manila, Philippines. It is important to note that the seeds of CMS and maintainers were available in limited quantities and this was a major constraint in planning experiments.

2.2. Methods

2.2.1. Callus induction and maintenance

2.2.1.1. Callus induction from mature seeds

Seeds of all the above mentioned rice lines were manually dehusked. Undamaged healthy seeds were surface sterilized in 70% (w/v) ethanol for 1 minute and in 0.1% (w/v) mercuric chloride ($HgCl_2$) for 5-7 minutes, followed by three washings in sterilized distilled water. Sterilized seeds (10 per 9 cm Petri dish) were plated on the following callus induction media:

1. LS medium (Linsmaier and Skoog, 1965) supplemented with 50.0 mg l^{-1} tryptophan, 4.0 mg l^{-1} 2,4-D (2,4- dichloro phenoxy acetic acid), 1.0 mg l^{-1} kinetin, 3% (w/v) sucrose and solidified with 0.8% (w/v) agar, pH 5.8 (designated LSA, Appendix-I) and

2. LS medium supplemented with 4.0 mg l^{-1} 2,4-D, 100.0 mg l^{-1} casein hydrolysate, 500.0 mg l^{-1} glutamine, and 3% (w/v) sucrose and solidified with 0.8% (w/v) agar, pH 5.8 (designated LSB, Appendix-I).

Seeds were placed on the media in such a way that the plumule-radicle side of the seeds was in contact with medium and the scutellum side up. Petri dishes were sealed with Parafilm (Whatman) and incubated at 28 ± 1°C in the dark for 2-3 weeks. After about 15-18 days, swollen scutella were separated from the endosperm and root and replaced on fresh medium with the scutellum surface facing up.

2.2.1.2. Callus initiation from immature embryos

Rice spikelets were collected at milk stage, 14-16 days after anthesis. Spikelets were surface sterilised in 70% (w/v) ethanol for 1 minute and 0.1% (w/v) mercuric chloride for 5-7 minutes followed by 5-6 washes in sterile distilled water. Immature embryos were dissected out from the spikelets and plated on callus induction medium LSA and LSB, in 90 mm Petri dishes, each containing 12 immature embryos with the scutellum surface facing up. Sealed Petri dishes were kept in dark at 28 ± 1°C for 10-14 days. After 2 weeks, swollen scutella were separated from the radicle and transferred to fresh callus induction medium.

2.2.1.3. Callus initiation from leaf base explants of seedlings

Sterilised rice seeds were cultured on modified MS medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose, and semi solidified with 0.8% (w/v) agar, pH 5.8 (designated MS0 medium, Appendix-II), in 175 ml capacity screw-capped glass jars (Beatson Clarke & Co. Ltd., Rotherham, UK). Cultures were kept at 28 ± 1^{0}C in dark for 7 days, whereupon the innermost leaf was excised from the basal 2 cm portion of each seedlings, just above the mesocotyl node. The leaves were cut into transverse section (2-3 mm) and placed on LSA medium. Petri dishes were sealed and incubated for 4 weeks in the dark at 28 ± 1^{0}C.

2.2.1.4. Callus initiation from stem base explants of seedlings

Sterilised rice seeds were germinated on hormone-free MS0 medium in 175 ml capacity screw-capped glass jars (Beatson Clarke & Co. Ltd., Rotherham, UK). Cultures were kept at 28 ± 1^{0}C in light for 7 days. Endosperm and radicle were excised from the 7-day-old seedlings. Approximately 2-3 mm thick segments were cut from the chlorophyllous tissues found at the base of the shoots. Segments were cultured on LSA semi-solid medium in 90 mm Petri dishes (15-20 segments per 25 ml medium) for further growth. Petri dishes were sealed and incubated in dark at 28±1^{0}C.

2.2.1.5. Maintenance of embryogenic calli

Embryogenic calli, which formed at the surface of the scutella of mature seeds and immature embryos, were separated from the scutellar tissue and any surrounding non-

embryogenic callus, then teased apart into smaller pieces and transferred to fresh medium. Care was taken to minimize any damage to the embryogenic tissue. After about 10-14 days, these primary embryogenic calli were transferred to modified N6 (Chu *et al.*, 1975) medium with 4 mg l^{-1} 2,4- D; 3.0% (w/v) mannitol and 2% (w/v) sucrose, solidified with 0.8% (w/v) agar (designated N6M, Appendix-I) in 90 mm Petri dishes. Dry, compact, nodular and white-pale yellow embryogenic calli were sub-cultured every 12-15 day interval. At each subculture, non-embryogenic callus was discarded; only embryogenic tissue was retained. Cultures showing severe browning or rhizogenesis were also removed at each subculture. Embryogenic calli obtained from the leaf base and stem base explants were selected and subcultured on N6M medium for further growth.

2.2.1.6. Plant regeneration directly from callus

For testing the regeneration potential of the callus, at each subculture, a small part of the friable embryogenic callus was transferred to regeneration medium. Five pieces of (3-4 mm diameter) embryogenic calli, were transferred to regeneration medium MSB [MS medium supplemented with 0.5 mg l^{-1} NAA, 3 mg l^{-1} kinetin, 3% (w/v) sucrose, pH 5.8 and solidified with 0.8% (w/v) agarose (Sigma type 1), designated MSB, Appendix-II]. Calli were incubated in dark at 27±1°C for one week and then transferred (without subculture to fresh medium) to light under continuous illumination (47 $\mu E\ m^{-2}\ s^{-1}$, cool White fluorescent tubes, Philips, India) at the same temperature for 3-4 weeks. The

shoot regeneration frequencies were recorded 15 days after transfer of tissues to MSB medium as the percentage of scutella-derived calli that produced one or more shoots.

2.2.1.7. Maintenance of regenerated plants

All the normal green plantlets and shoots regenerated from the calli in regeneration medium were separated and transferred individually to 175 ml jars containing semisolid rooting medium (MS0 1/2 strength) for hardening and rooting (respectively). Plants were maintained for 10-15 days in the light as described in Section 2.2.1.6.

2.2.1.8. Establishment of plants in the glass house

Regenerated plants, each approximately 5-7 cm in height, were removed from the rooting medium and their roots were washed thoroughly with tap water. About 40-50 plants of each cultivar were transferred to the glass house in plastic pots containing compost and soil mixture (1:1) and maintained until maturity (3-4 months).

2.2.1.9. Assessment of agronomic characters in somatic embryo and seed-derived plants

Ten numbers each of somatic embryo - and seed - derived plants were assessed for their agronomic characters, including the number of days to flowering, plant height, leaf length, leaf width, leaf length/width ratio, number of tillers, number of panicles, number of spikelets / panicle and spikelet fertility.

2.2.2. Cell suspension cultures – initiation, establishment and maintenance

2.2.2.1. Initiation and maintenance of cell suspension cultures of three Japonica rice lines (RCPL 1-3C, RCPL 1-2C and Nami)

Friable, embryogenic calli obtained from immature and mature embryos of different rice lines (RCPL 1-3C, RCPL 1-2C and Nami) were employed for the initiation of cell suspension cultures. Approximately 1 gm fresh weight of callus (45-60 day old) was transferred into a 50 ml conical flask containing 20 ml of liquid AA medium (Muller and Grafe, 1978) supplemented with 4 mg l^{-1} 2,4-D (designated AA4, Appendix-III). Cultures were incubated on a rotary shaker at 120 rpm in dark at 28 ± 1°C. During the initial stage of initiation of the cell suspension culture, two thirds of the old culture medium were replaced with fresh liquid medium every 2-3 days. When an adequate amount of small cell aggregates had been released into medium, they were transferred to 250 ml conical flask containing 50 ml of fresh AA4 medium.

Thereafter, the suspensions were subcultured on every fourth day and bigger cell clumps were sieved out once in a month. One-month-old suspension cultures were passed through a 500 μm pore size nylon sieve (Wilson Sieves, 2 Long Acre, Common Lane, Hucknall, Nottingham, UK) and smaller and fine cell clumps (<500 μm) were collected and maintained by regular subculturing. Growth of the cells in culture was regularly observed under an inverted microscope at the time of each subculture. Continuous subclturing and regular sieving of the cells gave rise to a highly cytoplasmic embryogenic cell suspension after 60-70 days.

2.2.2.2. Initiation and maintenance of cell suspension cultures of two Group I Indica rice (IR36 and IR65)

Embryogenic calli obtained from immature and mature embryos of rice lines (IR36 and IR65) were identified by regular observation of cultures using a stereo dissecting microscope. Compact calli were carefully teased apart into smaller pieces with adequate care to avoid wounding that led to excessive browning of the cultures, affecting the subsequent suspension initiation process. About 750 mg fresh weight of friable embryogenic calli (approximately 4-5 week old) were inoculated into 10-15 ml of liquid AA4. Cultures were incubated on a rotary shaker at 120 rpm in the dark at 28 ± 1°C. During the first one month, the medium was completely replaced with fresh medium every second day. After one month, suspension cultures were sieved through 500 μm pore size nylon sieve into a 90 mm Petri dish to collect the smaller cell clumps. Any suspension cells in the cultures showing severe browning or organogenesis were discarded during each subculture.

Microscopic observation of the cells was done regularly. After 6 weeks, there was substantial production of highly cytoplasmic cells in the suspension culture. A 10 ml aliquot of cell suspension was transferred to a 250 ml conical flask with 25 ml of fresh liquid medium (AA4). Thereafter, subculturing was carried out every 4 days with a replacement of 60% medium at each subculture until a fine suspension was obtained.

2.2.2.3. Initiation and maintenance of cell suspension cultures of the cytoplasmic male sterile lines and their respective maintainers (V20A, IR62829A, IR54752A, V20B, IR62829B and IR54752B)

Embryogenic calli were separated very carefully from the non-embryogenic tissue and cultured on modified N6M medium prepared with 0.8% (w/v) agarose (Sigma type 1) for one month. After two weeks, fast growing embryogenic calli were transferred to AA4 medium solidified with 0.8% (w/v) agarose (Sigma type 1) for further growth. The calli growing on AA4 medium became friable after one week of culture without loosing regeneration capability and were used for initiation of suspension cultures. Suspensions were initiated with 200-300 mg of friable embryogenic calli in a 50 ml flask containing 10-12 ml of liquid culture medium. V20A and V20B suspension cultures were initiated in AA4 liquid medium. However, due to the difficulties in initiating suspension cultures of IR62829A, IR62829B IR54752A and IR54752B in AA4 medium, cultures of these lines were initiated in AAP liquid medium (AA4 medium supplemented with $1gm^{-1}$ proline, Appendix-III). The density of the cell suspension and volume of liquid medium used was critical for all these varieties. Cultures were maintained by incubating on a shaker at 120 rpm in the dark at 28 ± 1°C. During the first one month of culture, the suspensions were sub-cultured on every alternate day and each time, medium was completely replaced. Thereafter, suspensions were maintained in 250 ml flasks containing 0.9-1 gm of cells in 50 ml of liquid medium. To obtain friable, embryogenic, small cell clumps, cell suspensions were regularly passed through 500 μm pore size

nylon sieve and small (< 500 μm) cell clumps were collected and maintained by sub-culturing on 4th day with replacement of 50% medium in the flask.

2.2.2.4. Determination of growth rate of cell suspension cultures

The growth of suspension cultures (6-8 month old) maintained in 250 ml conical flasks was determined by measuring settled cell volume (SCV) at daily intervals after subculture for 10 days. Graduated 10 ml centrifuge tubes were used for this purpose. Five replicate readings of the settled cell volume (SCV) (after 5 minutes) for 10 ml suspension were taken. The growth rate of the cultures was monitored over a 10-day period. Increase in volume of cell suspension cultures in liquid medium was used to determine the growth of cell suspension cultures.

2.2.2.5. Plant regeneration from cell suspension culture and maintenance of regenerated plants

To check regeneration potential of suspension cultured cells; they were transferred to the regeneration medium. On the third or fourth day of subculture, suspension cells were passed through a 500 μm pore size nylon sieve. Cell clumps retained on the sieve as well as those cells which passed through the sieve, were used separately for regeneration experiments. To ensure dehydration of the suspension cells, liquid media was completely removed, cells were transferred to a Whatman filter paper (Whatman International Ltd., Springfield Mill, Maidstone, Kent, UK) placed in a 90 mm Petri dish for 30-45 minutes under sterile conditions. Dried cell colonies were transferred to MSB

regeneration medium and solidified with 8% (w/v) agarose at pH 5.8 and kept in the dark for two weeks at 28 ± 1°C. After 2 weeks, cells were transferred to fresh regeneration medium and incubated under continuous illumination (47 μE m^{-2} s^{-1}, cool white fluorescent tubes, Phillips, India) at 28 ± 1°C for 3 weeks.

Establishment of the regenerated plants and maintenance in the glasshouse was performed as described in Sections 2.2.1.7 and 2.2.1.8 respectively.

2.2.3. Protoplast isolation, culture and plant regeneration from protoplast-derived calli

2.2.3.1. Protoplast source

Two to twelve month old embryogenic cell suspension cultures of all the rice lines (Section 2.2.2) were used for protoplast isolation, culture and plant regeneration. Further protoplasts isolated from these rice lines were used for donor-recipient protoplast fusion experiments.

2.2.3.2. Protoplast isolation

Protoplasts were isolated from established suspension cultures in their exponential growth phase, on the third day after subculture. Two incubation procedures were used for protoplast isolation as detailed below.

2.2.3.2.1. Overnight incubation

Approximately 1.0 g fresh weight of cells was transferred to a 90 mm Petri dish and incubated in 10 ml of enzyme solution (full strength) and diluted with 10 ml of CPW

13M (Frearson *et al.*, 1973, Appendix-VI) solution. The full strength enzyme mixture consisted of 1% (w/v) Cellulase RS (Yakult Honsha, Tokyo), 0.1% (w/v) Pectolyase Y-23 (Seishin Pharmaceuticals, Tokyo) and 5 mM MES buffer, in CPW 13M (Appendix-V). The Petri dish was sealed with Parafilm and incubated on a horizontal rotary shaker (30 rpm, 2 cm throw), in the dark at 28 ± 1^0 C for 12-15 hour followed by a 1 hour stationary incubation period.

2.2.3.2.2. Short-term incubation

The cell suspensions were sieved through a 500 μm pore nylon sieve into a 90 mm diameter plastic Petri dish to obtain small cell clusters suitable for enzymatic digestion. The liquid medium was removed using sterile Pasteur pipette or a sterile plastic pipette. About 1.0 g fresh weight of the cells was re-suspended in 20 ml of full strength filter-sterilized enzyme mixture without any dilution. The Petri dish was sealed and incubated in dark at 28 ± 1°C with gentle shaking (50 rpm) for 3 hours followed by one-hour stationary incubation (Bhattacharjee and Gupta, 1995).

2.2.3.3. Purification of protoplasts

After digestion, the enzyme-cell-mixture was filtered through nylon sieves of 64, 45 and 30 μm pore sizes to remove undigested cells and any large protoplasts resulting from spontaneous fusion and collected in a 90 mm Petri dish. The protoplast enzyme mixture was gently pipetted into glass centrifuge tube, using sterile Pasteur pipette, and pelleted by centrifugation at 80 x g for 10 minutes. The protoplasts were washed three times in

CPW 13M, heat shocked at 45° C for 8 minutes, then placed in ice cold water for 10 sec (Lin *et al.*1991). The protoplasts were then purified by floating over a 0.6 M sucrose gradient at 50 x g for 5 minutes and re-washed twice in CPW 13M with a final wash in protoplast culture medium [N6 medium (Chu *et al.*, 1975) supplemented with 1.5 mg l^{-1} 2,4-D, 0.2 mg l^{-1} zeatin, 500.0 mg l^{-1} casein hydrolysate and 0.7 M glucose, pH 5.8 and filter sterilized; designated N6PCMZ, Appendix-IV]; cells were pellated after washing by centrifugation at 80 x g for 5 minutes (Bhattacharjee and Gupta, 1995).

2.2.3.4. Determination of protoplast yield and viability

Protoplasts were resuspended in 10 ml N6PCMZ medium and a sample of suspension was transferred to a haemocytometer (Weber Scientific International Ltd., Teddington, Middlesex, UK). The number of intact protoplasts in five triple-lined squares was counted. Total yield was determined using the following formula:

$$\text{Total yield } (g\,f.wt.^{-1}) = \frac{n \times 5 \times 10^3 \times \text{volume of protoplast suspension}}{\text{weight of packed cells}}$$

where n = mean number of protoplasts in 1 triple-lined square

Protoplast viability was determined using the fluoroscein diacetate (FDA) staining method (Widholm, 1972). Ten ml of FDA stock solution (3.0 mg ml^{-1} in acetone) was diluted in 10 ml of CPW 13M solution. Protoplast samples were mixed with this diluted solution of FDA and incubated at room temperature (23 ± 2^0C) for 5 minutes. Protoplasts were observed with a Nikon 'Diaphot TMD' inverted microscope with a

high-pressure mercury vapor lamp, HBO 100 watt. Viable protoplasts labeled with FDA exhibited a green yellow fluorescence. Viability (%) was determined using the following formula:

$$\textit{Protoplast viability (\%)} = \frac{\textit{Number of fluorescing protoplasts}}{\textit{Total number of protoplasts}} \times 100$$

2.2.3.5. Protoplast culture

Protoplasts were cultured at a density of $1x10^6$ ml^{-1} in presence of feeder cells in N6PCMZ protoplast culture medium.

2.2.3.5.1. Preparation of feeder cells

Vigorously dividing young (2-3 month old) embryogenic cell suspension cultures of Japonica rice line, RCPL 1-2C and Indica rice IR36, were used for the preparation of feeder cells. Two different types of feeder plates were prepared. Suspension cells, 3-4 day after subculture, were mixed with N6PCMZ medium containing 0.8% (w/v) agarose (Loba chemicals, India) in a ratio of 1.5 ml packed cell volume (PCV) to 10 ml of N6PCMZ- agarose mixture and feeder culture plates were prepared as follows:

(i) one and half ml of this mixture containing uniformly suspended cells was plated in a form of a ring around the wall of a 35 mm Petri dish leaving a well in the center ('donut' holes) to accommodate 0.5-1 ml of culture medium containing protoplasts and

(ii) a 10 ml aliquot of this mixture was dispensed into a 5.5 cm diameter plastic Petri

dish. After solidification, a sterile 0.8 μm pore Whatman cellulose nitrate filter membrane (47mm diameter) was positioned on the surface of this feeder culture. Petri dishes containing the feeder cultures were sealed and kept in dark at 27 ±1°C for 24 hour before protoplast isolation and culture.

2.2.3.5.2. Culture of protoplasts

Protoplasts were cultured as follows:

(i) Protoplasts were suspended in liquid N6PCMZ medium (Appendix-), and 1 ml from this suspension was plated inside 'donut' holes formed by feeder cells at a density of 1 x 10^6 ml^{-1}.

(ii) Protoplasts were suspended in N6PCMZ 'AG' [liquid N6PCMZ medium mixed with (1:1) 0.15% (w/v) agarose (Sea Plaque, LGT, FMC. Rockland, USA; designated as N6PCMZ 'AG'] and 1 ml of this mixture was plated in a 35 mm Petri dish at a density of 1 x 10^6 ml^{-1} without feeder cells.

(iii) Protoplasts were suspended in N6PCMZ 'AG' and 1 ml from this mixture was plated on the membrane filter overlying the feeder cells at a density of 1 x 10^6 ml^1. Then protoplasts were spreaded on the surface of the membrane uniformly.

(iv) Protoplasts were suspended in liquid N6PCMZ medium and 1 ml of suspension was plated in a 35 mm Petri dish at a density of 1 x 10^6 ml^{-1} without feeder cells.

(v) Protoplasts were suspended in N6PCMZ 'AG' medium and 1 ml of this suspension was plated inside 'donut' holes at a density of 1 x 10^6 ml^{-1}.

(vi) The wells of controls were plated with 1 ml of liquid N6PCMZ and N6PCMZ 'AG' medium without protoplasts. Five plates of each were used as controls.

All the Petri dishes were sealed and cultures were incubated in dark at 27 ± 1°C for further growth. Cultures were fed with fresh medium every 7th day with reduced osmoticum of about 1/3rd at each feeding, to give a final concentration of 0.17M glucose.

2.2.3.6. Assessment of plating efficiency

Plating efficiency was calculated as percentage of plated viable protoplasts that had undergone sustained division leading to macroscopic colony (1 mm diameter) formation. For the first two methods of protoplast culture (Section 2.2.3.5.2), plating efficiencies were determined on the 7th, 15th and 30th day of culture. However, for the third method (Section 2.2.3.5.2) plating efficiency were determined only on the 15th and 30th day of culture. Protoplast plating efficiency was determined as follows:

$$\textit{Plating efficiency (\%)} = \frac{\textit{Total number of visible colonies}}{\textit{Total number of viable protoplasts plate}} \times 100$$

2.2.3.7. Callus proliferation from protopast-derived colonies

In the first two culture methods (Section 2.2.3.5.2), macro-colonies that had formed inside the 'donut' holes one month after plating were transferred with a wide mouth pipette onto a sterile 0.8 μm pore nitro-cellulose membrane filter (47 mm diameter), placed on the surface of agarose solidified LSA medium (Section 2.2.1.1). However, for the third culture method (Section 2.2.3.5.2), membranes with dividing protoplasts on top

were transferred to the surface of agarose solidified LSA medium without feeder cells. All these plates were sealed and incubated for 14 day in dark at 27 ± 1°C.

2.2.3.8. Plant regeneration from protoplast-derived calli

For testing regeneration potential, protoplast-derived calli (1-2 mm in diameter) were transferred to regeneration medium. Plant regeneration from protoplast-derived calli was assessed using regeneration medium MSB. Calli transferred to MSB medium were incubated in the dark at 27 ± 1°C for one week and then finally transferred (without subculture) to light (47 μE m^{-2} s^{-1}, cool White fluorescent tubes, Philips, India) at 28±1°C for 3 weeks. Plant regeneration frequency was calculated as follows:

$$\textit{Plant regeneration efficiency (\%)} = \frac{\textit{Number of calli producing plants}}{\textit{Total number of calli transferred to regeneration medium}} \times 100$$

2.2.2.9. Maintenance of regenerated plants

All normal green plantlets and shoots regenerated from the callus in regeneration medium were transferred to semisolid rooting medium and maintained as described in the Section 2.2.1.7

2.2.3.10. Transfer and acclimation of regenerated plants

Normal plants (10-15 cm tall at three-leaf stage) were removed from rooting medium. Roots were cleaned with tap water with the help of a paintbrush. Plants were transferred

to pots containing 1:1 sterile soil and compost and maintained in net house under natural condition where temperature ranged from 17- 28°C during crop growth period (April-May to September-October). Seed-derived control plants of each line were grown side by side of these protoclones.

2.2.3.13. Assessment of agronomic characters of protoplast derived plant and comparison with seed derived control plants

Agrobotanic data were recorded at the time of maturity. Evaluation of R1 and R2 progenies of the protoclones were conducted using seed-derived plants as control under low land during wet season of the year in a Randomized Block Design at the research farm of ICAR Research Complex for North Eastern Hill Region, Barapani situated at 950 meter above sea level. Row to row and plant to plant distances were 20 x 10 cm. A fertilizer dose of 60:60:40 Kg NPK per hectare was applied. Data on the yield and yield contributing characters *viz.* plant height, panicle length, panicle bearing tillers (PBT), number of spikelets/panicle, per cent spikelet fertility, 100 seed weight and yield/plant were recorded. Analysis of variance was performed and Duncan's Multiple Range Test [DMRT (Gomez and Gomez, 1976)] was used to compare the differences in the characters of R0, R1 and R2 with that of seed-derived controls. Since no significant difference was noticed among the three set of control that were grown with 3 generations of protoclones in three different years, therefore, an average performance of control (for various traits) was used to compare with those of R0, R1 and R2.

Plant characters, such as plant height, total number of tillers, leaf length, leaf width, leaf length/width ratio, number of days to flowering, number of panicle bearing tillers, panicle length, number of primary branches per panicle, number of secondary branches per panicle, number of spikelets and spikelet fertility were recorded for each plant, including seed-derived controls.

2.2.4. Electrofusion of rice protoplasts

2.2.4.1. Irradiation of donor protoplasts of CMS lines

2.2.4.1.1. Assessment of different doses of γ- rays on protoplasts

To determine the optimum dose of radiation that prevents division of protoplasts, freshly isolated protoplasts of donor lines V20A, IR62829A and IR54752A were exposed to several doses of γ- ray from a ^{60}CO source. Purified protoplasts were suspended in W5 solution [125 mM $CaCl_2$, 155 mM NaCl, 5 mM KCl and 5 mM glucose, pH 5.6 (Medgyesy *et al.*, 1980), Appendix-VII], centrifuged at 50 x g for 5 minutes, followed by two washings in the same solution. Protoplasts were then brought to a density of 1x 10^6/ml in W5 medium in several sterile tubes and plastic Petri dishes. These sterile tubes and Petri dishes were placed into a ^{60}CO source for the period calculated to give a desired dose (10 krad, 20 krad, 25 krad, 30 krad, 35 krad, 40 krad and 50 krad) to the protoplasts. Irradiated protoplasts were left undisturbed at room temperature for 15-30 minutes, then collected by centrifugation at 50 x g for 3 minutes.

2.2.4.1.2. Culture of irradiated protoplasts

Irradiated protoplasts were washed twice in W5 solution being collected at each stage by centrifugation at 50 x g for 3 minutes, then once in protoplast culture medium and cultured in the presence of feeder cells following the different methods of culture described in the Section 2.2.3.5.2. Plating efficiency was estimated as described earlier in the Section 2.2.3.6.

Protoplasts were observed under microscope to check cell division. The minimum dose of radiation that prevented cell division in culture medium but without killing the cells was selected as the required dose for the electro-fusion experiment.

2.2.4.2. Iodoacetamide treatment of the recipient protoplasts

2.2.4.2.1. Assessment of the effect of different doses of iodoacetamide (IOA) on protoplasts

To standardize the dose of iodoacetamide needed for inactivation, freshly isolated protoplasts of recipient lines, RCPL1-1C, RCPL1-2C, Nami, V20B, IR62829B, IR54752B, were incubated in different concentrations of iodoacetamide. Various concentrations of iodoacetamide solutions (5 mM, 10 mM, 15 mM, 20 mM and 25 mM) were prepared by dissolving iodoacetamide in W5 solution (Appendix-VIII). Before inactivation treatment, protoplasts were washed once in W5 solution, pellated by centrifugation at 50 x g for 5 minutes, resuspended in various concentrations of iodoacetamide and incubate for 15 minutes at 20^0C with occasional shaking. Treated protoplasts were pelleted by centrifugation at 50 x g for 3 minutes.

2.2.4.2.2. Culture of iodoacetamide treated protoplasts

Iodoacetamide-treated protoplasts were washed twice in W5 solution followed by one wash in protoplast culture medium and cultured in presence of feeder cells following the different culture methods as described in Section 2.2.3.5.2. Plating efficiency was determined as described in the Section 2.2.3.6. Minimum concentration of iodoacetamide needed to prevent division of protoplasts was selected as the required dose for fusion experiments.

2.2.4.3. Electro-fusion of inactivated donor and recipient protoplasts

2.2.4.3.1. Fusion of protoplasts

Inactivated protoplasts of donor and recipient lines were washed once in 0.6 M mannitol solution and collected by centrifugation at 50 x g for 2 minutes before exposure to electric field. Protoplasts of donor and recipient lines were mixed thoroughly in equal proportion (1:1) to give a final density of 5 x 10^5 ml^{-1}, 1 x 10^6 ml^{-1}, 2 x 10^6 ml^{-1} and 4 x 10^6 ml^{-1} in pre-cooled 0.6 M mannitol solution (pH 7.2). The mixture was further cooled for 10 minutes in ice cold water and then transferred to sterile cuvettes (pre-chilled) for fusion. Protoplast fusion was mediated through electric pulse (an AC amplitude of 25V/KHz for 25 sec for protoplast alignment, followed by a DC pulse of 900 Vcm^{-1} 30 μ sec for fusion) generated by a somatic hybridizer (BTX Inc., La Jolla, California, USA). Immediately after exposure to electric field, the protoplast mixture was cooled again for 10-15 minutes and left undisturbed for another 45 minutes at room temperature. Then

the protoplasts were collected in a centrifuge tube and pelleted by centrifugation at 50 x g for 5 minutes.

2.2.4.4. Protoplast culture followed by fusion

Fused protoplasts were washed twice in W5 solution, once in protoplast culture medium (N6PCMZ) and cultured in presence of feeder cells. Protoplasts were cultured according to the methods described in Section 2.2.3.5.2. To assess the effect of the feeder cells on fused protoplasts, in few experiments protoplasts were cultured in absence of feeder cells. Protoplasts were cultured at a density of 1 x 10^6 ml^{-1} and 2 x 10^6 ml^{-1}, then kept in the dark at 27 ± 1°C for further growth.

2.2.4.5. Culture of control protoplasts

As a control, inactivated and washed protoplasts of donor and recipient lines were mixed at a density of 1x 10^6 ml^{-1} and 2x 10^6 ml^{-1} and this mixture was cultured in presence of feeder cells following the same culture method as described in the Section 2.2.3.5.2.

2.2.4.6. Maintenance of cultured protoplasts

Dividing protoplasts in each plate was fed with fresh medium every 6-7 days of culture, reducing the osmoticum by about one third at each feeding, to a final concentration of 0.17 M of glucose.

2.2.4.7. Determination of plating efficiency

Plating efficiency of fused and physically mixed protoplasts were calculated as described in the Section 2.2.3.6.

2.2.4.8. Callus proliferation

Growth and maintenance of protoplast-derived colonies were performed as described in the Section 2.2.3.7.

2.2.4.9. Plant regeneration from fused protoplasts

Protoplast derived calli (1-2 mm. in diameter) were transferred to regeneration medium. Plant regeneration frequency from protoplast-derived calli was assessed as described in the Section 2.2.3.8.

2.2.4.10. In-vitro rooting and plant maintenance in the glass house

Shoots were separated from one another and transferred to rooting medium to stimulate root formation. Regenerated plantlets were maintained as described in the Section 2.2.1.7. Well-developed putative cybrid plants (10-15 cm tall at three-leaf stage) were removed from rooting medium; roots were cleaned with water and transferred to pots containing 1:1 mixture of soil and compost. Plants were further maintained as in Section 2.2.3.12.

2.2.5. Analysis and characterization of putative cybrids

2.2.5.1. Pollen grain staining to confirm sterility

Pollen grains were collected from the mature plants of putative cybrids. At the same time pollen grains were also collected from all the parental lines (fertile and sterile) used in the protoplast fusion experiments. Pollen grains were stained with 1% KI + I_2 and examined under the light microscope for starch staining.

2.2.5.2. Pollen germination test to confirm sterility

Mature pollen grains were collected from all putative cybrids and the fusion partners. Pollens were incubated in pollen germination medium (Bino *et al.*, 1987) for two hours at 25 ± 1^0 C in light to test for pollen germination and observed under the light microscope. The percentage of pollen grains producing pollen tubes after incubation was recorded for each rice line.

2.2.5.3. Test for sterility of putative cybrids

The putative cybrid plants growing in the glasshouse were selfed by bagging to check sterility of cybrids. Morphological characters of plants were recorded after selfing.

2.2.5.4. Maintenance of putative cybrids

Putative cybrids were hand crossed with known maintainer to obtain seeds of CMS lines.

2.2.5.5. Test for fertility restoration of putative cybrids

Each sterile putative cybrid plant was hand-crossed with a known restorer line IR36 to check restoration of fertility in cybrids. Plants were maintained until maturity after crossing and agronomic characters were recorded at maturity.

2.2.5.6. Molecular analysis of putative cybrids

2.2.5.6.1. Extraction of mitochondrial DNA

Mitochondria were isolated from young leaves and shoots of potted putative cybrids and the fusion partners. Isolation of mitochondria and mtDNA was carried out as described by Nath *et al.* (1993) with some modifications. Before mtDNA extraction, plant materials were kept in the dark for 3-4 days. Approximately 20g of leaves were used for each extraction. All procedures were performed at 4^0 C, unless otherwise stated. The leaves and young shoots were harvested, cut into small pieces and homogenized with a pre-chilled pestle and mortar in 100 ml of isolation buffer (0.4 M mannitol, 50 mM TES, pH 7.5, 1 mM EGTA, 0.1% (w/v) BSA and 0.1% (w/v) cysteine). The homogenate was filtered through four layers of cheese cloth or muslin and one layer of Miracloth (Calbiochem) into 50 ml centrifuge tubes (Nalgene) and centrifuged at 5,000 rpm for 10 minutes, using a JA20 rotor in a Beckman J2-21 centrifuge. The supernatant was transferred to a fresh tube. The pellet was resuspended in 30 ml isolation buffer using a fine brush and centrifuged at 5,000 rpm for 10 minutes.

The low speed centrifugation pelleted nuclei, chloroplasts and other cellular debris. Both supernatants collected from the first and second low speed centrifugation were

combined and centrifuged at 12,000 rpm for 10 minutes at 4^0C to pellet the mitochondria. The mitochondrial pellet was suspended in 3 ml of extraction buffer (without BSA and cysteine) and any remaining cellular debris was pelleted by low speed centrifugation at 5000 rpm for 10 minutes at 4^0C. The supernatant containing the mitochondria was incubated on ice for one hour in presence of DNase I (10units/10gm tissue) and 5 mM $MgCl_2$. The DNase treated sample was carefully layered over five volumes of wash buffer (15 ml) containing 0.6 M sucrose, 10 mM TES pH 7.5, and 20 mM EDTA. The sample was then centrifuged at 16,000 rpm. The washed pelleted mitochondria was re-suspended in 15 ml wash buffer and re-centrifuged at 16,000 rpm for 20 minutes. The mitochondrial pellet obtained was then lysed in 3 ml lysis buffer comprising of 0.5% (w/v) SDS in 50 mM Tris-HCl pH 8.0, and 50 mM EDTA at 55^0C for 1 hour. The lysate was extracted with equal volume of equilibrated phenol: chloroform (1:1) and then extracted twice with an equal volume of chloroform, at room temperature. The mtDNA was precipitated from the aqueous layer with 2 volumes of ethanol and 0.1 volume of 0.3 M sodium acetate at -20^0C (overnight). The mtDNA was pelleted by centrifugation at 13,000 rpm for 15 minutes, washed with 70% (v/v) chilled ethanol, air dried and dissolved in 400 µl of urea-NaCl solution (6.5 M urea and 1 M NaCl). The solution was heated at 65^0C for 10 minutes, cooled and the mtDNA was re-precipitated by the addition of two volumes of chilled ethanol. After pelleting, the precipitated DNA was dissolved in TE buffer (10 mM Tris-HCl; pH 8.0 and 1 mM EDTA) and treated with RNase (10 µg/10 g tissue) at 37^0C for 1 hour. The sample was again extracted with phenol/chloroform as described above and precipitated with 0.3 M

sodium acetate and two volumes of ethanol at -20^{0}C for 1 hour. The DNA was pelleted at 12,000 x g in a refrigerated microfuge, dissolved in 100 µl of TE buffer and used for further analysis.

Quantification of the mtDNA was carried out by electrophoresis of 1 µl of each DNA preparation adjacent to lambda DNA standards (100, 250, 500, 750, 1000 ng) on a 100 ml, 0.8% (w/v) agarose gel for 1 hour at 100V. The gel was stained in ethidium bromide solution (0.5 mg l^{-1}) for 30 minutes and DNA was visualized on a U.V. transilluminator. Comparison of band intensity with the lambda DNA standards allowed an approximation of the quantity of plant DNA.

2.2.5.6.2. Synthesis of primers

Synthesis of primers was carried out at Dr John. Kyte's laboratory at the Biopolymer Synthesis and Analysis Unit, School of Bio-medical Sciences, University of Nottingham, Nottingham, UK. Based on the published sequences of several mitochondrial genes, namely rice cytochrome oxidase subunit II (*coxII*), rice ATPase subunit 6 (*atp6*), rice pseudogene *urf79*, wheat gene *orf156 and orf299* and sunflower genes *orf522* (Kao *et al.*, 1984; Kadowaki *et al.*, 1990; Akagi *et al.*, 1995; Gualberto *et al.*, 1991; Laver *et al.*, 1991), specific oligo-nucleotides were synthesized to act as primers for the polymerase chain reaction (PCR). Description of different mitochondrial gene probes, their origin and sequences of primers are given in the Table 2.

Table 2.1: Description of different mitochondrial gene probes, their origin and sequences of primers

Probes	Origin	Primer sequences 5'-3'	Reference
atp6	Rice	Forward ATGGGTTTGAATCAGAGAGA Reverse ATTCAATTATGAAATTACTC	Kadowaki *et al.* (1990) Akagi *et al.* (1995)
urf79	Rice	Forward ATGGCAAATCTGGTCCGATG Reverse ACTTACTTAGGAAAGACTAC	Kadowaki *et al* (1990) Akagi *et al.* (1995)
orf156	Wheat	Forward TTTCTTATTTGAAATCCAAATCG Reverse CGAGATTCGAAGCAAGCAAGTT	Gualberto *et al.* (1991)
coxII	Rice	Forward CCTCACAATCGCTCTTTCTG Reverse CGCCTAACCCATAGGTTAGT	Kao *et al.* (1984) Laver *et al.* (1991)
orf522	Sunflower	Forward CCGAATTCTGAAATCACTACAGGC Reverse AAGAATTCTACTAATTCCAAG	
orf299	Wheat	Forward GCCGAATTCTGAAATCACTACAGGC Reverse AAGAATTCTACTAATTCCAAG	Gualberto *et al.* (1991)

2.2.5.6.3. Screening of putative cybrid plants by PCR

Standard PCR was performed using a programmable temperature cycler (Perkin Elmer 480 DNA thermal cycler). The reaction was carried out using substrate mtDNA from each putative cybrid plant and all parents. The amplification reaction mixture in a 0.5 ml polypropylene tube consisted of 0.50 µl of (0.5 units) thermostable Taq DNA polymerase (Boehringer Mannheim Lewis, UK), 2.5 µl of 10x reaction buffer [100 mM Tris HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3 (20^0C)] (Boehringer Mannheim, Lewis, UK), 0.50 µl of 5 mM dNTP mixture (Promega Ltd., Southampton, UK), 1.0 µl of forward and 1.0 µl of reverse primer, 2.0 µl of extracted plant mtDNA and 17.5 µl distilled water to give a total volume of 25 µl. To avoid contamination from other DNA

sources or possible bacterial contamination, all reagents were dispensed in a laminar flow cabinet. Finally, the reaction mixture was overlaid with 25 µl of mineral oil (Sigma), to avoid evaporation of the reaction mixture during the PCR cycles, as high temperatures are required for denaturation of DNA strands. The tubes were briefly centrifuged (13000 rpm; 30 seconds). All samples from cybrids (maximum 20 samples in each experiment) plants and their parents were checked at the same time. PCR amplification of DNA segments was carried out by running 30 cycles. Each cycle contained three different thermal periods. Specifically, denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and polymerisation at 72°C for 1minute.

2.2.5.6.4. Electrophoretic analysis of amplified DNA fragments

Five µl of tracking dye was added to each reaction mixture. Tubes were briefly centrifuged (13000 rpm, 30 sec). From each mixture 15 µl was electrophoresed for 2 hours at 50 V in a 0.8% (w/v) agarose horizontal slab gel using TBE buffer (90 mM Tris HCl, 90 mM boric acid and 2 mm Na_2EDTA). The gel was stained with 0.5 mg l^{-1} ethidium bromide in TBE. The amplified fragments were observed using a U.V. transilluminator (TM-20 model; UVP Ltd., San Gabriel, California, USA) and photographed.

2.2.5.6.5. Restriction endonuclease digestion of DNA

Rice mtDNA was restricted with different restriction enzymes viz. *BamHI*, *HindIII* and *EcoRI* (Boehringer Mannheim, Lewis, UK). Five µg of mtDNA was digested in 50 µl

reaction mixture with 25 units of enzyme at 37^0C for 16 hour. To check digestion, 5 µl of the reaction was electrophorased on a 50 ml 0.8% (w/v) agarose gel for 1 hour at 75 V. The gel was stained in ethidium bromide solution (0.5 mg l^{-1}) for 30 minutes and DNA bands were visualized on a U.V. transilluminator. If the samples were not fully digested, they were re-precipitated with 0.1 volume of 3 M sodium actetate (pH 5.2) and 2.5 volumes of absolute ethanol, (Sambrook *et al.*, 1989) and then digested with fresh enzymes for a further 16 hours.

2.2.5.6.6. Agarose gel electrophoresis of restriction digested DNA

Restricted DNA was fractionated on 0.8% (w/v) agarose (molecular biology grade; NBS Biologicals, Huntingdon, UK) horizontal slab gel in TBE buffer for 12-16 hours. The gel was stained with 0.5 mg l^{-1} ethidium bromide in TBE buffer for 30 minutes and then photographed using a U.V. transilluminator.

2.2.5.6.7. Southern hybridisation analysis of mtDNA

2.2.5.6.7.1. Non-radioactive labelling, Southern hybridisation and chemi-luminescent detection

DIG-labelled different mitochondrial gene probes were prepared by PCR (Lion and Haas, 1990). A small amount (0.5 µl) from this PCR mixture analysed by electrophoresis to check amplification of the desired DNA product. The amplified PCR product was precipitated, dissolved in 50 µl of distilled water and kept at -20^0C for further use. Capillary blotting of restricted fragments of mtDNA was carried out using

alkaline transfer with 0.4 N NaOH (Sambrook *et al.*, 1989) onto positively charged nylon membrane (Boehringer Mannheim, Lewis UK) according to the manufacterer's instructions. The membrane was neutralised in 2 X SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0) for 5 minutes, air dried on a sheet of Whatman 3 mm paper and stored in a plastic bag at 4^0C.

Pre-hybridisation, hybridisation and post-hybridisation washes were performed in 200 ml roller tubes in a hybridisation oven (SI 20H, Stuart Scientific Ltd, Redhill, Surrey, UK). Pre-hybridisation of the membrane was done at 37°C in DIG Easy Hyb (Boehringer Mannheim, Lewis, UK) for 1 hour. Hybridisation was performed at 42°C for 16 hours in DIG labelled probe solution (40 µl DIG PCR labelled probe in 10 ml DIG Easy Hyb; probe was denatured immediately prior to use by heating to 94°C for 10 minutes then chilling on ice for 10 minutes). Post-hybridisation washes were done twice for 5 minutes each at room temperature in 2 x SSC, 0.1% (w/v) SDS, then again two times for 15 minutes each at 65°C in 0.5 x SSC, 0.1% (w/v) SDS. Following washing the membrane was blocked by non-specific high molecular weight protein (powdered milk).

Chemiluminescent detection was performed using anti-DIG AP fragments (Boehringer - Mannheim, Lewis, UK) and CDP-*Star*™ (Tropix, Massac-husettes, USA). The hybridised probe was immuno-detected by alkaline phosphatase-conjugated anti-DIG antibody and visualised using enzyme activated chemiluminescent substrate (CDP-*Star*™).

Filters were exposed to Hyperfilm-MP (Amersham) in an X-ray cassette with an

intensifying screen for 15 minutes to 1 hour, depending on the intensity of the signal.

2.2.5.6.7.2. Radioactive labelling of probe, Southern blotting, hybridisation and autoradiography

^{32}P labelled DNA probes were generated by random primer labelling using the Oligolabelling kit (Pharmacia Biotech). Purified PCR product (2.5µl) and 31.5 µl sterile distilled water was boiled for 5 minutes to denature the template DNA, then briefly centrifuged and placed on ice for 2 minutes. After cooling, 10 µl reagent mix (250 mM Tris-HCl pH 7.2, 500 mM DTT, 1 mg ml^{-1} BSA, 15.5 mg ml^{-1} random hexamers, 125 mM dATP, 125 mM dGTP, 125 mM dTTP), 5 µl [α-^{32}P] dCTP (3000Ci $mmol^{-1}$) and 1 µl Klenow enzyme (2 U μl^{-1}) were added. After gentle mixing, the reaction mixture was incubated at 37^0C for one hour.

The labeled probe generated was purified to remove unicorporated nucleotides using a NICK™ Column (Pharmacia Biotech) containing Sephadex® G-50. The column was washed with 400 µl sterile distilled water before adding the labeled probe. A further 400 µl aliquot of sterile distilled water was added and the fraction eluting between 0.5 and 1.0 ml was recovered and used as the probe. This was boiled to denature the probe DNA prior to addition to the hybridization buffer.

Restriction digestion of mtDNA and agarose gel electrophoresis was done according to the method described in the sections 2.2.5.6.8 and 2.2.5.6.9.

After electrophoresis, restricted mtDNA was denatured by placing the gel in a bath of 0.5 N NaOH, 1 M NaCl on a moving platform shaker for 30 minutes at room

temperature. The gel was neutralized by bathing it in 0.5 M Tris-HCl, pH 7.4, 3 M NaCl for 30 minutes at room temperature on a moving platform shaker. The gel was capillary blotted onto a Zeta-Probe® nylon membrane (BioRad, USA) using 10 X SSC as a transfer buffer, according to the method described by the suppliers. After DNA transfer, the membrane was rinsed briefly in 2 X SSC and UV crosslinked at 1200 kJ.

Membranes were pre-hybridized for 1 hour at 37^0C in 20 ml of hybridization solution (3 x SSPE, 50% (v/v) formamide, 5 x Denhardt's solution, 0.5% (w/v) SDS, 5% (w/v) dextran sulphate) containing 100 $\mu g\ ml^{-1}$ of heat denatured herring sperm DNA [1 x SSPE = 180 mM NaCl, 10 mM NaH_2PO_4, 1 mM NaEDTA (pH 7); 1X Denhardt's solution= 0.02% (w/v) BSA (Pentax fraction V), 0.02% (w/v) ficoll, 0.02% (w/v) PVP. These were made up as 20 x SSPE and 5 x Denhardt's]. Hybridization with mitochondrial gene probe was carried out by placing the pre-hybridized filter in fresh hybridization solution containing 50 µl of the labeled probe (denatured before use) and incubation at 37^0C overnight in oven.

Following hybridization, the filter was washed twice in 30 ml per cm^2 area of 2 x SSPE, 0.1% (w/v) SDS for 5 minutes at room temperature, then twice in 30 ml per cm^2 area of 0.1 x SSPE, 0.1% (w/v) SDS for 15 minutes at 65°C. The nylon filter was kept moist in a plastic bag for autoradiography to prevent irreversible binding of the probe.

Autoradiography of the damp nylon filter was performed at -70^0C for various exposures times using Fuji RX x-ray film with intensifying screens. The film was developed in 500 ml Kodak LX 24 x-ray developer (1:4 water : developer) and fixed in 500 ml Ilford Hypam fixer (1:4 water : fixer).

2.2.5.6.7.3. Removal of probes from nylon membrane

Probes were stripped from the nylon filter by boiling in 1 litre 0.1% SDS (w/v) for 15-30 minutes. The filter was kept at 4^0C for further use.

2.2.5.7. Assessment of inheritance of female fertility

2.2.5.7.1. Establishment of plants in the glass house and back-crossing

Cybrids obtained from different fusion experiment (BC0) were backcrossed with their respective maintainer lines and maintained in the glasshouse until maturity. Seeds were collected at maturity. Seeds were germinated in MS0 medium in culture jars and kept in a 12 hour photoperiod at 27 ± 1°C (day/night) for 3 weeks. When the plantlets (BC1) were big enough with a good rooting system they were transferred to the glasshouse and grown in separate pot. Plants were maintained in the glasshouse until maturity and agronomic characters of these plants were recorded.

2.2.5.7.2. Pollen grain and pollen germination test

Pollen grain staining and the pollen germination test was performed for all BC1 plants as described in the sections 2.2.5.1 and 2.2.5.2, respectively.

2.2.5.7.3. Molecular analysis of BC1 plants

Mitochondrial DNA extraction from BC1 plants and hybridization analysis was done as described in the section 2.2.5.6.

CHAPTER 3

RESULTS

CHAPTER 3 : RESULTS

3.1. Callus induction, establishment of cell suspension and plant regeneration from rice lines/varieties

3.1.1. Callus induction and plant regeneration

3.1.1.1. Callus induction from different explants

Calli were induced from different explants, such as mature seeds, immature embryos, and leaf- and stem bases. Morphology of the callus was highly related to the origin of explants. Variation in the initial response of callus induction was observed among the explants of different origin. In mature and immature embryos, callus was produced on the upper surface of the scutellum (Fig.2.a.). The embryos germinated within 2 days and the shoots attained the height of 2-3 cm by day 3. While further development of shoot was inhibited, enlargement of the scutellum occurred and callus was gradually formed on the surface (Fig. 2.a.) Occasionally callus was produced from the mesocotyl and the basal region of the coleoptile too, but those calli were usually fragile and watery with numerous root primordia. The initial response of callus induction from leaf base and stem base explants was related to the age of the source material. Explants from young sources were more responsive than those from older sources and usually only the younger sources produced good calli. Calli from scutella of mature seeds and immature embryos were usually compact, organized and nodular. Callus induction was highest (61-95%) from the scutella of immature embryo, where as it was the lowest (9-20%) from the stem bases. (Table: 3).

Legend for figures

Fig. 2.(a - f). Plant regeneration from embryogenic suspension cells of RCPL1-2C initiated from embryogenic calli of mature seed derived scutellum.(**a**) Scutellum obtained from mature seeds of RCPL 1-2C (**b**) Close up view of scutellum with embryogenic and non-embryogenic calli on the surface (**c**) Fine cell suspension culture of RCPL 1-2C (**d**) Photomicroghaph of densely cytoplasmic suspension cells with prominent starch grains (**e & f**) Plantlets regenerated from suspension cells.

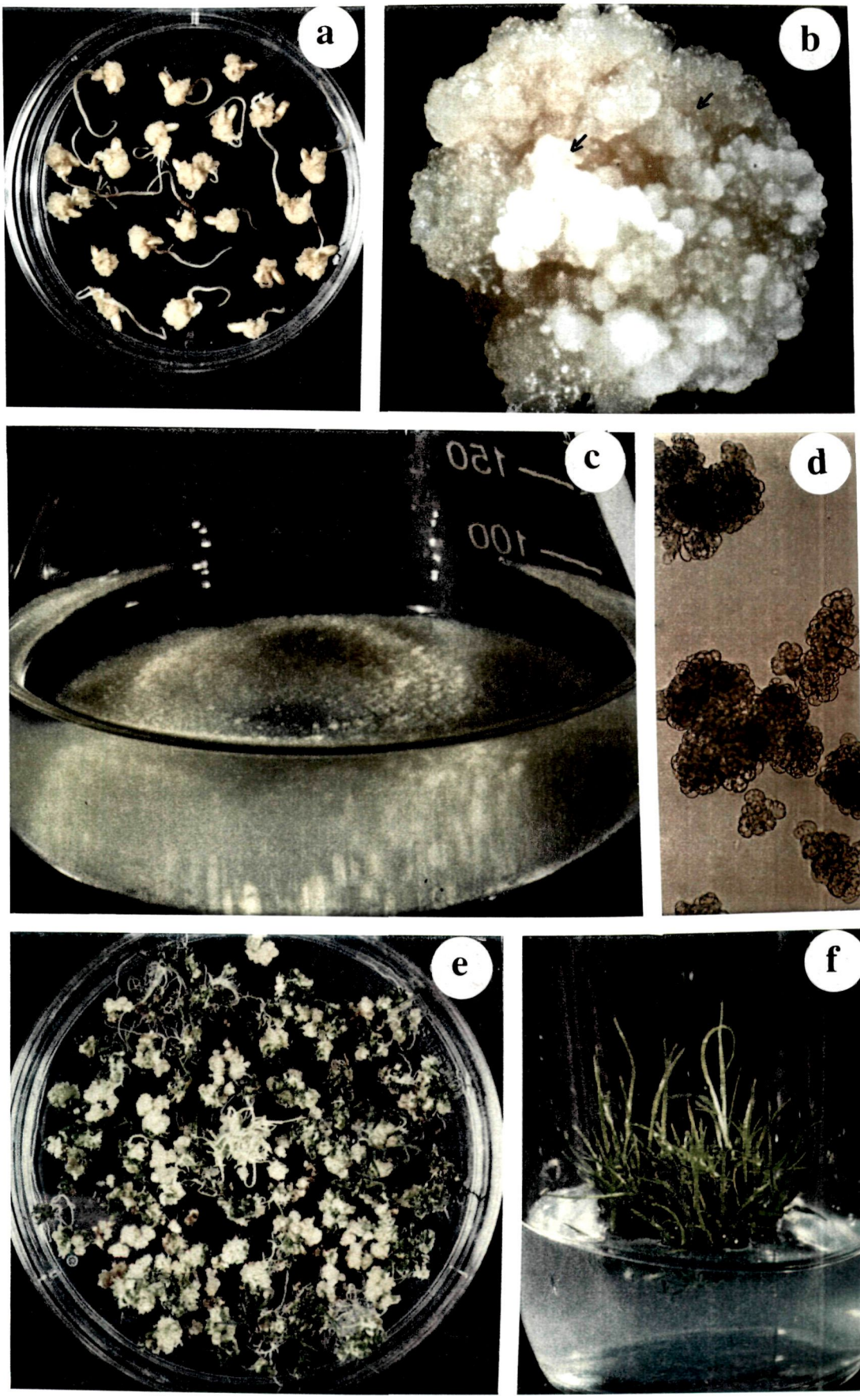

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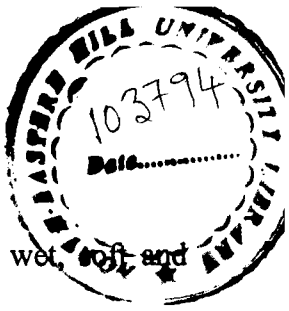
Table 3. : Per cent embryogenic callus production from different explants on two different medium**

Serial No	Lines/ varieties	Percent embryogenic callus production (%)							
		LSA				LSB			
		Immature embryos	Mature seeds	Leaf bases	Stem bases	Immature embryos	Mature seeds	Leaf bases	Stem bases
1	RCPL1-2C	86	77	28	14	95	90	35	20
2	RCPL1-3C	89	80	30	17	94	89	33	20
3	Nami	82	79	29	12	93	86	31	19
4	IR36	93	85	30	20	74	77	25	12
5	IR64	93	88	29	19	72	69	21	17
6	IR65	85	68	20	16	69	63	16	12
7	IR74	86	65	23	20	73	61	19	11
8	V20A	80	77	21	20	62	53	12	16
9	V20B	79	73	20	19	63	58	17	12
10	IR62829A	NA*	76	21	16	NA*	56	19	16
11	IR62829B	81	79	20	15	61	53	12	09
12	IR54752A	NA*	69	23	16	NA*	59	21	14
13	IR54752B	71	65	20	18	62	52	13	16

*** Not Available**
**** Numerical average of five experiments**

3.1.1.2. Effect of different media on callus induction

Two different types of callus-induction media were used in this study. Calli became visible 6-9 days after inoculation. Two types of calli, embryogenic and non-embryogenic were obtained from all the explants, viz., mature seeds, immature embryos, leaf bases and stem bases. The embryogenic calli were identifiable by pale yellow color, compact, dry and globular in appearance (Fig.2.b.). In contrast, the non-embryogenic



calli were composed of less organized elongated cells, sometimes with wet, soft and mucilaginous appearance. Per cent callus induction from immature and mature seeds in Japonica lines (RCPL1-2C, RCPL1-3C and Nami) was ranged from 93% to 95% on LSB whereas it ranged from 82% to 86% on LSA. In Indica lines (IR36, IR65, IR64 and IR74) per cent callus induction from mature seeds and immature embryos was ranged from 85% to 93% on LSA medium and 69% to 74% in LSB medium. CMS and maintainer lines (V20A and V20B, IR62829A and IR62829B, IR54752A and IR54752B) showed higher callus induction percentage from mature seeds and immature embryos on LSA medium and ranging from 65% to 81%. In contrast, callus induction percentage ranged from 52% to 63% on LSB medium. There was a significant difference in per cent callus induction among various cultivars on different media irrespective of the source material used (Table 3). However, in all the cases callus induction percentage was higher from immature embryos either on LSA or on LSB medium. In the present investigation, callus induction percentage from leaf base and stem base explants was found very less. Due to high response of callusing from mature seeds and immature embryos on LSA and LSB media, these two media were only used as callus induction media for all the rice lines, throughout the study.

3.1.1.3. Maintenance of embryogenic calli

The calli were regularly subcultured to maintain their embryogenicity and continuous supply. At each subculture, embryogenic calli were separated from the nonembryogenic calli because embryogenic calli showed tendency to convert to nonembryogenic calli.

All the calli became friable after few subcultures on the same medium where they were induced. The calli obtained from immature embryos were mostly embryogenic where as they were mixture of embryogenic as well as non-embryogenic in case of other explants.

3.1.1.4. Plant regeneration from embryogenic calli

In order to check the embryogenicity; the embryogenic calli obtained from different rice lines were transferred to regeneration medium, MSB. They were required an initial culture period in the dark (7-10 days) before transfer to light for regeneration. Regeneration percentage differed significantly among the cultivars. Regeneration percentage in japonica rice lines was higher when they were initiated and maintained on LSB medium. In contrast, in Indica and CMS rice lines regeneration percentage was higher when calli were initiated and maintained in LSB medium. However, in all the rice lines, callus obtained from immature embryos showed higher plant regeneration percentage and was irrespective of callus induction medium used. Per cent regeneration of the calli is presented in Table 4.

3.1.2. Cell suspension cultures - initiation, establishment, maintenance and plant regeneration

3.1.2.1. Initiation of cell suspension culture

Suspension cultures were initiated from the fast growing, friable, embryogenic calli derived either from mature seeds or from immature embryos (Fig.2.c.). When observed under inverted microscope, single cells as well as small cell clumps were seen

Table 4. : Per cent plant regeneration from embryogenic calli of different explants obtained from two different callus induction media**

Serial No	Variety / Lines	Plant regeneration percentage (%)							
		LSA				LSB			
		Immature Embryos	Mature seeds	Leaf bases	Stem bases	Immature embryos	Mature seeds	Leaf bases	Stem bases
1	RCPL1-2C	93	84	53	47	98	90	68	58
2	RCPL1-3C	91	80	54	36	97	90	72	61
3	Nami	89	75	52	40	98	93	81	49
4	IR36	97	90	63	60	89	84	58	42
5	IR64	89	78	48	39	72	68	42	30
6	IR65	93	90	69	60	89	81	62	53
7	IR74	78	69	49	31	70	57	30	28
8	V20A	83	78	62	59	65	62	56	52
9	V20B	90	87	68	60	78	65	59	54
10	IR62829A	NA*	83	65	54	NA*	78	62	50
11	IR62829B	92	89	64	50	85	69	56	47
12	IR54752A	NA*	90	69	62	NA*	85	60	54
13	IR54752B	92	89	64	61	84	76	56	57

*** : Not Available**
**** : Numerical average of five experiments**

dissociating from the calli during the first 3-4 weeks of initiation of suspension culture. Some of these cells or cell clumps that dispersed into the liquid medium during this period were thin walled and cytoplasmic where as most of them were composed of elongated and vacuolated cells. Small cell clumps composed of cells with dense

cytoplasm were actively dividing and multiplied fast (Fig.2.d.). The proportion of these cells gradually increased in the cultures as the suspension grew older with regular subculturing and gave rise to a fine cell suspension within 3-4 months of regular subculturing.

3.1.2.2. Effect of origin of calli on establishment of suspension cultures

Origin of calli played an important role in establishment of fine suspension cultures. It was observed that initiation and maintenance of suspension cultures from mature and immature embryo-derived calli were comparatively easier and faster than stem base- and leaf base-derived calli. The calli from the latter sources were composed largely of root primordia and the extent of browning of such cultures was higher than the calli derived from immature and mature embryos. Therefore, longer period was required for the establishment of suspension cultures from leaf base and stem base-derived calli.

3.1.2.3. Effect of culture medium composition on establishment of suspension cultures

Composition of the culture medium was another important factor that affected initiation of suspension cultures. AA4 was found to be the best medium for initiation and maintenance of suspension cultures of Japonica rices lines RCPL 1-3C, RCPL 1-2C and Nami; Indica rice lines IR36 and IR65; CMS line V20A and the maintainer line V20B. AA4 medium was, however, not suitable for the other rice lines viz. IR62829A, IR62829B, IR54752A and IR54752B. The cell cultures of these lines exhibited severe

browning in AA4 medium. However, when proline was incorporated in the AA4 medium (AAP), cell growth of these lines were faster in AAP and the cultures consisted of smaller cell clumps that were suitable for protoplast isolation.

3.1.2.4. Maintenance of embryogenic cell suspensions

After 6-7 weeks of initiation, suspension cultures either in AA4 or in AAP media were found composed of actively dividing small cell clumps with dense cytoplasm (Fig.2.d.). At this stage, cultures were maintained by subculturing on fourth day, which was found optimum for maintaining the embryogenic cell suspensions. Sieving the cultures at an interval of 4-5 weeks was necessary to eliminate bigger cell clumps and to recover higher yield of protoplasts

3.1.2.5. Growth curve of cell suspension cultures

Packed cell volume (PCV) of 10 ml suspension culture doubled within 4 day of transfer in all the cultures initiated and maintained in liquid culture medium. On 4^{th} day, subculture was found necessary for the maintenance of the embryogenic nature of the cell suspension cultures as it kept them in exponential phase. The growth curve for seven rice lines is presented in (Fig. 3).

3.1.2.6. Plant regeneration from suspension cells

Embryogenicity of the suspension cells was checked by transferring the cell colonies to regeneration medium MSB where they became dry and compact in morphology after 10-

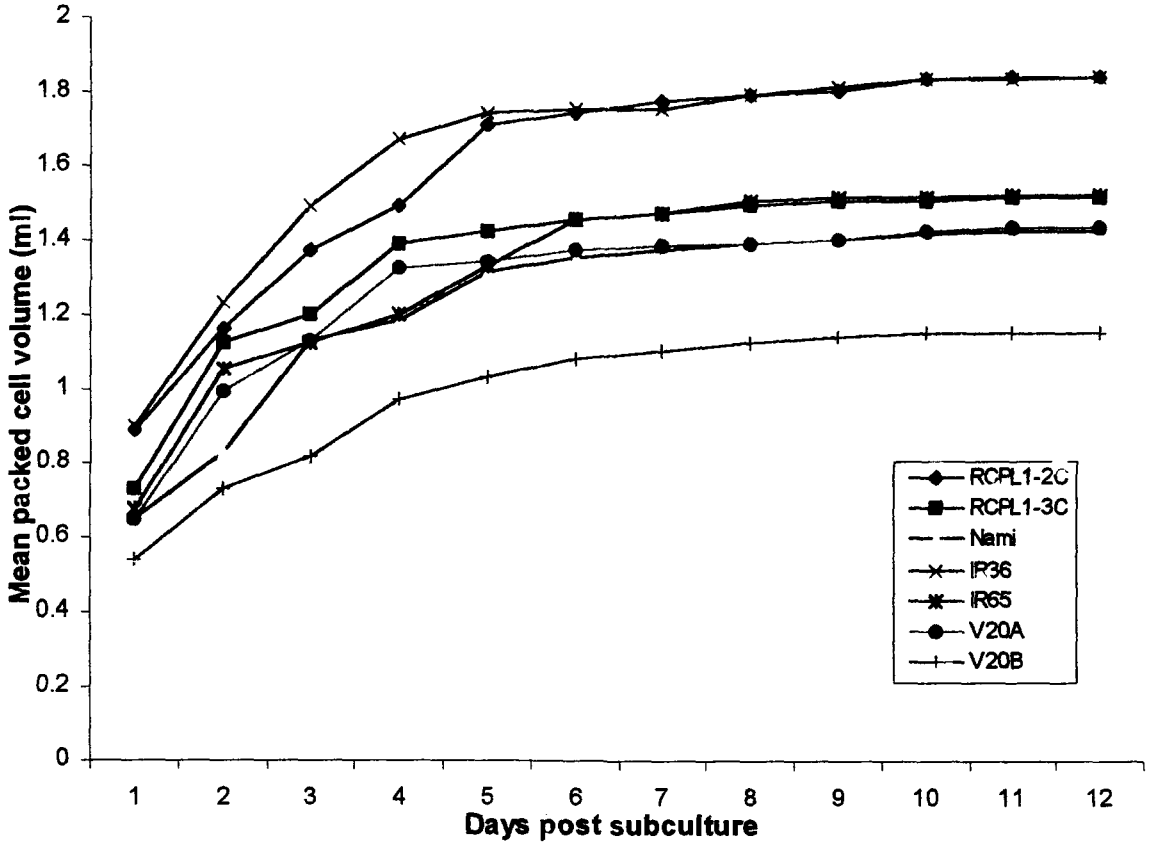


Fig. 3. Growth curves of cell suspension cultures of cvs. RCPL 1-2C, RCPL 1-3C, Nami, IR36, IR65, V20A and V20B

12 days of culture in the dark. These calli on transfer to light in the same regeneration medium regenerated into plantlets after 10-15 days of culture (Fig.e.&f.). Differences in regeneration frequencies among all the cell lines were observed. Cells showed 65-73% plant regeneration in different rice lines used (data not shown).

3.2. Protoplast isolation, culture and plant regeneration

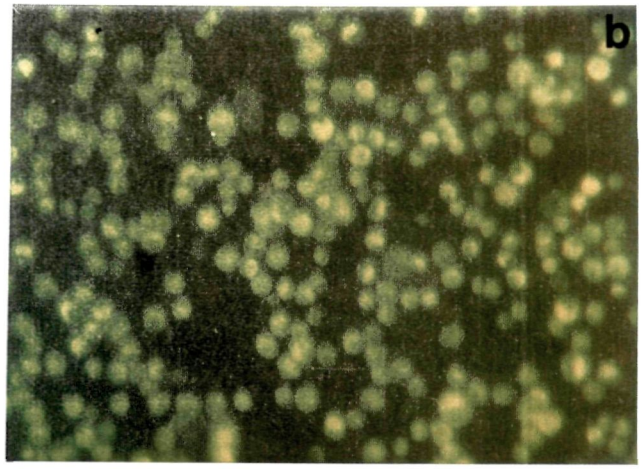
3.2.1. Protoplast isolation

Protoplasts were isolated routinely from cell suspension cultures of all the cultivars, using overnight and short-term incubation methods. Overnight isolation was done for fusion experiments whereas short-term method was used for plant regeneration and other routine experiments. Small cell clumps started releasing protoplasts after 2-3 hour of incubation in enzyme mixture. However, maximum release was observed when the mixture was kept stationary. At the end of the enzyme treatment, when observed under inverted microscope, the Petri dish was full of protoplasts. Some undigested cell clumps were also found. If the isolation was done from a cell suspension in exponential phase, most of the cell clumps were found digested. In contrast, if the suspension had attained stationary phase, a number of undigested clumps were seen at the end of enzyme treatment. Some times, when isolation was done shortly after subculture, many fusion products were found. If the frequency of such multiple fusion products exceeded 10%, the isolation was abandoned. In the overnight isolation, the suspension started releasing protoplasts after 5-6 hour of incubation and the process was complete after 8-10 hour. When these two methods of isolations were compared for their effectiveness on

Legend for figures

Fig. 4. (a - g). Plant regeneration from protoplasts of Japonica rice line RCPL1-2C. **(a)** Freshly isolated protoplasts. **(b)** Fluorescein diacetate-stained protoplasts showing viability. **(c)** Initial divisions. **(d)** Colonies resulting from sustained divisions in presence of feeder. **(e)** Proliferation of small colonies on membrane filter. **(f)** Plantlets regenerated from protocalli. **(g)** Fertile plant with filled grains.


a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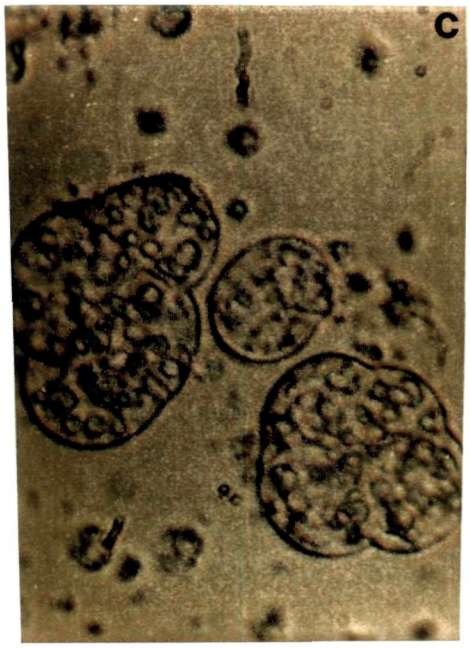
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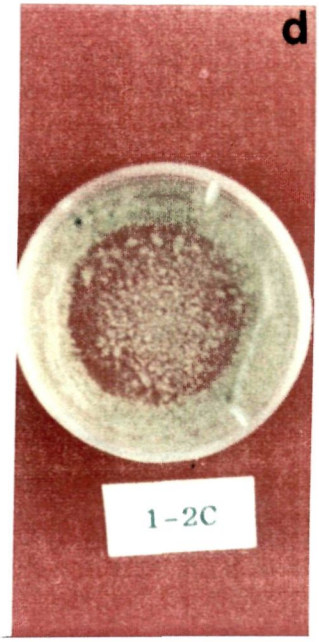
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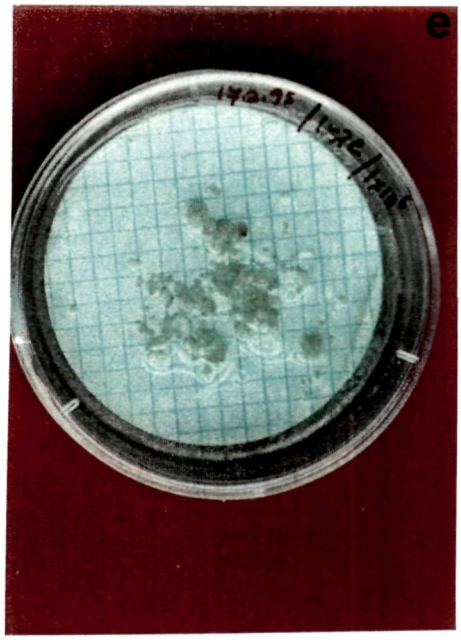
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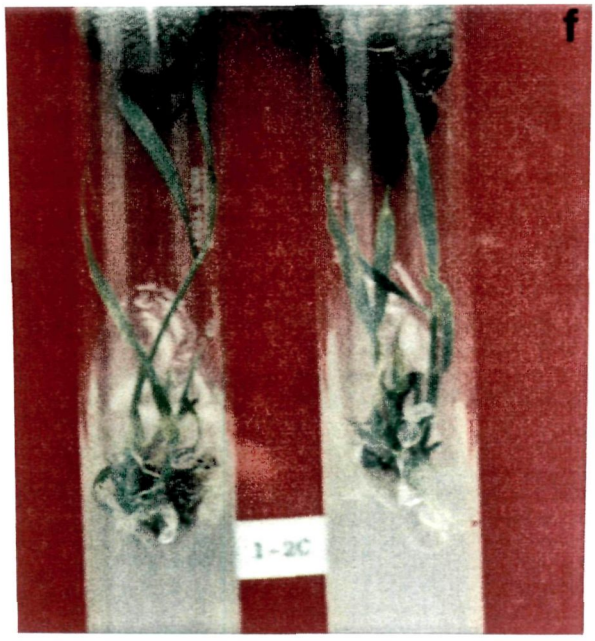
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g

protoplast isolation, no significant difference in yield as well as viability was observed between the two methods.

3.2.1.1. Purification

The enzyme mixture, when passed through a stack of sieves, separated the undigested cell clumps and the filtrate contained mostly the protoplasts, broken cells and the cell debris. Repeated washings with CPW13M removed the enzymes. Protoplasts, when floated over 1M sucrose and centrifuged at a low speed (50 rpm for 5 minutes), a thick band was formed over the sucrose layer and the vacuolated protoplasts and other debris settled down thus, separating the intact and highly cytoplasmic protoplasts. The band of protoplasts when washed twice in CPW13M removed the sucrose. When checked under microscope, the purified protoplasts were found uniform in size (Fig.4.a., 5.a. & 6.a). They did not fluoresce in the presence of Calcoflour White indicating the absence of cell wall. Size of the freshly isolated protoplasts ranged from 9-12 μm in diameter. The purified protoplasts were highly cytoplasmic with distinct starch grains (Fig.4.a., 5.a. & 6.a)

3.2.1.2. Test for viability

Freshly isolated protoplasts, stained with FDA, fluoresced green under UV (Fig.4.b.& 5.b.). When observed under bright field, some of protoplasts did not fluoresce indicating nonviability. The viability of isolated protoplasts ranged from 85 ± 1.15% to 90 ± 1.07%.

3.2.1.3. Yield of protoplasts

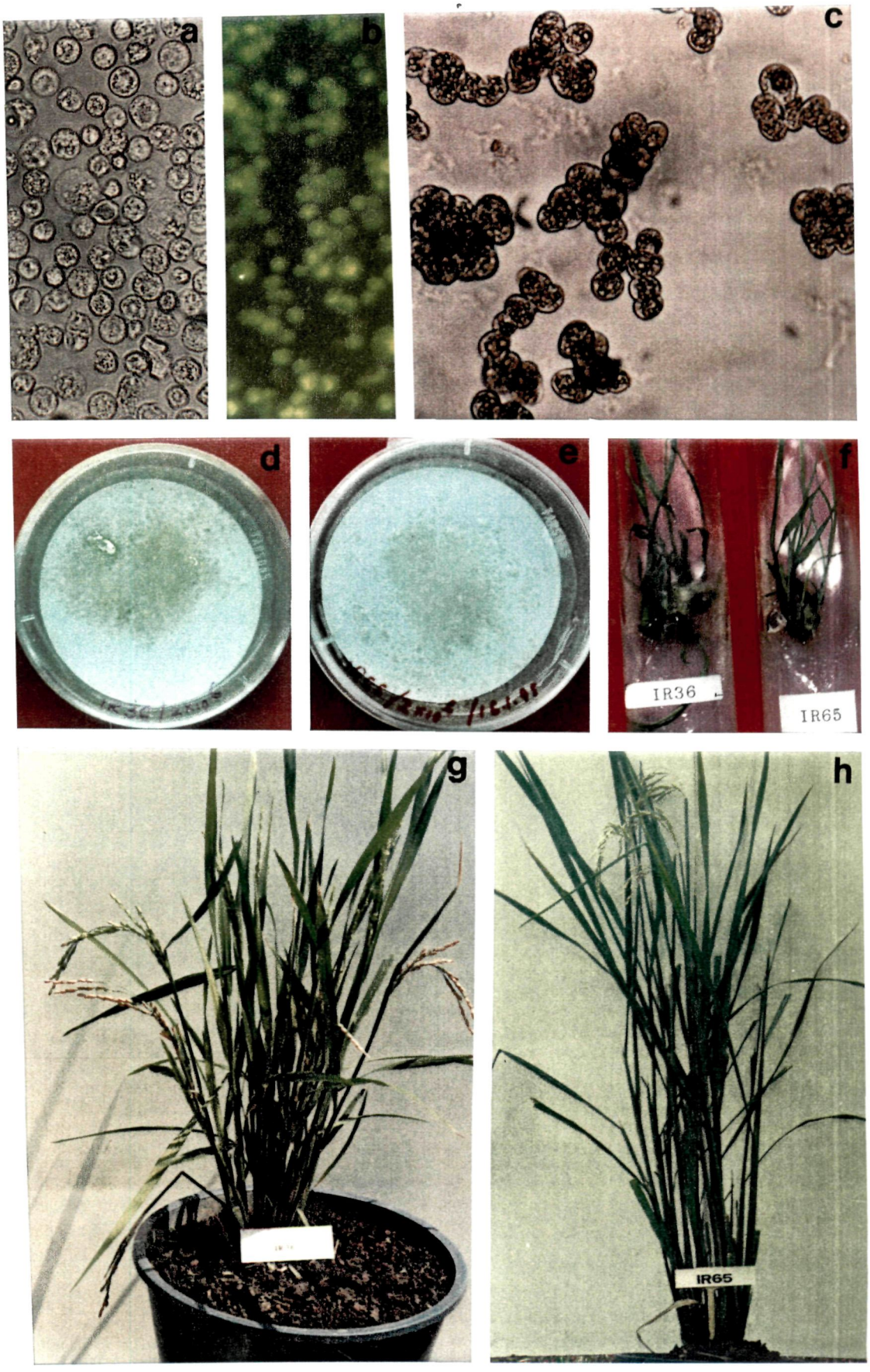
Protoplast yield was low from very young (50-60 days after initiation) cell suspensions. However, after 80-90 days in liquid culture, the yield of protoplasts increased dramatically and it ranged from 5 x 10^5 to 4 x 10^7 g $^{-1}$ f wt of tissue. Protoplast yield varied from 5 x 10^6 to 2 x 10^7 (SD = 1.11) g $^{-1}$ f wt of tissue in Japonica rice lines. In RCPL 1-2C, protoplast yield varied from 9 x 10^6 to 2 x 10^7 (SD = 1.21) g $^{-1}$ f wt of tissue whereas in RCPL 1-3C it ranged from 5 x 10^6 to 4 x 10^7 (SD = 1.11) x 10^6 g $^{-1}$ f wt of tissue. Yet in another Japonica rice line Nami, it ranged from 7 x 10^6 to 2 x 10^7 (SD = 1.21) g $^{-1}$ f wt of tissue.

Among Indica rice lines, protoplast yield ranged from 8 x 10^6 to 11 x 10^6 (SD = 1.23) in IR36 and 7 x 10^6 to 9 x 10^6 (SD = 1.11) in IR65 g^{-1} f wt of tissue. Protoplast yield was higher in Japonica lines as compared to Indica lines.

In CMS line V20A, the yield of protoplast ranged from 5 x 10^6 to 10 x 10^6 (SD = 1.20) g^{-1} f wt of tissue and in V20B it was ranged from 8 x 10^6 to 12 x 10^6 (SD = 1.10) g^{-1} f wt of tissue. In IR62829A it ranged from 1 x 10^6 to 5 x 10^6 (SD = 1.15) g^{-1} f wt of tissue and in IR62829B it ranged from 2 x 10^6 to 7 x 10^6 (SD = 1.11) g^{-1} f wt of tissue. In IR54752A, protoplast yield ranged from 1 x 10^6 to 5 x 10^6 (SD = 1.15) g^{-1} f wt of tissue and in IR54752B it ranged from 3 x 10^5 to 8 x 10^6 (SD = 1.20) g^{-1} f wt of tissue. Yield of the protoplasts was greatly influenced by the age of the cell suspension culture. Good quality protoplasts were obtained from 3-12 months old suspension cultures of different rice lines. In general, 5-6 months after the establishment, older the suspension cultures higher the protoplast yield.

Legend for figures

Fig 5. (a – h). Plant regeneration from protoplasts of Indica rice lines IR36 and IR65 **a:** Freshly isolated protoplasts of IR36 after purification (x 350) **(b)** Fluorescein diacetate-stained protoplasts showing viability **(c)** Microcolonies formed 15 - 20 days after protoplast culture of IR36 (x 350) **(d & e)** Macrocolonies of IR36 **(d)** and of IR65 **(e)** resulting from culture of the protoplasts for 6-7 weeks at a plating density of 1 x 10^6 and 2 x 10^6/ml, respectively **(f)** Plant regeneration from protocalli of IR36 and IR65 **(g & h)** Fertile plants of IR36 **(g)** and of IR65 **(h)** with filled grains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IR65

3.2.2. Protoplast culture and induction of division

Protoplasts of all the cell lines, except CMS lines IR62829A and IR54752A, when cultured in presence of nurse (section; 2.2.3.5.2), regenerated cell wall within 24-36 hour and first cell division was recorded between 4-8 day after culture (Fig.4.c.). Viable protoplasts remained cytoplasmic and continued to divide, but the daughter cells tended to separate from each other causing an increase in the number of viable cells. At this stage, reduction in the osmoticum helped in subsequent division, which led to the formation of micro-colonies of 32-64 cells (Fig.5.c.). After 4 weeks of culture, the colonies became visible with naked eye inside 'donut' holes (Fig. 4.d.). On the other hand, the protoplasts plated on the membrane kept over feeder cells (Section 2.2.3.5.2) began to expand during the first week of culture and micro-colonies formed after 18-19 days. However, it was not possible to observe the first few divisions of protoplasts on membrane, although protoplast-derived cell colonies were visible with stereo microscope. These colonies grew further and 200-250 macroscopic colonies/10^6 protoplasts were observed (Fig.4.e., Fig.5.d.& e. and 6.b.) after 25-30 days of culture. Sustained division and colony formation were recorded in all the three methods of protoplast culture but the highest plating efficiency were recorded in the 2^{nd} method (section 2.2.3.5.2).

Protoplasts of CMS lines IR62829A and IR54752A, when cultured in presence of nurse, (section: 2.2.3.5.2), started dividing after 18-19 days of culture. Although, most of the protoplasts cultured in presence of nurse remained viable for a longer period of time (2-3 months) after culture, yet 90 to 95% of them failed to undergo sustained division as

revealed by microscopic observation. Only some of the dividing protoplasts (5-10% of the total protoplasts cultured) underwent sustained divisions and led to the formation of microcolonies after 50-60 days of culture. Few of the microcolonies grew further and became macroscopic after 70-80 days.

Protoplasts, cultured in absence of nurse cells (4th and 5th method of culture, section 2.2.3.5.2) did not undergo sustained division. In case of RCPL1-2C, only few divisions were observed in absence of nurse cells. Indica and CMS lines, however, did not show any division in absence of nurse and even after serial reduction in the osmoticum of the culture medium, these protoplasts failed to divide.

In the negative control (6^{th} method of culture, section 2.2.3.5.2) where, N6PCMZ medium was used in 'donuts' surrounded by nurse, no leakage of nurse cells was observed even after two month of culture with regular reduction in osmoticum.

3.2.2.1. Effect of heat shock on division of protoplasts

Heat shock treatment to the freshly isolated protoplasts was essential to obtain high frequency division in all the cell lines. Without heat shock treatment, frequency of protoplast division was low. In Japonica rice line RCPL1-2C, division frequency was notably higher in the heat shocked protoplasts as compared to the protoplasts, which were cultured without heat shock. In contrast, the protoplasts of Indica and CMS rice lines did not show any sign of division when cultured without heat shock treatment.

3.2.2.2. Effect of protoplast density on division

A significant variation was observed in the frequency of division at different plating densities that ranged from 1 x 10^5 to 5 x 10^6 ml^{-1} of protoplasts. Maximum division was recorded at plating density of 1 x 10^6 ml^{-1} in presence of nurse cells. When the plating density was raised to 2 x 10^6 ml^{-1}, sustained division was followed by formation of micro-colonies, however, only 1/3rd of these colonies became macroscopic, thus, reducing the plating efficiency (Table: 5).

Table 5. : Plating efficiency at different plating densities of protoplasts of RCPL1-2C, IR36 and IR65*

Plating densities	Plating efficiencies on different days after plating (%)								
	RCPL 1-2C			IR36			IR65		
	7th day	15th day	30th day	7thday	15thday	30thday	7thday	15thday	30thday
5x10^5	2.31	4.03	0.003	3.03	1.12	0.05	3.78	1.23	0.008
1x10^6	29.1	37.1	0.27	23.42	5.180	2.31	20.00	5.660	1.72
2x10^6	15.32	10.89	0.03	18.79	9.34	0.08	15.86	3.09	0.087
5x10^6	10.06	6.05	0.004	8.03	1.19	0.012	11.07	1.28	0.05

*** Numerical average of five experiments**

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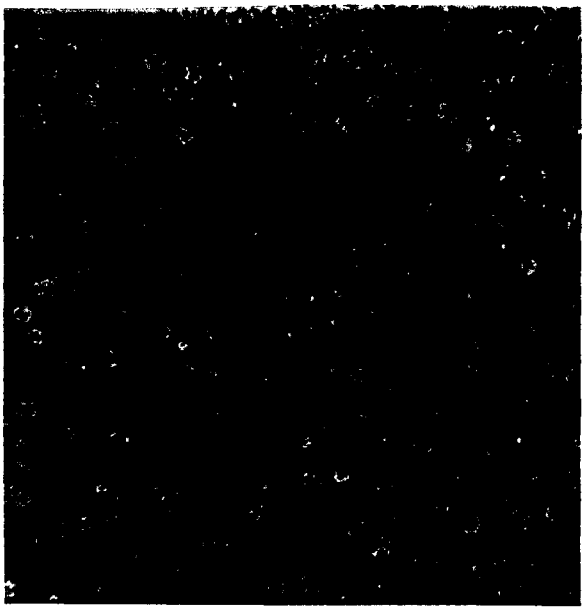
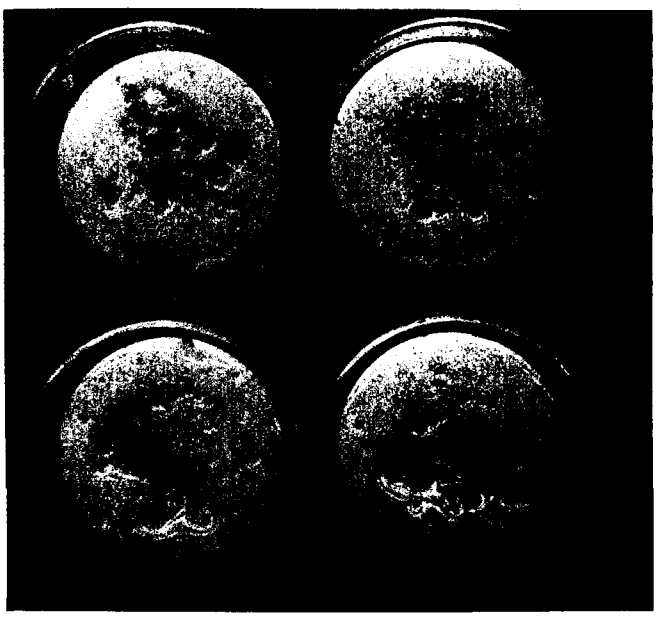
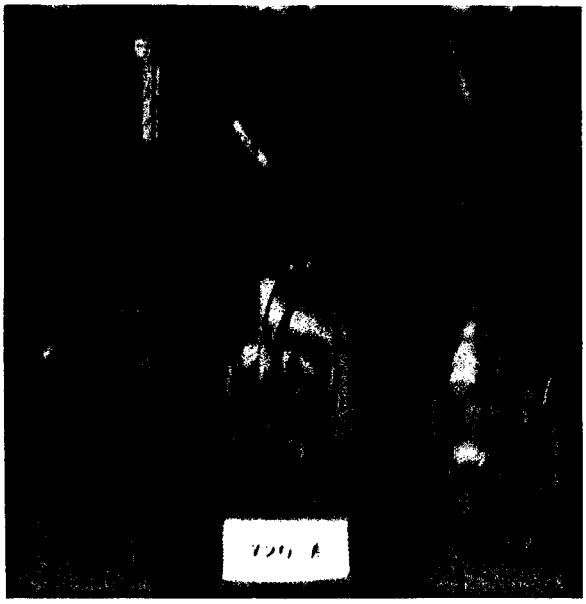
Fig 6. (a – e).: Plant regeneration from protoplasts of CMS line V20A **(a)** Freshly isolated protoplasts of V20A after purification (x 700) **(b)** Macrocolonies of V20A resulting from culture of the protoplasts for 6-7 weeks at a plating density of 1×10^6 /ml **(c)** Plant regeneration from protocalli of V20A **(d)** Plants of V20A **(e)** Sterile pollen grains of V20A







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3.2.2.3. Effect of osmolarity on protoplast division

N6PCMZ culture medium with 0.7M glucose provided the best osmoticum for protoplast culture. When cultured in low osmoticum, the protoplasts tended to burst and at higher osmoticum they shrank and did not divide sustainedly. Division of protoplasts could be sustained only when the cultures were replenished with 0.5 M, 0.35 M and 0.17 M N6PCMZ medium at weekly interval. Gradual reduction in the osmolarity of cultures was found essential for sustained division of the protoplasts.

3.2.3. Plant regeneration from protoplast-derived calli

After transfer of the protoplast-derived calli to regeneration medium, white embryo like structures developed on the surface of the micro calli after 7-10 days of culture in dark. Such calli, when transferred to light, differentiated into shoots and roots very rapidly to produce plants in 7-20 days (Fig.4.f., 5.f. and 6.c.).

Microtocalli obtained from the protoplasts of Japonica rice lines, on transfer to light differentiated into shoots and roots only after 7-12 days whereas protocalli calli of all other rice lines differentiated only after 10-20 days of transfer to light. Plant regeneration percentage from protoplast-derived calli of Japonica rices ranged from 30% to 35% and it ranged from 12% to 49% in IR lines on MSB medium. In case of CMS line V20A, per cent regeneration was 64.26% and in maintainer line V20B, it was 63.40% (Table: 6). Highest plant regeneration percentage was obtained from V20A and V20B lines compared to any other lines of rice.

Table 6. : Plating efficiency and plant regeneration from protoplasts of V20A & V20B*

Variety	Plating efficiency on different days after plating			Regeneration %
	7th day	15th day	30th day	
V20A	15.8	23.71	3.19	64.26 (69)**
V20B	18.8	31.4	5.6	63.40 (137)**

*** numerical average of 10 experiments**
**** total number of plants regenerated**

3.2.4. Effect of the age of cell suspension on plating efficiency and plant regeneration

Age of the cell suspension that was used as source for the protoplasts played an important role in plant regeneration from the protoplast-derived calli. Effect of age of the cell suspension on plating efficiency and plant regeneration was studied in details for a Japonica rice line RCPL1-2C, which is presented in the Table 7. Protoplasts isolated from very young suspensions (1-3 month old) showed low plant regeneration percentage (3-9%). With the increase in the age of cell suspension (4-12 month old) plant, regeneration percentage also increased (23.75% to 41.56%) and it attained a peak when the cell suspension was 6-8 months old. As, the suspensions grew older, per cent plant regeneration from protoplast-derived calli decreases and it was less than 2.41% in case of 16-18 month old suspensions. Protoplasts isolated from 18-month-old suspension

showed no plant regeneration. In case of some cultivars, a few albino plants were obtained from protoplasts isolated from older cell suspension cultures.

Table 7. : Age of the cell suspension, plating efficiency and plant regeneration from protoplasts of RCPL 1-2C cultured in N6PCMZ (AG)*

Expt No.	Age of cell suspension (days)	Plating efficiency (%)			No. of calli transferred to regeneration medium	No. of green plantlets	Percent regeneration percentage (%)	No. of mature plants
		7th day	15th day	30th day				
1	85	29.9	37.4	0.21	37	5	13.5	3
2	103	30.1	39.3	0.24	41	7	17.1	9
3	127	31.1	36.4	0.22	39	9	23.1	7
4	133	29.1	33.3	0.25	46	10	21.7	12
5	137	29.1	37.1	0.27	51	13	25.5	16
6	138	28.1	29.6	0.22	31	10	31.3	18
7	141	32.0	39.1	0.23	46	11	23.9	9
8	143	28.6	37.6	0.21	43	12	27.9	8
9	145	29.2	38.9	0.22	51	14	27.4	21
10	146	29.1	38.2	0.23	40	12	30.0	12

***Numerical average of five experiments**

3.2.5. Effect of different culture methods on plating efficiency and plant regeneration

Among the six different culture methods described in the section 2.2.3.5.2, sustained division was observed only in presence of nurse cells. Therefore, the three methods of protoplast culture, viz.1st, 2nd and 3rd (in which nurse was used) were compared for plating efficiency and plant regeneration for IR36 and IR65 rice lines. Both plating

Table 8. : Plating efficiency of protoplasts and frequency of plant regeneration from protoplast-derived calli of IR36 and IR65 following different culture methods

Variety	Protoplast culture method	Plating efficiencies on different days after plating			Plant regeneration percentage	Total no. of plants regenerated
		7th day	15th day	30th day		
IR36	1*	13.84	2.130	0.96**	23	12**
	2*	23.42	5.180	2.31**	49	52**
	3*	Not visible	0.081	1.10**	40	18**
IR65	1*	9.12	4.160	0.47**	12	11**
	2*	20.00	5.660	1.72**	39	71**
	3*	Not visible	0.030	0.94**	29	19**

1* Liquid N6PCMZ inside ring of feeder.
2* Liquid N6PCMZ + Agarose 0.15% inside ring of nurse cells.
3* Liquid N6PCMZ + Agarose 0.15% on membrane filter.
** Correlation (r) of plating efficiency with total number of plants regenerated**
r = 0.823*

efficiency and plant regeneration percentage was found highest in 2nd method where protoplasts were cultured in N6PCMZ medium solidified with agarose in 'donuts' surrounded by nurse cells. Plating efficiency was the lowest in the 1st method where protoplasts were cultured in liquid N6PCMZ in 'donuts' surrounded by nurse cells. Effect of different culture methods on plating efficiency and plant regeneration was thoroughly investigated for the protoplasts of IR36 and IR65 and the results are presented in Table 8.

The highest frequency of regeneration (49% in IR36 and 39% in IR65) was recorded in the protocalli obtained from the 2nd method of protoplast culture followed by method-3 (40% in IR36 and 29% in IR65) and method-1 (23% in IR36 and 12% in IR65). Direct correlation of plating efficiency (on day 30) with per cent plant regeneration was found significant and positive ($r = 0.823^{*}$, Table 8).

3.2.6. Transfer and establishment of regenerated plants in green house

Protoplast-derived plantlets of all the lines, when transferred to 1/2 strength MS medium, showed a relatively reduced rooting frequency (67% to 85%) as compared to 100% rooting frequency in case of tissue-culture-and suspension culture-derived plantlets. Some of the protoplast-derived plants showed tendency to produce small roots that were brown.

However, after 2-4 weeks, when a good rooting system was established, plants with 10-12 cm height were transferred to glasshouse for further growth. The timing of transfer to the glasshouse determined their transition to the flowering state. All the plants except

CMS lines set seeds at maturity (Fig.4.g., 5.g.& h. and 6.d.). Number of protoplast-derived plants established in the glasshouse varied in different rice lines. From ten individual experiments, 135 plants of RCPL 1-2C, 20 plants of RCPL 1-3C, 18 plants of Nami, 82 plants of IR36, 101 plants of IR65, 69 plants of V20A, 2 plants of IR62829A, 3 plant of IR54752A, 137 plants of V20B, 12 plants of IR62829B and 15 plants of IR54752B were obtained. Plant regeneration percentage from some rice lines (IR54752A, IR54752B, IR62829A and IR62829B) was very poor and only few plantlets could be recovered from the protocalli. Even with some modification in the regeneration medium, by using maltose as carbon source, regeneration percentage was not enhanced from the protocalli of these lines. Ten plants each of RCPL 1-2C, RCPL 1-3C, Nami, IR36, IR65, V20A and V20B were established in the glasshouse and grown until maturity.

3.2.7. Protoclonal variation

In order to assess protoclonal variations in the protoplast-derived regenerants and their progeny, agrobotanic characters of R0, R1 and R2 plants of IR36 and IR65 were compared with those of the seed-derived controls and the data are presented in Table 9 and 10). Plant height and spikelet fertility of R0 plants in IR36 was significantly lower than controls (Table 9). On the other hand, no significant differences were observed between R0 and control of IR65 in plant height, panicle length, number of spikelets/panicle and per cent spikelet fertility (Table 10).

Average spikelet fertility in the present investigation was 55.2% in R0 of IR36 and 71.87% in control. It was, however, almost like control in IR65 (76.4 and 77% in R0

Table 9. : Agrobotanic characters of protoplast-derived R0, R1, R2 and seed-derived control plants of IR36 *

Plants	Characters							
	Plant height (cm)	Panicle length (cm)	PBT	Spikelets /panicle	Spikelet fertility (%)	100 seed weight (gm)	Yield/ plant (gm)	Plot yield (q/ha)
IR36 control	64.4^{a}	20.44^{a}	5.51^{b}	75.25^{a}	71.87^{a}	2.56^{b}	4.50^{ab}	27.5^{a}
IR36 protoclone (R0)	57.21^{b}	19.93^{ab}	**	65.35^{a}	55.2^{b}	NR	**	NR
IR36 protoclone (R1)	59.03^{b}	17.46^{bc}	6.1^{a}	72.63^{a}	59.85^{b}	2.48^{b}	4.28^{b}	22.82^{b}
IR36 protoclone (R2)	58.73^{b}	17.06^{c}	5.8^{ab}	78.86^{a}	65.54^{ab}	2.86^{a}	5.27^{a}	23.17^{b}

***: Average of five plants in R0 and three replications with ten plants/replication in R1 and R2. Within each column, means followed by a common letter are not significantly different at 5% level by Duncan's Multiple Range Test.**

****: Data were not recorded because protoplast-derived plants are generally a cluster of multiple shoots.**

NR: Not recorded.

and control, respectively). R1 progenies of IR36 and seed-derived control showed significant difference in plant height, panicle length, spikelet fertility and plot yield as revealed by Duncan's Multiple Range Test (Table 9). Out of these, plant height, panicle

length and plot yield remained significantly different even in R2 but spikelet fertility attained parity with control (in R2).

Yield/plant and 100 seed weight of IR36 showed positive shift in R2. In contrast, all the characters *viz.* plant height, panicle length, panicle bearing tillers, number of spikelets / panicle, 100 seed weight, yield/plant and plot yield. (except spikelet / panicle in R1) were at par with seed-derived control in the R1 and R2 progeny of IR65 (Table 10).

Table 10. : Agrobotanic characters of protoplast-derived R0, R1, R2 and seed-derived control plants of IR65 *

Plants	Characters							
	Plant height (cm)	Panicle length (cm)	PBT	Spikelets / panicle	Spikelet fertility (%)	100 seed weight (gm)	Yield / plant (gm)	Plot yield (q/ha)
IR65 control	80.38[a]	21.42[a]	6.31[a]	87.17[a]	77.00[a]	2.53[a]	5.66[a]	26.12[a]
IR65 protoclone (R0)	82.05[a]	21.76[a]	**	87.44[a]	76.4[a]	NR	**	NR
IR65 protoclone (R1)	70.2[a]	19.60[a]	5.43[a]	69.21[b]	66.45[a]	2.43[a]	4.66[a]	25.71[a]
IR65 protoclone (R2)	72.86[a]	19.6[a]	6.6[a]	90.33[a]	67.76[a]	2.49[a]	4.96[a]	24.53[a]

***: Average of five plants in R0 and three replications with ten plants/replication in R1 and R2. Within each column, means followed by a common letter are not significantly different at 5% level by Duncan's Multiple Range Test.**

****: Data were not recorded because protoplast-derived plants are generally a cluster of multiple shoots.**

NR: Not recorded.

In general, there was reduction in plant height, panicle length and spikelet fertility, which was significantly, lower in IR36, however, they were at par with control in IR65 (Table 10).

3.3. Transfer of CMS through protoplast fusion

3.3.1. Scheme for cytoplasmic gene transfer

The scheme for cytoplasmic gene transfer involves inactivation of the protoplasts of the donor (CMS line) by a physical mutagen to eliminate the nucleus (Kyozuka *et al.*, 1989, Ozias-akin *et al.*, 1987). Cytoplasm (mitochondria) of the recipient is inactivated by chemical treatment viz. iodoacetamide (Kyozuka *et al.*, 1989, Ozias-akin *et al.*, 1987). Protoplasts treated with physical mutagen and iodoacetamide separately ceased to divide on culture. The fusion products of the inactivated protoplasts should have three types (Fig.7). Out of them those with cytoplasm of the donor and nucleus of the recipient will divide and form colonies. Plants obtained from these colonies are expected to be male sterile with intact female fertility (Fig.7).

3.3.2. Inactivations of protoplast, protoplast fusion, culture of fusion product and plant regeneration

3.3.2.1. Inactivation of protoplasts

Since the protoplasts of the donor CMS lines (V20A, IR62829A and IR54752A) as well as fertile recipient lines (RCPL 1-2C, RCPL1-3C, Nami, V20B, IR62829B and IR54752B) were undergoing sustained division, in order to make the selection effective,

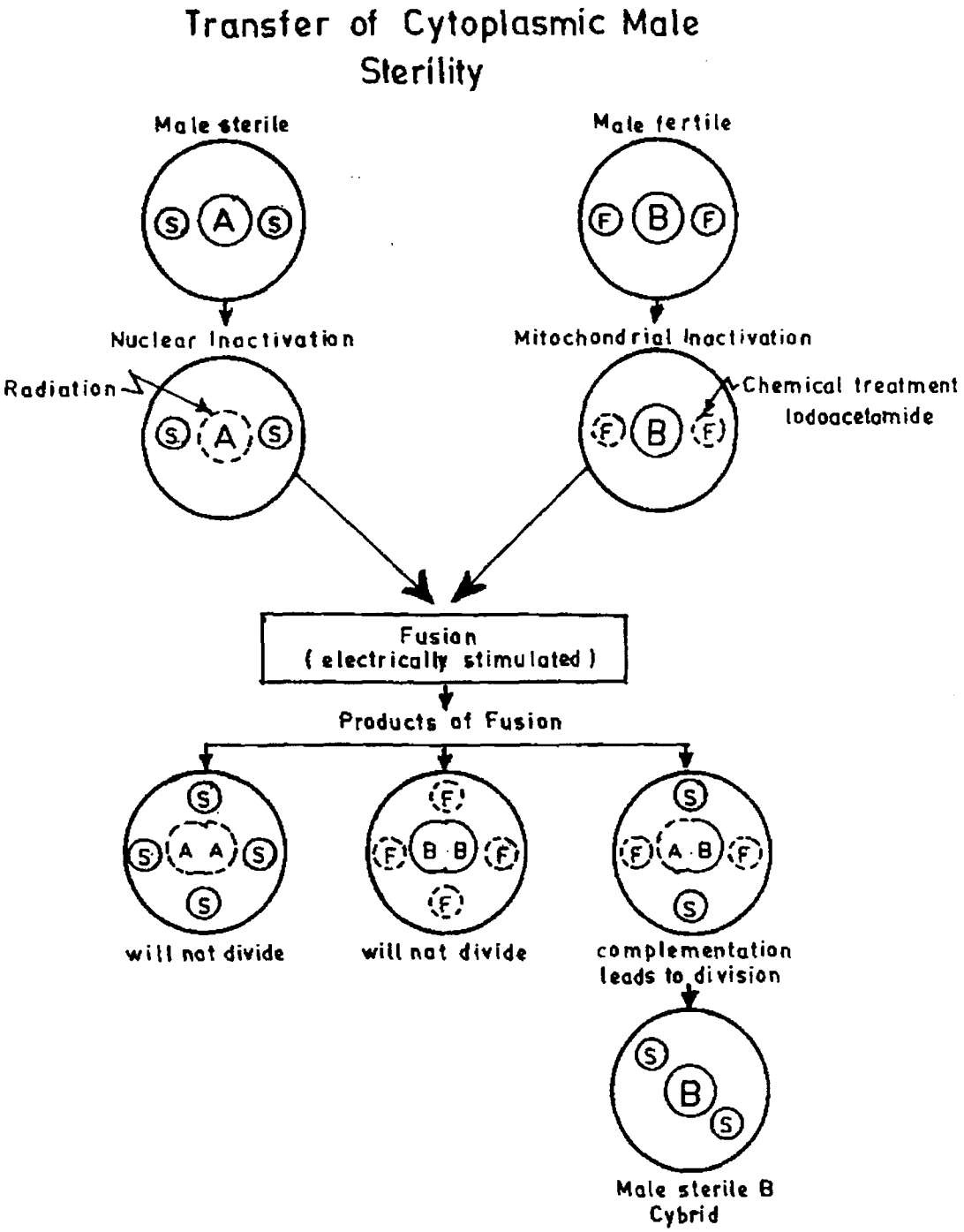


Figure 7.: Schematic diagram illustrating the donor - recipient protoplast fusion for cytoplasmic gene transfer: Line A is the donor and Line B is the recipient. A, V20A; B, RCPL 1 - 2C; S, mitochondria of male sterile line; F, mitochondria of male - fertile line

protoplasts of CMS lines were irradiated with 30 krad of γ-rays that completely inhibit the division in the protoplasts of CMS lines (Fig.8.b.). Treatment with 10mM iodoacetamide for 15 minutes at room temperature was optimal to stop the division of protoplasts of the fertile maintainers (Fig. 8.c.). Effects of different doses of radiation and various concentrations of iodoacetamide on the protoplasts are presented.

3.3.2.1.1. Effect of different dosages of γ-ray on protoplasts

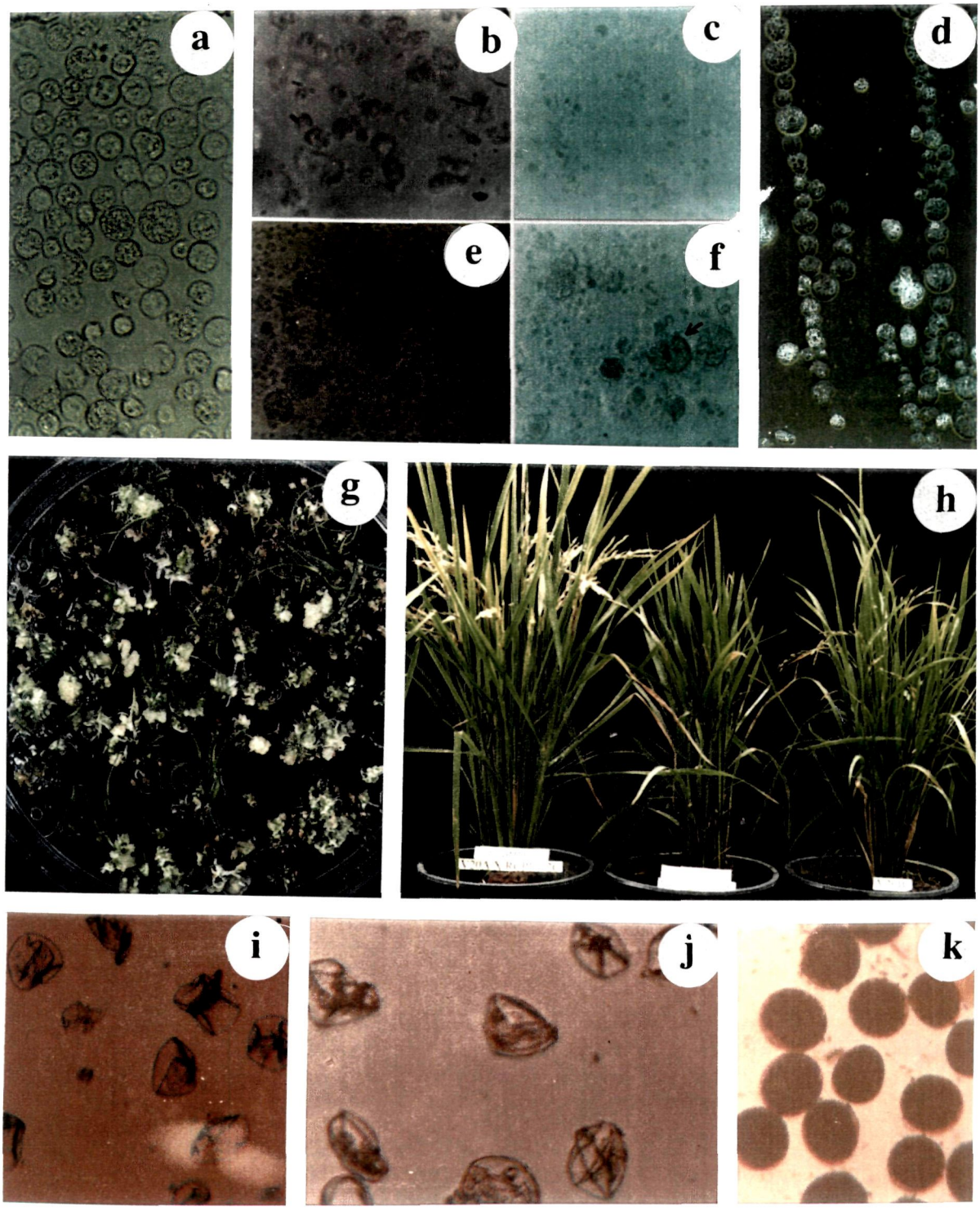
Protoplasts of CMS lines, irradiated with 10, 15, 20 and 25 krad of γ-ray, started dividing between 4^{th} to 6^{th} day of culture. Subsequent division of the protoplasts irradiated with 10 and 15 krad led to the formation of microcolonies that became macroscopic after 20-30 days of culture. Frequency of division reduced drastically in the protoplasts irradiated with 20 and 25 krad of γ-rays without formation of micro colonies. Protoplasts irradiated with 35, 40, and 50 krad of γ-ray did not divide even after 2-3 weeks of culture. These protoplasts became vacuolated after 15-20 days of culture and turned brown. Some of the protoplasts appeared elongated and some of them bursted and died. The results showed that 30 krad of γ-ray were adequate to inactivate the protoplasts of CMS lines (Fig. 8.b.). Therefore, this dose was used routinely for inactivation of protoplasts.

3.3.2.1.2. Effect of iodoacetamide treatment on protoplasts

Protoplasts of fertile lines (RCPL1-2C RCPL1-3C, Nami, V20B, IR62829B and IR54752B), treated with 5, 10, 15, 20 and 25 mM iodoacetamide solution, were assessed

Legend for figures

Fig. 8. (a - k) Cybridisation and plant regeneration from cybrid calli. **(a)** Freshly isolated protoplasts **(b)** Protoplasts of V20A inactivated with gamma ray. **(c)** Protoplasts of RCPL1-2C inactivated with iodoacetamide **(d)** Inactivated protoplasts of RCPL 1-2C and V20A are arranged in a chain in an AC electric field **(e - f)** Colonies resulting from the fusion products **(g)** Plantlets regenerated from the putative cybrid between V20A X RCPL1-2C **(h)** Mature plant of cybrid between V20A X RCPL1-2C (left), between V20A X V20B (center) and V20B (right) **(i)** Sterile pollen grains of the cybrid between V20A X RCPL1-2C **(j)** Sterile pollen grains of the cybrid between V20A X V20B **(k)** Fertile pollen grains of V20B showing starch filled pollen grains.


a
b
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for inhibiting cell division. At 5 mM concentration, protoplasts started dividing 10-12 days after culture and formed microcolonies after 30-35 days. The protoplasts treated with 10 and 15 mM iodoacetamide appeared healthy for 7-10 days in culture but there was no division. Later the treated protoplasts turned brown and died after 20-30 days. Higher concentrations of iodoacetamide (20 and 25mm) were found toxic to the protoplasts. Therefore, 10 mM concentration of iodoacetamide was used routinely to inactivate the protoplasts of all the fertile recipient lines.

3.3.2.2. Protoplast fusion

Inactivated protoplasts of V20A and V20B and V20A and RCPL 1-2C (mixed in 1:1 ratio), when brought under electric field, they established a protoplast to protoplast contact and led to the formation of chains as revealed under inverted microscope (Fig. 8.f.). At this stage, a DC pulse broke the thin cell membrane surrounding the protoplasts and the adjacent protoplasts fused. Frequency of fusion ranged from 5 to 7%.

3.3.2.2.1. Effect of protoplast density on fusion

Density of protoplasts influenced the frequency of fusion. When parental protoplasts (donor and recipient) were mixed in 1:1 ratio at the different densities ranging from 0.5 x 10^5 ml^{-1} to 4 x 10^6 ml^{-1}, fusion frequency was recorded highest at the density of 1 x 10^6 ml^{-1}. At low density (< 1 x 10^6 ml^{-1}), fusion frequency was very low (0.01-0.1%) and after post fusion washing, only few fused products could be recovered. When density of protoplast was higher (> 1 x 10^6 ml^{-1}), fusion frequency was again very low (0.01-0.3%)

and very less number of fused products were obtained. Therefore, protoplast fusion experiments were conducted at a density of 1 x 10^6 ml^{-1}.

3.3.2.2.2. Effect of pre and post fusion cooling treatments to the protoplast

When protoplast mixture were maintained at room temperature before and after exposure to electrical field, large number of protoplasts bursted. In contrast, when protoplasts were kept at low temperature (in ice cold water) for 10 minutes before and after fusion and left undisturbed for 45 minutes at room temperature after fusion; large number of fusion products could be recovered after washing. Therefore, ten minutes pre and post fusion cooling treatment to the protoplast mixture in ice was given for stability of the fused products.

3.3.2.2.3. Culture and division of fused products

Fused products were cultured without preferential selection of cybrid cells in donut holes as well as on membrane filter in presence of nurse (2^{nd} and 3^{rd} method of culture, section 2.2.3.5.2). The fusion products regenerated cell walls within 24-36 hours of culture in the donut holes. First cell division was observed within 5-6 days. Further divisions led to the formation of micro-colonies after 20-30 days (Fig.8.d & e.). Fused products, cultured on the membrane over feeder cells (3^{rd} method of section 2.2.3.5.2), began to expand after the 7-9 day of culture and microcolonies formed after 25-30 days. Microcolonies obtained from the membrane filter became macroscopic after 35-40 day of culture after serial reduction in osmoticum at 7-day interval. Fused products, cultured

in absence of nurse cells (4th and 5th method of section 2.2.3.5.2) did not undergo sustained division. Instead, they became vacuolated and died after 3-4 days of culture. Plating efficiencies recorded on 7th, 15th and 30th day after culture of fused products following 2nd method of culture (Section 2.2.3.5.2) from two sets of the fusion experiments viz. V20A x V20B and V20A x RCPL1-2C are presented in Table 11. Plating efficiency, number of macrocolonies obtained and plant regeneration percentage was higher in the fused products obtained from V20A x RCPL1-2C fusion experiment than the fusion experiment where protoplasts of V20A were fused with V20B.

Table 11. : Plating efficiency, number of putative cybrid plant, plant regeneration percentage and number of mature plants obtained from fused products of two fusion experiments*

Serial No	Fusion partners	Plating efficiency (%)			Total no of putative cybrid plant	Plant regeneration percentage	No of plants grown to maturity
		7th day	15th day	30th day			
1	V20A X V20B	18.67	9.46	0.22	33	22.03	23
2	V20A X RCPL1-2C	28.99	7.82	0.31	39	37.00	27

*: **Numerical average of five experiments**

3.3.2.3. Plant regeneration from putative cybrid calli

Putative cybrid calli (1-2 mm diameter in size) on transfer to regeneration medium produced creamy white, dry, nodular and compact embryo-like structures on the surface after one to two weeks of culture in dark. Calli became harder with prominent shoot and root primordia after 10-12 days. On transfer to light, the calli showed green pigmentation after 10-12 days and then differentiated into shoots and roots after another 15-20 days (Fig.8.g.). Sometimes, green pigmentation on the putative cybrid calli was noticed after 15-20 days of exposure to light. Occasionally, few calli that showed green pigmentation did not differentiate into shoots and roots even after keeping them in light for more than one month. Some of them became brown and died after few days.

Putative cybrid calli obtained from the fusion experiments between V20A and RCPL 1-2C showed higher plant regeneration percentage (26 -37%) whereas plant regeneration percentage from putative cybrid calli obtained from fusion between V20A x V20B ranged from 17% to 23%. Regeneration from the putative cybrid calli was obtained only in the medium solidified with agarose. No regeneration was recorded on the medium solidified with agar. The data on plant regeneration of the putative cybrid calli from different fusion experiments is presented in the Table 11.

3.3.2.4. Maintenance of putative cybrid plants and their establishment in the green house

Putative cybrid plants, on transfer to rooting medium (1/2 strength MS), showed normal root development after 10-15 days of transfer. Healthy plantlets with normal root

growth were ready for transfer to the glasshouse after 3-4 weeks of culture. The rate of survival of putative cybrid plants in the glasshouse ranged from 65% - 70%. In total, 27 putative cybrid plants between V20A x RCPL 1-2C and 23 plants from V20A x V20B were recovered and all of them were grown to maturity.

3.3.2.5. Agronomic characters of putative cybrid plants at maturity

Putative cybrid plants between V20A x RCPL1-2C were taller (Fig.8.h.) than V20A as well as putative cybrid between V20A x V20B. In contrast, putative cybrids between V20A and V20B were semidwarf (Fig. 8.h.) and morphologically similar to V20B. At maturity, neither the putative cybrid plants between V20A x RCPL1-2C nor those of V20A x V20B set seeds and all were sterile. They were shorter than RCPL 1-2C line, which is a tall cultivar. Other morpho-physiological characters of the cybrids appeared normal.

3.3.3. Characterization of putative cybrids

3.3.3.1. Pollen grain staining and germination

Pollen grains from cybrids and CMS line, V20A were shrivelled, empty, and smaller in size (Fig.8.i.& j.). When stained with KI and I_2 none of the pollen grains from cybrids between V20A x V20B showed staining for starch (Fig. 8.j.). In contrast, 6-8% of the pollen grains from 15 putative cybrid plants between V20A x RCPL1-2C showed deep brown staining for starch. However, when grown on pollen germination medium, none of the pollen grains from the putative cybrids (including those showing staining for

starch) germinated and all of them failed to form pollen tubes indicating sterility. On the other hand, pollen grains from the fertile lines, V20B and RCPL 1-2C showed normal starch-filled, round pollen grains. When stained with KI and I_2 they showed deep brown staining for starch (Fig.8.k.). These pollen grains, when cultured in pollen germination medium, germinated and formed normal pollen tube indicating fertility.

3.3.3.2. Molecular analysis of putative cybrids

3.3.3.2.1. Mitochondrial DNA restriction profiles of cybrids

Restriction digestion with *HindIII* and *BamHI* did not show any difference between the mtDNA of the cybrids and that of the CMS line. *HindIII* digested mtDNA from leaves of all sterile cybrids, CMS line, V20A and fertile lines, V20B and RCPL1-2C showed apparently identical restriction profiles.

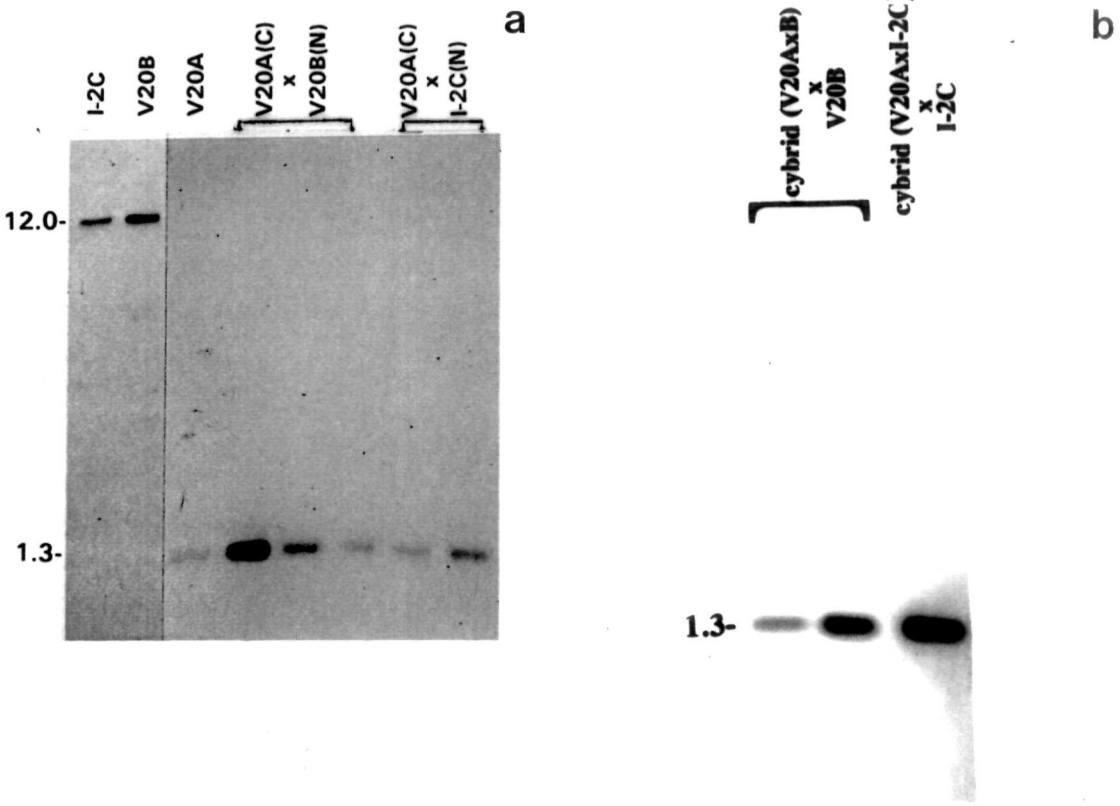
3.3.3.2.2 Southern hybridization analysis of rice mtDNA of the fusion partners and of the cybrids

Probing of *Hind*III-digested mtDNA with *orf155* gene probe exhibited polymorphism between mtDNA of the CMS and that of fertile maintainers. In case of *Hind*III-digested mtDNA of CMS line, V20A, *orf155* gene was located on 1.3 kb fragment whereas in case of fertile maintainers, V20B as well as RCPL1-2C, *orf155* gene was located on a 12 Kb fragment in *Hind*III digested mtDNA. The mtDNA from five cybrid plants (three of V20A x V20B and two of V20A x RCPL 1-2C) were digested with *Hind*III and probed with *orf*155, which hybridized with 1.3 kb fragment in all the cybrid plants (Fig.9.a.).

Legend for figures

Fig. 9. (a & b) **(a)** Southern blot of *Hind* III-digested mitochondrial DNA from the maintainers, RCPL1-2C (lane 1) & V20B (lane 2); CMS line V20A (lane 3); putative cybrids between V20A X V20B (lane 4,5,6) and V20A X RCPL1-2C (lane 7, 8) probed with *orf155* showing the location of *orf155* in 12 kb fragments in the maintainer lines and 1.3 kb fragment in CMS as well as putative cybrid lines.

(b) Southern blot of *Hind* III-digested mitochondrial DNA (from BC1 progenies) probed with *orf155* showing the location of *orf155* in the 1.3 kb fragment


a
I-2C
V20B
V20A
V20A(C) x V20B(N)
V20A(C) x I-2C(N)
12.0-
1.3-
b
cybrid (V20AxB) x V20B
cybrid (V20Axl-2C) x I-2C
1.3-

This showed that mtDNA of male sterile line V20A has been transferred in the nuclear background of RCPL1-2C and of V20B.

3.3.3.3. Inheritance of female fertility

Female fertility of the cybrids was detected by crossing them with respective maintainer lines. Fifty-five seeds from the cross between male sterile cybrid (V20A x RCPL1-2C) x RCPL1-2C (BC1) and 58 seeds from the cross between cybrid (V20A x V20B) x V20B (BC1) were obtained. This indicated that the female fertility of the cybrids remained unaffected during transfer of CMS. BC1 plants were grown to maturity and were found sterile.

3.3.3.3.1. Characterisation of BC1 plants

3.3.3.3.1.1. Pollen grain and pollen germination test

Pollen grains of BC1 plants, when stained with KI and I_2, did not show staining for starch. When germinated in pollen germination medium they failed to develop pollen tube. Morphologically, the pollen grains of BC1 plants appeared shrivelled, empty, and smaller as seen in CMS lines.

3.3.3.3.1.2. Mitochondrial DNA analysis

In order to find out the inheritance of mtDNA from cybrid (V20A x RCPL 1-2C) to the BC1 plants, mtDNA from three BC1 plants were isolated and purified. The mtDNA from all three BC1 plants [two of (V20A x V20B) x V20B and one of (V20A x RCPL 1-

2C) x RCPL 1-2C] were digested with *Hind*III and probed with *orf*155, which hybridised with 1.3 kb fragment in all the plants (Fig. 9..b.). This confirmed the presence of WA cytoplasm in the BC1 plants.

3.3.3.4. Assessment of fertility restoration in cybrids

Male sterile cyrids were, when pollinated with the pollen grains IR36, a known restorer line of WA CMS; set seeds and the resultant seeds were used to raise F1 plants. In total, six plants were grown to maturity and all of them set seeds. Percent spikelet fertility ranged from 75-88%. This showed that WA CMS in the fusion-derived male sterile cybrids (V20A x RCPL1-2C) behaves as that of WA CMS line V20A plants in its response to restorer genes.

3.3.4. Production of new CMS lines by transfer of WA cytoplasm through protoplast fusion and molecular characterization of CMS, maintainers and putative cybrids

3.3.4.1. Production of new CMS lines

3.3.4.1.1. Protoplast fusion and culture and division of fused products

Having standardised the protocol for transfer of WA CMS from V20A to RCPL1-2C and V20B through donor-recipient protoplast fusion, we used the same protocol to transfer CMS from WA CMS line, V20A to the nuclear background of RCPL1-3C and Nami. Further we transferred CMS from WA CMS line, IR62829A to the nuclear background of IR62829B (used as positive control), RCPL1-2C, RCPL1-3C and Nami.

and from WA CMS line, IR54752A to the nuclear background of IR54752B (used as positive control), RCPL1-2C, RCPL1-3C and Nami.

Inactivated protoplasts of donor CMS and fertile recipient lines (Section 3.3.2.1.), mixed in 1:1 ratio, when brought under electric field, an established protoplast to protoplasts contact led to the formation of a chain (Fig.10.a.). A DC pulse, at this stage, broke the thin cell membrane surrounding the protoplasts and the adjacent protoplasts fused.

Fusion frequency was ranged from 3 to 7%, when the protoplasts of V20A were fused separately with those of RCPL1-3C and Nami. However, the fusion frequency ranged from 7 to 9% when inactivated protoplasts of IR62829A were fused separately with IR62829B, RCPL1-2C, RCPL1-3C, Nami. Fusion frequency was lower and ranged from 2 to 5% when, IR54752A protoplasts were fused separately with those of IR54752B, RCPL 1-2C, RCPL1-3C and Nami.

Frequency and rate of fusion was influenced by the density of protoplasts as reported in the Section 3.3.2.2.1. Frequency of fusion was found optimum at the density of 1 x 10^6 ml^{-1} of protoplasts. Therefore, protoplast fusion was conducted at a density of 1 x 10^6 ml^{-1} for all set of fusion experiments.

Large number of fused products could be recovered when a ten minutes pre and post fusion cooling treatment was given to the protoplast mixture in ice as reported in the previous case (Section 3.3.2.2.2).

The fused products regenerated cell walls as usual, within 24-36 hours of culture in the 'donut holes'. First cell division was observed within 5-6 days. Further divisions led to the formation of micro colonies after 20-30 days. Fused products, cultured on the

Legends for figures

Fig. 10. (a - i) Production of new CMS lines through protoplast fusion . **(a)** Inactivated proplasts of donor (IR62829A) and recipient line (RCPL1-2C) are arranged in a chain in an AC electric field **(b & c)** Proliferation of small putative cybrid colonies on membrane filter **(d)** Plantlets regenerated from the putative cybrid **(e)** Putative cybrid plants of IR62829A and RCPL1-2C (left); V20A and Nami (middle) and IR54752A and Nami (right) growing in jars **(f)** Putative cybrids between V20A and Nami growing in pot **(g)** Putative cybrid between IR62829A and RCPL1-2C growing in pot **(h)** Putative cybrid between IR54752A and Nami (left) and IR54752A and RCPL1-2C (right) growing in pot **(i)** Sterile pollen grains of the cybrid between IR62829A and RCPL1-2C.

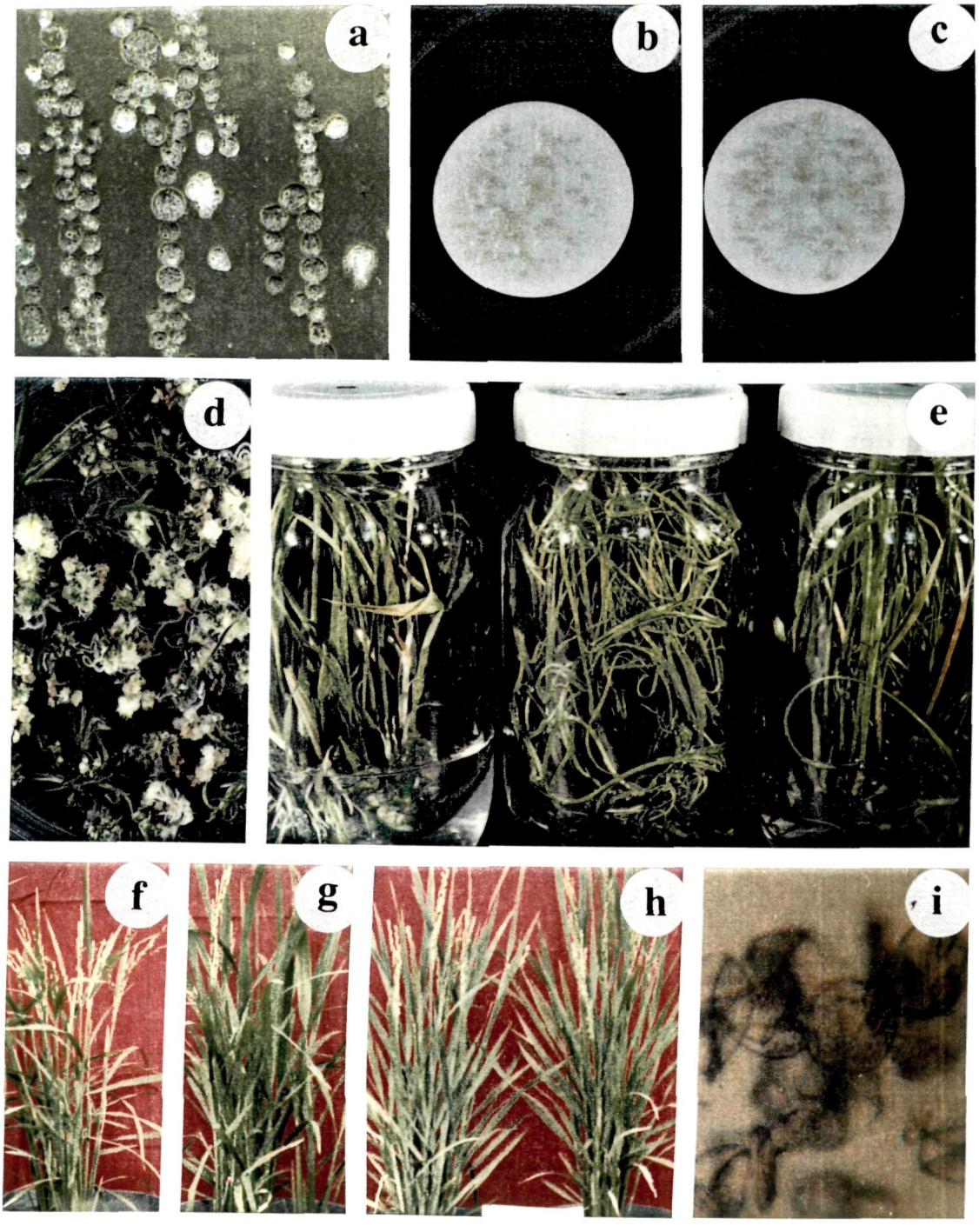

a
b
c
d
e
f
g
h
i

Table 12. : Plating efficiency, total no of plant regenerated, plant regeneration percentage and number of mature plants obtained from fused products of different fusion experiments*

Serial No	Fusion partners	Plating efficiency (%)			Total No of putative cybrid plant	Plant regeneration percentage (%)	No of mature plants
		7th day	15th day	30th day			
1	V20A X RCPL1-3C	17.43	1.69	0.12	28	18.99	16
2	V20A X Nami	16.31	3.46	0.21	26	16.92	15
3	IR62829A X IR62829B	11.46	2.61	0.10	14	11.06	6
4	IR62829A X RCPL12C	13.92	7.73	0.31	20	12.99	8
5	IR62829A X RCPL1-3C	10.96	3.41	0.10	15	9.10	7
6	IR62829A X Nami	12.82	7.31	0.21	19	10.31	6
7	IR54752A X IR54752B	10.91	2.21	0.17	10	10.92	6
8	IR54752A X RCPL1-2C	19.43	1.32	0.23	13	12.46	5
9	IR54752A X RCPL1-3C	8.63	0.41	0.08	17	8.36	6
10	IR54752A X Nami	9.82	2.34	0.10	14	9.31	5

***Numerical average of five different experiments**

membrane kept over feeder cells began to expand after 7-9 day of culture and led to the formation of microcolonies after 25-35 days. These microcolonies became macroscopic after 40-45 days of culture with serial reduction in osmoticum at an interval of 7 days Fig. 10.b.& c.). Fused products, plated in absence of nurse cells (4^{th} and 5^{th} method of culture of section 2.2.3.5.2) did not undergo sustained division in culture. They became vacoulated after 3-4 days of culture, turned brown and slowly died.

Plating efficiency recorded on 7th, 15th and 30th day after culture of the fused products following 2nd method of culture (Section 2.2.3.5.2), number of macro-colonies obtained, number of plantlets regenerated and per cent plant regeneration from all the fusion experiments are presented in the Table 12. Plating efficiency, number of macrocolonies recovered and plant regeneration percentage was considerably higher in the fused products where V20A was fused with RCPL1-3C and Nami as compared with other sets of fusion.

3.3.4.1.2. Plant regeneration from putative cybrid calli and establishment of plantlets in glasshouse

Putative cybrid calli, on transfer to regeneration medium, showed compact embryo-like structures on the surface after 10-14 days of culture in dark. On transfer to light, they differentiated into shoots and roots after another 15-20 days (Fig.10.d.). All the calli showed green pigmentation was not differentiated into shoots and roots even after keeping them in light for more than one month. Some of them became brown and died after few days. No of mature plants obtained from different fusion experiments ranged from 5 to 16 (Table: 1.11). Putative cybrid calli obtained from the fusion experiments between V20A x RCPL 1-3C and V20A x Nami showed higher plant regeneration percentage that ranged from 16.92 to 18.99%. Whereas plant regeneration percentage from putative cybrid calli obtained from other fusion experiments ranged from 9.10% to 12.99% (Table: 3.10). Regeneration could be obtained only, when the regeneration

medium was solidified with agarose and used. No regeneration was recorded on the medium solidified with agar.

Healthy putative cybrid plantlets (Fig. 10.e.) with normal root growth were ready for transfer to the glasshouse after 3-4 weeks of culture. The rate of survival of putative cybrid plants in the glasshouse ranged from 60% - 70%.

3.3.4.1.3. Morphological characterisation of putative cybrids, CMS lines and their fertile maintainers

Putative cybrid plants between V20A x RCPL1-3C and V20A x Nami were of the same height as in case of V20A line (Fig.10.f.). Putative cybrids between IR62829A x IR62829B, IR62829A x RCPL1-2C, IR62829A x 1-3C and IR62829A x Nami were of the same height as in case of IR62829A lines (Fig.10.g.). At maturity, the putative cybrid plants between IR54752A x IR54752B, IR54752A x RCPL1-2C, IR54752A x RCPL1-3C and IR54752A x Nami also showed the same height as in case of IR54752A lines (Fig. 10.h.).

None of the cybrids set seed at maturity and they were sterile (Fig. 10.f.g.&h.). Other morpho-physiological characters of the cybrids appeared normal.

Pollen grain staining from all the cybrids and CMS lines V20A, IR62629A and IR54752A and pollen germination test confirmed sterility(Fig. 10.i.). On the other hand, pollen grains from the fertile lines, RCPL1-2C, RCPL1-3C, Nami, V20B, IR62829B and IR54752B, when stained with KI and I_2 showed normal starch-filled, round pollen grains and they germinated in pollen germination medium indicating fertility.

3.3.4.2. Molecular characterisation of CMS, maintainers and putative cybrids

3.3.4.2.1. Molecular characterization CMS lines

3.3.4.2.1.1. PCR analysis

Employing PCR technique, mitochondrial gene fragments were amplified from WA CMS lines V20A, IR62829A and IR54752A by using a pair of oligo-nucleotides specific for mitochondrial gene of rice. In the first set of reaction primed with oigo-nucleotides pair (P1→P2) specific for rice *atp6* gene, a single fragment of size 1.2 kb was amplified in all the CMS lines. Amplification was also attempted using primers P3→P4, specific for rice gene *urf79*; S1→S2 specific for sunflower gene *orf522* and O3→O4 specific for wheat gene *orf299* but no amplification was observed in any of the CMS lines. However, with primer BM1→BM4, specific for whole *coxII* gene of rice, a 3 kb fragment was amplified in all the CMS lines. Primer O1→O2, specific for rice gene *orf155* yielded a single fragment of size 1 kb. In all the cases, no amplification was observed when λDNA was used as a template (negative control).

3.3.4.2.1.2. Southern hybridisation

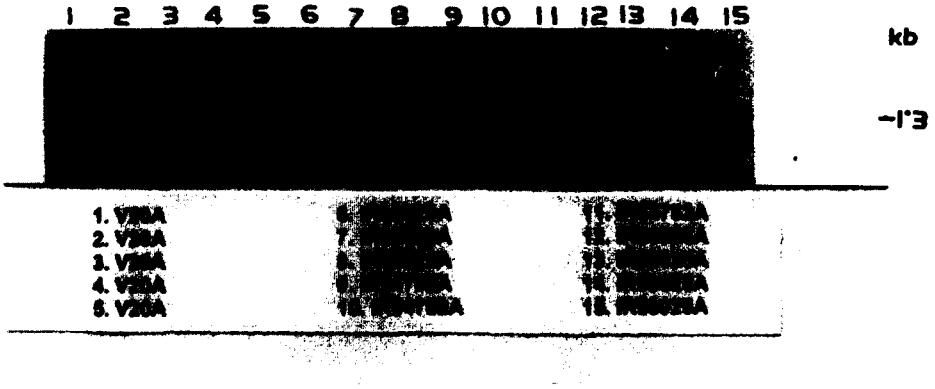
Following restriction endonuclease digestion and electrophoresis, rice mtDNA from CMS lines were transferred to nylon membranes. Hybridization was done using several heterologous mitochondrial gene probes. Hybridization of *atp6* gene probe to *HindIII*-digested mtDNA from all the CMS lines produced identical hybridization signals (1 kb fragment). Hybridization of *coxII* gene probe to the *Hind*III-digested mtDNA showed a 3 kb fragment in all the CMS lines. When *Hind*III-digested mtDNA of CMS line,

Legend for figures

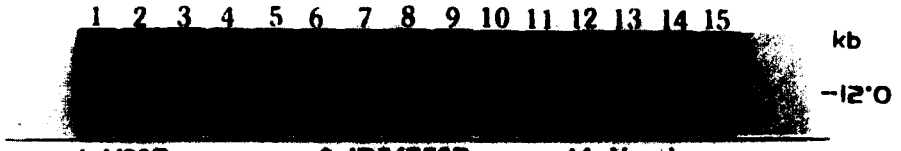
Fig. 11. (a & b) **(a)** Southern blot of *Hind* III-digested mitochondrial DNA from the CMS line V20A (lane 1-5), IR62819A (lane 6-10) and IR54752A (lane 11-15) probed with *orf155* showing the location of *orf155* in 1.3 kb fragment in CMS lines.

(b) Southern blot of *Hind* III-digested mitochondrial DNA from the fertile maintainer lines V20B (lane1-2), IR62819B (lane 3-4) IR54752B (lane 5-6), RCPL1-2C (lane 7-8), RCPL1-3C(lane 9-10), Nami (lane 11-12), IR58025B (13-14) and Taipai 309 (15) probed with *orf155* showing the location of *orf155* in 12 kb fragment in maintainer lines.

a



b



1. V20B	6. IR54752B	11. Nami
2. V20B	7. 1-2C	12. Nami
3. IR62829B	8. 1-2C	13. IR58025B
4. IR62829B	9. 1-3C	14. IR58025B
5. IR54752B	10. 1-3C	15. Taipai 309

V20A, IR62829A and IR54752A were probed with rice *orf155* gene probe, it was located on a 1.3 kb fragment (Fig.11.a.).

3.3.4.2.2 Molecular characterization of maintainers

3.3.4.2.2.1. PCR analysis

Mitochondrial gene fragments were amplified from fertile lines V20B, IR62829B, IR54752B, RCPL1-2C, RCPL1-3C and Nami by using oligo-nucleotides specific for mitochondrial gene(s) of rice and wheat. A single amplified fragment of 1.2 kb size was found when PCR reaction was primed with a pair of oigo-nucleotides (P1→P2) specific for rice *atp6* gene in all the fertile lines. No amplification was observed in the rice lines when PCR reaction was primed with pair of oigo-nucleotides, (P3→P4) specific for rice *urf79* gene; (S1→S2) specific for sunflower gene *orf522* and (O3→O4) specific for wheat gene *orf299*. A 3 kb fragment was amplified from all the lines when (BM1→BM4) specific for whole *coxII* gene of rice was used. (O1→O2) specific for rice *orf155* gene also yielded a single fragment of size 1 kb. No amplification was observed when λDNA was used as a template (negative control).

3.3.4.2.2.2. Southern hybridisation

Three mitochondrial gene probes, viz. *atp6*, *coxII* and *orf155* were used for the hybridization with mtDNA of the maintainer lines. After transfer of the restricted DNA to positively charged membrane, mtDNA was hybridized to non-radioactive labeled probes from three mitochondrial genes. Southern hybridization analysis of *atp6* gene

Legend for figures

Fig. 12. (a - c) **(a)** Southern blot of *Hind* III-digested mitochondrial DNA from the putative cybrids between V20A X V20B (lane 1-3), V20A X RCPL1-2C (lane 4-6), V20A X RCPL1-3C (lane 7-9) and V20A X Nami (lane 10-12) probed with *orf155* showing the location of *orf155* in 12 kb fragments in 1.3 kb fragment in cybrid lines.

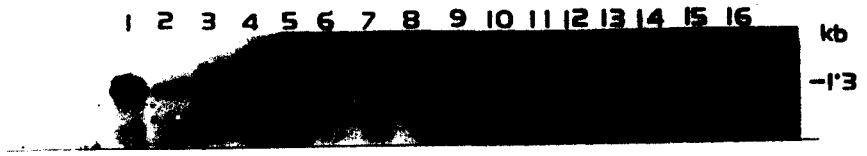
(b) Southern blot of *Hind* III-digested mitochondrial DNA from the putative cybrids between IR62829A X IR62829B (lane 1-4), IR62829A X RCPL1-2C (lane 5-8), IR62829A X RCPL1-3C (lane 9-12) and IR62829A X Nami (lane 13-16) probed with *orf155* showing the location of *orf155* in 1.3 kb fragment in cybrid lines

(c) Southern blot of *Hind* III-digested mitochondrial DNA from the putative cybrids between IR54752A X IR54752B (lane 1-5), IR54752A X RCPL1-2C (lane 6-10), IR54752A X RCPL1-3C (lane 11-14) and IR54752A X Nami (lane 15-18) probed with *orf155* showing the location of *orf155* in 1.3 kb fragment in cybrid lines

a

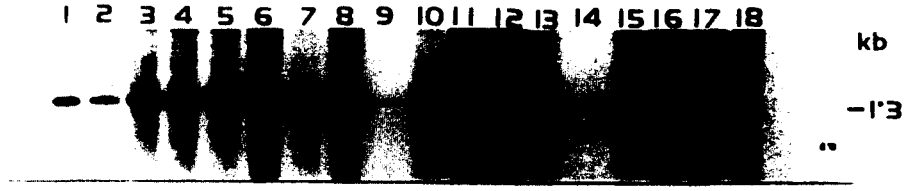
1. V20A X V20B
2. V20A X V20B
3. V20A X V20B
4. V20A X 1-2C
5. V20A X 1-2C
6. V20A X 1-2C
7. V20A X 1-3C
8. V20A X 1-3C
9. V20A X 1-3C
10. V20A X Nami
11. V20A X Nami
12-14. - do -

b



1. IR62829A X IR62829B
2. IR62 -A X IR62 -B
3. IR62 -A X IR62 -B
4. IR62 -A X IR62 -B
5. IR62 -A X 1-2C
6. IR62 -A X 1-2C
7. IR62 -A X 1-2C
8. IR62 -A X 1-2C
9. IR62 -A X 1-3C
10. IR62 -A X 1-3C
11. IR62 -A X 1-3C
12. IR62 -A X 1-3C
13. IR62 -A X Nami
14. IR62 -A X Nami
15. IR62 -A X Nami
16. IR62 -A X Nami

c



1. IR54752A X IR54752B
2. IR54 -A X IR54 -B
3. IR54 -A X IR54 -B
4. IR54 -A X IR54 -B
5. IR54 -A X IR54- B
6. IR54 -A X 1-2C
7. IR54 -A X 1-2C
8. IR54 -A X 1-2C
9. IR54 -A X 1-2C
10. IR54 -A X 1-2C
11. IR54 -A X Nami
12. IR54 -A X Nam
13. IR54 -A X Nami
14. IR54 -A X Nami
15. IR54 -A X 1-3C
16. IR54 -A X 1-3C
17. IR54 -A X 1-3C
18. IR54 -A X 1-3C

probe to *HindIII*-digested mtDNA in all the maintainers produced identical hybridization signals (1 kb fragment). Hybridization of *coxII* gene probe to the *HindIII*-digested mtDNA from all the maintainers also produced identical hybridization signals of 3 kb fragment. Probing of *Hind*III-digested mtDNA of all fertile maintainers V20B, IR62829B, IR54752B, RCPL1-2C, RCPL1-3C and Nami of to a specific gene probe of *orf155* exhibited hybridization signal of 12 kb fragment (Fig.11.b.) that was different from CMS lines in which it hybridized with 1.3 kb fragment of mtDNA digested with *Hind*III restriction enzyme.

3.3.4.2.3. Molecular characterization of putative cybrids

3.3.4.2.3.1. PCR analysis

Mitochondrial gene fragments were amplified by using different pair of oligo-nucleotides, which were specific for the mitochondrial gene(s) of rice. In case of *atp6* gene, PCR analysis primed with a pair of oigo-nucleotides (P1→P2) specific for rice *atp6* gene, a single fragment of 1.2 kb was amplified in all the putative cybrids. In amplifications, using primers P3→P4 specific for rice gene *urf79*; S1→S2 specific for sunflower gene *orf 522* and O3→O4 specific for wheat gene *orf299;* no amplification was observed in any of the cybrids. In case of BM1→BM4 primers specific for rice *coxII* gene, a 3 kb fragment was amplified in all the putative cybrids whereas in case of O1→O2 primers specific for rice *orf155* gene, only one fragment of 1 kb was amplified. In all the cases, no amplification was observed when λDNA was used as a template (negative control).

3.3.4.2.3.2. Southern hybridization

Three different restriction enzymes (*Hind*III, *Eco*RI and *Bam*HI) digested mtDNA from leaves of sterile cybrids showed apparently identical restriction profiles. Restriction endonuclease-digested mtDNA from the cybrids were electrophoresed and transferred to positively charged nylon membranes. Hybridisation was done using several heterologous non-radioactive labelled mitochondrial gene probes. Hybridisation of *atp6* gene probe to *Hind*III-digested mtDNA from all the cybrids produced identical hybridisation signals of 1 kb fragment (similar to CMS and maintainer lines) whereas hybridisation of *coxII* gene probe to the *Hind*III-digested mtDNA from the cybrids showed an identical 3 kb fragment (similar to CMS and maintainer lines). Probing of *Hind*III-digested mtDNA of cybrid lines with *orf155* gene showed a 1.3 kb fragment which is similar to hybridisation pattern of CMS lines (Fig.12.a.,b.&c.).

CHAPTER 4

DISCUSSION

CHAPTER 4: DISCUSSION

4.1. Callus induction, maintenance of embryogenicity and plant regeneration from rice lines

Establishment of embryogenic cell suspension cultures from embryogenic calli is a base line for obtaining cytoplasmic protoplasts with morphogenic potential. In the present investigation, a reproducible plant regeneration system was established directly from embryogenic calli of four Japonica, four Indica, three cytoplasmic male sterile lines and their maintainers. Embryogenic calli were induced from mature seeds, immature embryos, leaf bases and stem bases on LSA and LSB medium. Source of explant, genotype and chemical composition of callus induction medium were found responsible for high frequency embryogenic callus production from all the rice lines.

Response of different explant types viz. mature seeds, immature embryos, leaf bases and stem bases, on callus production was significantly different. Among all the explants, mature seeds and immature embryos were most suitable source of embryogenic calli. Immature embryos were although more responsive than mature seeds, the later were used frequently because of their availability round the year. Immature embryos have been used as source explants in wheat (Harris *et al.*, 1988; Vasil *et al.*, 1990), maize (Cai *et al.*, 1988; Shillito *et al.*, 1989; Morocz *et al.*, 1990) and rice (Lai and Liu, 1976; Lai and Liu, 1982; Peng and Hodges, 1989; Wu and Zapata, 1992; Bhattacharjee and Gupta, 1995, Bhattacharjee *et al.*, 1998). Immature embryos in strictly defined stages of development were found suitable for production of embryogenic calli. Frequencies of callus induction from stem and leaf base explants were poor and it was difficult to

produce enough embryogenic calli from these explants as only basal meristematic regions of leaf and stem bases were able to produce calli with embryogenic potential. This is in an agreement with the results of Barcelo *et al.* (1991) on callus induction. Choice of genotype and media composition have been reported to play important role in production of embryogenic calli from rice (Raina, 1989). In the present study, large genotypic variations were observed in callus induction and somatic embryo formation among different cultivars. Japonica rice lines (RCPL 1-2C, RCPL 1-3C and Nami) were found suitable for embryogenic callus production in different media as compared to Indica and CMS rice lines. This is in accordance with other reports that Japonica rice lines are more responsive to callus induction as well as plant regeneration than the Indica rice lines (Hartke and Lörz, 1989; Chowdhry *et al.,* 1993; Rance *et al.,* 1994; Rout and Lucas, 1996). Although, a number of media were used for callus induction, LSA and LSB media solidified with agar were found more suitable to induce embryogenic calli from mature and immature embryos of all the rice lines. LS medium supplemented with casein hydrolysate and glutamine in presence of 4.0 mg l^{-1} 2,4-D was found very responsive for the induction of embryogenic calli from japonica rice lines. In contrast, LS medium supplemented with tryptophan and kinetin in presence of 4.0 mg l^{-1} 2,4-D was the best callus induction medium for Indica and CMS rice lines. Effectiveness of LS medium in callus induction is in agreements with the earlier reports in Taipai 309 (Abdullah *et al.*, 1986), *Japonica* CMS lines (Xue and Earle, 1995), the wild rice (Baset, 1992) and the *Indica* rice lines (Jain *et al.*, 1996; Azhakanandam, 1999). The minor variations in callus induction could be attributed to factors such as

differences in the constituents of the medium, concentrations of endogenous phytohormones and differential susceptibility of Indica and Japonica varieties to auxins (2,4-D) as reported by Sundaru *et al.*, 1983.

In the present investigation, frequency of efficient plant regeneration directly from callus was found genotype-dependent. Regeneration frequency was higher in Japonica rices (RCPL1-2C, RCPL1-3C and Nami) than Indica lines (IR36, IR64, IR65 and IR74) and Indica CMS lines (V20A, IR62829A and IR54752A). Presence of low concentration of auxin (NAA) and higher amount of cytokinen (kinetin) in the regeneration medium was found essential for plant regeneration directly from callus without adversely effecting their friability. Except in the number of tillering, other agronomic characters of seed-derived and somatic embryo-derived plants were similar. Number of tillers were, however, higher in somatic embryo-derived plants than the seed-derived plants. It is, thus, possible to conclude that the frequency of embryogenic callus induction and efficient plant regeneration is mainly influenced by the genotype and the composition of culture media including the plant growth regulators.

4.2. Initiation, establishment, maintenance of cell suspension and plant regeneration

Protoplasts isolated directly from meristemetic cells of rice are extremely recalcitrant in culture and slow multiplication rate of calli does not allow availability of adequate amount of calli for protoplast isolation. Therefore, in order to overcome this problem, several workers reported the need for establishment of cell suspension cultures (Torrizo

and Zapata, 1992; Datta *et al.*, 1992b). In the present investigation, suspensions were initiated and maintained in amino acid medium supplemented with 4 mg l^{-1} 2,4-D that helped in establishing the suspension faster than any other liquid culture medium. A number of factors like composition of culture medium, genotype and nature of the inoculum were found important for establishment and long term maintenance of embryogenic cell suspension cultures. Several other researchers reported similar observations (Abdullah, 1987, Tang, 1995) who established and maintained embryogenic cell suspensions for isolation of rice protoplasts.

Our success in establishing cell suspension cultures of Indica and CMS rice lines, was mainly due to use of AA medium with high concentration of 2,4-D. In an earlier attempt to initiate cell suspensions of IR lines, Kyozuka *et al.*, (1988) reported formation of type I callus from IR36, but suspension cultures could not be established due to compact nature of calli obtained from the IR lines. Increase in 2,4-D concentration (4 mg l^{-1}) in AA medium helped in establishing the suspension faster. AA medium, which contains amino acids as the sole source of nitrogen, has widely been used in initiation and maintenance of cell suspensions. It has also been shown to be effective in faster dissociation of calli and helps in checking the browning of cell suspensions (Abdullah *et al.*, 1986; Datta *et al.*, 1992; Gupta *et al.*, 1993b; Gupta and Bhattacharjee, 1995). Availability of higher concentration of 2,4-D prevented rhizogenesis and an irreversible conversion of potentially embryogenic cells to non-embryogenic state. This result is in an agreement with Mitsouka *et al.* (1994)'s observation that availability of intracellular 2,4-D suppresses re-differentiation process and, therefore, higher concentration of 2,4-D

causes lesser browning. Vasil (1983) described similar requirements for the establishment of embryogenic cell suspension cultures of graminaceous species especially of grasses. Suspension cultures of some Indica rice lines (IR62829A, IR54752A, IR62829B and IR54752B), however, could not be established in AA medium even with higher concentration of 2,4-D. However, the presence of proline in AA4 medium helped in the enrichment of the suspension cultures with actively dividing cytoplasmic cells. In addition, the presence of proline improved growth of suspensions and lead to higher yield of protoplasts. Protoplast yield improved further when suspension cells were transferred to AA4 liquid medium and maintained for three weeks before isolation. Proline in AA4 medium supported maintenance of the embryogenic potential of suspension cells and this is in agreement with the results from maize, rice and tall fescue *Festuca arundinaceae* (Armstrong and Green, 1985; Ozawa and Komamine, 1989; Datta *et al.*, 1992).

In the present investigation, genotype of the plant was an important factor in initiation and establishment of suspension cultures. It was comparatively easy to initiate and maintain the suspension culture of Japonica lines, RCPL1-2C, RCPL1-3C and Nami than that of Group I Indica lines IR36, IR65, CMS lines V20A, IR62829A, IR54752A and maintainers V20B, IR62829B, IR54752B. In Japonica rice lines, suspension cells dissociated very fast in liquid medium and gave rise to a fine cell suspension because the calli became friable after few subcultures. In contrast, in case of Group I Indica rice lines, IR36, IR65; Indica CMS lines, V20A, IR62829A and IR54752A and maintainers V20B, IR62829B, IR54752B the calli remained compact even after many subcultures.

When the calli were transferred to liquid medium, various degrees of browning was observed during the initiation presumably because of leaching of phenolics. In some cases, once callus of Indica lines were transferred to liquid medium, cell suspensions became viscous and large cell clumps were formed. Therefore, in order to avoid this, alternate day subculturing with regular sieving and microscopic observation was found the best way to enrich the cultures with actively dividing small groups of round and cytoplasmic cells. Regular replacement of culture medium minimized phenolic oxidation of the culture and regular sieving helped in removing bigger and non-embryogenic cell clumps. In contrast, Japonica rice lines did not show any kind of browning during initiation and establishment. Similar results have been reported by Zhang, (1995) for establishment of cell suspension of Group 1 Indica rice, IR64.

Plant regeneration from suspension cells was essential to confirm the embryogenicity of the cultures and only regenerating suspensions were used for protoplast isolation. Regeneration potential of suspension cells was affected by the age of suspension culture. Highest (68-90%) plant regeneration was obtained from five to twelve months old suspension cultures but as the culture grew older (>12 month), plant regeneration percentage declined. Reduction in frequency of plant regeneration with increase in the age of cell suspensions has been reported in *Japonica* lines (Finch, 1991), wild rices (Baset, 1992) as well as in barley (Lührs and Lörz, 1988; Karp and Lazzeri, 1992). In the present investigation, it was observed that efficient plant regeneration from suspension cultures was also genotype-dependent. Frequency of plant regeneration was remarkably higher in Japonica rice lines, RCPL1-2C, RCPL1-3C and Nami than the

Group I Indica, CMS lines and the maintainers. This is in agreement with the results reported by Ozawa and Komamine, (1989) that genotype was an important factor affecting regeneration potential *in vitro*.

4.3. Protoplast isolation culture and plant regeneration

4.3.1. Protoplast isolation, culture and division

Protoplasts were obtained by enzymatic digestion of the embryogenic suspension cells of all the rice lines using either short-term incubation or overnight incubation method. Protoplast yield and viability remained unaffected by the two methods of incubation, which is understandable in view of the fact that concentration of enzymes was reduced to ½ in overnight incubation. Cellulase R10 contains β-1,4-glucasae and digests cell wall materials that comprises of cellulose and hemicellulose. Pectolyase is a protein with high activity of pectin lyase and polygalacturonase. Pectolyase Y23 digests the pectic substances that comprise about 5% of the primary cell wall and are important in maintaining the structure of the cell and in establishing connection between plant cell. Protoplast isolation from cell suspension cultures of rice following enzymatic digestion of suspension cells have been reported by several groups (Abdullah *et al*, 1987, Zhang, 1990, Su *et al.*, 1992; Wu and Zapata, 1992; Baset, 1992; Bhattacharjee and Gupta, 1995; Jain *et al.*, 1995; Bhattacharjee *et al.*, 1998; Azhakanandam, 1999, Bhattacharjee *et al.*, 1999) and the results of the present investigation are in agreement with them.

Protoplast yield was greatly influenced by the age of culture. Very young cell suspension cultures (less than 2 month old) were normally composed of large cell

clumps, consisting of irregular and elongated cells, therefore, higher yield of protoplasts could not be obtained from these cell suspensions. In contrast, higher yield was obtained from the suspension cultures that were between 4-12 months old. Cell suspensions older than 12 month, although gave high yield of protoplasts but the plating efficiency and plant regeneration percentage both were poor. Similar observations were reported by Abdulla *et al.*, 1987, Zhang, 1990, and Datta *et al.*, 1992b. With an increase in the age (more than 12 month), cell suspensions became less cytoplasmic and consisted of more irregular (non-isodiametric) cells, which caused reduction in protoplast yield. This is in accordance with the results of Bhattacharjee and Gupta 1995, Tang, 1995. Therefore, in the present investigation, use of 4-12 month old cell suspensions was optimum for protoplast isolation and high frequency plant regeneration. Protoplast yield and viability were found genotype-dependent in the present investigation. Amongst all the rice lines used here, the highest protoplast yield was recorded from cell suspensions of Japonica rice lines as compared to cell suspension of Group I Indica and CMS lines. Bright green staining of freshly isolated protoplasts with FDA under UV light confirmed the viability of protoplasts. FDA is a non-fluorescing and non-polar stain, and freely permeates across the plasma membrane. Inside the living cell it is cleaved by esterase activity, releasing the fluorescent polar portion fluorescein. Since fluorescein is not freely permeable across the plasma membrane, it accumulates mainly in the cytoplasm of intact cell, but in dead and broken cells it is lost. When illuminated with UV light viable cells fluorescence green (Widholm, 1972).

An improvement in the plating efficiency was observed by heat shock treatment to the protoplasts before culture. In Japonica rice lines (RCPL 1-2C, RCPL 1-3C, and Nami), heat shock-treated protoplasts exhibited a significant increase in the division frequency of plated protoplasts on day 7 and day 15 as compared to untreated protoplast. However, in case of Indica and CMS rice lines, protoplasts did not divide without heat shock treatment before culture. Thompson *et al.*, (1987) evaluated the effect of heat shock treatment to rice protoplast and reported improvement in the stability and division of cultured rice protoplasts by combining the application of heat shock treatment with embedding in agarose. Similar result was reported by Zhang (1990). Lin *et al.*, (1991) investigated biochemical changes in heat shock treatment and suggested that heat shock protein (70, 59, 51 and 34 KD) might play a role in the enhancement of cell division and protoplast stability. Methods of protoplast culture play an important role in increasing plating efficiency and subsequent plant regeneration in Japonica as well as Indica rice lines. Plating efficiencies were higher when culture medium was semi-solidified with Sea Plaque agarose and protoplasts were cultured in presence of nurse in 'donut' holes. Division frequency of prototoplasts plated in presence of nurse, in liquid culture medium was less than that of agarose-solidified culture medium. Agarose solidified media have been reported beneficial in a number of plant species as a means of improving plating efficiencies, formation of embryo-like structure and plant regeneration from protoplast-derived calli. Although, the role of agarose in improving protoplast division is not well understood but, culturing protoplasts in agarose-solidified media reduces protoplast expansion and consequent budding during the early stages of culture. It has also been

suggested that surrounding protoplasts with agarose reduces the diffusion of cell wall precursors and other metabolites through the medium (Takeuchi and Komamine, 1982, Thompson, 1986).

The results of the present investigation show that suspension cells either in the form of nurse (in direct contact with culture medium) or in the form of feeder (separated by membrane filter), help initiate and sustain the division of protoplasts. Feeder cells were found essential for protoplast culture of all the Indica rice lines studied here. None of the protoplasts divided in absence of feeder cells except in case of Japonica rice line RCPL 1-2C where division frequency was less than 10% in absence of feeder cells. As reported by other researchers too, presence of feeder is essential for induction of sustained cell division and colony formation in the protoplasts of the *Japonica* rice varieties, viz. Taipei 309 and Nipponbare (Li and Murai, 1990), Radon and Baldo (Wen *et al.*, 1991), Nortai (Su *et al.*, 1992), Miara (Guiderdoni and Chaïr, 1992) and Taipei 309 (Jain *et al.*, 1995) as well as in *Indica* rice varieties, viz. Chinsurah Boro II, Cyokoto (Kyozuka *et al.*, 1988), IR54 (Lee *et al.*, 1989), IR52 and IR54 (Su *et al.*, 1992), IR58 (Torrizo and Zapata, 1992), IR43 (Ghosh Biswas and Zapata, 1993), IR50 (Timothy and Rangasamy, 1993) and Pusa Basmati and Jaya (Jain *et al.*, 1995). Nurse culture helps in supporting sustained division either through the production of physiologically active compounds or through the prevention of leaching of vital compounds out of the cells (Eigel and Koop, 1989; Jelodar, 1996). The exact components released from nurse cells and their physiological mechanisms are, however, not adequately understood (Jelodar, 1996). It has also been suggested that nurse or feeder cells help in maintaining critical

density of active cells (Funatsuki *et al.*, 1992) which release growth promoting factors, such as organic acids or oligo-saccharides (Birnberg *et al.*, 1988; Jorgensen *et al.*, 1992) into the medium, promoting protoplast division and further development (Ludwig *et al.*, 1985; Eigel and Koop, 1989). It may also be possible that nurse cultures reduce or modify certain deleterious components of the culture media (Krautwig and Lörz, 1995). The overall action of feeder cells is believed to supply growth factors, which promote sustained division in the neighboring cells. Protoplast division and colony formation in the present investigation were found dependent on the age of the cell suspensions that were used in the preparation of feeder cells. Young cell suspensions (2-3 month old) were the best for supporting sustained division in protoplasts.

Yet, another factor, which affects plating efficiency, is the plating density of the protoplasts. In general, the choice of protoplast plating density depends on the species and the size of the protoplasts. As for example, the optimum plating densities reported for Japonica rices vary from 3 to 5 x 10^5 ml^{-1} (Abdullah *et al.*, 1986; Thompson *et al.*, 1986; Wu and Zapata, 1992). In contrast, it varies from 1 x 10^5 ml^{-1} (Ghosh Biswas and Zapata, 1991) to 3 x 10^5 ml^{-1} (Lee *et al.*, 1989; Su *et al.*, 1992) in Indica rices. In the present study however, relatively higher plating densities (1.0-2.0 x 10^6 ml^{-1}) gave better plating efficiencies. At lower densities, protoplast division declined and this resulted in low plating efficiency. Similar requirements of high plating density have been reported for some other varieties of Japonica rices viz., Shuangbai A, Tai 2A and IR58024A (Xue and Earle, 1995), Indica rices viz. Pusa Basmati 1 and IR36 (Tang, 1995), IR72 (Datta *et al.*, 1992b) and the allied species *Hordeum vulgare* L. (Jähne *et al.*, 1991b; Yan *et al.*,

1990). Beneficial effect of high plating density on plating efficiency has also been reported in protoplast culture of Iranian rice lines (Jelodar, 1996). Our results are thus, in agreement with the reported results of Japonica and Indica rice protopolast culture.

The plating efficiency of different rice lines very greatly because the developing colonies were counted (by various researchers) at different time intervals by different workers. Plating efficiency after 10-15 days is usually high and range from 3.4 to 9.8 % (Kyozuka *et al.*, 1987) and 5 to 42 % (Abdulla *et al.*, 1987) in Japonica rice lines. Plating efficiency after four weeks is although generally low but it reflects sustained division leading formation of colony that were bigger than 1 mm diameter and therefore, appear to be more meaningful. In the present investigation, the plating efficiencies, on day 30 for Group I Indica rice IR36 ranged from 0.96 to 2.3% and for IR65 it ranged from 0.47 to 1.72% which are lower than that of IR54 (0.001 to 3.0%; Lee *et al.*, 1989) and much higher than that of IR72 (0.0004 to 0.04%) as reported by Datta *et al.*, 1992. In Japonica rice line, RCPL 1-2C, plating efficiencies ranged from 0.21 to 0.27% on 30th day. The reported plating efficiency after 3-4 weeks ranged from 0.1 to 1.2% in Japonica rices (Abdulla *et al.*, 1987). The plating efficiencies in the present investigation, in Indica and Japonica rice lines are comparable to the reported plating efficiencies of suspension derived protoplast of rice.

4.3.2. Plant regeneration from protocalli

A relatively simple and reproducible protocol procedure for the regeneration of plants from protoplasts of the rice varieties is essential for genetic manipulation. One of the major objectives in this study was to maximize the frequency of plant regeneration from

protoplast-derived calli (protocalli) of different rice lines. Plantlets were regenerated from protocalli obtained from protoplasts of three Japonica rices (RCPL1-2C, RCPL1-3C and Nami), two Group I Indica rices (IR36 and IR65), three WA CMS line (V20A, IR62829A, IR54752A) and its fertile maintainers (V20B, IR62829B, IR54752B). As reported by other researchers, plant regeneration and their frequency is dependent on genotype as well as combinations of plant growth regulators used during regeneration (Tsukahara and Hirosawa, 1992). In the present study, plant regeneration frequency in Japonica lines, RCPL1-2C ranged from 13.5 to 31.2% which is comparable to earlier reports of regeneration in Japonica rices where, frequency of plant regeneration has been reported to vary from 2 to 34% (Lee *et al.*, 1989) and 2.7 to 13.3% (Datta *et al.*, 1990). Plant regeneration frequency in *indica* rice line, IR54, has been reported to range from 2 to 34% (Lee *et al.*, 1989) which is lower than the frequency reported here from Group I Indica rice lines IR36 and IR65 (12-49%). Higher plating efficiency and higher frequency of plant regeneration helped us to couple the present protocol with transfer of CMS through protoplast fusion (Bhattacharjee *et al.*, 1999). Plantlets could be regenerated from protoplast-derived colonies of three *Japonica* rice varieties and four *Indica* rice cultivars on regeneration medium MSB that contained 3.0 mg l^{-1} kinetin and 0.5 mg l^{-1} NAA. This is in agreement with previous reports, where low concentration of auxin (NAA) in combination with moderate level of cytokinin (kinetin) were found suitable for plant regeneration from protocalli of *Japonica* rice lines (Abdullah *et al.*, 1986, Bhattacharjee and Gupta, 1995) and wild rices (Baset *et al.*, 1992). Similarly a combination of kinetin and NAA in the regeneration medium have also been reported to

be beneficial for plant regeneration from protocalli of *Indica* rice (Lee *et al.*, 1989; Ghosh-Biswas and Zapata, 1991; Datta *et al.*, 1992b, Azhakanandam, 1999). The type and concentration of plant growth regulators in the regeneration medium have also been reported to be critical factors in plant regeneration from protocalli of rice (Lee *et al.*, 1989; Datta *et al.*, 1992b). No significant difference was however, observed between BAP and kinetin on regeneration from protoplasts of Nortai, a *Japonica* cultivar (Su *et al.*, 1992).

4.3.3. Protoclonal variation in the regenerants

Insignificant protoclonal variations were found in Japonica rice line, RCPL1-2C, RCPL 1-3C and Nami except in fertility, which ranged from 20-32% in RCPL1-2C; 45-69% in RCPL1-3C and 31-78% in Nami against 80-85% in seed-derived plants. In case of Group I Indica rice, IR36 and IR65 plant height and panicle length of R0 plants were shorter than the respective controls. On the average, protoclones of IR36 and IR65 showed lower number of spikelets/panicle than the controls. Reduction in the number of spikelets/panicle has also been reported in R0 plants of Nipponbare, Fujisaka 5 and Iwaimochi and this was accompanied by marginal reduction in spikelet fertility of Nipponbare and Fujisaka 5 but was substantial in Iwaimochi (53.9 and 75.4%) in R0 and controls respectively (Ogura *et al.*, 1987). Average spikelet fertility in the present investigation was 55.2% in R0 of IR36 and 72.65% in controls. It was, however, almost like the control in IR65 (76.4 and 77% in R0 and control, respectively). Other researchers have reported severe to marginal reduction in spikelet fertility of R0

protoclones, which ranged from 3.4 to 93% in Nipponbare (Li and Murai, 1990) and 0 to 80% in Tepi Boro (Alam *et al.*, 1994) with an average of 65.4 to 70% respectively. Fertility in the protoclones seems to depend on the quality and age of cell suspension used for protoplast isolation. In the present investigation, only young cell suspensions were used therefore, fertility level was satisfactory in the protoclones.

No significant difference in PBT, spikelet fertility, 100 seed weight and yield/plant was observed between R2 progeny of IR36 & IR65 and seed-derived controls. Plant height, nevertheless, continued to show significant difference between the control and R2 plants of both the lines. Panicle length and spikelets/panicle were significantly different in the R2 progenies of IR36, however, they remained at par with control in the R2 lines of IR65. Over all, significant negative shift was noticed in most of the characters (recorded in this investigation) of R0 plants. All these characters, however, recovered in R1 and R2.

Consequently, plot yield of R1 and R2 progeny in both the lines remained at par with that of seed-derived controls. Abdullah *et al.*, 1989 studied protoclonal variation in R1 progenies of Taipei 309 and reported unidirectional positive shift for spikelets/panicle and negative shift for panicle length. In the present study, however, unidirectional negative shift was recorded for both the characters in both the lines. The outcome of the present investigation are broadly in agreement with that of Ogura *et al.*, 1987 and Alam *et al.*, 1994 who studied protoclonal variations in R0 plants. In the present investigation, marginal protoclonal variations in protoplast-derived plants and controls indicates that, rice protoplasts can be utilised in protoplast-mediated genetic manipulations.

4.4.1. Protoplast fusion

In the present investigation, γ-ray-inactivated protoplasts of donor lines and iodoacetamide-inactivated protoplast of recipient lines were fused electrically in separate experiments to transfer CMS from donor lines to the nuclear background of fertile recipient lines. Metabolic complementation between irradiated and IOA-treated protoplasts was successfully used here to produce rice cybrids. As reported earlier, X-ray or γ-ray irradiation of protoplasts cause fragmentation of nuclear DNA (Yang *et al.*, 1988; Akagi *et al.*, 1989; Kyozuka *et al.*, 1989). In order to transfer CMS from A58 rice line, protoplasts of CMS line, A-58 were irradiated with a range of γ-rays doses that varied from 0.7 to10.7 krad delivered by a ^{60}Co source (350 rad/min) to inactivate them. Inhibition of division and colony formation were observed only after treatment with more than 5.3 krad γ-rays (Yang *et al.*, 1988). In another report by Akagi *et al.*, 1989, high doses of X-rays (125 krad) was required to inactivate the protoplasts of donor Japonica CMS line, MTC-9A. Yet in another experiment, protoplasts of Indica CMS line, Chinsursh Boro II (CB II) were inactivated by 70 krad X-rays before fusion (Kyozuka *et al.*, 1989). In the present investigation, protoplasts of Indica CMS lines, V20A, IR62829A and IR54752A required irradiation of 30 krad γ-rays for inactivation as evidenced by complete inhibition of colony formation. However, most of the protoplasts remained alive after irradiation (with 30 krad γ-ray) as revealed by microscopic examination. This is in agreement with the results of Akagi *et al.*, 1989 and Fujimura *et al.*, 1996. Inactivation of protoplasts with high doses of γ-ray used in this experiment is supported by the study of Vardi *et al.*, 1987. A dose of 50 to 60 krad

gamma rays (60-Cobalt) was required for virtually complete division-arrest of Citrus protoplasts (Vardi *et al.*, 1987). Radiation induced elimination of donor chromosome was found to increase with increasing the dose of γ-ray for all doses tested (5-50 krad) was confirmed by dot-blot hybridization in *N.tobacum* (+) *N. plumbaginifolia* hybrids, but that donor chromosome elimination is an inherently variable process as reported by Trick *et al.*, 1994.

Iodoacetamide (IOA) has widely been used in cybrid production. However, lower concentrations of IOA did not prevent division and colony formation. Therefore, higher concentration of IOA was required to minimize uniparentally derived callus formation. Protoplasts of 'Fujiminori' were treated with 4.0 mM IOA to stop division (Yang et *al.*, 1988). In another study, Akagi *et al.*, 1989 reported formation of many colonies when IOA was used at concentration lowers than 25mM. Similarly, the protoplasts of fertile recipient line, N8 were inactivated at a concentration of 30 mM IOA to eliminate the possibilities of colony formation from non-cybrid cells after fusion (Akagi *et al.*, 1989). In the present investigation, 10mM concentration of iodoacetamide (IOA) was found optimum to inactivate the protoplasts of fertile recipient lines for cybrid production. Lower concentration of IOA could not prevent the division of protoplasts and higher concentrations showed deleterious effect on the protoplasts. The results of IOA inactivation in this study are in agreement with those reported earlier (Yang *et al.*, 1988 and Akagi *et al.*, 1989).

Electrofusion has extensively been used in protoplast fusion (Zimmermann and Schleurich, 1981; Zimmermann and Vienken, 1982; Jones *et al.*, 1994) for the

production of somatic hybrid as well as cybrids. Electrofusion has several advantages over chemical fusion: (i) higher frequency of binary fusion (ii) non-toxicity and (iii) minimal loss of fusion products in course of washing which is necessary to remove fusogens (Tempelaar and Jones, 1985; Waara and Glimelius, 1995). The recovery of maximum electrofusion value depends on a number of factors including protoplast origin, size and chain length. Leaf mesophyll protoplasts fused much more readily than suspension–derived protoplasts (Kao and Michayluk, 1989). Large protoplasts tended to fuse more readily than small protoplasts. In short chains (composed of ≤ five protoplasts), the fusion frequency was lower, but products of one-to-one fusion were greater than in long chains composed of ≥ 10 protoplasts (Tempelarr and Jones 1985; Bates 1985, Kao and Michayluk, 1989). Other factors that affect pair formation and fusion frequency are the AC and DC voltage and pulse lengths. Under AC field, the protoplasts formed aggregates or bridges of two or three on the electrodes. This 'pearl chain' arrangement of the cells was stable only for the duration of the applied field. By the additional application of a single DC field pulse, it was possible to induce cell fusion within the aggregates or bridges. As reported by Yang *et al.*, 1988; voltage of AC fields effect the protoplast pair formation. When AC fields of various strengths were applied for 60 sec at 500 kHz, the respective pair formation was 5.8%, 12%, 32% and 21.0% for fields of 25, 50, 100 and 200 Vp-p/cm. Due to the higher percentage of pair formation (32%), 100 Vp-p/cm at 500 kHz for 60 sec was used as the AC field for pair formation. In the present investigation, an AC field of 25V/kHz strength for 25 sec was found optimum for pair formation (20.08-35.54%). Close contact between membranes of

adjacent protoplasts was attained by further momentary increase in the AC field strength to 30V/kHz for 25sec, whilst protoplast fusion occurred following DC pulses. Higher percentage of pair formation resulted in higher fusion frequency.

Effect of DC field strength on fusion frequency and colony formation was studied by Yang *et al.*, 1988 for a fixed period of 40 μsec. Fusion frequency peaked at 1 and 1.25 kV/cm at 40 μsec. However, colony formation decreased with an increase in field strength. Therefore, a DC pulse of 1kV/cm at 40 μsec was applied to obtain 14% fusion frequency of protoplasts and colony formation of 1.6% relative to the total protoplast population (Yang *et al.*, 1988). In the present investigation, the maximum fusion frequency ranged from 5 to 6%, which was achieved using a DC field strength of 900 Vcm^{-1} 30 μsec and a colony formation of 7-15% relative to the total protoplast population.

4.4.2. Culture of fusion product and induction of division

The fusion products between V20A x V20B, IR62829A x IR62829B and IR54752A x IR54752A served as control for the transfer of CMS from V20A to the nuclear background of RCPL 1-2C, RCPL 1-3C and Nami; IR62829A to the nuclear background of RCPL 1-2C, RCPL 1-3C and Nami and IR54752A to the nuclear background of RCPL 1-2C, RCPL 1-3C and Nami, respectively. Protcolonies formed only after irradiated protoplasts of CMS lines were fused with IOA-treated protoplasts of recipient lines and no colonies were observed from unfused cells. Metabolic complementation between nucleus and cytoplasm is known to restore the capacity for cell division in the

fused protoplasts. (Zelcer *et al.*, 1978). In the present study, fused products, cultured in presence of nurse, regenerated cell wall within 24 to 36 hours when cultured in the 'donut' holes. First cell division was observed within 5 to 6 days. On the other hand, the fused products, cultured on the membrane over feeder cells started dividing only on 7 to 9 day of culture. Further divisions led to the formation of microcolonies after 20 to 25 days, both in the 'donut' holes as well as on the membrane filter and they became macroscopic after 30 to 40 day of culture after serial reduction in osmoticum at 7 day interval. Fused products cultured in absence of nurse did not divide and they became vacuolated after few days of culture. This is understandable in view of requirements of nurse for undergoing sustained division in the protoplasts. In absence of nurse, the fusion products did not undergo division.

4.4.3. Plant regeneration from putative cybrid calli

Plant regeneration percentage from the putative cybrid calli was dependent on the age of the suspension culture used for protoplast isolation and fusion. Protoplasts isolated and fused from young suspension (4 - 8 months) cultures showed significant difference in plant regeneration from putative cybrid calli than the protoplasts from older (8 months) cell suspensions. Frequency of regeneration was higher in case of fusion events where V20A CMS line was used as cytoplasm donor then the other sets of fusion where IR62829A and IR54752A CMS lines were used as cytoplasm donor. It may be due to the fact that protoplast to plant regeneration from V20A line was much easier than the other two CMS lines (IR62829A and IR54752A). Even, when compared with V20A

protoplasts, frequency of protoplast division was too low in CMS lines IR62829A and IR54752A when cultured in presence of nurse.

4.4.4. Morphology and molecular analysis of putative cybrid plants

Cybrids plants produced through protoplast fusion showed normal characteristics except that the cybrids between V20A & RCPL1-2C showed their resemblance with that of RCPL1-2C in its tall stature presumably due some contribution of the nuclear genome by RCPL1-2C. On the contrary, the cybrids between all other lines were of the same height as that of the respective CMS line. The progenies did not segregate for agronomic characters showing thereby the stability in the constitution of the progenies obtained from fusion-derived cybrids. Pollen grain staining and pollen germination tests from all the cybrid plants confirmed sterility of cybrids.

Cybridity was confirmed by mtDNA analysis of cybrids. *orf155* has been shown to exhibit polymorphism between CMS and fertile cytoplasm by distinguishing them through the presence of *orf155* in 1.3 kb fragment in *Hind* III-digested mtDNA of the CMS line and 12 kb fragment in the mtDNA of maintainer lines. In the present investigation, the localization of *orf155* gene in 1.3 kb fragment of the *Hind* III-digested mtDNA of the cybrids confirmed transfer of mitochondrial genome from WA CMS lines to the cybrids. All the cybrid plants were completely male sterile but they set seeds on crossing with the respective maintainers. Recombination in the mtDNA during cybridization and somatic hybridization has been reported in many plants (Belliard *et al.*, 1979, Ozias-akins *et al.*, 1987, Akagi *et al.*, 1989). Conversely, lack of

recombination within the mitochondrial genome has also been reported in *Brassica* (Barsby *et al.*, 1987). In the present investigation, the results of mtDNA analysis from all the cybrid lines did not show alteration in the molecular weight of *Hind* III-digested DNA fragment that hybridized with *orf155*. This indicated absence of recombination in the mitochondrial genome at least in and around *orf155*. In addition, the appearance of complete sterility in the cybrids suggested absence of change in the area conferring CMS trait. The results of the present investigation on transfer of mitochondrial genome through asymmetric fusion are in agreement with the reported results of Yang *et al.*, 1988 and Kyozuka *et al.*, 1989.

4.4.5. Molecular identification of CMS and maintainer lines

Following PCR technique, it was not possible to differentiate the WA CMS lines V20A, IR62829A and IR54752A from fertile lines V20B, IR62829B, IR54752B, RCPL1-2C, RCPL1-3C and Nami using different sets of primers specific for different mitochondrial genes. One thing is very clear from this study that the WA cytoplasm is very different from the Chinsura Boro II (BT) cytoplasm. The earlier studies with BT cytoplasm have specially dealt with polymorphism in the *atp6* gene and generation of a chimeric *atp6* gene designated as *urf-rmc* (Kadwaki *et al.*, 1990). Further, altered processing and incomplete editing of the *atp6* transcript (Iwabuchi *et al.*, 1993) and the expression of anti-sense transcripts from an open reading frame downstream from *atp6* (Akagi *et al.*, 1994) has also been reported. This region downstream from *atp6* in the mitochondrial genome of BT cytoplasm is closely related to the appearance of CMS. This region has

been shown to code *orf79*, which is a chimera of another mitochondrial genome region (Akagi *et al.*, 1994). Using a primer pair located in a unique sequence downstream from *atp6* of BT cytoplasm (*orf79*), specific amplification was observed in CMS lines but not in fertile lines, which could distinguish the sterile and fertile cytoplasm (Akagi *et al.*, 1995).

However, in case of WA CMS either *atp6* gene or *orf79* gene could not differentiate the sterile WA cytoplasm from the fertile lines. For rice *atp6* gene, a single fragment of size 1.2 kb was amplified from all the CMS and maintainer lines. Using primer specific for rice gene *urf79* no amplification was observed in CMS as well as in fertile lines which confirms that *atp6* gene or *orf79* gene may not play any role in conferring CMS trait to WA cytoplasm. Even when, primer specific for whole *coxII* gene of rice was used, a 3 kb fragment was amplified in all the CMS and maintainer lines. Using a primer pair specific for rice gene *orf155* yielded a single fragment of 1 kb in CMS as well as in maintainer lines. Therefore, molecular identification of fertile and sterile lines was not possible following PCR amplification of mtDNA using different primers specific for several mitochondraial genes.

Southern hybridisation of *atp6* and *coxII* gene probe to *Hind* III-digested mtDNA from all the CMS and maintainer lines produced identical hybridisation signals (1kb and 3kb fragment respectively) without distinguishing the sterile and fertile cytoplasm. However, when *Hind* III-digested mtDNA of CMS line, V20A, IR62829A and IR54752A were probed with *orf155* gene probe; it was located on a 1.3 kb fragment in CMS lines and 12 kb fragment in the maintainer lines viz. V20B, IR62829B, IR54752B,

RCPL1-2C, RCPL1-3C and Nami. Thus, the localisation of this gene could distinguish the mtDNA of CMS and maintainer cytoplasm.

Among all the mitochondrial genes studied here, the only gene that revealed differences in the cytoplasm of sterile and fertile cytoplasm was *orf155* which was supported by the studies done by Seth *et al.*, 1996. This gene has been found in all the plant species studies and is known as *orf* B in *Oeonothera* and sunflower (Seth *et al.*, 1996). This open reading frame encodes for a 155 amino acids membrane bound polypeptide (Srivastava *et al.*, 1997) in rice unlike in wheat where it is believed to encode for a 156 amino acids membrane bound polypeptide of unknown function (Gualberto *et al.*, 1988). No differences were observed in the coding region of *orf155* between the sterile and fertile lines. Further studies showed that *orf155* revealed differences in transcription between WA CMS and fertile lines. All the fertile maintainers and restorer lines exhibit a single transcript of 0.7 kb in size. While WA CMS lines exhibit an additional 1.1 kb transcript of lower intensity. The 3'flanking sequence of *orf155* (which is homologous to 3' flanking sequence of *coxII*) is transcribed as part of the additional 1.1 kb transcript in the sterile lines but not in the fertile lines (Srivastava *et al.*, 1997). This additional transcript of 1.1kb in the sterile lines may thus represent either an unprocessed form or co-transcription with an open reading frame either upstream or downstream from *orf155*

4.4.6. Female fertility and characterisation of BC1 plant

Seed set in the cybrids crossed with maintainers showed that the female fertility of the cybrids remained intact. In addition, when crossed with the maintainer, male sterility

was inherited to the backcrossed progenies. Presence of sterile pollen grains and localization of *orf155* in 1.3 kb fragment of the *Hind* III-digested mtDNA in backcrossed progenies confirmed the inheritance of the CMS through protoplast fusion.

4.4.7. Fertility restoration in cybrids

Restoration of fertility of the cybrid lines was confirmed by crossing them with IR36, a known restorer of fertility of wild abortive CMS. Seeds obtained from these crosses, when germinated, the F1 plants showed fertility ranging from 75-88%. Fertilities ranging from 61 to 100% have been classified as fully fertile (Virmani, 1996). Therefore, the fertility-restored plants of the present investigation can be treated as fully fertile demonstrating thereby the restoration of fertility in the cybrids obtained through protoplast fusion. These cybrids behaved as that of WA CMS in V20A in their response to restorer genes. In addition, the restoration of fertility in fusion-derived cybrids shows that WA CMS transferred to new genetic background through protoplast fusion can be employed in hybrid seed production.

The results of the present investigation demonstrate that protoplast fusion can be a powerful aid to plant breeding the enhancing its efficiency by expediting transfer of CMS to new genetic backgrounds. In addition, it will also help in expanding the genetic base, which has hitherto not been possible through conventional breeding.

CHAPTER 5

SUMMERY

CHAPTER 5 : SUMMARY

Cytoplasmic hybridisation, a method for transferring mitochondrial-encoded traits like cytoplasmic male sterility, requires a donor-recipient protoplast fusion based on efficient protoplast-to-plant regeneration system. The genetic engineering of economically important cereal, rice through cytoplasmic hybridisation depends, primarily on the ability of the protoplasts to regenerate into fertile plants. The major objectives of the present investigation were, therefore, to establish a reproducible plant regeneration system from the protoplasts of Indica, Japonica, CMS lines of rice and their respective maintainers followed by production of cybrids therefrom. The summary of the results is as follows:

1. Callus induction, establishment of cell suspension, culture of protoplasts and plant regeneration

Since protoplasts isolated directly from meristemetic cells of rice are extremely recalcitrant in culture; embryogenic cell suspension cultures were used as a source of protoplasts in this investigation. The cell suspensions were initiated and established from the embryogenic calli obtained from various explants viz. scutella of mature seeds and immature embryos, leaf and stem bases. Scutella of mature seeds and immature embryos were the best sources of explants for the initiation of embryogenic calli.

Although immature embryos in a strictly defined stage, were more suitable for the initiation of embryogenic calli but mature seeds were the most commonly used explants because of their availability throughout year. The results of the present investigation

showed that genotype and culture media influence the production of embryogenic calli to a great extent. Embryogenic calli were produced from mature seeds and immature embryos of different lines including Japonica rice (cvs. RCPL 1-2C, RCPL1-3C and Nami), Group I Indica rices (cvs. IR36, IR64, IR65 and IR74), CMS lines (cvs. V20A, IR62829A and IR54752A) and their maintainers (cvs. V20B, IR62829B and IR54752B). The results of this investigation are in agreement with that of others (Abdullah *et al.* 1986; Finch 1991; Baset 1992; Bhattacharjee and Gupta, 1995; Jelodar, 1996, Bhattacharjee *et al.*, 1998; Azakanandam, 1999) who reported formation of two types of calli (embryogenic and non-embryogenic) from the scutella of mature and immature embryos of rice. High percentage of plant regeneration from the embryogenic calli of all the rice lines studied here is also in accordance with the previous reports, in which efficient plant regeneration was reported from the embryogenic calli of Indica, Japonica and CMS rices (Tang, 1995; Bhattacharjee and Gupta, 1995; Xue and Earle, 1995; Jelodar, 1996; Lee, 1996; Bhattacharjee *et al.*, 1998; Khanna and Raina, 1997; Xue and Earle, 1995; 1998, Azakanandam, 1999, Bhattacharjee *et al.*, 1999). Plant regeneration was, however, influenced by many factors including the genotype of the plant, origin of explants and composition of the basal medium used for regeneration.

The production of embryogenic calli from all the rice lines was essential for initiation and establishment of suspension cultures. A number of factors including genotype and culture media were found important for the establishment of cell suspension cultures. In addition, selection of callus type was a crucial first step, coupled with regular replacement of culture medium to minimise phenolic oxidation during the early culture.

Choice of genotype is one of the important factors for the establishment of embryogenic suspension cultures (Abdullah, 1987). It was observed here that the callus obtained from scutella of mature seeds and immature embryos were the best explants for initiation of suspension cultures of Japonica, Indica and CMS lines. While Indica and CMS lines showed varying degrees of browning during the initiation of cell suspension cultures, calli of japonica lines did not show browning and dissociated faster in liquid medium. Similar results have been reported by Thompson (1986) on establishment of cell suspension of Japonica line, Taipei309. Indica rices are known to be more difficult concerning the establishment of cell suspension cultures. Once embryogenic callus of Indica rice was transferred to liquid culture medium, cell suspensions became viscous and large cell clumps were formed. The removal of large cell clumps by sieving resulted in the enrichment of the suspension cultures with small groups of round, actively dividing cytoplasmic cells. Similar results were also reported by Zhang, 1995 and Blackhall *et al.*, 1999 for Group I Indica rice.

Besides plant genotype, the present study also demonstrated the importance of culture medium in initiation and establishment of cell suspensions. AA medium supplemented with high concentration of 2, 4-D was suitable for the initiation and maintenance of suspension cultures of all the rice lines. Higher concentration of 2,4-D was found essential in preventing rhizogenesis and an irreversible conversion of potentially embryogenic cells to the non-embryogenic state. A similar phenomenon was observed by Mitsouka *et al.* (1994), who reported that high intracellular concentration of 2,4-D suppressed redifferentiation process after each subculture, and, therefore, the higher

concentration of intracellular 2,4-D caused lesser browning. Vasil (1983) described similar requirements for the establishment of embryogenic cell suspension cultures of other graminaceous species. AA medium, which consists of four amino acids as the sole source of nitrogen, has been largely used for the initiation of fine embryogenic cell suspension cultures in rice by several researchers (Ghosh Biswas and Zapata, 1991; Baset *et al.*, 1991; 1993; Su *et al.*, 1992; Guiderdoni and Chaïr, 1992; Torrizo and Zapata, 1992; Yin *et al.*, 1993; Giri and Reddy, 1994; Jain *et al.*, 1995, Bhattacharjee and Gupta, 1995, Bhattacharjee *et al.*, 1998. Bhattacharjee *et al.*, 1999). The amino acids as the sole nitrogen source for rice cell suspension appeared beneficial for the growth of small actively dividing cell clumps. In the present study, for the establishment of cell suspension cultures of Indica rice lines IR74 and IR64 and CMS lines IR62829A and IR54752A, presence of proline in the AA medium was, however, found essential for the establishment of a fine suspension culture. This is in agreement with the results of Datta *et al.*, (1992) for Indica rice line IR72.

The regeneration capacity of such cell suspension cultures lasted only for 8-12 months. This imposed a practical constraint on the use of rice protoplasts for plant genetic manipulation purposes. Similar reduction in per cent plant regeneration with increase in the age of cell suspensions has been reported in barley (Lührs and Lörz, 1988; Karp and Lazzeri, 1992), Japonica as well as wild rices (Finch, 1991; Baset, 1992). Cell suspensions were, therefore, progressively initiated from the calli of mature seed and immature embryos to ensure on-going availability of totipotent suspension cultures. Once embryogenic cell suspension cultures were established, such cultures were used as

source for the isolation of viable protoplasts. In the present study, the isolation of large numbers of highly viable protoplasts was possible from embryogenic cell suspension cultures of rices by enzymatic digestion of the cultured cells using an overnight incubation method. This procedure has been successfully employed by many workers for isolating protoplasts from cell suspensions of rice (Su *et al.*, 1992; Wu and Zapata, 1992; Baset *et al.*, 1993; Jain *et al.*, 1995). Many factors were found to determine the protoplast yield, plating efficiency and, eventually, plant regeneration. The age of culture was, however, the most important and greatly influenced protoplast yield. Older cultures (5-12 months), although, gave higher yields but the frequency of regeneration was low. Therefore, older suspensions proved good provided they were capable of satisfactory plant regeneration. It was not possible to obtain high protoplast yields from newly initiated cell suspension cultures (less than 3 months after initiation). This might have been due to the heterogeneous nature and large amounts of irregular and elongated cells present in such young suspensions. Similar observations were reported by Zhang (1990), Tang (1995), Jelodar (1996), Lee (1996) and Azakanandam (1999). With passage of time, the suspensions consisted of more and more densely cytoplasmic cell clusters and therefore protoplast yields increased accordingly. However, after a period of 12 months of culture, depending on the cultivars, cell suspensions began to become less and less densely cytoplasmic and contained more irregular cells, with corresponding decrease in protoplast yield.

In the present investigation, protoplasts all the rice lines were cultured using both 'donut' culture method and the membrane filter method in presence of nurse. Sustained

protoplast division and colony formation were obtained by the use of both the culture methods. Nurse cells were, therefore, absolutely required for sustained protoplast division and colony formation from all the rice lines. Protoplast plating density is important in initiating and sustaining division of protoplast-derived cells. The influence of protoplast plating density on plating efficiency indicated that the optimum density was 1.0×10^6 protoplasts ml^{-1} for all the cultivars. The beneficial effect of high plating density on plating efficiency was also reported in protoplast culture of the Indica rice cv. IR72 (Datta *et al.*, 1992b) and barley (Yan *et al.*, 1990).

Protoplast-derived calli of all the varieties regenerated plants after 3-4 weeks of culture. As studied by other researchers, a wide range of factors including genotype of the cultivar and specific combinations of plant growth regulators was found to influence plant regeneration from the protocalli. In general, Japonica rice varieties are more responsive to *in vitro* culture than are Indica rices. Differences in culture response have also been observed among different groups of Indica rice cultivars. These facts support the general principle of genotypic dependency for totipotency in rice (Tsukahara and Hirosawa, 1992). The type and concentrations of plant growth regulators in the regeneration medium are critical factors for plant regeneration of rice (Lee *et al.*, 1989; Datta *et al.*, 1992b). In the present investigation, a combination of kinetin and NAA in the regeneration medium was found beneficial for plant regeneration from protoplast-derived calli of all the rice lines. This is in agreement with the results of other researchers (Abdullah *et al.*, 1986, Lee *et al.*, 1989, Baset *et al.*, 1991, 1993, Ghosh-Biswas and Zapata, 1991; Datta *et al.*, 1992b, Bhattacharjee and Gupta, 1995,

Bhattacharjee *et al.*, 1998, Azakanandam, 1999, Bhattacharjee *et al.*, 1999). The results obtained in the present investigation suggest that genetic manipulations like somatic hybridization and cytoplasmic hybridization, which involve protoplast fusion and therefore, requires large amounts of viable, densely-cytoplasmic protoplasts, should be carried out as soon as embryogenic cell suspensions become usable. This will avoid loss of totipotent protoplasts, reduction in protoplast yield, efficient colony formation and eventually, efficient plant regeneration due to prolonged subculture of the suspensions.

2. Production of new CMS lines through protoplast fusion and molecular characterisation of the cybrids

Having standardised the method for protoplast-to-plant regeneration, from CMS lines (V20A, IR62829A and IR54752A), the fertile maintainers (V20B, IR62829B and IR54752B) and Japonica rice lines (RCPL 1-2C, RCPL1-3C, and Nami), several cybrid lines were produced following the donor-recipient protoplast fusion method. In the present investigation, the CMS lines were used as donor and the fertile maintainers like Japonica rice lines were used as recipient. Here, CMS trait was transferred to the nuclear background of Japonica rice lines through protoplast fusion. Since the protoplasts of the donor as well as recipient lines were undergoing sustained divisions, therefore, in order to make the selection effective, protoplast of CMS line were irradiated with 30 krad gamma ray that completely inhibited the divisions. Treatment of 10 mM iodoacetamide for 15 minutes at room temperature was optimum to stop the division of protoplasts of the fertile maintainers and of the restorers. Treated protoplasts

on culture did not undergo division and started showing signs of degeneration after 20-30 days after plating. In contrast, untreated protoplasts, under similar condition, divided sustainedly and formed microcolonies after 25-30 days. Inactivated protoplasts were fused electrically. The resultant protoplasts along with fusion products, on culture in protoplast culture medium in presence of feeder, started dividing. Sustained divisions gave rise to colonies after 30-40 days that grew on the membrane filters and formed calli. These calli, on transfer to regeneration medium, differentiated and gave rise putative cybrid plants from all the fusion combinations. Morpho-physiological characters of the cybrids appeared normal except for the pollen grains that were shrivelled, empty and smaller in size as well as in number in all the putative cybrids against normal starch-filled pollen grains in the fertile maintainer and Japonica rice lines. Few pollen grains from some putative cybrid plants (between V20A & RCPL1-2C), although showed staining for starch, however, none of the pollen grains from the putative cybrids (including those showing staining for starch) germinated on culture and all of them failed to form pollen tubes indicating sterility. The cybrids plants produced through protoplast fusion between V20A & RCPL1-2C showed resemblance with that of RCPL1-2C in its tall stature presumably due the contribution of the nuclear genome by RCPL1-2C which is a tall variety.

Cybridity was confirmed by localisation of *orf155* in 1.3 kb fragment of the *Hind* III-digested mtDNA of the cybrids. *orf155* has been shown to exhibit polymorphism between CMS and fertile cytoplasm by distinguishing them through presence of *orf*155 in 1.3 kb fragment in *Hind* III-digested mtDNA of the CMS line and 12 kb fragment in

the mtDNA of maintainer lines. All the cybrid plants were completely male sterile but they set seeds on crossing with the respective maintainers. This showed that the female fertility of the cybrids remained intact. In addition, when crossed with the maintainer, male sterility was inherited to the backcross progenies. Presence of sterile pollen grains and localisation of *orf155* in 1.3 kb fragment of the *Hind* III-digested mtDNA in backcross progenies confirmed the inheritance of the CMS through protoplast fusion. The progenies did not segregate for agronomic characters that showed stability in the constitution of the progenies obtained from fusion-derived cybrids.

3. Future outlook

CMS is a prized agricultural trait that has been employed in hybrid seed production of several vegetables, like carrot, onion, and brassica and field crops like sorghum, sugar beet, sunflower, corn and rice. Hybrid rice has been released for commercial cultivation on a large scale in China and similar work has been initiated in other countries as well. In China, few million hectares of land has been brought under hybrid rice (Yuan and Mao, 1991) and nearly all the hybrid rice production is based on use of cytoplasmic male sterility (CMS) for control of pollination. According to an estimate, more than 90% of the Indica hybrid rice area currently planted carries the WA type of CMS (Yuan and Virmani, 1988). In India almost all the CMS lines possess WA cytoplasm and therefore carries a risk of pest epidemic due to cytoplasmic homogeneity as in case of maize by Southern Corn leaf blight (caused by *Helminthosporium maydes* Nisikado Miy race T) in 1970 in USA and pearl millet by downy mildew and ergot (caused by

Sclerospora grainicola and *Claviceps microcephala* respectively), in India. This has alarmed plant breeders world over about the risk of maintaining cytoplasmic homogeneity and has induced them to diversify the source of CMS in important crops. Although, there are diverse sources of CMS in rice, yet only two sources BT and WA has so far been used world wide in hybrid rice seed production programme which makes the crop vulnerable to epidemics. Thus, there is an urgent need to diversify the source of CMS in rice. At the same time, very little is known about molecular mechanism of CMS in rice; although there are few isolated reports available. The genetic determinants controlling CMS, the molecular mechanism and its expression will provide a deeper insight into the process which will be useful in diversifying the cytoplasmic base thereby making hybrid seed production safer.

A successful hybrid seed production programme for a new region requires routine transfer of cytoplasmic male sterility in one of the well-adopted varieties of that particular area/region. This is usually accomplished by 5-6 recurrent backcrossings, which requires 5-6 years, if one crop is grown each year. This process can, however, be hastened through biotechnological manipulation using protoplast fusion technique. These new CMS lines can be used as a female parent in the hybrid seed production programme for a new region. In future research, protoplast fusion programme in rice should be directed towards cytoplasmic hybridisation through protoplast fusion to produce new CMS lines that can be used for hybrid seed production.

In view of the new intellectual property right, the plant breeders as well as the respective institutions releasing new hybrid varieties of crop would like to undertake the molecular

characterisation /profiling of the hybrids. In order to accomplish this, molecular markers are being identified. In addition, in order to help identify the restorer lines used for hybrid seed production from the germplasm, the molecular markers need to be identified for rapid screening and selection. Therefore, research work in this area will prove rewarding.

CHAPTER 6

APPENDICES

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Appendix I: LSA and LSB callus induction medium (Modified from Linsmaier and Skoog (1965) and N6M callus maintenance medium (Modified from Chu *et al.*, 1975).

Component	Concentration (mg l^{-1})		
	LSA medium	LSB medium	N6M medium[2]
Macronutrients			
KNO_3	1900.0	1900.0	2830.0
NH_4NO_3	1650.0	1650.0	
$CaCl_2$	440.0	440.0	166.0
KH_2PO_4	170.0	170.0	400.0
$MgSO_4$	370.0	370.0	185.0
$(NH_4)_2SO_4$			463.0
Micronutrients			
KI	0.83	0.83	0.8
H_3BO_3	6.20	6.20	1.6
$MnSO_4$	22.30	22.3	4.4
$Na_2MoO_4.2H_2O$	0.25	0.25	
$ZnSO_4.7H_2O$	8.60	8.60	1.5
$CuSO_4.5H_2O$	0.025	0.025	
$CoCl_2.6H_2O$	0.025	0.025	
$FeSO_4.7H_2O$	27.85	27.85	27.85
Na_2EDTA	37.25	37.25	37.25
NaFeEDTA			
Vitamins			
Myo-inositol	100.0	100.0	
Nicotinic acid		0.5	0.5
Pyridoxine HCl		0.5	0.5
Kinetin	1.0		
Thiamine HCl	1.0	0.1	1.0
Glycine	2.0		
amino acids			
Glutamine		500.0	2.0
Other Supplements			
Casein hydrolysate		100.0	
Tryptophan	50.0		
2,4-dichlorophenoxyacetic acid	4.0	4.0	4.0
Sucrose	30000	30000	20000
Mannitol			30000
pH 5.8			

LSA, LSB and N6M media were prepared as single strength in distilled water and made solidified with 0.8% (w/v) agar; autoclaved at 121°C for 20 minutes.

Appendix II : MS0 seed germination medium an and MSB plant regeneration medium (Modified from Murashige and Skoog, 1962)

Component	Concentration (mg l^{-1}) MS0 medium	MSB
Micronutrients		
NH_4NO_3	1650	1650
KNO_3	1900	1900
$CaCl_2.2H_2O$	440	440
$MgSO_4.7H_2O$	370	
KH_2PO_4	170	
Macronutrients		
KI	0.83	0.83
$CoCl_2.6H_2O$	0.025	0.025
H_3BO_3	6.2	6.2
$Na_2MoO_4.2H_2O$	0.25	0.25
$MnSO_4.4H_2O$	22.3	22.3
$CuSO_4.5H_2O$	0.025	0.025
$ZnSO_4.7H_2O$	8.6	8.6
$FeSO_4.7H_2O$	27.85	27.85
Na_2EDTA		
	37.25	37.25
Vitamins		
Inositol	100	100
Nicotinic acid	0.5	0.5
Pyridoxine HCl	0.5	0.5
Thiamine HCl	0.1	0.1
Glycine		
	2.0	2.0
Carbon Source		
Sucrose		
	30000	30000
Growth Regulators		
Kinetin		3.0
NAA		0.5
pH 5.8		

MS0 medium was made up as a single strength solution in distilled water and autoclaved at 121°C for 20 min made solidified with 0.8% (w/v) agar. MSB medium was made up as a single strength solution in distilled water and autoclaved at 121°C for 20 minutes made solidified with 0.8% (w/v) agarose (Sigma Type 1)

Appendix III: AA4 suspension culture medium (Modified from Muller and Grafe 1978)

Component	Concentration (mg l^{-1}) AA4 medium	AAP medium
Macronutrients		
$CaCl_2$	440.0	440.0
KH_2PO_4	170.0	170.0
$MgSO_4$	370.0	370.0
KCl	2940.0	2940.0
KNO_3		
Micronutrients		
KI	0.83	0.83
H_3BO_3	6.20	6.20
$MnSO_4$	22.3	22.3
$Na_2MoO_4.2H_2O$	0.25	0.25
$ZnSO_4.7H_2O$	8.60	8.60
$CuSO_4.5H_2O$	0.025	0.025
$CoCl_2.6H_2O$	0.025	0.025
$FeSO_4.7H_2O$	27.85	27.85
Na_2EDTA	37.25	37.25
Vitamins		
Myo-inositol	100.0	100.0
Nicotinic acid	0.5	0.5
Pyridoxine HCl	0.1	0.1
Thiamine HCl	0.5	0.5
Glycine	75.0	75.0
L-Glutamine	877.0	877.0
L-Aspartic acid	266.0	266.0
L-Arginine	228.0	228.0
L-Proline		1.0
Other Supplements		
2,4-dichlorophe-noxyacetic acid	4.0	4.0
Gibberellic acid	0.1	0.1
Kinetin	0.2	0.2
Sucrose	30000	30000
pH 5.8		

AA4 and AAP medium was made up as a single-strength solution in reverse osmosis water and filter sterilized through a 0.2 μm pore size membrane.

Appendix IV : N6PCMZ protoplast culture medium (Modified from Chu *et al.*, 1975)

Component	Concentration (mg l^{-1}) N6PCMZ medium
Macro nutrients	
KNO_3	2860
$(NH_4)_2SO_4$	463
KH_2PO_4	400
$MgSO_4.7H_2O$	185
$CaCl_2.2H_2O$	166
Micro nutrients	
H_3BO_3	1.6
$MnSO_4.4H_2O$	4.4
$ZnSO_4.7H_2O$	1.5
KI	0.8
$FeSO_4.7H_2O$	27.85
Na_2EDTA	37.25
Amino acid	
Glycine	2.0
Vitamins	
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	1.0
Carbon Source	
Sucrose	20000
Glucose	90000.08
Growth Regulator	
2,4-D	1.5
Zeatin	0.4
Casein hydrolysate	500
pH 5.8	

N6PCMZ medium was made up as single strength solutions in distilled water and filter sterilized by passage through a 0.2 μm membrane.

Appendix V: Composition of Enzyme Mixture (Bhattacharjee and Gupta, 1995)

Component	Concentration (mg l^{-1})
Cellulase RS	10000
Pectolyase Y23	1000
MES	11000
pH 5.8	

Enzyme Mixture was made up in CPW13M solution and filter sterilised by passing through a 0.2 μm pore size membrane.

Appendix VI: CPW 13M Solution (Modified from Frearson *et al.*, 1973)

Component	Concentration (mg l^{-1})
KH_2PO_4	27.2
KNO_3	101.0
$CaCl_2.2H_2O$	1480.0
$MgSO_4.7H_2O$	246.0
KI	0.16
$CuSO_4.5H_2O$	0.025
Mannitol	130000
pH 5.8	

CPW 13M solution was made up as a single strength solution in reverse osmosis water and autoclaved at 121^{0}C for 20 min.

Appendix VII : W5 solution for protoplast fusion (After Medgyesy *et al.*, 1980)

Component	Concentration (mM)
$CaCl_2$	125 mM
NaOH	155 mM
KC	15 mM
Glucose	5 mm
pH 5.6	

W5 solution was made up as a single strength solution in reverse osmosis water and autoclaved at 121^{0}C for 20 min.

Appendix VIII : Iodoacetamide solution for inactivation of protoplasts (Bhattacharjee *et al.*, 1999)

Component	Concentration (mg ml^{-10})
Iodoacetic Acid	18.6
pH 5.6	

18.6 gm of iodoacetic acid was dissolved in 10 ml of 10mM W5 solution and filter sterilized by passing through a 0.2 μm pore size membrane.

CHAPTER 7

REFERENCES

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II. Educational qualifications beginning with the first degree or equivalent

Sl.No.	Examination	Subjects	Year	Univ./Board
1.	High School	Bengali, English, History, Geography, Mathematics Science, Social studies	1982	Board of Secondary Education, Assam
2.	Intermediate	Bengali, English, Biology, Chemistry, Mathematics, Physics	1984	do
3.	B.Sc.	Foundation, Botany, Chemistry, Zoology Major in Botany	1987	Gauhati University Gauhati,
4.	M.Sc.	Botany	1990	North Eastern Hill University, Shillong Meghalaya

III. Employment details: Nature and duration of present and past employment :

(Designation, duration, organisation, Employment record)

Serial No	Designation	Organisation	Duration Joining	Leaving	Scale of pay
1.	Research Fellow	ICAR Research Complex for NEH Region, Barapani	23.11.90 to 31.7.93		Rs.2200+allowances
2.	Research Associate	-do-	1.8.1993 – 16.4.1997		Rs. 2500+ allownces
3.	Research Associate	University of Nottingham, U.K.	17.4.1997- 30.4. 1999		US $ 1000 pm
4.	Research Associate	ICAR Research Complex for NEH Barapani	1.6.1999- to date		Rs.10,120 pm

IV. Awards :

1990 - Gold Medal for 1st class first in M.Sc.

1997- Indian Council of Agricultural Research's Team Award (Team associate) for Outstanding Research in Crop Improvement for the Triennium1994-96.

1997 - Ph. D Dissertation fellowship of the Rockefeller Foundation, USA. for a period of two years at the University of Nottingham, U.K.

V. LIST OF PUBLICATIONS:

1. Bhattacharjee, B., Sane, A.P. and Gupta, H.S. (1999). Transfer of Wild Abortivecytoplasmic male sterility in rice through asymmetric protoplast fusion. *Molecular Breeding*.5 : 319-327.

2. Bhattacharjee, B., Pattanayak, A. and Gupta, H.S. (1998) Fertile plant regenerationfrom suspension culture-derived protoplasts of two indica rice lines and field evaluation of the seed progeny. *J. Genet. & Breeding.* 52 : 135-141.

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VI. LIST OF PAPERS PRESENTED IN NATIONAL AND INTERNATIONAL CONFERENCES

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2. Gupta H.S., **Bhattacharje, B.**, Kang, H.G. and Gynheung, An. (1997). Early flowering and dwarfing due to expression of *OsMADS*1 in rice. In 1997 Meeting of International Program on Rice Biotechnology. Malacca. Malayasia, September 15-19.

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6. Bhattacharjee, B. Pattanayak, A. and Gupta, H.S., (1996). Transfer of cytoplasmic male sterility in Indica rice through protoplast fusion. In: 5th Annual Meeting of National Rice Biotechnology Network, IARI, New Delhi, November 13-16,1996

7. Bhattacharjee, B., Gupta, H.S., Kang, H.G. and Gynheung, An (1996). Fertile transgenic IR36 produced by co-transformation of protoplasts with *BAR* and *OsMADS1* genes. In:2nd international Crop Science Congress, New Delhi, 17-24 November, pp-25.

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10. Gupta, H.S. Pattanayak, A. and **Bhattacharjee, B**. (1993). Protoplast mediated manipulation in rice. In: Sixth Annual Meeting of The International Program on Rice Biotechnology. February 1-5, Chiang Mai, Thailand.