REGENERATION OF PLANTS FROM ANTHER, CALLUS AND PROTOPLAST CULTURES OF RICE (*ORYZA SATIVA* L.)

Department of Life Science
The University of Nottingham

By
Maksuda Anjuman Nahar B.Sc., M.Sc.
The University of Dhaka, Bangladesh

Thesis submitted to the University of Nottingham for the degree of
Doctor of Philosophy
September 1994

Dedicated to my loving parents for their continuous inspiration, encouragement and help

Mrs. Hafiza Khatun and the late Md. Alimuddin Miah

"Science is not merely a collection of facts and formulas. It is pre-eminently a way of dealing with experience." Thus wrote the late Leslie A. White, when he concluded in an important essay* that "Science is sciencing."

Acknowledgements

I am deeply grateful, would like to acknowledge and thank Professor Edward C. Cocking, my supervisor, for his advice, support, patience, encouragement and guidance throughout my entire course of study and research and for the critical reading of this thesis.

The financial support from the British Council on behalf of the Overseas Development Administration is gratefully acknowledged. I would like to express my gratitude and thank to the Ministry of Agriculture, Research and Development, the members of staff of the Overseas Training Programme, the Government of Bangladesh for approving me study leave abroad (on deputation) for this course of study. I am indebted to my research institute, the Bangladesh Rice Research Institute, especially to Dr. A.K.M. Shahjahan, Dr. Nilufar Hye Karim and Dr. S.A. Miah for their moral support, encouragement and to provide me the opportunity to do a further study in England.

I would like to express my deepest gratitude and special thanks to Mr. N. W. Blackhall and Dr. M. R. Davey for their kind assistance, cooperation, help and advice in various aspects of my work and critical reading of my thesis. Special thanks are due to Dr. Norihan M. Saleh, Dr. Inez H. Slamet, Dr. R. P. Finch, Dr Abdul Baset, Dr. Asma Khatun and H. T. Huang for their cooperation and help. The friendship and support of all my other colleagues in PGMG (Plant Genetic Manipulation Group), both past and present, especially Dr. Norzulaini Khalid, Dr. Villa Pillai, Phillip Eyles, Song-Ho-Lee, K. Tang, R. S. Patil, G. S. Khera and Mrs. J. Jotham is gratefully acknowledged.

The technical assistance of Janet Rayner, Dave Wilson, Paul Anthony, June Jones and John Hawlins is much appreciated. Thanks and appreciation are also due to John Gaskin and Ian Gilder for their technical help and caring of plants in all glasshouse. I am also grateful to Brian case and Pat Crosby for their close attention, skilful work, expert advice and support in connection with all photographic matters.

I am particularly grateful to my loving mother and sons, Mrs. Hafiza Khatun, Muztaba Sharif, Murtaza Sharif and my husband Abdur Rouf Sharif for their constant moral support, encouragement, patience and help during my studies abroad.

Finally, my deepest gratitude and special thanks to my two Pakistani brothers, Khan Gul Jadoon and Iftikhar Mehdi in the Dept. of Mining Engineering, Indonesian brother's family, Ikram Azhari and Mrs. Moon Canyani, Bangladeshi brother's families, especially Dr. M. A. Matin and Mrs. Nasima Matin for their continuous encouragement, moral support and technical help in the preparation of my thesis.

Last but not least, I am grateful to my intimate friends Dr Malika Nebbali from Algeria, Mrs. Rukaiya Ahmed from Nigeria and Dr. Fakhreia Al Sughayer from Kuwait for their friendship, help, accompany, patience, encouragement and moral support during my stay in Nottingham.

Contents

Abbreviations

ABA	abscisic acid
ACC	accession
Al	aluminium
APH(3´)II	aminoglycoside phosphotransferase II (gene product)
aph(3´)*II*	aminoglycoside phosphotransferase II gene
BAP	6-benzylamino purine
BC	before Christ
∝-BN	∝-bromonaphthalene
°C	degrees Celsius
cDNA	chloroplast DNA
CIAT	International Center of Tropical Agriculture, Columbia
cm	centimetre
CMS	cytoplasmic male sterility
cv.	cultivar
d	day/s
2, 4-D	2, 4-dichlorophenoxyacetic acid
DNA	deoxyribonucleic acid
µE	micro Einstein/s
EDTA	ethylene diamine tetra acetic acid
e.g.	exempli gratia (Latin; for example)
EPICS	electronically programmable induced cell sorter
et al.	et alia (Latin; and others)
F_1	first filial generation
F_2	second filial generation
F_3	third filial generation
FAC	factor analysis of correspondences
FAO	Food and Agriculture Organization
FDA	fluorescein diacetate
Fig.	figure
FW	fresh weight
g	gram/gravitational force
G 418	Geniticin 418
GUS	β glucuronidase (gene product)

gus	β glucuronidase gene
h	hour/s
ha	hectare
hph	hygromycin phosphotransferase gene
8-HQ	8-hydroxyquinoline
IE	immature embryo
ISE	immature seed-embryo
IAA	indole-3-acetic acid
IOA	iodoacetamide
i.e.	id est (Latin; for instance)
IRGC	International Rice Germplasm Centre
IRRI	International Rice Research Institute
IR varieties	rice varieties released by IRRI
isozyme	isoenzyme
KHz	kilohertz
l	litre(s)
LBM	leaf-base meristems
log.	logarithmic
LS	Linsmaier and Skoog (1965) basal medium
lux	unit of illumination
LI90	log integral of 90° light scatter
m	metre
mm	millimetre
µm	micrometer
mg	milligram
µg	microgram
MHz	megahertz
ml	millilitre
µl	microlitre
M	molar
mM	millimolar
µM	micromolar
MES	2 [N-Morpholino] ethanesulfonic acid
mW	milli Watt

min	minute/s
MOPS	4-[N-morpholino] propanesulphonic acid
MS	Murashige and Skoog (1962) basal medium
MSS	mature seed-scutellum
mtDNA	mitochondrial DNA
MW	molecular weight
°N	degrees north
NAA	α-naphthalene acetic acid
ng	nanogram
nm	nanometer
No.	number
NPTII	neomycin phosphotransferase (gene product)
nptII	neomycin phosphotransferase gene
oz	ounce(s)
pc	personal communication
pcv	packed cell volume
PEG	polyethylene glycol
pg	pico gram
p-grain	pollen grain
pH	negative logarithm of hydrogen ion concentration [$-\log(H^+)$]
PI	propidium iodide
pKAN	the neo gene
PMC	pollen mother cell
PMT	photo multiplier tube
pats	protoplasts
psi	pound per square inch
pPUR	the gusA gene
R_o	primary regenerant generation
R_1	The first generation seed progeny derived from primary regenerant generation
R_2	The second generation seed progeny derived from the first generation seed progeny
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid

rpm	revolutions per minute
s/sec	second(s)
µs	micro second
°S	degrees south
scv	sedimented cell volume
SE	standard error
t	tonne(s)
To	primary transgenic generation
T_1	The first transgenic seed progeny
T_2	The second transgenic seed progeny
TCCP	Tissue Culture for Crops Project
T309	Taipei 309 (*Japonica* rice variety)
TN1	Taichung Native 1(*Japonica* rice variety)
UK	United Kingdom
USA	United States of America
uv	ultraviolet light
V	Volt(s)
v	variety
Vol.	volume
v/v	volume to volume ratio
W	Watt(s)
w/v	weight to volume ratio
%	percentage

Abstract

The work described in this thesis focuses on the development of fully reproducible systems of plant regeneration through embryogenic callus and totipotent protoplast cultures of rice (*Oryza sativa* L. cv. Taipei 309). The calli originated from different explants, including excised anthers, isolated microspores, mature seed-embryos, immature seed-embryos and leaf-base meristems. In the callus culture and plant regeneration studies, excised anther-derived callus produced higher numbers of green (90.5%) and albino (9.5%) plants, both haploids (81.2%) and diploids (18.8%), compared to other explants. The plant regeneration frequency from mature seed-embryo-derived callus varied with callus age and embryogenicity of the callus. A maximum of 20% plant regeneration was obtained with approx. 2% of the regenerated plants being albinos. In the case of mature or immature seed-embryos and leaf-base meristems, plant regeneration frequency from callus was dependent on some more specific factors, in addition to callus age and embryogenicity. These factors were the age of seedlings grown *in vitro* and the age of immature embryos after anthesis for leaf-base meristem and immature embryo cultures, and also the scutellar surface orientation of mature or immature embryos during callus culture initiation.

The present study also focused on the plating efficiencies and plant regeneration frequencies obtained from culture of embryogenic cell suspension-derived protoplasts. These varied with the explant source, the embryogenic nature and age of the callus during suspension initiation, the age of the embryogenic suspension culture, the time of enzymatic digestion, the plating density, viability of protoplasts and use of the suspensions at their exponential (actively dividing) growth stage. The results from this study indicated that leaf-base meristem and anther calli-derived suspensions retain their regenerability for a longer time compared to suspensions derived from mature seed-scutellum and immature seed-derived embryos. Maximum plant regeneration frequency (45%) was obtained from embryogenic suspensions derived from mature seed-embryos. A detailed study of different agronomic characteristics of 57 protoclonal plants (mature seed-scutellum-derived) was performed and the statistical analysis of the data indicated a range of variability among the protoclonal plants and also between the protoclonal and seed-derived plants.

A detailed study of the ploidy levels of regenerated plants obtained through different culture systems (callus and protoplasts) was performed by flow cytometry and chromosome counting of meristematic cells. The results showed that anther calli-derived plants were mostly haploids, protoplast-derived plants of leaf-base meristem, anther, and mature seed-scutellum origin were mostly tetraploids and in between these two groups, diploids, triploids and aneuploids were also present.

Chapter 1

General Introduction

1.1 Introduction

Rice (*Oryza sativa* L.) is ranked as the world's most important cereal food crop, being grown in an area of 144.641 million ha and with a production of over 468.275 million tons (IRRI Rice Facts 1988). It is the major source of calories for about 40% of the global population; more than 90% of the world's rice is grown and consumed in Asia, where it contributes about 50% of dietary energy (Khush 1984; Juliano 1985). Rice grows from more than 3000 m elevation in the Himalayas to sea level in the deltas of the great rivers of Asia, especially Bangladesh, India, Thailand and Vietnam. Consequently, it is also one of the world's most intensively investigated and versatile crop plants (Raina 1989). Rice is also a staple food source in Latin America, parts of Africa, and the Middle East. Yields of rice are disappointingly low in tropical Asia (2.0 t/ha), as compared with yields from temperate countries (5.0 t/ha), e.g. Japan, Korea, USA, Australia, Spain and Italy.

Tremendous effort and progress have been made at IRRI for more than 3 decades, the achievements have resulted in the release of cultivars like Taichung Native 1, IR 8, Mashuri and IR 36. The cultivar IR 36 is now being grown over large areas to serve the Asian population (Khush and Coffman 1977). Because rice is grown under a wide range of agroclimatic conditions, a number of natural enemies and adverse growing conditions cause enormous loss in yield. The main objectives in rice breeding at International Rice Research Institute (IRRI) and other countries are disease resistance, improvement of grain quality, increased protein content and tolerance to insects, adverse soils, water and drought. For speedier, or even immediate solution to a whole range of problems, the attention of rice breeders has been diverted to innovative approaches like the techniques of rice cell and tissue culture, which offer a number of areas to the challenges of rice crop improvement.

1.2 The Genus *Oryza*

The name of the genus *Oryza* was given by Linnaeus, who specified Asian cultivated rice as *Oryza sativa*. The genus is a member of the grass family *Gramineae* (*Poaceae*),

belonging to the tribe *Oryzeae* under the subfamily *Oryzoideae* (*Pooideae*). It consists of 21 wild species and two cultivated *Oryza* species (Vaughan and Sitch 1991). The two cultivated *Oryza* species are *Oryza sativa*, the Asian or cosmopolitan rice species, now cultivated worldwide and *Oryza glaberrima*, the African rice, which is endemic to West Africa; each species has a large number of varieties. The remainder of the species grow as weeds or wild populations on the continents of Asia, Africa, Australia and South America. It has been estimated that more than 100,000 varieties of *Oryza sativa* are being cultivated world wide (Chang 1985a). Most of the wild *Oryza* species are diploids and seven of them are tetraploids. These wild species have 2n = 24 and or 48 chromosomes with six different genome types, AA, BB, BBCC, CC, CCDD, EE, and FF on the basis of chromosome pairing in the meiosis of F1 plants (Second 1985). The genomic constitution of some species (*O. ridleyi*, *O. longiglumis*, *O. meyeriana*, *O. granulata*, *O. indandamanica*) is not known. At the International Rice Research Institute, The International Rice Germplasm Centre (IRGC) has registered approximately 85,000 accessions of rice germplasm (both wild and cultivated species at the end of 1990 (Vaughan and Sitch 1991).

The species names, genome group, chromosome numbers and geographical distribution are shown in Table 1A. The nomenclature of the *Oryza* species has been an issue of great controversy, particularly for those with the genome A which are closely related. So far, there are 85 names documented for the 23 species of the genus *Oryza* and 33 synonyms have been published for *Oryza sativa* alone (Oka 1988). There has been particular confusion in naming the common Asian wild rice *O. rufipogon*, for which 19 synonyms have been used. The name *O. perennis*, as a general name for different forms of common wild rice around the world has been preferred by a number of workers. However, *O. rufipogon* has been recognised and established as the name fitting for the common wild rice of the Asian and American taxa (Tateoka 1964). *Oryza longistaminata*, is now widely accepted as being the correct nomenclature for the African taxon of common wild rice, (Clayton 1968).

A number of well known species of the subfamily *Oryzoideae* have been moved from the genus *Oryza* in recent years. *Oryza angustifolia*, *O. perrieri* and *O. tisseranti* were placed in the genus *Leersia* to join *L. hexandra*, a common companion of *Oryza* species. An important salt-tolerant grass, originally known as *Oryza coarctata* and then

Schlerophyllum coarctatum was finally placed in the genus *Porteresia* (Tateoka 1965). Some taxa in the genus *Oryza* have been described as consisting of complexes of closely related species.

1.3 Origin, Classification and Distribution

1.3.1 Origin and distribution of the genus *Oryza*, cultivated and wild

The problem of the origin and evolution of rice has been studied for a long time. Information on the origin of domesticated rice is still unsatisfactory and undetermined, it will probably never be traced with certainty. It is presumed that the cultivated species have originated from certain of the wild species, and it is unlikely that any of the wild rices are descended from cultivated rice. From the evidence it is assumed that the habitats of the wild rices are probably the places of origin of cultivated rices (Grist 1983). The distribution of wild rices suggests the centres of domestication of *O. sativa* were somewhere in the piedmont zone of Assam, upper Burma, northern Thailand, Laos, North Vietnam and Southwest and South China (Chang 1976b). In the savanna areas of West Africa and Tanzania, *O. glaberrima*, the less important of the two African cultivated species was most likely domesticated in the habitats of its wild annual progenitor *O. barthii* (formerly *O. breviligulata*) (Miezan and Second 1979; Chang 1976b). The geographical isolation of the African cultivated species (*O. glaberrima*) suggests that these two species (*O. sativa* and *O. glaberrima*) were independently domesticated from their respective wild progenitors. Although there is confusion about the synonyms, these wild progenitors can be determined without much controversy (*O. rufipogon* gave rise to *O. sativa*, and *O. barthii*, formerly known as *O. breviligulata*, gave rise to *O. glaberrima*), since they are so closely related to the cultivated species and coexisting in the same habitats (Oka 1974).

The sites in Asia where most of the common wild rices have been found were in eastern India, Thailand and southern China, and a few in Malaysia, Indonesia and Philippines. These sites are distributed in tropical to subtropical zones; most of them are at low altitudes. A few of them were found at about 1,000 m (from the sea level) in the Jeypore Tract, India where the minimum temperatures in winter are about 10°C or higher (Oka 1988). The wild progenitor of *O. sativa* is thought to be *O. rufipogon*, or the Asian form of *O. perennis* complex, and its relatives are distributed in the humid

tropics of Africa, America, and Oceania. In the Yangtze basin of China, Jiangxi Province (28.1°N) where the minimum temperatures in winter are below zero and the marsh is frozen, some of common Asian wild rices were also found. However, the wild rices found there were perennial types which regenerated from the old stubbles in spring. They have a special cold tolerance character, possibly because of dormancy of certain vegetative tissues which other varieties do not have.

The habitats of perennial types are in deep swamps of both India and Thailand, which remain moist throughout the whole year, whereas the habitats of annual types are in temporary swamps which are parched in the dry season. The habitats of intermediate perennial-annual types are in a range of water depths in early dry season, and they may be considered as ecotypes (Morishima et al. 1984a). Water conditions in swamps are always changeable and stresses due to drought and submergence are probably the most critical for plants like wild rices in these areas.

The African form of the *O. perennis* complex or *O. longistaminata*, which is rhizomatous, is distributed throughout the savanna areas of West to East Africa and Madagascar. *Oryza breviligulata*, an annual type, is thought to be the progenitor of African cultivated rice, *O. glaberrima* and is distributed abundantly in the savanna areas of West Africa and sparsely in Tanzania (Miezan and Second 1979) and Zambia (Bezancon and Second 1979). In West Africa, the higher the latitude, the rainfall is more uncertain and the dry season is more parched. The wild-rice species are also found in depressions and ponds which are inundated in the rainy season. The American form of the *O. perennis* complex is perennial and is found in swamps and creeks, surrounded by forest in the Amazon and other river basins in northern South America. The habitats are deeply flooded when the water level rises, and in these areas there is no defined dry season (Oka 1988). The Oceanian form of *O. perennis* complex is annual, while *O. australiensis* is perennial. They are found in natural swamps near Port Darwin (Western Australia), and some parts in Queensland (Oka 1978b). A few strains of the Oceanian form with some perenniality are found in New Guinea, where drought is not severe.

Table 1A Species of *Oryza*, their chromosome numbers, genome symbols, and geographical distributions (adapted from Vaughan and Sitch 1991, Bajaj 1991)

Species name (synonym)	Chromo-some no.	Genome group [a,b]	Geographical distribution
***Oryza sativa* complex :**			
***O. sativa* L.**	24	AA	Worldwide
O. nivara* Sharma & Shastry** (O. fatua*, *O. sativa* f. *spontanea***)	24	AA	South and Southeast Asia and southern China
O. rufipogon* W. Griffith** (O. perennis*, *O. fatua*, *O. perennis* subsp. *balunga***)	24	AA	Tropical Asia, southern China and Australia
***O. glaberrima* Steud.**	24	A^gA^g	Primarily West Africa
O. barthii* A. Chev.** (O. breviligulata***)	24	A^gA^g	Tropical West Africa
O. longistaminata* A. Chev. & Roehr.** (O. barthii***)	24	A^lA^l	Tropical Africa
O. glumaepatula* Steud.** (O. perennis* subsp. *cubensis***)	24	$A^{gp}A^{gp}$	Central and South America and West Indies
***O. meridionalis* N. Q. Ng**	24	A^mA^m	Tropical Australia
***O. officinalis* complex :**			
***O. officinalis* Wall. ex Watt**	24	CC	Tropical Asia, New Guinea
***O. rhizomatis* (new)**	24	CC	Sri Lanka
***O. australiensis* Domin**	24	EE	Tropical Australia
***O. eichingeri* A. Peter**	24, 48	CC, BBCC[c]	Sri Lanka, tropical Asia, East and central Africa
***O. punctata* Kotschy ex Steud**	24, 48	BB, BBCC[c]	Tropical Africa
***O. minuta* J.S. Presl ex C.B. Presl**	48	BBCC	Southeast Asia, Philippines
***O. latifolia* Desv.**	48	CCDD	Central and South America
***O. alta* Swallen**	48	CCDD	Central and South America
***O. grandiglumis* (Doell) Prod.**	48	CCDD	South America
***O. meyeriana* complex :**			
***O. granulata* Nees & Arn. ex Hook f.**	24	Diploid [b]	South and Southeast Asia
***O. meyeriana* (Zoll. & Morrill ex Steud.) Baill.**	24	Diploid [b]	Southeast Asia, southern China
***O. ridleyi* complex :**			
***O. longiglumis* Jansen**	48	Tetraploid [b]	Indonesia, New Guinea
***O. ridleyi* Hook f.**	48	Tetraploid [b]	Southeast Asia, New Guinea
Species not in any complex :			
***O. Schlechteri* Pilger**	24	Diploid [b]	New Guinea
***O. brachyantha* A. Chev.& Roehr**	24	FF	West and central Africa

[a] Superscripts refer to species names.

[b] The absolute source of the D genome remains unknown.

[c] Diploid (annual) and allotetraploid (perennial) forms exist under the same specific name.

Natural dispersal and human selection have extended the present span of rice cultivation from 53°N to 40°S latitude. From a report on the agroclimatic distribution of cultivated rice, it is revealed that rice can grow in a diversity of environments and it has originated in the hot, humid tropics, where monsoon rain and flood water create an aquatic environment in some parts of the year (Lu and Chang 1980). It can also adapt very well to diverse growing conditions and performs better than any other cereal grain crops in areas with unfavourable soil conditions (saline, alkaline and acid sulphate soils). Cultivated rice is grown in cool climates in high mountainous regions of Nepal and India, and in the hot deserts of Pakistan, Iran and Egypt. In parts of Asia, Africa and Latin America, it is also grown as a dry land crop (Swaminathan 1984). Rice can be grown under a wide range of agroclimatic conditions and a broad spectrum of natural enemies and adverse growing conditions. It is grown across various water regimes, ranging from upland hill slopes to a maximum water depth of about 5 m in river deltas of Bangladesh, India, Thailand and Vietnam (Khush 1984a).

1.3.2 Type differentiation, type classification and morphology of rice cultivars

Type differentiation

For long time, the existence of two types of rice (Hsien and Keng) has been recognised by the Chinese and they correspond with the rice subspecies, *Japonica* and *Indica* (Ting 1949). Later, these two 'subspecies' were considered as 'types' or 'varietal-groups' and proper names were given to them as *Japonica* and *Indica* (used in this thesis), since there is no single criterion to distinguish them reliably (Oka 1987). The *Japonica* group of varieties is thought to have originated in China, not in Japan, and some workers preferred the name '*Sinica*' to this group. Although few significant differences were found between the *Japonica* and the *Javanica* types, the latter was considered as a third, less important varietal-group of rice and given a different name.

Furthermore, it has been regarded as a tropical subgroup of the *Japonica* type (Oka 1953, 1987) to designate the *bulu* and *gundil* varieties of Indonesia (Oka 1958; Morinaga and Kuriyama 1958). Some workers have compared the plant and grain characteristics of the three 'ecogeographic' races (Table 1B) and considered the *Javanica* type at the same rank as the other types (Chang 1976b; Lu and Chang 1980).

Table 1B. Comparative morphology and physiology of the three ecogeographic races of *Oryza sativa* (adapted from Chang 1988)

Organ :	*Indica*	*Japonica* (*Sinica*)	*Javanica*
Leaf	Pale green, narrow → broad, slightly pubescent, the upper leaf forming an acute angle with the culm.	Dark green, narrow, the upper leaf forming an obtuse angle (in many cases an acute) with the culm	Pale green, broad and stiff
Grain	Caryopsis short → long, slender, narrow, slightly flattened, length to width ratio 3.1:1 → 3.5:1	Caryopsis short, thick, rounded in transverse section, length to width ratio 1.4:1 → 2.9:1	Long, broad and thick
Awns	Usually awnless or possessing short and smooth awns	Awnless or long awned, also intermediate forms	Awnless or long awned
Palea & Lemma	Have thin, short hairs	Have long, fairly thick, and dense hairs	Have long hairs
Character :			
Shattering	Easy	Low	Low
Photoperiod sensitivity	Varying	None → low	Low
Amylose content	23 - 31%	10 - 24%	20 - 25%
Gelatinization temperature	Low → intermediate	Low	Low

The *Indica* subspecies is the most predominant, primary type of *O. sativa* with the greatest diversity and is grown commercially in south and central parts of China, tropical monsoon regions of south and Southeast Asia, the Malaysian peninsula and the major islands of Southeast Asia. The *Japonica* subspecies is grown mainly in the temperate zone of Japan, north and eastern China, Korea and USSR (Chang 1976b), while the *Javanica* types are grownespecially in Madagascar, northern Luzon and recently on the islands of Bali. On the basis of morphological and physiological characters (plant morphology, hybrid sterility) and geographic distribution, a group of Japanese botanists first attempted to distinguish the different types of rices and recognised the distinction between *Indica* and *Japonica* types in the late 1920s (Bray 1986).

Eleven out of 41 characters examined, showed wide variation between these two main types and were used for their classification. Subsequently based on plant characters, especially if two or more characters are used in combination, it was difficult to do type evaluation since variation is greatly increased (Oka 1953). However, the characters like spikelet morphology and the phenol reaction are still considered as moderately reliable criteria for type designation.

Type classification

The interspecific variation in *Oryza sativa* is remarkably extensive and subspecific classification has always been a matter of importance for rice breeders and geneticists. Biochemical methods of investigation, especially isozyme analysis, has provided valuable tools for rice geneticists. Electrophoretically identifiable isozymes have been utilised for the classification of varieties within *O. sativa* (Glasmann 1987). Starch gel electrophoresis for the detection of enzyme variation was used to investigate the genetic structure of the *O. sativa* L. species. Nowadays, isozyme analysis is being used routinely as a proven diagnostic tool for classifying rice germplasm (Second 1982; Glaszmann 1987). Using this tool, six varietal groups of rice have been identified in a representative sample of 120 Asian cultivated rice varieties based on gene polymorphism at 21 presumed loci (Glaszmann 1985). This result was confirmed from a survey of 1688 varieties of Asian cultivated rice (*O. sativa* L.) by 'factor analysis of correspondences (FAC)' of isozyme data for 15 polymorphic loci coding for 8 enzymes (Glaszmann et al. 1984).

Varietal groups I and VI were the two major ones, groups II and V were minor and groups III and IV were considered to be 'satellites'. Varietal group VI corresponds with the temperate and tropical *Japonica* and *Javanica* types, when these varietal groups were compared based on previous morphological cassifications of rice varieties (Oka 1958). Group I corresponds to the typical *Indicas*, but a number of varieties fall in the remaining groups (Group II to V) and may be considered as intermediate types which were previously classified as '*Indicas*'. In this thesis, *Indica* varieties of varietal group I, for example, commercially important varieties are referred to as 'true-*Indicas*' and those in groups II to V are referred to as '*Indica*-types'.

Another recent biochemical technique of varietal classification, nuclear restriction fragment length polymorphism (RFLP), is more reliable than the conventional isozyme analysis method and can detect and monitor genetic variation in plants at the DNA level by assaying both coding and non-coding regions of the genome. This technology is of great importance in crop improvement and is particularly useful as a means of assessing genetic variation in natural populations, thus revealing the evolutionary relationships among plant taxa (Song et al. 1988; Tanksley et al. 1989). RFLP analysis was used for a phylogenetic study and to estimate the level of DNA variation in a mixed population of 70 rice varieties. The results showed that all the classical *Japonica* type varieties formed a single group and they had a strong genetic affinity for each other, which also corresponded with the enzymatic group VI in the isozyme analysis (Glaszmann 1987; Wang and Tanksley 1989). The conventional *Indica* types were not clearly grouped as a single unit and some of them, previously classified as '*Indicas*' were grouped with the '*Japonica*-types' strongly suggesting initial misclassification.

Morphology of the rice plant

The rice plant is classified as an annual grass with a round hollow jointed culm, flat sessile leafblades and a terminal panicle. A mature rice grain is comprised of a husk and an enclosed caryopsis. The embryo remains in the caryopsis and consists of an embryogenic axis, a plumule and a radicle. The plumule is enveloped by the scutellum and the epiblast. The radicle remains enclosed in the coleorhiza. When a rice grain germinates, the coleorhiza and the coleoptile extrude from the grain. From the coleorhiza, the radicle develops into a root system and the young leaves emerge from the coleoptile. The primary leaf is the first leaf to emerge and has no blade. The second and the subsequent leaves are differentiated into sheath, blade, ligule and auricle. The uppermost leaf, which is just below the panicle, is termed the flag leaf. There are several nodes and internodes in the culm which is enveloped by the leaf sheaths.

During the whole growth period of the rice plant, some of the shoots at the basal internodes of the culm grow into tillers under suitable conditions. The height of rice plants varies according to the variety and the conditions under which they are grown. The height ranges from 100 to 120 cm at maturity for most of the commercial varieties. A rice plant usually matures in 90 to 200 days, from seed germination to

grain ripening. A mature plant produces a branched panicle on the uppermost internode of its culm, just above the flag leaf. Many spikelets grow on the panicle, each of which contains a flower with six stamens and one pistil enveloped in two glumes, the lemma and the palea. During anthesis, the two glumes of a mature spikelet open for a short period of time and the anthers dehisce. After pollination, the ovary develops gradually into the caryopsis of a mature seed which consists of an embryo and the endosperm.

1.4 The economic importance and history of rice cultivation in Southeast Asia, Africa and Europe

The economic importance

Rice occupies a unique place among the cereals, it is one of the world's most intensively grown cereal food crops, nutritionally provides more calories and carbohydrate per hectare than any other cereal crop (De Datta 1981). Rice ranks only next to wheat in terms of total cultivable area and total acreage harvested, it shares equal importance with wheat in feeding the human population of the world. In fact, it provides most of the daily food for one in every three people on Earth and more than half of the world's population uses rice as staple food (Lu and Chang 1980; Chang 1985a). Rice has sustained mankind since prehistorical age and provides 20% of the calories and 13% of the protein for human consumption on a world wide basis (Yamada and Loh 1984).

Brown rice has more calories per 100 g (447) than wheat (436), it is slightly below maize (461). Brown rice has also the highest digestible energy (96.3%) among six cereal grains. One hectare of rice can sustain 5.63 persons annually, while a hectare of wheat or maize can only support 3.67 and 5.06 persons respectively (based on 1977 world average yield). In terms of protein yield per hectare, rice (0.16 t/ha) compares favourably with wheat (0.17 t/ha) and surpasses maize (0.14 t/ha). Milled rice has a very high true protein digestibility (85%) against 90% for wheat and it is almost exclusively used as a direct source of food (Chang 1987). Because of its high biological value (75%), cooked rice powder can be used as a supplement for milk for babies during the weaning period. Until 1981, in some Asian countries like Bangladesh, Burma, Vietnam, Laos, Thailand and Kampuchea the relative importance of rice as a

staple food is further recognised by its high dietary calorie supply (almost 80% of the total) (IRRI 1987).

Rice is very popular as a food in tropical Asian countries, because of its excellent palatability when simply boiled, its versatility and its high grain yield to seed ratio. This crop has also a wide range of uses other than as a direct source of food. Rice is generally considered to be a semiaquatic species best suited with monsoon climate, but the plants are widely adaptable under variable climatic conditions. Among the cereal crops, it is the only crop that can be grown under prolonged waterlogging conditions, and in low-lying areas of the humid tropical countries it is the natural choice for millions of subsistence farmers. Between 30 and 40% of the rural population subsist on incomes insufficient to insure minimum calorie intake (Chang 1985a; Akhmed 1989). In addition, only the rice plants can derive nitrogen in flooded soil in association with nitrogen-fixing leguminous crops used as green manuring crops and with biofertilizer sources, such as *Azolla* and blue green algae (Rogers and Watanabe 1986; Ponnamperuma 1978).

The submergence of certain unproductive soils increased nutrient availability, the rice plants could benefit from this, and thus they could be grown in poor soils without applying expensive fertilizers but with continuous low yields (Ponnamperuma 1978; Chang 1985a). In 1987, the world's total harvested rice area exceeded 1.44×10^8 ha and total production stood 4.57×10^8t (IRRI 1987). Before this report, The Food and Agriculture Organization (FAO) had estimated that it would be necessary to increase annual rice production by 3% by the end of this century to keep feeding the world's rising population (Swaminathan 1984). The world's human population in mid-1983 (4.72 billion) would be expected to increase to 8.0 billion by the year 2020, and of these 4.3 billion would be primary rice consumers. However, it was estimated that the world's annual rough rice production must increase from 1987s 4.6×10^8t to 5.6×10^8t by 2000, and to at least 7.6×10^8t by 2020 (a 65% increase) just to feed and to keep up with the population growth (IRRI Rice Facts 1988; IRRI 1989). In order to achieve this gigantic goal, new strategies and innovative approaches, for example, recent *in vitro* biotechnological development for rice needs to be incorporated into traditional rice breeding programmes to generate new genetic variability to face the major challenge.

The history of rice cultivation

Information on the history of rice cultivation is fragmentary and unsatisfactory. The oldest agriculture began in the Near East about 10,000 years ago, probably rice has been cultivated less than 9,000 years and it seems that wild rice cultivation preceded domestication. During the Neolithic age, cultivation of annuals at different elevations in east India, northern Southeast Asia and Southwest China was influenced by alternating periods of drought and variations in temperature (Whyte 1972). The antiquity of rice cultivation in China has long been a subject of debate. It is evident from the earliest archaeological findings that the oldest sample of domesticated Hsien rice found in the lower Yangtze Delta is from the Chinese village, Ho-mu-tu in Yü-yao-hsien, Chekiang. Carbon-dated stratum of the rice grains, hulls, and stems excavated from Ho-mu-tu dates back to 5008 BC (Chang 1983). Recent carbon dating of archaeological findings where rice glumes were found at Miao-ti-kou site in Honan places, the date of rice cultivation at 3280 BC. Excavations from the upper and lower basins of Yangtze River were dated between 3395 and 2000 BC (Cheng 1974; Ho 1975). In central and southern China, several fourth millennium sites in Yangtze Delta and two sites in Guangdon, which date back to between 2000 and 3000 BC contain remains of domesticated rice (Yang 1978).

The earliest specimens of rice glumes excavated from Non Nok Tha of Thailand were dated at 3500 BC or slightly earlier (Bayard 1970; Solheim 1972). Archaeological evidence dated to approx. 5000 BC, from two early non-marshy sites in north Thailand strongly suggests the presence of rice-farming in this region prior to 4500 BC. In upland Southeast Asia, rice domestication began in natural marshes about 9,000 years ago and early rice farmers were able to move to drier areas as their skills improved (Gorman 1977). Ancient India is undoubtedly one of the oldest regions where cultivation of *O. sativa* began. From the earliest evidence, it suggests that rice cultivation in India began shortly before the Aryan invasion between 1500 BC and 2000 BC (Candolle 1886; Vishnu-Mittre 1974). The oldest carbonized grains found in India and the oldest grain sample excavated at Mohenjodaro of Pakistan dated back to about 2300 BC and 2500 BC respectively (Allchin 1969; Andrus and Mohammed 1958). The archaeological survey of India dates back to the Ice Age, where it suggests that rice terraces were purposely built on the banks of the Ravi River in Kashmir and it

is more likely that the Southeast zone is the original home of domesticated rice (Bray 1986).

Sri Lanka was once the granary of the East and an export trade developed with neighbouring countries, it has also been indicated that rice has been grown in this island as dryland and as wetland crop since at least 540 BC and 420 BC respectively (Paul 1945). The earliest evidence obtained from the southern island Kyushu suggests rice cultivation was introduced to Japan much later, dated between 300 and 400 BC (Tamake and Hatade 1974). Rice pollen found in clay below Lake Biwa indicates that rice cultivation may have begun in this area of Japan about 2,900 years ago. However, its cultivation did not reach Hokkaido, the most northern islands of Japan until the eighteenth century (Grist 1983). Historical records indicate that cultivation in North Vietnam began sometime after 2000 BC. Evidence of the establishment of wetland rice culture in the Red River Delta of Vietnam was reported by the third millennium BC (Chang 1976c; Higham 1984). Cultivation in Indonesia, Malaysia, and the Philippines began sometime after 1500 BC (Spencer 1963).

In the Philippines, the impressive system of rice terraces in the mountains of Luzon island is of great age and is thought to date back to the second millennium BC. Although rice was known to the ancient Romans as a result of their Eastern conquests, it did not reach Western Asia, Greece or Egypt until much later. Its cultivation may have existed in Sicily in very early times, although it did not exist in the north of Italy and the Mediterranean basin until the fifteenth century (Grist 1983). Rice was introduced into Central and South America at the same time. From the mid-seventeenth century, it was introduced into the state of Virginia and commercial crops are now being grown in Arkansas, California, Louisiana and Texas almost exclusively. Since the 1850s, rice has been grown in Australia but its production did not reach a commercial scale until 1924 and as an important export product, both Australia and the USA now rely on rice (Grist 1983). Records are lacking concerning the evolutionary changes of rice cultivation in Africa. From the report it is evident that the cultivation of African rices is more recent and probably not more than 3,500 years old (Portères). The cultivated species, *O. glaberrima*, was most likely domesticated from the wild annual *O. barthii*; the latter is primarily adapted to water holes in the savannas and secondarily adapted to the forest zone (Harlan 1973).

1.5 Conventional rice breeding

At present, rice production in the world is increasing steadily. World's rough rice production was 4.73 x 10^8 t in 1986, harvested from about 1.45 x 10^8 ha (FAO 1988). However, world population has also been increasing especially in the rice growing regions. The urgent need for food is the most potential problem in more than half of the nations in the world today. Although 4-6 t/ha are produced in some areas where modern varieties are grown and improved agronomic or cultural practices are followed, normally rice yields range only between 1.5-2.0 t/ha or even lower in the tropics and subtropics (Sun and Zheng 1990). However, progress has easily exceeded that previous achievement since the first rice breeding programs began in the early part of the 19th century. From 1965 to 1985, rice production in Asia increased by over 187% with the highest increase, over 300% increase occurring in Indonesia, mainly due to the establishment of rice research centres in India, Bangladesh and Indonesia (IRRI 1987). Such increases have been achieved largely due to the major advances in rice improvement and production through conventional breeding methods.

Major increases in rice yields in tropical Asia have been achieved by the introduction of improved, fertilizer-responsive, semi-dwarf, high yielding varieties like Taichung Native 1 and IR8, in the late 1960s. This is the so called 'Green revolution' and the great success of rice breeders was marked with the adoption of improved varieties, cultural practices and increased cropping intensity in many Asian countries. This was the major breakthrough in the (IRRI), which was established in early 1960. Later IR varieties, with improved grain quality, higher levels of resistance to pests and diseases, photoperiod sensitivity and early maturity have subsequently replaced IR8 (Chang 1984). The qualities of tolerance to adverse soil conditions and nutritional improvement have been incorporated into the recently released IR varieties (Khush 1977). The rice breeding programme of IRRI is one of the biggest crop improvement programmes in the world. It endeavours to develop and help to develop improved rice varieties for the diverse rice growing conditions. It is an interdisciplinary team effort, where plant breeders work with scientists in other disciplines such as plant pathologists, entomologists and agronomists in improving the rice plant in many important attributes (Khush 1984).

For the production of varieties compatible with local climates, IRRI has concentrated on its efforts to provide germplasm to the local research effort. Currently, the introduction of the semi-dwarf IR8 and TN1 has resulted in the development of modern high yielding varieties (Yamada and Loh 1984). The quality of this improved plant type of IR8 has encouraged most of the national rice improvement programmes for immediate initiation of crossing programmes to develop new varieties with short stature. Improvement of yield potential has received major attention in the early years. This technique of sexual rice breeding has enabled early rice breeders to develop many new varieties for disease resistance, for tolerance to drought, flood, salinity and soil alkalinity mainly by crossing carefully selected parents and through mass selection and introduction methods (Yamada and Loh 1984). The major shortfall in the development of semi-dwarf rices was the indepedent use of a common recessive gene, *sd*1, which conferred short plant stature on TN1, IR8 and its derivatives from the taller varieties. The genetic uniformity of these new rice varieties produced by sexual crossing made them potentially vulnerable to diseases and insect epidemics and led to devastating yield losses due to tungro virus, grassy stunt virus, blast and brown planthopper infestation (Chang 1984).

Another impact of conventional rice breeding on the improvement of rice production is hybrid breeding. In China, the development of F_1 hybrid rices through the use of a 'wild abortive' type of cytoplasmic male sterility (CMS) donated from a weedy species of rice (*O. sativa* f. *spontanea*) was a major breakthrough. This achievement has increased rice production substantially and the yields of current hybrid rices can exceed those of the best high yielding varieties by 20-30% (Virmani and Edwards 1984; Yuan and Virmani 1986). At present, about 6 million hectares are planted with hybrid rices in China alone.

1.5.1 Objectives of the conventional rice breeding and its limitations for rice improvement

Objectives of rice breeding

The major objectives in rice improvement have been suggested by plant breeders (Khush and Virmani 1985; Ling et al. 1986) are as follows :

- High yield,

- Early maturity,
- Good grain quality,
- Incorporation of genetic resistance to biological stresses, e.g., diseases, pests, nematodes and viruses,
- Incorporation of genetic tolerance to environmental stresses, e.g., low or high temperature, soil toxicities, moisture stress, or nutritional imbalances,
- Incorporation of novel traits from alien species,
- Streamlining of current breeding methods and selection procedures.

It is obvious that the breeders in different rice-growing areas face different kinds of problems, so that they modify the objectives to meet the local requirements.

Limitations for rice improvement

Although 50% of the crop yield increase has been attributed to genetic improvement, the efficiency of conventional rice breeding is low because of the long term selection and backcross (generally seven generations), the large scale of field trials (usually 4,000-5,000 F_2 plants or F_3 lines), and the labour intensity. Thus, for each new variety release can cost up to 1 million dollars (Oram 1982). Progress in the development of improved rice varieties has been very rapid during the past two decades in terms of increased production, better quality, and higher level of resistance to pests and diseases. As a consequence, significant advances in food production have been achieved (Barker et al. 1985). However, with increasing pressure for further improvements with limited land resources for the growing population, there is requirement to develop rice varieties with even higher yield potentials. The success of any crop improvement programme depends on the extent of genetic variability in the base population, which should be readily available to the breeders (Khush and Virmani 1985; Bajaj 1990). There is a lack of genetic variability in most agricultural crops, and in many cases, variability in the cultivated species for important economic traits is limited (Khush 1991).

The urgency of need for the production of more food has been forcing plant breeders to look for certain valuable traits that are not found in cultivated species. The application of any conventional rice breeding method specifically relies on the

combination of important useful traits through sexual hybridization, depending on the reproductive mechanism of the crop species and on the specific breeding goals or objectives (Khush and Jena 1986). Sometimes, a particular breeding objective might indicate a certain method to use, but the reproductive mechanism of the species may not permit its use. Another great problem has been experienced in the past with many of the existing high yielding IR rice varieties, as they gradually became more and more susceptible to disease and insect epidemics. One of the main reasons for this increase in susceptibility is genetic uniformity, since all the IR varieties were bred from common ancestral species, i.e., using the same maternal parents (Hargrove et al. 1980). The same method has been followed to develop hybrid rices in China, using the same sources of CMS lines, which occupied 90% of the cultivable area (Virmani and Edwards 1984). This might lead to devastating yield losses, as experienced in maize by southern corn blight disease epidemics during 1970-71 in Mexico, because of cytoplasmic uniformity.

The most obvious limitations in conventional pureline breeding methods are summarized as follows :

- the plant breeders are always confined to naturally occurring genetic variability,
- the breeders are also confined to work within the primary gene pool of the species,
- in conventional breeding methods, when recessive genes are being persued, dominance delays progress,
- when a population or a variety needs to be evaluated against biotic or abiotic stresses, both efficiency and accuracy of the method are affected,
- the capacity of conventional breeding methods is always inadequate to handle large populations and environmental factors affect the results,
- the time taken to develop a new variety by this method is always long, at least 6-7 years,
- these methods are very labour intensive, the extent of land required for any of these methods is also large.

1.5.2 Objectives and role of wide hybridization in rice improvement

Diseases and insects, particularly new races or strains, are the major threats even for the new improved rice varieties to continued yield stability. Many rice improvement programmes in different countries have concentrated on the development of improved varieties with multiple resistances to major diseases and insects; indeed at IRRI varieties possessing multiple resistances have already been developed (Khush 1984a, b; Khush and Virmani 1985). However, it is quite possible that the genetic variability for economically important traits present within the improved varieties of *Oryza sativa* may either be exhausted or will be very limited in the near future. Wide hybridization is an important plant breeding tool for the introduction of alien genetic variations and the transfer of useful traits from wild species of *Oryza* to commercially useful improved varieties. The wild relatives of *Oryza* are an extremely rich source of useful genes and genetic diversity for further varietal improvement (Table 1C). Important genes for disease and insect resistance have already been transferred into a few crop species through wide hybridization. This gene transfer technique can be utilized for broadening the primary gene pool of the crop species and the creation of additional genetic variability into crop improvement programmes (Khush and Jena 1986).

For the improvement of certain important traits, characteristics that are present in related species or genera can be exploited and donated to the cultivated species (Jena and Khush 1987). However, for growing rice under different agroclimatic conditions and natural stresses, there was an urgent need to develop improved varieties with wider genetic bases. Although the wild species of *Oryza* are an important reservoir of desirable genes, they have rarely been used in rice improvement programmes in the past, mainly due to the problem of sexual incompatibility. A few significant exceptions were the transfer of a resistance gene to the grassy stunt virus donated from a single strain of *O. nivara* to *O. sativa* and the cytoplasm of *O. perennis* to develop new cytoplasmic male sterile (CMS) lines for hybrid seed production (Khush et al. 1977; Lin and Yuan 1978). After successfully overcoming the post-zygotic incompatibility barrier, 12 different monosomic alien addition lines having the complete chromosome complement of *O. sativa* plus an additional alien chromosome of *O. officinalis* have been developed (Jena and Khush 1986). Such lines are also being developed and characterized in *O. australiensis*, *O. latifolia*, *O. minuta* and *O. brachyantha*. It was

possible to overcome this barrier by utilizing the *in vitro* techniques of 'embryo rescue' or hybrid-embryo culture.

The introgression of genes for brown planthopper (BPH) and white backed planthopper (WBPH) resistance from *O. officinalis*, blast and bacterial leaf blight

Table 1C. Some wild species of *Oryza*, and a few grass species with useful traits of economic importance (adapted from Khush 1991)

Species name	Chromo-some no.	Useful traits[a]
O. nivara	24	Drought avoidance, resistance to GSV & blast
O. rufipogon	24	Tolerance to acid sulphate soil, stagnant flood; source of CMS
O. glaberrima	24	Drought tolerance, resistance to GLH
O. barthii	24	Drought avoidance, resistance to BLB
O. longistaminata	24	Resistance to SB, drought tolerance, floral characters for outcrossing and high pollen production
O. glumaepatula	24	Elongation ability
O. meridionalis	24	Elongation ability
O. officinalis	24	Resistance to BPH, WBPH and GLH
O. australiensis	24	Drought tolerance, resistance to BPH
O. brachyantha	24	Tolerance to laterite soil, resistance to YSB and LF
O. rhizomatis **(new)**	24	Drought tolerance
O. granulata	24	Shade tolerance, drought resistance
O. meyeriana	24	Shade tolerance and adaptation to aerobic soil
O. eichingeri	24, 48	Shade tolerance, resistance to BPH, WBPH and GLH
O. punctata	24, 48	Resistance to BPH
O. minuta	48	Resistance to BPH, WBPH, GLH, BLB and blast
O. latifolia	48	High biomass production, resistance to BPH, WBPH, GLH
O. alta	48	High biomass production
O. grandiglumis	48	High biomass production
O. ridleyi	48	Shade tolerance, resistance to RWM, SB, BLB & blast
O. longiglumis	48	Resistance to BLB and blast
Grass species :		
Leersia perrieri	-	Resistance to SB
Leptochloa fusca	20	Salt tolerance
Porteresia coarctata	48	Salt tolerance

[a]GSV : Grassy stunt virus; GLH : Green leafhopper; BLB : Bacterial leaf blight; SB : Stem borer; YSB : Yellow stem borer; BPH : Brown planthopper; WBPH : White-backed planthopper; CMS : Cytoplasmic male sterility; LF : Leaf-folder; RWM : Rice whorl maggot.

resistance from *O. minuta*, BPH and bacterial blight resistance from *O. australiensis* and *O. latifolia*, and CMS from *O. perennis* have successfully been made into cultivated rices. At IRRI and in other national rice breeding programmes, these elite breeding lines produced from wide crosses are being used extensively without any problem of sexual incompatibility [Jena and Khush 1990; Khush 1991; Brar 1993 (personal communication)].

1.6 The needs of plant cell and tissue culture in the 21st Century

The needs of plant cell and tissue culture in the next century will increasingly be enormous and linked to molecular aspects of plant cell biology. During the past thirty years, plant cell and tissue culture has emerged from its purely descriptive and cataloguing phase. The understanding of the fundamental processes involved in growth and development of plants is in urgent need of further research. The challenge for plant cell and tissue culture in future is to try to improve our understanding of the activities of plants *in vitro* and *in vivo*, manipulated in a scientific manner. In the 21st century, plant cell biology will have many aspects, and these will be related mainly to the needs of plant cell and tissue culture (Cocking 1987). It has already been recognized that genetic diversity of cytoplasmic factors is very important. In this context, emerging recombinant DNA technologies through appropriate tissue culture techniques, may help in the near future, not only by eliminating unwanted genes from mitochondria but also by allowing the transfer of re-engineered mitochondrial CMS genes across sexual barriers. Protoplast fusion techniques may also help to generate cytoplasmic variability. Of the various techniques involved in plant cell and tissue culture, the culture of immature hybrid embryos seems to be the most effective for wide hybridization, and there will be a tremendous need for this technique in the next century for rescuing valuable hybrids having useful traits of economic importance (Swaminathan 1986).

1.6.1 Application of cell and tissue culture in rice crop improvement

During the last 4 decades, considerable progress has been achieved in rice cell and tissue culture studies since Ameniya and his group first successfully attempted *in vitro* culture of immature rice embryos in Japan (Ameniya et al. 1956). Since then, a number of new techniques of potential usefulness have been developed and utilized effectively in rice improvement programmes. Some of the techniques are becoming economically

viable technologies of proven benefit, and certain others appear very promising but need further investigation (Raina 1989). Although rice tissue cultures were initiated first from excised roots and young hybrid embryos, the first plant regeneration was achieved from rice callus derived from nodal segments; a few years later complete plants from rice callus were regenerated for the first time. This was also the first success with monocots (Furuhashi and Yatazawa 1964; Nishi et al. 1968). In the same year, the production of haploid rice plants through *in vitro* culture of anthers was another remarkable achievement, and the use of anther culture breeding had led to the release of more than 100 improved rice varieties and lines in China by 1986 (Niizeki and Oono 1968; Zhang and Chu 1986).

The most fascinating area in rice cell and tissue culture is protoplast technology; a better understanding and utilization of this technology in rice improvement is necessary. Genetic transformation of rice using protoplast technology holds enormous potential for plant genetic improvement. The main areas of potential usefulness of protoplast technology in rice improvement are the transfer of CMS in varieties with good combining ability, the transfer of disease resistance to and tolerance for various environmental stresses from wild species, and obtaining greater vigour in hybrid rice through mitochondrial recombination. Most of these objectives are too difficult or even impossible to accomplish through conventional techniques. For the production of cybrids through somatic cybridization, and for the transfer of alien genes, somatic hybridization could be attempted in less time where incompatibility barriers are severe and the possibility of producing hybrids through embryo rescue techniques are also limited (Raina 1989). However, cell and tissue culture techniques in rice have also opened up the possibility of obtaining cell lines, and eventually plants, with increased amino acid levels, a higher total protein content and resistance to diseases. This could be done by induction and selection of mutants at the cellular level for resistance to amino acid analogues, disease toxins or toxin analogues, and subsequent regeneration of plants from the resistant calli (Zapata 1985; Schaeffer 1986; Chen and Meng 1986).

Rice cell culture techniques are also expected to offer the possibility of generating plants with increased tolerance to various environmental stresses, such as soil salinity, soil acidity, metal toxicity, cold temperature, drought and flood. The group at Tissue Culture for Crops Project (TCCP), at Colorado State University have reported the

regeneration of about 4,000 plants from NaCl-tolerant cell cultures of a number of rice varieties, and also claimed that the tolerance is stable and inheritable (Nabors and Dykes 1985). The IRRI group also reported some interesting results, and found that the growth curves of a salt-tolerant variety (Pokkali) and that of a susceptible variety (IR28) were similar at different NaCl concentrations (Abrigo et al. 1985). They identified a number of salt-tolerant and Al-tolerant mutants utilizing somaclonal variant seedlings derived from self regenerants in their screening test for salt and aluminium tolerance. However, from the results so far, it seems that a callus-mediated tissue culture system, using different explants, could be a novel source of useful genetic variability in rice, thus shortening the breeding cycle to develop improved varieties. The current status of genetic manipulation of rice cell and tissue culture to transfer specific economic traits is discussed separately (see section 1.7 of this Chapter).

1.6.1.1 Organogenesis and Embryogenesis

Rice plants can be regenerated from *in vitro* culture induced callus tissues, using different explants either through organogenesis or embryogenesis. Organogenesis and morphogenesis can also be induced from cell suspension cultures, especially when the cells are transferred from high auxin to high cytokinin concentration culture medium (Flick et al. 1983). Plant regeneration from rice callus tissues through organogenesis has been reported by several authors (Kawata and Ishihara 1968; Nakano and Maeda 1979). Since the achievement of the first rice plant regeneration from anther-derived callus through embryogenesis in 1973, several other reports on plant regeneration by somatic embryogenesis using different explants have been published over the past twenty years (Guha and Mukherjee 1973; Heyser et al. 1983; Abe and Futsuhara 1985). During somatic embryogenesis, somatic embryoids or globular structures form on the surface of callus and germinate like zygotic embryos when transferred to a regeneration medium. Somatic embryoids always have a bipolar structure with shoot and root meristems, connected with each other and not interrupted by undifferentiated callus tissue. In contrast, when regeneration occurs by organogenesis, shoots and roots are formed from a callus, and these organs develop independently in respect of localization. Somatic embryogenesis, was considered to be rare in the Gramineae, is now thought to be the most common method of plant regeneration *in vitro*.

Embryogenesis and morphogenesis are very important events in rice tissue cultures, mainly to generate somaclonal variations *in vitro* for creating more genetic variations in the base populations to complement the efforts of plant breeder. They are also useful to provide embryogenic callus tissues for initiating and establishing suitable, embryogenic cell suspension cultures; and to rescue hybrids of wide crosses by culturing immature embryos and regenerating them to overcome post-zygotic incompatibility (Abdullah et al. 1986; Jena and Khush 1986b). For mutational breeding purpose, selected mutants from somaclonal variants also provide important genetic variations, and identification of a number of salt-tolerant and Al-tolerant mutants, mutants with higher level of protein and lysine content in their grains are examples (Nabors and Dykes 1985; Abrigo et al. 1985; Schaeffer 1986).

Usually, the initiation and growth of embryogenic callus requires one medium, and the development of embryos into plants requires another medium. The importance of optimal levels of auxin, particularly 2,4-D, for embryogenic callus initiation has been stated by several workers. The regeneration of rice plants was found to be enhanced by media containing lower levels of cytokinins (NAA, BAP or IAA) and without auxin depending on the cultivar (Abe and Futsuhara 1985; Raghava-Ram and Nabors 1985). In some cases, yeast extract and coconut water were found to be useful additions to the media to improve somatic embryogenesis. In two recent reports, the use of maltose as the sole carbon source in the liquid cell suspension medium and osmoticum in protoplast culture has been emphasized [Biswas and Zapata 1993; Jain et. al. 1994 (personal communication)]. It was also found that regeneration frequency increased 8 to 12-fold when maltose was used in place of sucrose as a carbohydrate source.

1.6.1.2 Androgenesis

Microspores just before, or at the time of first mitosis exhibit various modes of development in cultured anthers to form haploid proembryos or callus, which ultimately give rise to haploid or spontaneous diploid plants. This mode of development of microspores leading to the formation of haploids either directly by embryogenesis, or indirectly via callus formation is termed as androgenesis. The first event in androgenesis is a shift of the microspores from gametophytic to sporophytic development. After this induction phase, microspores of *Datura* and *Nicotiana*

develop into plants directly via embryogenesis, but androgenesis in rice usually has an intermediate step of callus formation; this callus subsequently gives rise to plants through organogenesis and/or embryogenesis (Guha and Maheshwari 1966; Nitsch and Nitsch 1969; Niizeki and Oono 1968). In *in vitro* culture of rice anthers, microspores generally undergo various modes of androgenesis which lead to the development of haploid plants either directly by embryogenesis, or indirectly via callus formation. There are two distinct modes of androgenesis in the division of rice microspore nucleus, the direct and the indirect, irrespective of the early events.

- *Direct androgenesis* : In this mode, the microspore behaves like a zygote and undergoes various stages of embryogeny like those *in vivo*, as in *Atropa*, *Datura*, and *Nicotiana*. The embryos are released from the exine mostly at the globular stage and develop further. Finally, the cotyledons develop and the plantlets emerge from the anthers in 4-8 weeks.

- *Indirect androgenesis* : The microspore divides a few times to form a callus (instead of undergoing embryogenesis), which bursts out through the anther wall. This mode of development is quite common and is usually caused by complex media. Finally, the callus either differentiates to form embryos, or roots and shoots on the same medium, or it needs to be transferred to another medium for differentiation. The callus-derived plants exhibit genetic variations and polyploidy (Bajaj 1990).

Although androgenesis can be induced in cultured anthers at the tetrad stage, or at the binucleate pollen stage, the most suitable stage for the induction of androgenesis in rice is mid-uninucleate stage (microspores just before first mitosis) or late uninucleate stage (microspores at the time of first mitosis) depending on the cultivars (Gresshoff and Doy 1972; Kameya and Hinata 1970; Nitsch and Nitsch 1969).

1.7 Importance and objectives of genetic manipulation in cereals

The economically important cereal and grass crops have generally been considered to be recalcitrant to *in vitro* genetic manipulation. High frequency regeneration of plants from single cells or tissues, which is a prerequisite for cellular and molecular manipulation, has proven to be especially difficult for the major cereal crops (Vasil 1982; Bright et al. 1985). Since 1980, significant progress has made possible cellular

and molecular genetic manipulation and improvement of crop species of economic importance. In particular, during 1986 to 1988, the remarkable discovery and exploitation of embryogenic tissue and single somatic cell cultures led to the development of efficient procedures for plant regeneration in almost all of the important species of grasses and cereals such as maize, rice and sugarcane (Abdullah et al. 1986; Vasil and Vasil 1986; Kyozuka et al. 1987; Rhodes et al. 1988). These results, as well as success in somatic hybridization and transient, as well as stable, expression of introduced genes in cereal cells and plants, provided challenging opportunities for the genetic manipulation and improvement of this group of crops.

The objectives and importance of cell culture and genetic manipulation are to produce plants which have added valuable and useful agronomic qualities. These qualities are improved nutrition, resistance to herbicides or insect pests and diseases. Unique and useful sexual (by embryo rescue) somatic or cytoplasmic hybrids which have been obtained by protoplast fusion, combine desirable characters of sexually incompatible species. Additionally, plants have been produced in which useful foreign genes have been stably integrated by genetic transformation. In most cases, these combinations cannot be readily incorporated into new varieties by conventional breeding methods (Vasil 1988; Peng et al. 1992).

1.7.1 Current status of genetic manipulation in cereals with special emphasis on rice

The direct or indirect transfer and insertion of a specific gene or group of genes into a recipient genome has wide application in the genetic manipulation of crop plants. Recent advances in cell and tissue culture, somatic hybridization and cybridization, recombinant DNA technology, the availability of well defined selectable markers and efficient gene vectors (Ti and Ri plasmids) and direct DNA transfer methods have opened new avenues to precisely transfer foreign genes into plants (Schell 1987; Willmitzer 1988). At present, a number of indirect and direct transfer methods are also available for genetic manipulation and production of transgenic plants. The indirect methods include a modified Ti and Ri plasmid systems (*Agrobacterium*-mediated gene transfer), somatic hybridization involving distantly related species to overcome sexual incompatibility and to transfer valuable agronomic traits, cybridization to transfer

useful cytoplasmic traits. The direct gene transfer methods include PEG-induced DNA uptake, microinjection of DNA into cultured cells and plant organs, electroporation, microprojectile bombardment (Uchimiya et al. 1988).

Genetic manipulation using indirect gene transfer method

Cereals, particularly rice, do not respond well to *Agrobacterium*-mediated gene transfer. There are a few reports of rice transformation using Ti and Ri plasmids which lack plant regeneration. In 1986, the Ti plasmid was introduced into rice by PEG-induced fusion of rice protoplasts with *A. tumefaciens* spheroplasts and the colonies formed (1 in 1000) which it was claimed were able to synthesize T-DNA specific opines (Baba et al. 1986). Recently, transgenic callus was produced after inoculating mature embryos of the cultivar Nipponbare with the wide host range, supervirulent *Agrobacterium* strain A281 (pTiBo542). Embryos of another cultivar, Fujisaka 5, gave a hypersensitive response when inoculated with strain A281, but showed extensive root production following inoculation with the limited host range bacterial strain A856. The calli from the wide host range inoculation experiment grew on kanamycin containing medium, indicating that they were carrying the *nptII* (neomycin phosphotransferase II) gene and fluoresced blue upon incubation with β-glucuronidase indicating substrate GUS activity. Hybrid plants could not regenerate, probably because of the inhibitory effects of kanamycin selection system (Reineri et al. 1990).

Somatic hybridization through protoplast fusion is an alternative procedure for the transfer of useful desirable genes from an alien species to cultivated species. Several successful achievements of somatic hybridization in rice for transferring novel genetic traits across sexual incompatibility barriers were encouraging. A number of putative hybrid calli and morphologically distinct hybrid plantlets were obtained following electrofusion between cell suspension-derived protoplasts of *Japonica* rice Nipponbare and those of barnyard grass (*Echinochloa oryzicola*) which exhibits C4 photosynthesis. The selection of hybrid cells was based on inactivation of rice protoplasts by iodoacetamide (IOA) and the inability of barnyard protoplasts to divide. The hybridity of these materials were confirmed by isozyme and chromosome analysis, but hybrid plants could not grow to maturity (Terada et al. 1987). The first somatic hybrid plants were obtained between *Japonica* rice and four wild rice species (see Table 2D,

Chapter 2) using similar fusion and selection systems. However, only one of the hybrid plants (*O. sativa* (+) *O. officinalis*) produced a single seed and was fertile (Hayashi et al. 1988).

In the same year, somatic hybrid plants were recovered from fused haploid cell suspension-protoplasts of two *Japonica* rice cultivars and identified using anatomical and morphological markers (Toriyama and Hinata 1988b). From the Plant Genetic Manipulation Group at Nottingham, putative somatic hybrid callus between *Japonica* rice (T309) and a salt-tolerant wild relative of rice (*Porteresia coarctata*), somatic hybrid plants between T309 and a 'true-*Indica*' rice cultivar IR54, and putative somatic hybrid plants between T309 and a wild rice species *O. rufipogon* possessing genes tolerance to acid sulphate soil and stagnant flood were also recovered during the last 3 years (Finch 1991; Slamet 1991; Baset 1992). There is great potential for the rapid production of new CMS rice lines through the use of protoplast fusion technology. The transfer of maternally inherited CMS-traits by protoplast fusion has been reported by few workers.

The first report of the production of cybrid callus followed by cybrid plants was from a laboratory in Japan, in which protoplasts of one of the parents was from a CMS line or cultivar (Yang et al. 1989). Following electrofusion, as many as 560 cybrid plants were regenerated using a CMS donor variety (MTC-9A), which is a nuclear mutant and a fertile *Japonica* variety (Norin 8) and the source of the nucleus was determined in putative somatic cybrid progeny by a specific enzyme assay (Akagi et al. 1989). Cybrid plants were also obtained from protoplast fusion between *Japonica* rice (Nipponbare), having normal cytoplasm and '*Indica*-type' rice (Chinsurah Boro II), having CMS-cytoplasm (Kyozuka et al. 1989). Recently, putative cybrid cell cultures and plants were produced through protoplast fusion, aiming to transfer the mitochondrial genome of a CMS *Japonica* rice (WU10A) into the nuclear background of fertile *Japonica* rice T309 (Finch 1991).

Genetic manipulation using direct gene transfer method

The first direct and stable gene transfer to cereal protoplasts was achieved by incubation of *Triticum monococcum* protoplasts with plasmid pBL1103-4, which was carrying chimeric genes encoding the *npt*II gene as a selectable marker (Lorz et al.

1985). Later, the technique was extended to rice and simple *E. coli*-based vectors were developed for protoplast transformation (Potrykus et al. 1985). The first stable transformation in *O. sativa* was reported by a Japanese group in 1986. In this study, cell suspension-derived protoplasts were incubated with PEG and the bacterial plasmid pT2T3, which carries aminoglycoside phosphotransferase II gene (*APH*(3′)II; *npt*II) conferring resistance to the two antibiotics kanamycin and genticin 418 (G418). Plasmid uptake and gene introgression was confirmed using Southern blot analysis and an *APH*(3′)II enzyme assay (Uchimiya et al. 1986). Later on, plasmid uptake into rice protoplasts induced by PEG, electroporation, and PEG combined with electroporation were compared. Transgenic rice plants from kanamycin resistant calli of *Japonica* rice (T309) were regenerated, the expression presence of the *npt*II gene were confirmed by enzyme activity and DNA hybridization (Yang et al. 1988; Zhang et al. 1988).

Furthermore, other workers had noted the inhibitory effect of kanamycin on the regeneration of green plants from callus growing on selection medium. For the selection of transformed cells of *Japonica* rice Yamahoushi following electroporation of protoplasts in the presence of pCN carrying the *npt*II gene, G418 was more effective and gave rise to a higher frequency of green plant regeneration (Toriyama et al. 1988a). After surveying a range of selectable markers for rice transformation, it was concluded that kanamycin was not an efficient selection agent, although transformed cells were selected in the past at the early stage of protoplast culture (Dekeyser et al. 1989; Zhang et al. 1988). In another study, PEG-induced uptake of pAI_1GusN (carrying the non-selectable *gus* gene) into rice protoplasts was carried out and plants were regenerated from plasmid-treated protoplasts without using any selection system to avoid antibiotic inhibition. The transgenic nature of the plants was confirmed by Southern blot and slot blot analyses (Zhang and Wu 1988).

In 1989, the bacterial *hph* gene encoding hygromycin B resistance was introduced into *Japonica* rice protoplasts by electroporation to produce transgenic rice plants and the transgenic nature was confirmed by Southern blot analysis. In addition, emphasis was given on the feasibility of co-transformation of a non-selectable gene with a selectable marker and fertile plants were regenerated from protoplasts co-transformed with an unlinked *gus* gene. In both cases, fertile seed production from selfing hygromycin-resistant plants, germination of seeds and survival of seedlings on hygromycin B

containing medium confirmed the transmission of the active *hph* gene to seed progeny (Shimamoto et al. 1989). In 1990, the first transgenic *Indica* rice plants were recovered after incubating cell suspension-derived protoplasts with pKAN, carrying the *npt*II gene in the presence of PEG (Peng et al. 1990). However, all the transformed plants (T_o) recovered from G418-resistant calli, containing the foreign gene were sterile. In a subsequent study in 1991, transgenic *Indica* rice plants were regenerated from G-418-resistant protoplast-derived calli from the laboratory at Purdue University, USA (Dept. of Botany and Plant Pathology) (Peng et al. 1991). Introgression and presence of the *neo* gene (pKAN) and the *gusA* gene (pPUR) were confirmed, and transmission of the foreign gene (*gusA*) to the successive progenies (T_1 and T_2) were verified and confirmed by histochemical analysis of GUS expression (Peng et al. 1992).

Several other reports have appeared on the production of both *Indica* and *Japonica* transgenic rice plants and isolation of an efficient actin promoter for use in rice transformation and now genetic transformation, gene transfer are routine in many laboratories (Hayashimoto et al. 1990; Tada et al. 1990; Terada et al. 1990; Datta et al. 1990c; McElroy et al. 1990a, b). At present, among the other investigated new techniques, direct DNA uptake into protoplasts mediated by chemical treatment, electroporation, or a combination of both procedures is the only reproducible method for inserting foreign genes into cereals including rice (Davey et al. 1989; Potrykus 1990a, b). Although great progress has been made in the manipulation of rice protoplasts in the last 5 years, the overall process of transforming and regenerating rice protoplasts is complex. It is now possible to initiate studies in relation to the mechanism, or mechanisms of gene integration into the host genome, which would facilitate the targeting of foreign gene(s) into specific sites in the rice genome or insertion mutagenesis of native genes. Developments in this field of study should be extremely exciting during the next few years (Hodges et al. 1991; Cao et al. 1991).

1.8 Haploids from gametophytic cells-recent developments and future prospects

The term 'haploids' refer to those plants which posses the gametophytic number of chromosomes in their sporophytes. In flowering plants, such as angiosperms, the flower contains specialized structures, the anthers and the pistil or gynoecium, in which

the male and the female gametophytes (pollen grains and embryo sacs respectively), are formed. In these groups of plants, a diploid, spore-producing generation (sporophyte) alternates with a haploid, gamete-producing generation (gametophyte). Normally, the male gametophyte completes its early development within the anther (Mascarenhas 1989). Haploids are induced by meiosis or somatic reduction (generally reducing the chromosome number to half of the original). Haploid calli and plants can be regenerated from both male and female gametophytic cells through *in vitro* culture of anthers, isolated microspores or pollen grains, excised ovaries and ovules, and elimination of chromosomes by the 'bulbosum technique' in barley (Kasha and Kao 1970). Regeneration and production of haploid green plants have great potential in plant breeding for two main reasons :

- the presence of one set of chromosomes facilitates the isolation of mutants and
- homozygous diploids can be obtained by chromosome doubling.

In the early 1980s, with the availability and the introduction of *in vitro* techniques, especially anther culture for the induction of androgenesis, a renewed interest was gained all over the world in the production of haploids for crop improvement (Bajaj 1983a; Hu 1985). Subsequently, it has become increasingly evident that these techniques considerably accelerate the production of haploids for plant breeding programmes. Although it is possible to produce homozygous pure lines by conventional inbreeding and backcrossing, these methods are cumbersome, laborious, time-consuming processes and rather inefficient. Since the last decade, *in vitro*-produced haploids have been introduced into breeding programmes of many agricultural crops, and useful positive results have been obtained especially with major cereals, such as rice, wheat, barley and maize. Among these, rice and wheat are the best examples. Since the first release of commercial rice varieties derived from haploid rice breeding in 1976, more than 100 new rice varieties and lines have been developed through anther culture breeding in China, are currently grown on 250,000 hectares (Yin et al. 1976; Oono 1988).

Currently, haploids can be regenerated in a matter of weeks in some important agricultural crops through anther culture, and by doubling their chromosome number homozygous diploids can be procured in a single generation. Subsequently, these

fertile homozygous plants can be used for developing the inbred lines for hybrid production and they offer the advantage that they do not loose desirable characters by normal meiotic segregation. The culture and potential of haploid protoplasts or cells have been well emphasized, because they may help to understand many difficult problems of somatic cell genetics, especially in the induction of mutations and genetic variability (Bajaj 1983b, 1989b). Since most of the mutations are recessive, they are difficult to detect in the presence of an unmutated dominant gene. The haploid system, with one set of chromosomes, makes their detection easier. A number of mutants of various types, including auxotrophic, autotrophic and resistant ones have been induced through this culture system (King 1986; Raina 1989) (see section 3.5, Chapter 3). Another potential application of haploids in breeding is in the production of desirable genetic translocations, substitution and addition lines through the culture of intergeneric and interspecific hybrid anthers (Wang and Hu 1985).

In addition to other advantages, haploids can be used to obtain homozygosity for genes in cases where it is normally difficult to achieve, such as self-incompatible alleles in rye (Snape 1989). The most remarkable use of cereal haploids is the one-step transfer of cytoplasm from one line to another. Using *Zea mays* monoploids, it was possible to transfer successfully the genotypes of inbred lines into their cytoplasm which caused male sterility (Kermicle 1973). For the uptake and genetic manipulation studies, haploid protoplasts are an ideal tool. Their foremost potential use lies in mutation and complementation studies; they have the advantage of early detection and easy isolation of mutants. For these studies, although pollen grains or somatic embryos are able to take up DNA, the efficiency is low. This can be improved by the use of isolated pollen protoplasts, which are natural haploids and which lack the thick wall, the exine. For the induction of mutants that are resistant to pests, pathotoxins, herbicides, and tolerant to various environmental stresses, haploid systems would be the most rewarding for investigations of crop improvement in the future (Bajaj 1990). The recent developments and future prospects of the haploid system are summarized in more detail in the Chapter 3 of this thesis (see section 3.1 and 3.5).

1.8.1 Anther or microspore culture

Anther or microspore culture is the most extensively used technique in plant tissue culture, especially in rice. The main attractions of this technique are, the excision and

culture of anthers is relatively simple and efficient, and there is every possibility of obtaining haploid cells, tissues and complete plants which have only a single dose of genetic information (Raina 1989; Bajaj 1990). In this *in vitro* technique, the normal gametophytic development of the microspores within the anther can be diverted to a sporophytic pathway. After this diversion or induction phase, the cultured microspores of *Datura* and *Nicotiana* develop into plants directly via embryogenesis, where in *Oryza* they continue to divide to form callus or embryoids on a specified culture medium. Finally, embryogenesis and/or organogenesis of the callus or embryoids leads to plant formation (Guha and Maheshwari 1966; Nitsch and Nitsch 1969; Niizeki and Oono 1968). In some rice cultivars, a lower concentration of 2,4-D, combined with higher levels of NAA and kinetin can stimulate direct plant regeneration from developing microspores, bypassing the intermediate step of callus formation, which is not desirable. However, generally rice plantlets must be indirectly regenerated from the induced callus, and this can be achieved by removal, or reduction of 2,4-D concentration, although NAA, IAA and kinetin also play an important role in plantlet regeneration. The absolute growth factor requirements for both callus induction and plantlet regeneration are genotype dependent (Guha-Mukherjee 1973; Liang 1978; Cornejo-Martin and Primo-Millo 1981).

The production of haploids and subsequently homozygous diploid plants through *in vitro* anther or microspore culture has advanced dramatically in the last 25 years. Since the first report of the production of haploid plants from rice microspores through *in vitro* culture of immature anthers, more than 100 varieties and lines were developed by 1986 through 'anther culture breeding' in China (Niizeki and Oono 1968; Zhang and Chu 1986). Since 1974, isolated microspores or microspore-derived embryos, and pollen tetrad or haploid cell suspension-derived protoplasts have been used as an alternative experimental system for cell fusion, genetic transformation and mutation studies. These studies have been carried out using different species by several investigators and they could successfully produce fertile gametosomatic hybrids in *Petunia* and *Nicotiana* by protoplast fusion, and transgenic rapeseed plants by microinjection of DNA into microspore-derived embryoids (Pirrie and Power 1986; Lee and Power 1988; Neuhaus et al. 1989). Another example is the production of fertile, transgenic 'Indica-type' rice plants following uptake of foreign DNA into the

haploid (microspore-derived) cell suspension protoplasts (Datta et al. 1990c). All these primary transformants were homozygous, lacking segregation and also showed an identical DNA-integration pattern, presumably because of the use of haploid protoplasts and spontaneous doubling of their chromosomes.

Since 1968, subsequent studies have shown that, in angiosperms, various cultural conditions and even genetic factors, either singly or in an interacting manner, affect microspores in their new developmental pathway (Maheshwari et al. 1980; CC Chen et al. 1991). These factors affecting androgenesis, any of which can be limiting, especially in rice, are discussed in detail in Chapter 3 (see section 3.1) (Zapata et al. 1983; CC Chen et al. 1986, 1991; Loo and Xu 1986, 1991; Lee et al. 1988; Mercy and Zapata 1988; Raina 1989; Bajaj 1990). Optimization of these conditions and maximum control of the genetic factors leads to high frequency green plant regeneration with a minimum occurrence of albino plants. The future prospects of anther or microspore culture in cereals, with special emphasis on rice for producing hybrids to overcome incompatibility barriers, transgenic rice to obtain stable integration of desirable gene(s), for detecting and isolating valuable recessive mutants, together with somaclonal and gametoclonal variants, are discussed in detail in Chapter 3 (see section 3.4).

1.8.2 Ovary or ovule culture

The production of haploids from cultures of unpollinated ovaries has been reported in a number of plant species, especially in rice, where a reliable system has been provided (Asselin de Beauville 1980; Zhou and yang 1980, 1991). Embryological studies traced that proembryos and calli originated from the embryo sac. Later, it was found that the embryo sac, like the pollen, can be induced *in vitro* to pass through a sporophytic pathway, via proembryo development, callus formation and organ differentiation, to plant regeneration (Zhou and Yang 1981).

The technique of ovary/ovule culture, after identifying the key factors affecting the process, have been described in detail (Zhou et al. 1986; Yang et al. 1986). The natural limitation of one embryo sac per ovule for inducing callus is the major disadvantage in ovary/ovule culture research. Most of the plants that regenerated from ovary culture were green, although the efficiency was still rather poor compared to anther culture (Yang et al. 1984; Liu and Zhou 1984; Zhonglai and Chang 1984). Most of the ovary-

culture derived plants were haploids with a few diploids in comparison to anther culture derived plants showing a wide range of ploidy level (Zaonglai and Chang 1984). The only similarity was a better induction frequency of gynogenic calli in *Japonica* cultivars than in the *Indicas*, as in anther culture. However, a low-responding anther culture cultivar could do very well, whilst a high-responding one was not necessarily remarkable in ovary/ovule culture; (Zhou et al. 1986).

In 1986, some elite lines were selected after they had regenerated diploid green plants directly from the culture of unfertilized ovaries of the hybrids between *Indica* and *Japonica* crosses (Lai 1986). With the improvement of culture techniques, ovary culture in rice appears to be drawing attention as a potential technique for the production of haploids and double haploids in sufficient numbers for utilazation in the breeding programmes. This has opened a new avenue to genetic research and haploid breeding.

1.8.3 Haploid cell suspension and protoplast culture

Haploid cell suspension cultures can be initiated directly from microspores, megaspores, or immature embryos of certain wide crosses undergoing chromosome elimination. They may also be induced from explants of tissues from haploid plants (Kasha 1974). For the study of somatic cell genetics, especially for mutations, haploid cells or protoplasts are also useful materials. They offer the advantage of detecting mutations readily, whether dominant or recessive. The other advantage of the haploid cells or protoplasts is that the mutants can be easily diploidized to form homozygous diploids.

Emphasis was given on the culture and potential of haploid protoplasts in early 1980s and a number of mutants of various types, auxotrophic, autotrophic and the resistant cell lines have been induced through this system (Bajaj 1983b; King 1986). Later, haploid cell suspensions of rice were initiated by a number of workers using callus induced from immature anthers or young intact panicles of cytoplasmic male sterile double mutant lines of *Japonica*, *Indica*-type and true-*Indica* varieties (Toriyama and Hinata 1985, 1986; Datta et al. 1990b; Biswas and Zapata 1991). A viable system for the isolation of totipotent haploid protoplasts has been established. Diploid somatic-hybrid plants and transgenic, fertile *Japonica* and *Indica*-type rice plants were

recovered recently using this system (Toriyama and Hinata 1988a; Toriyama et al. 1988b; Datta et al. 1990c). There were great advantages in using a haploid protoplast system for producing transgenic plants of *Indica*-type rice. All of these regenerated plants demonstrated an identical DNA-integration pattern. In addition, no segregation of the hybridizing bands was observed on the Southern blots. The lack of segregation in the offsprings indicated the homozygosity of the primary transformants. This result strongly suggested the production of first self-pollinated, homozygous R1 progeny as a result of spontaneous doubling of the chromosome number, following uptake of the foreign DNA into the haploid, microspore-derived protoplasts (Datta et al. 1990c)

1.8.4 Isolated microspore or pollen culture

Isolated microspore or pollen culture is an unique, experimental system which offers opportunities not just for production of haploids for basic genetic studies and crop breeding, but especially in studies related to androgenesis, mutagenesis, genetic manipulation, and transformation. The culture of isolated pollen also offers some additional advantages :

- a large quantity of haploid plants from fewer anthers could be obtained;
- the sequence of androgenesis can be observed starting from a single cell;
- the uncontrolled interference of somatic callus development from the anther wall and other associated tissue can be avoided;
- various factors governing androgenesis can be better regulated and
- microspores or pollen are ideal for uptake, transformation and mutagenic studies, as pollen may be evenly exposed to chemicals or physical mutagens.

Since 1974, when Nitsch made her report on microspore/pollen culture, this technique has generated a lot of hope. After the achievement of successful plant regeneration in *Nicotiana* and in certain other members of the *Solanaceae* (Nitsch 1981), it was thought that microspore culture might be a substitute for anther culture. Rice plants were obtained from isolated microspore culture as early as 1980 (Chen et al. 1980). However, in many other cases, only limited success has been achieved in isolated microspore culture of rice (Chen et al. 1981a; Chen 1983). Intact green rice pollen plantlets were also obtained from isolated pollen, but the rate of success was very low.

Until now, the technique for isolation of rice pollen from anthers for culturing in shallow layers of liquid medium involves two different methods : mechanical isolation (crushing or disruption by magnetic stirring) and natural pollen shedding in liquid medium. In fact, before isolation of rice pollen, cold-shock of panicles at 8-10°C for 10-15 days and preculture of anthers for at least 3-4 days were found to be very helpful and important. These two factors were found to be the most important for altering the frequency of pollen initiation and induction to form callus or embryos, in particular, the absence of preculture resulted in very few pollen undergoing division and subsequent development (Chen 1983; Chen 1986a). Many other investigations have shown that the basic factor involved in successful culture of isolated microspores in the development stage of microspores prior to isolation. It was also found that only embryogenically determined pollen could develop further under *in vitro* culture condition and that the determination of pollen grains had to take place within the anthers. During this period, part of the pollen within the anthers divided twice or more. This showed that the anther wall may played a critical role during the early stage of culture (Chu CC 1982; Chen 1983).

In 1987, plants were successfully regenerated from three *Japonica* rice varieties, Sasanishiki, Nipponbare and Reimei using isolated rice pollen culture (Jia et al. 1987). At IRRI, success has also been achieved in regenerating plants from a *Japonica* variety Taipei 309 and a true-*Indica* variety IR36 (Cho and Zapata 1988; Zapata et al. 1990). In these studies, high initial percentage of p-grain and microspore viability were recognized as important prerequirements for improving efficiency in isolated microspore culture. Although plants have been regenerated from isolated microspore culture in *Indica* varieties, a major problem was the very low regeneration frequency and the fact that forty three percent of the regenerants were albinos (Zapata et al. 1990). However, this technique has potential uses in transformation, since microinjection of DNA into single microspore-derived proembryos may be feasible for rice transformation as it has been achieved for *Brassica* (Neuhaus et al. 1987). Although the plant regeneration frequency is much lower than that obtained with anther culture, isolated microspore culture provides a useful experimental system to study the effect of the anther wall and the nutrition for the cultured microspore (Loo and Xu 1991).

1.8.5 Production of homozygous diploid and haploid rice plants and utilization in rice breeding programmes

Homozygous haploid and diploid rice plants can be produced from excised anther, isolated microspore and ovary cultures in a relatively short time (with doubling of their chromosome numbers in the case of diploid plants). However, it is possible to obtain pure lines by conventional inbreeding and backcrossing, but it is a long and labour intensive process. The duplication of the chromosomes can be achieved by a number of methods : endomitosis, colchicine treatment and fusion of pollen nuclei.

In culture, haploid rice cells are generally unstable and have a tendency to undergo endomitosis to form diploid cells (chromosome duplication without nuclear division). This property of cell cultures has been exploited to produce homozygous diploid plants in many species. As a spindle inhibitor to induce chromosome duplication, colchicine has been extensively employed to produce homozygous diploid rice plants from haploid cultures. Completely homozygous and diploid rice plants can also be obtained when spontaneous diploidization occurs as a result of fusion of pollen nuclei (Bajaj 1983a).

For more than 15 years, anther culture techniques have been utilized in rice breeding programmes, mostly in China. The first two rice varieties developed through haploid breeding were released for cultivation in 1976. Since then, more than 100 varieties and lines were reported to have been developed through anther culture and are currently grown on 250,000 hectares in China (Yin et al. 1976; Zhang and Chu 1986; Oono 1988). In Taiwan, the application of anther culture was mainly to study the intersubspecific and interspecific F_1 hybrids. This technique was also successfully utilized to isolate brown plant hopper-resistant lines from pollen plants derived from F_1 hybrids (Woo and Huang 1980; Woo and Chen 1982). In Korea, varieties resistant to blast, stripe virus diseases, brown plant hopper and having good grain quality were developed within just 4 years.

Since 1983, IRRI's programme of anther culture application was mainly focused on the development of cold-tolerant rice varieties using F_1 hybrids of *Japonica-Indica* and *Indica-Japonica* crosses through a collaborative project between IRRI and Korea (Zapata 1985; Zapata et al. 1986a,b). Perhaps the biggest anther culture programme

outside China is the one at International Center of Tropical Agriculture, Columbia (CIAT) and as many as 20,000 diploid R_2 lines were raised from approx. 100 triple crosses. These lines were ready for observation nurseries within just 1 year, thus saving 3 years time from conventional breeding methods (Pulver and Jennings 1986).

The production of desirable genetic translocations, substitution and addition lines through the culture of anthers of interspecific and intergenic hybrids is another potential application of haploid in rice breeding (Keller et al. 1987). Anther, microspore or ovary cultures can be used to identify agronomically-desirable variants without the use of mutagens and specific *in vitro* selection strategies (Schaeffer et al. 1984; Parisi and Picard 1986). Another advantage of the haploid technique lies in the higher selection efficiency compared with that of diploid breeding. The homozygosity in doubled haploid lines is higher than that in inbred lines and it is possible to produce hybrids with maximum heterosis because of such high purity. It can also be useful in the production of hybrid seeds in self-incompatable wild rice species (Chu 1982).

The haploid technique, especially anther culture, is very useful in a breeding programme where distant hybridization is utilized. Stable fertile lines from hybrids between rice subspecies, *Japonica* and *Indica* can be obtained and the segregation and partial sterility of the hybrids may be effectively controlled by anther culture. Haploidy is especially useful for the study of mutagenesis, because of the presence of one set of chromosomes. Once a gene mutation takes place, it is immediately expressed (Chu CC 1982). It is obvious that in rice breeding, successful utilization of haploids mainly depends on the induction frequency of haploids.

1.9 Current status of protoplast techniques in rice

Impressive progress has been made in the field of rice protoplast research and techniques since the 7th International Protoplast Symposium 4 years ago, especially with the *Japonica* subspecies. The basic processes which are involved with *Indica* or *indica*-type subspecies are not known exactly, so that high frequency plant regeneration cannot be achieved routinely. It is still empirical and only a few reports of plant regeneration and transgenic rice production have been published (Datta et al. 1990a, b, c; Lee et al. 1989; Peng et al. 1992; Biswas and Zapata 1991a, b; Torrizo and Zapata 1992; Datta et al. 1992). For plant regeneration from *Japonica* or *Indica*

rice protoplasts, the choice of donor tissue plays an important role, in combination with the culture techniques. Using an embryogenic cell suspension culture or embryogenic callus as donor tissue, green and fertile plants were recently obtained for a number of important cereal crops, such as barley, *Indica* and *Indica*-type rices, sorghum, maize and wheat (Puite1992).

1.9.1 Isolation, culture and plant regeneration from cell suspension culture of different explant origin

In the early 1970s, protoplasts were successfully isolated from various tissues using enzyme mixtures, but these protoplasts were not cultured (Maeda and Hagiwara 1974; Harn 1973). Sustained division and colony formation were first achieved from protoplasts isolated and cultured from embryogenic rice callus (Deka and Sen 1976; Niizeki and Kita 1981). Later, the isolation of large numbers of totipotent rice protoplasts was possible from cell suspension cultures (Wasaka 1984). High frequency callus formation and green plant regeneration was achieved from protoplasts isolated from anther callus-derived cell suspension cultures. It is important to note that use of a medium containing amino acids as the sole nitrogen source was necessary and critical for the culture of rice protoplasts (Toriyama and Hinata 1985). Successful rice plant regeneration was also reported from protoplasts isolated from cell suspension cultures in the same year from four different laboratories (Coulibaly and Demarly 1986; Toriyama et al. 1986; Yamada et al. 1986; Abdullah et al. 1986). In these studies, significant variations in plant regeneration frequencies were observed (1-24%).

For the first time, reproducible regeneration of fertile rice plants from embryogenic cell suspension-derived protoplasts culture through somatic embryogenesis was demonstrated from the Plant Genetic Manipulation Group at Nottingham, UK. Emphasis was also given on routine production and maintenance of embryogenic suspension cultures to retain their regenerability, the use of agarose-solidified culture medium and a heat shock treatment before culture for enhancing rice protoplast division and for increasing protoplast plating efficiencies effectively (Abdullah et al. 1986; Thompson et al. 1986, 1987). In these studies, the regeneration frequency was not more than 20%, but the culture technique was rather simple without any requirement of nurse cells or feeder layers. High frequency fertile plant regeneration

(up to 50%) was reported from two other laboratories, using nurse culture and different protoplast preparation techniques (Kyozuka et al. 1987, 1988; Masuda et al. 1989). In most of the cases, plant regeneration was obtained from *Japonica* and *Indica*-type rice varieties (Wang et al. 1989; Datta et al. 1990a).

The first protoplast-to-plant regeneration system for true-*Indica* rice (IR54) was reported in 1989. In that report, four factors have been identified as critical for the successful plant regeneration (Lee et al. 1989; Hodges et al. 1990). Following this report, recently four other reports on high efficiency fertile plant regeneration from protoplasts of true-*Indica* rices (IR43, IR58, IR72), transgenic *Indica* rice plant production (IR54) have been published with encouragement for work on *Indica* rices in the near future (Biswas and Zapata 1991; Datta et al. 1992; Peng et al. 1992; Torrizo and Zapata 1992). Plant regeneration from protoplasts isolated from embryogenic cell suspension cultures of most of the important species of cereals and grasses have also been reported during the last six years (Vasil 1992) {Table 1D; Chapter 2, Part II (Table 2C)}.

However, embryogenic suspension cultures are sometimes difficult to establish and maintain if suitable growth facilities are not available. As an alternative, extensive efforts were made for a long time to induce sustained cell divisions in, and regenerate plants from, mesophyll protoplasts of graminaceous species. Remarkable success was achieved only last year using a commercial rice variety, progeny lines and *Indica* rices and plant regeneration was obtained from mesophyll protoplasts isolated from leaf-bases and sheaths of rice seedlings (Gupta and Pattanayak 1993). The development of such techniques has opened a new avenue towards the production of transgenic plants through transformation and somatic hybridization with *Indica* rices.

1.9.2 Somaclonal variation in rice tissue and cell culture

Somaclonal variation, as one aspect of biotechnology which has been applied to rice breeding, includes genetic, epigenetic and other kinds of variation. Genetic variation results from preexisting variation in the donor explants and/or from *in vitro* culture. Plants regenerated from rice tissue culture frequently exhibit genetic aberrations, and thus the culture of rice tissues *in vitro* may be considered as the simplest form of genetic manipulation. Comparing this technique with the normal backcross program-

Table 1D. Plant regeneration from cell suspension and mesophyll-derived protoplasts of the important cereals

Cereal species	Plant regeneration	Reference
***Oryza sativa* L. (rice):**		
***Japonica* (CS-protoplast)**	Fertile plants	Fujimura et al. 1985
***Japonica* & *Indica* („)**	„	Abdullah et al. 1986
***Japonica* (CS-protoplast)**	„	Yamada et al. 1986
***Japonica* (AC-CS protoplast)**	„	Toriyama et al. 1986
***Japonica* (CS-protoplast)**	Plantlets	Coulibaly & Demarly 1986
„	Fertile plants	Kyozuka et al. 1987
***Indica* (CS-protoplast)**	„	Kyozuka et al. 1988
***Japonica* (CS-protoplast)**	„	Masuda et al. 1989
***Indica* (CS-protoplast)**	„	Lee et al. 1989
***Japonica* (CS-protoplast)**	„	Jenes & Pauk 1989
***Indica*-type & CMS („)**	Plants	Wang et al. 1989
***Indica*-type & *Indica* („)**	Fertile plants	Datta et al 1990a, 1992
***Japonica* (CS-protoplast)**	„	Biswas & Zapata 1990
***Indica* (CS-protoplast)**	„	Biswas & Zapata 1991
***Indica* (CS- „)**		Biswas & Zapata 1993
***Indica* (CS-protoplast)**	„	Torrizo & Zapata 1992
Indian-*Indica* (ME-protoplast)	„	Gupta & Pattanayak 1993
***Indica* [protoplast (ST of IE)]**	„	Biswas et.al. 1994
Indian-*Indica* (CS-protoplast)	„	Jain et al. 1994 (in press)
***Zea mays* (maize)**	Green plants	Rhodes et al. 1988
	„	Cai et al. 1988
	Fertile plants	Shillito et al. 1989
	„	Prioli & Sondahl 1989
	„	Gordon-Kamm et al. 1990
	„	Morocz et al. 1990
Hordeum vulgare	Green plants	Lazzeri & Lörz 1989
(barley)	Fertile plants	Jähne et al. 1991a, b
Triticum aestivum	Plantlets	Harris et al. 1988
(wheat)	Green plants	Vasil et al. 1990
Saccharum officinarum	„	Srinivasin & Vasil 1986
(sugarcane)	„	Chen et al. 1988

CS - cell suspension-derived; AC-CS - anther callus-cell suspension-derived; CMS - cytoplasmic male sterile; ST of IE - scutellar tissue of immature embryos; ME - mesophyll-derived.

me, the potential benefit of somaclonal and game-toclonal variations observed in tissue cultures of rice offers a potential route to broaden the genetic variability and to accelerate the breeding process of this important crop (Larkin and Scowcroft 1981;

Evans et al. 1984). The advantages of somaclonal variation in rice breeding programmes can be considered as wider spectrum and higher frequency, rapid stabilization, small population and high efficiency (Sun and Zheng 1990).

1.9.2.1 Phenotypic variation

Rice is one of the systems which has been extensively studied, and somaclones have been obtained from cells, callus, protoplasts, anthers, endosperm and embryos. Phenotypic variation in rice can be observed in callus and regenerated plants, in progenies of regenerated plants and in grain quality. Morphological differences have also been reported in rice cell suspension cultures (Raghava and Nabors 1984; Zou et al. 1986). Phenotypic variation, in *in vitro* regenerated plants and in progenies of regenerated plants has been reported to be induced by variation of ploidy and chromosome number, mutation in nuclear gene(s), chloroplast mutation, homozygous mutations and variation in grain quality (Sun and Zheng 1990). Variations in the number of tillers per plant, plant height, flag leaf length, heading date, panicle length, fertility and number of seeds produced are commonly observed in rice tissue culture-derived plants (Zakri 1986). Variations in plant height, heading date, fertility and grain number were also reported from an analysis of the seed progeny of tissue culture-derived rice plants, confirming that they were carried through to the subsequent seed generation (Oono 1983).

Dwarfism, another phenotypic variation has been associated with some specific cultivars. The first generation seed progeny (15-36%) were shorter than control in anther-derived plants of the cultivar Calrose 76. It was possible to identify male-sterile somaclones in the *Indica* rice cultivars IR24 and IR54; fertility segregation in the R_2 progeny showed that male-sterility was controlled by two independent nuclear genes (Schaeffer et al. 1984; Ling et al. 1988). Recent analysis of regenerated plants from seed-derived callus of four Chinese cultivars revealed that the grain length and weight of somaclones decreased compared to that of control plants. Variation in grain quality in somaclones of three of the cultivars was also apparent (Zheng et al. 1989). From the American rice cultivar Labelle, three R_2 somaclonal lines have been selected which showed high resistance to sheath blight disease. One of the somaclones exhibited good

agronomic characteristics compared to existing resistant cultivars and the resistance was stable during 4 years field and glasshouse trials (Xie and Rush (1990).

For the selection of somaclonal variants, for example, callus lines of Norin 8 which were resistant to 5-methyltryptophan (a tryptophan analogue), were selected and the resistance was inherited in two subsequent generations as a dominant nuclear mutation (Wasaka and Widholm 1987). Using sea water and sodium salts as stressing agents, salt resistant calli have been recovered from a number of rice cultivars and by this means salt resistant plants of Norin 8 were regenerated. Resistant regenerants of the *Indica* cultivars IR8 and IR54 were selected after screening the induced calli against a specific toxin (H-toxin), produced by a fungus, *Helminthosporium oryzae* (Dykes and Nabors 1986; Ling et al. 1986). Some other promising results, for example, a pathogenic strain of a bacterium, *Xanthomanas oryzae* was inoculated onto calli derived from mature embryos of a susceptible variety and forty four bacterial leaf blight resistant plants were regenerated. In another attempt, the pathogenicity of culture filtrate from a fungus, *Pyricularia oryzae* on rice was studied in detail and twelve mutants with high resistance were selected out of the regenerated plants derived from 810 resistant calli of 10 cultivars (Zheng et al. 1985; L. Sun et al. 1986; C. Li et al. 1986).

1.9.2.2 Protoclonal variation

Plant regeneration from tissues and protoplast cultures is a complex morphogenetic phenomenon in which both natural and unnatural factors play important roles (Vasil 1987). However, much less is known about the molecular and biochemical events associated with *in vitro* morphogenesis. During the last six years, significant progress has been achieved in plant regeneration from protoplasts of rice and other important species of cereals and grasses (Vasil and Vasil 1992). Previously, it has been shown in the protoplast regeneration system that protoplast-derived microcolonies differentiating by somatic embryogenesis can regulate somaclonal (protoclonal) variation and this pathway of regeneration is desirable (Abdullah et al. 1986; Sun and Zheng 1990). Since genetically uniform and normal plant populations can be raised from protoplasts derived from embryogenic suspension cultures through somatic embryogenesis, compared to those regenerated by organogenesis, it is assumed that

there is less chance of variation in the regenerated progeny because of single cell origin. For applied research and also for understanding the genetic and physiological basis of totipotency of protoplasts, these results are useful.

The successful establishment of protoplast-to-plant regeneration systems for rice has recently facilitated the assessment of protoclonal variation in protoplast-derived plants and their seed progeny. In an attempt to investigate the field performance and cytological variation of protoplast-derived rice plants of four *Japonica* cultivars (Nipponbare, Fujisaka 5, Norin 14 and Iwaimochi) under submerged paddy field conditions, encouraging results have been obtained (Ogura et al 1987). In total, 80% of the protoplast-derived plants exhibited normal characters and the rest of the plants showed variation. The variant plants produced more tillers, which resulted in more panicles per plant, a slightly shorter culm length and fewer spikelets per panicle with slightly lower seed fertility compared to respective contorol (seed-derived) plants. Grain yield was higher than that of respective control plants in protoclones of all four cultivars, although 1000 grain weight was lower, may be because it was mainly related with the increased number of panicles per plant. However, variants with lower seed fertility had normal chromosome complements; triploid, tetraploid and aneuploid plants were also found among the variants (Ogura et al. 1987).

Among the first seed progeny plants derived from the protoclones of Fujisaka 5, about 25% of semi-lethal segregants were observed in a subsequent study. This result suggested that the progeny plants obtained from protoplast-derived rice plants, except for Fujisaka 5, exhibited a similar yielding ability and phenotypic uniformity to those of the control (seed-derived) plants (Ogura et al. 1989). In another study, a total of thirteen phenotypic characters were evaluated for first seed progeny of protoplast-derived plants of *Japonica* rice cultivar T309 under field conditions at IRRI. Protoclones exhibited delayed flowering, flag leaf and panicle lengths were decreased with increase in flag leaf width and number of branches per panicle. It appeared from this study that protoclonal variation shifted means away from the direction of the previous selection history of the cultivar, and the positive variability exhibited by the protoclones can be exploited by the improvement of the cultivar (Abdullah et al. 1989). Later, the phenotypic characteristics and molecular analysis of the protoplast-derived transgenic R_0 plants and their first generation R_1 seed progeny were described (Davey

et al. 1991). For the first time, it was shown that transgenic R_o and R_1 plants were shorter, took longer time to flower, and had reduced pollen viability compared to non-transformed R_1 protoplast-derived plants. Southern hybridization and dot blot analysis confirmed the presence of the *npt*II gene in transgenic R_o plants and in their R_1 seed progeny, because the seeds germinated on kanamycin-containing medium (Davey et al. 1991). In 1992, rice protoclonal variation, using cultivar Norin 8, was tried in order to investigate morphogenetic potential. In this study, protoclones with different regenerative capabilities were identified from protoplasts derived from a single homozygous seed; the polypeptide composition suggested the presence of specific polypeptides related to regeneration potential. Analysis of ploidy level based on plant morphology and pollen size suggested the predominance of tetraploids among the regenerated plants (Kawata et al. 1992). In 1993, the phenotypic characterisation of R_2 generation transgenic rice plants, obtained from the seed progeny produced by self-pollinating three R_1 generation transgenic plants of *Oryza sativa* L. var. Taipei 309, were conducted under field conditions at Maricopa Agricultural Experimental Station, Arizona, USA. Significant phenotypic differences were observed between individuals within the group of R_2 transgenic plants and the *npt*II gene was present in all transgenic plants (Schuh et al. 1993).

1.9.2.3 Molecular variation

From a study related to the stability of mitochondrial DNA in cell suspension cultures of T309, it was apparent that there was no significant alteration in the organisation of mitochondrial DNA up to 19 months, as analysed by DNA restriction and Southern analysis (Saleh et al. 1990) However, clear differences were observed in the relative abundance of specific DNA sequences when a 30-month-old suspension culture was used. In the same year, variations in the genomic DNA of rice plants regenerated from embryogenic callus, cell suspensions and protoplasts were characterised by RFLP analysis using the actin gene as a probe. It was interesting to note that the degree of variation was lower in many lines of protoplast-derived plants than in callus regenerants, although a significant increase in variation for callus regenerants was observed with increase in time in culture (Müller et al. 1990). Also, in an attempt to determine the induction of DNA polymorphism, directly regenerated protoplast-derived rice plants were examined by RFLP analysis. The result showed significant

increase in levels of DNA polymorphism compared with those in non-tissue culture control plants. Analysis with gene sequences revealed that such polymorphisms were apparently widespread and not associated with any particular region of DNA (Brown et al. 1990).

1.9.2.4 Variation at ploidy level

The number of chromosome variations, especially triploid, tetraploid, polyploid and aneuploid; and variations in nuclear DNA content in cultured plant tissues, cell suspension cultures, cells and regenerated plants result in phenotypic changes and ploidy changes. This kind of variability has been observed by many workers, especially chromosome number variations in regenerated plants i.e. ploidy changes, deletion, duplication, and rearrangements (Orton 1980; Murata et al. 1983; D'Amato 1989). It was reported many years ago that chromosome aberrations influenced agronomic traits and the ploidy level of rice plants (L. Zhang and Chu 1984). However, in most of the cases, a proportion of tissues or induced callus, cell suspension cells or regenerated plants exhibited phenotypic and genotypic variation compared to the parental type (Müller et al. 1990). A number of plant regeneration studies in which somatic embryogenesis was employed have been made, however only a few reports are available on cytological analysis or chromosomal variability in cell and tissue culture-derived plants in cereals (Orton 1980). Amongst such reports, eudiploid regenerants were reported in rice (Wernicke et al. 1981).

Chen and Chen (1980) found that 74% of of microspore-derived callus of rice were mixoploid, and the rest of them were uniform haploid, diploid, tetraploid and hexaploid. Chen and Lin (1976) observed that only 14% of the regenerated plants of five *Japonica* rice cultivars were tetraploids. Futhermore, polyploids and aneuploids amongst the regenerantes which were regenerants derived from rice embryo callus were found (Bajaj and Bidani 1980, 1986). Spontaneous polyploidy and chromosomal rearrangements in callus, suspension cultures and regenerated plants were observed to be frequent phenomena in barley. High frequency chromosomal abnormality in oat plants regenerated from callus cultures and chromosomal aberration in dividing protoplast suspensions of wheat were other findings (Orton 1980; McCoy et al. 1982; Karp et al. 1987). In a cytological study, among 126 protoplast-derived regenerants of

4 *Japonica* rice cultivars, 1 triploid, 10 tetraploids and 1 aneuploid (trisomic, 2n = 25) were found (Ogura 1987). The remaining 114 plants were diploids, but 12 distinct somaclonal variants were found among them. Current developments in this area of research are discussed in detail in Chapters 4 and 5.

1.9.3 Aims and the plan of the thesis

The main aims of the work described in this thesis were :

- to develop an efficient and reproducible plant regeneration system either directly from rice anther-derived callus, from rice anther-derived somatic embryoids, or directly from rice microspore-derived callus (see Chapter 3, Part I),
- to develop an efficient and reproducible plant regeneration system from protoplasts isolated from cell suspension cultures derived from anther-callus of the *Japonica* rice cultivar Taipei 309 (T309) (see Chapter 3, Part II).

Various changes were detected in the ploidy of the regenerated plants obtained from anther-callus origin, which necessitated a detailed analysis using both flow cytometry (see Chapter 4) and karyotyping (see Chapter 5). This involved determining the ploidy level both by flow cytometry and by chromosome counting, using preparations from the vigorously growing root-tips of regenerated plants. Regenerated plants from both callus cultures (see Chapter 2, Part I and Chapter 3, Part I) and from cell suspension-derived protoplast cultures (see Chapter 2, Part II and Chapter 3, Part II), initiated from a range of both haploid and diploid explants were used. It was also found necessary to compare and correlate the accuracy of determination of the ploidy level determined by flow cytometry with that determined from the chromosome counting of root-tip squashes.

The successful production of improved rice cultivars through cell and tissue culture, protoplast culture and genetically manipulated callus or protoplast culture depends on efficient transfer of regenerated plants from each culture system to growth-room, and glasshouse environment. On the other hand, intensive care and maintenance of these plants to adjust with the partially controlled environment for the first time are immediate needs for their successful growth towards maturity. At the same time, it is also necessary to create favourable growing conditions, especially maintenance of

temperature, humidity and light intensity together with the supply of proper nutrients for their favourable growth. For the present study, an efficient procedure has been developed to transfer protoplast-derived regenerants to the growth-room and subsequently to the glasshouse for their successful growth and development towards maturity. The detailed procedures are discussed in Chapter 2 (see section 2.6.9). Further details in this area of research to measure different agronomic traits of regenerated plants from protoplasts compared with seed-grown (control) plants are discussed in Chapter 6.

Chapter 2

Development of callus and protoplast-to-plant regeneration systems for the *Japonica* (*Sinica*) rice cultivar Taipei 309 (T309)

Part : I Plant regeneration from callus cultures initiated from different explants

2.1 Introduction I

2.1.1 Regeneration of plants from induced calli of different explant origin using the variety Taipei 309 (T309)

Many reports during the last two decades have stated that genotype and explant source are important parameters in determining the success of rice plant regeneration from in vitro cultures (Lai and Liu 1982, 1986, 1988; Abe and Futsuhara 1984, 1986). In *Japonica* rices, callus cultures can be produced relatively easily from almost any part of the plant, including roots, shoots, leaves, leaf-base meristems, mature and immature embryos, young inflorescences, pollen grains, ovaries and endosperms. Such cultures can be induced to regenerate plants (Bajaj 1991). In comparison to *Japonica* rices, 'true' *Indica* type I and *Indica* type II rices are more recalcitrant in culture (Lee et al. 1989; Kyozuka et al. 1988). The mode of differentiation in both *Japonica* and *Indica* types is either via callus to plants, or callus to somatic embryos and then to plants (Abe and Futsuhara 1985; Jones and Rost 1989). Thus, it is necessary to understand the requirements and factors important for differentiation. In general, the addition of 2,4-D to the medium causes callus induction, and its subsequent removal often results in morphogenesis (Bajaj and Bidani 1980). A problem is that often initial high regenerative ability is rapidly lost after a few subcultures (Inoue and Maeda 1980; Lai Liu 1986). However, high frequency long-term regeneration has also been reported when the cultures have been stressed (Heyser et al. 1983; Ozawa and Komamine 1989). Many studies have been conducted on the effect of exogenous phytohormones (Saka and Maeda 1969; Inoue and Maeda 1980). Some of these have indicated the need for the medium to be enriched with yeast extract (Yatazawa et al. 1967), or

particular amino acids, such as tryptophan (Siriwardana and Nabors 1983). It has been suggested that it is helpful for the calli showing regenerative potential to be carefully separated from non-regenerating ones and transferred to fresh medium during each subculture (Heyser et al. 1983). The composition of the culture media, the pretreatment of the tissue explants, culture conditions, such as temperature and light intensity, also have a direct effect on callus induction and plant regenerative ability (Croughan and Chu 1991). It has been observed that an important factor for inducing callus from rice embryos is to inoculate the embryos with the plumule-radicle axis side facing the medium (scutellum side up) (Lai and Liu 1982). Recently, it has been reported that the water content of rice callus tissues is an essential factor for inducing plant regeneration; a poorly regenerating callus can be changed to a vigorously regenerating one by 'water stress' and 'salt stress' (Liu and Lai 1991; Binh et al. 1992; Tsukahara and Hirosawa 1992).

Plant cell cultures are known to undergo various changes, especially in chromosome number and ploidy level (D'Amato 1965). Studies conducted over the last decade on many crops have shown that there are in vitro induced changes which can be used in breeding improvement programmes (Bajaj 1990a). In rice, extensive work has been carried out on the induction of somaclones arising from in vitro cultures. Plants have been obtained with a varying number of chromosomes, ranging from haploid (n = x = 12) to polyploids and aneuploids (Bajaj and Bidani 1980).

The aims of the work described in Part I of this Chapter are :

- to develop and to establish an efficient and reproducible plant regeneration system from rice callus cultures using different explants as source materials,
- to compare the variation in callus induction and plant regeneration frequencies from various explants of the *Japonica* rice cultivar T309.

In addition, variation in the ploidy level of the regenerated plants arising from these explants was determined using flow cytometry (Chapter 4). Changes in chromosome numbers were determined by chromosome counting of root-tip squash preparations (Chapter 5).

2.2 Materials and Methods

2.2.1 Source of working materials

The seeds of *Oryza sativa* cv. Taipei 309 (Acc. no. 425761) used in these experiments were kindly supplied by Dr. T. T. Chang, International Rice Germplasm Centre (IRGC), International Rice Research Institute (IRRI), Philippines.

2.2.2 Callus induction from mature seed-scutellum

Linsmaier and Skoog (1965) basal medium (LS 2.5) was used for callus induction for all the explants. This medium was prepared by using Murashige and Skoog (1962) basal salts supplemented with the vitamin thiamine 1.0 mgl^{-1}, 3% (w/v) sucrose and 2.5 mgl^{-1} 2,4-D. Before culturing any explant for callus induction, the solidified medium was freshly prepared by mixing equal volumes of autoclaved double strength liquid LS 2.5 medium with 0.8% (w/v) agarose (Sigma Type I) aqueous solution. The mixture was then dispensed into 9 cm Petri dishes, each containing 25 ml of solution.

Dehusked rice seeds were surface sterilized in 30% (v/v) 'Domestos' bleach (Lever Bros. Ltd., London, UK.) for 1h and washed 5 times in sterile distilled water. The seeds were inoculated onto the LS 2.5 medium (see Appendix A1.3) with 10 seeds per Petri dish with the plumule-radicle side in contact with the medium (scutellum side up) The Petri dishes were sealed with Nescofilm (Nippon Shoji, Kaisha Ltd. Osaka, Japan) and incubated in the dark at 27°C.

2.2.3 Callus induction from seedling leaf-base meristems

Dehusked seeds were sterilized as described in section 3.2.2 Ten seeds were germinated on the surface of 30 ml of agar-solidified Murashige and Skoog (1962) medium supplemented with 3% (w/v) sucrose, but without any growth substances (designated MSO, see Appendix A1.3) in 6 oz (175 ml capacity), powder round glass jars (Beatson Clarke and Co. Ltd., Rotherham, Yorkshire, UK.) and incubated in the dark at 27°C. After 7 days of incubation, the coleoptile and the first leaf of each seedlings were removed. The innermost leaf was excised from the basal 1 cm portion of each seedlings, just above the mesocotyl node, and transverse sections of 2-4 mm were serially inoculated onto LS 2.5 medium (4 dissected leaves per 9 cm Petri dish). The dishes were sealed and incubated as above.

2.2.4 Callus induction from immature embryos

Immature spikelets were collected between 7-9 d after anthesis at the soft dough stage from glasshouse-grown Taipei 309 plants. The lemma and palea of each spikelet were carefully removed and the caryopsis was surface sterilized in 10% (v/v) 'Domestos' solution for 10 min, followed by 5 washes with sterile distilled water. The immature embryos were dissected from the caryopsis, and inoculated onto the surface of agarose-solidified LS 2.5 medium (8-10 embryos per 9 cm Petri dish). The dishes were sealed and incubated as above.

2.2.5 Maintenance of embryogenic callus cultures

Embryogenic calli which developed at the scutellar surface of mature seeds, from the basal-most segments of seedling leaves, and on the surface of the immature embryos, were selectively subcultured onto fresh LS 2.5 medium at 4-5 week intervals. Five cycles of subculture were necessary to increase the friability of the initially compact embryogenic calli to make them suitable for the initiation of cell suspension cultures.

2.2.6 Transfer of embryogenic calli to regeneration medium for plant regeneration

At the time of each cycle of subculture, the embryogenic calli were carefully separated from non-embryogenic ones and transferred to fresh induction medium (LS 2.5). Four to six subcultures of mature seed-scutellum, immature embryos and seedling leaf-base meristem callus were needed to produce adequate supplies of callus. When the callus was between 90 and 105 days old , suspension cultures were initiated from friable, globular, embryogenic clusters; clusters between 1 and 2 mm in diameter were placed on the regeneration medium (MSKN, see Appendix A1.7). Three clusters were placed on 3 ml aliquots of solidified medium in the separate chambers of 25-well plastic Sterilin dishes (100 mm x 100 mm). The dishes were sealed with Nescofilm and incubated in the dark at 27°C ± 1°C. Somatic embryos, which formed on the surface of the callus after 5 to 6 days, began to germinate after a further 7-12 days, producing distinct radicles and coleoptiles. Approximately 1 to 2 cm long emerging shoots were transferred to MSBP2 medium (see Appendix A1.7). One or two shoots were placed in each 6 oz jar containing 40 ml medium and incubated for further development (16 h day length, 125 μEm-2 S-1 PAR, at 25°C). After one month, the regenerated plants

were transferred to MSN1.5 medium (see Appendix A1.7), for healthy and functional root production and incubated similarly.

2.2.7 Transfer of cells from suspension cultures after sieving to regeneration medium for plant regeneration

Suspension cultures were initiated from friable, globular, embryogenic callus that was 3 to $3^1/_2$ months-old and which had originated from various explants. After 3 months, the suspensions were established and contained small cell clusters. At the exponential growth phase of the suspension cultures, 4 to 6 days after subculture, cell clusters from a 250 ml conical flask culture were filtered through a 45 µm nylon sieve. Cell clusters (1.0 to 1.5 mg F.W.) were transferred onto regeneration medium (MSKN, see Appendix A1.7). Ten clusters of cells were placed onto 25 ml of solidified medium (9 cm Petri dish). The dishes were sealed and incubated in the dark at 27°C. After one month of culture, they were assessed for shoot and root production. This experiment was repeated every month to observe whether there had been any change in the regenerative potential depending on the age of the cell suspensions.

2.2.8 Care and maintenance of the regenerated plants for ploidy determination and chromosome analysis

The shoots of regenerated green plants (5 to 7 cm tall) obtained from various callus cultures of different explant origin were micropropagated first in MSBP2, and then in MSN1.5 media (see Appendix A1.7). These micropropagated plants had healthy shoots and white, functional roots. This method of micropropagation was adapted from the micropropagation procedure developed (in the PGMG laboratory, Nottingham, England) for wild rice species (Finch et al. 1992). Five to six successive subcultures (one month intervals) were necessary to establish adequate growth.

2.3 Results and Discussion I

2.3.1 Callus production from different explants using the standard induction medium

The response of the different explants is summarized in Table 2A. Embryos from mature and immature rice seeds (T309) cultured on standard LS 2.5 medium, became swollen after several days. The growth of plumules and radicles was suppressed,

although scutella and the mesocotyl tissues proliferated. The proliferation of the scutella was due to expansion of parenchymatous cells and division of epidermal cells. After 15 to 21 days of culture, rapid cell division began and callus masses grew from these proliferating cell areas (Fig 2.1b & e). Initially, two types of callus formed from each explant; non-embryogenic callus was composed of rooty, elongated cells, sometimes with a wet, soft and mucilaginous appearance. Embryogenic callus was identifiable by its compact, dry and globular appearance and pale yellow colour (Finch et al. 1991).

The immediate response of the leaf-base segments was the enlargement of the two cut ends after 2-3 days. The degree of callus formation was directly related to the relative position of the basal segments from the meristematic region of the leaf-base. In most of the cases, the basal-most plated segments from the innermost (youngest) seedling leaves, which is the real meristematic region, produced maximum embryogenic calli at a low frequency. The morphology and the texture of the callus produced was also variable among the explant sources. In most of the cases, callus formed from mature seed-scutellum and immature embryos was normally dry and compact, with numerous globular embryoids on the surface. However, most of the callus formed from the leaf-base meristem segments was mucilaginous, watery and sometimes produced roots (Finch et al. 1991).

Variations in the initial growth rates, the frequency of callus initiation, the amount of callus production, callus texture (whether compact or friable) were observed among the explants of different origin. During the first passage of callus induction from their mature embryos or from 10 to 12-day-old immature embryos, the orientation of the embryos, (i.e. whether inoculated with the plumule-radicle axis facing the medium or the scutellum facing the medium) was found to be the most important feature in determining the callus induction frequency and the type of callus formation (Lai and Liu 1982) (Table 2B). It was also found that separating the germinating mature or immature embryos completely from the endosperm and reorienting them, resulted in the production of a higher proportion of embryogenic calli on the surface of the swollen scutellum (Abdullah et al. 1986) (Figs 2.1c & f). Another important factor involved during each subculture was the need to separate carefully the embryogenic

Fig 2.1 Embryogenic callus and somatic embryo formation from callus culture of different explants of *O. sativa* (cv. Taipei 309)

a) A full-grown mature rice caryopsis (dehusked seed) showing mature embryo (me) (x 6.3).

b) Formation of globular, embryogenic callus from the scutellar surface of mature seed embryo after four weeks of culture on LS 2.5 medium (left plate). Initial growth of embryogenic (ec) and non-embryogenic (nec) callus after removal of radicle and plumule and culturing them on the same medium for a further two weeks (right plate) (x 2.3).

c) Three embryogenic calli (ec)/somatic embryos (one lateral view, two top view) from the scutellar surface of a mature seed embryo under stereo-microscope (x 7.4).

d) An immature green rice caryopsis after 10 days of anthesis of spikelet and two dissected out immature embryos (ie) (x 5.3).

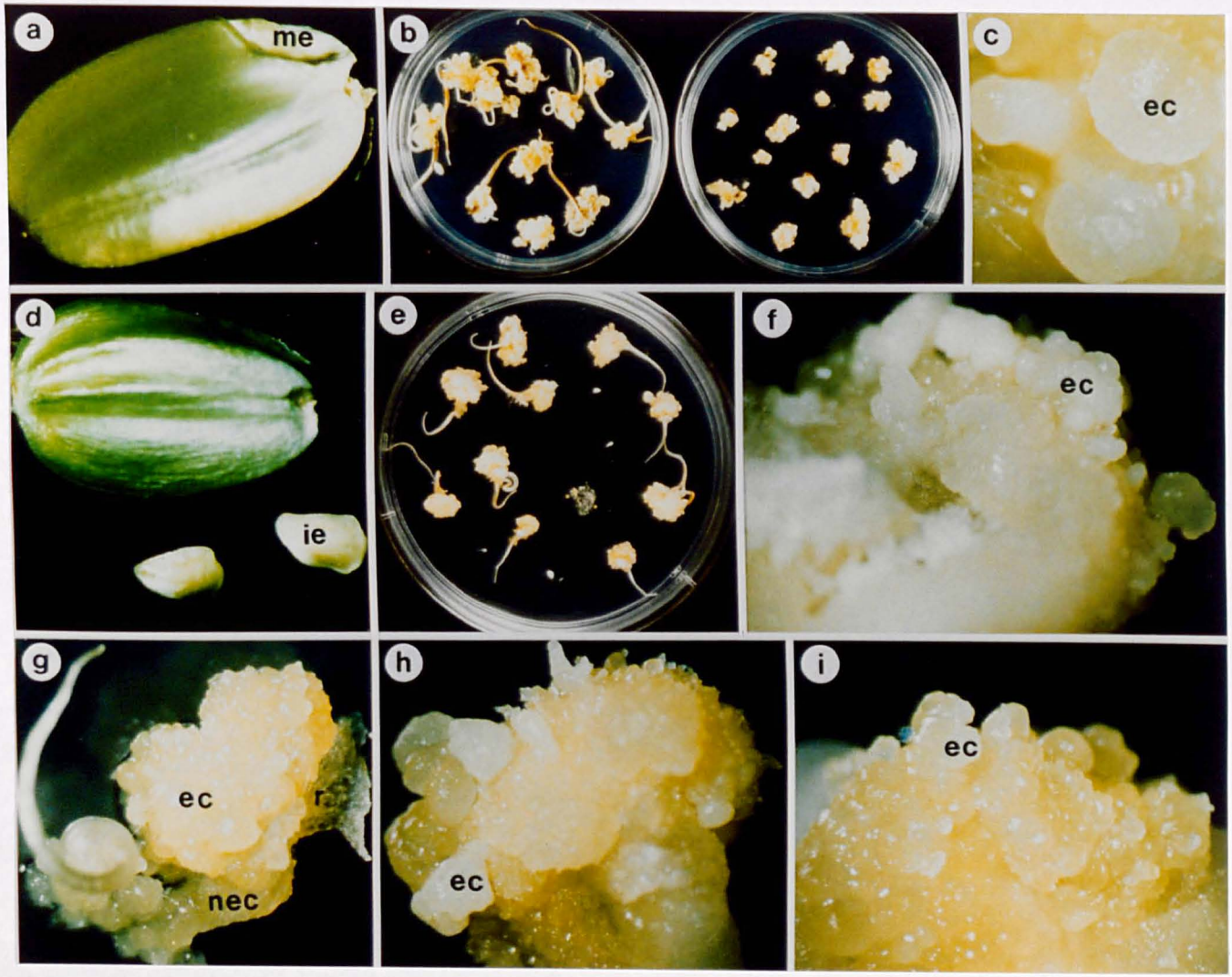
e) Embryogenic and non-embryogenic callus formation from dissected immature seed-embryos after four weeks of culture on LS 2.5 medium (x 3.5).

f) Embryogenic callus (ec)/somatic embryo formation from dissected immature seed-embryo after four weeks of culture on the same medium (x 7.4).

g) Embryogenic (ec) and non-embryogenic callus (nec) formation from dissected leaf-base meristem after four weeks of culture on LS 2.5 medium (x 3.5).

h) Embryogenic callus (ec) formation from dissected leaf-base meristem after first subculture of the embryogenic callus on the same medium (x 5.3).

i) Embryogenic callus (ec) formation from dissected leaf-base meristem after second subculture of the embryogenic callus on the same medium (x 7.4).


a
me
b
c
ec
d
ie
e
f
ec
g
ec
r
nec
h
ec
i
ec

calli from the non-embryogenic ones and to transfer them to fresh medium to ensure adequate amount of callus production.

2.3.2 Comparison of callus formation efficiency from the different explants

The response of various explants with respect to callus formation is summarized in (Table 2A).

Table 2A. The response of various explants: callus induction frequency, the amount of callus production and the type of callus on LS 2.5 medium (T309)

Explant source	Callus fresh weight[3] (mg)	% of explants producing callus[1]	% of embryogenic callus production[1]	% of non-embryogenic callus production[1]
Immature embryo[2]	125.0 ± 40.3	93.12 ± 3.45	89.35 ± 4.97	0.533 ± 0.07
Mature embryo[2]	129.5 ± 37.5	86.23 ± 6.23	82.15 ± 6.27	0.711 ± 0.04
Leaf-base meristem[2]	105.2 ± 34.8	74.65 ± 5.37	22.34 ± 2.75	77.66 ± 6.21

[1]Data represent the means ± standard error based on 3 replicate experiments.

[2]Refers to those embryos which had germinated.

[3]Each mean and its standard deviation were calculated from 20 samples of 4-week-old cultures.

Immature embryos were found to be the most responsive, producing 80 to 100% callus compared to all other explants. A large proportion of the calli produced from the seedling leaf-base meristem segments was non-embryogenic compared to those produced from mature and immature embryos. However, the callus induction frequency, and the relative proportion of embryogenic callus production compared to non-embryogenic callus production, were different for the different explants.

2.3.3 Comparison of the plant regeneration frequency using pieces of calli or cell clusters from suspension cultures derived from different explants

Small cell-clusters from embryogenic cell suspensions (3 to $3^1/_2$ months-old) which had originated from various explants were transferred to regeneration medium. On MSKN medium, cell clusters formed only roots at a low frequency and never developed any shoot-like structures (Fig 2.2i). However, somatic embryoid-like structures, whitish

green in colour, were visible in differentiating regions of the pieces of calli within 8-10 days of culture on MSKN medium. After 2 to 3 weeks of culture, a distinct bipolar structure with shoots and roots had developed (Figs 2.2b, e, h). These structures developed into plantlets and then into plants upon transfer sequentially to the shoot and root forming media MSBP2 and MSN1.5 (see Appendices A1.3 & A1.7). The regenerated plants were transferred to an illuminated culture room, after 3 weeks of dark treatment in the incubator at 28°C ± 1°C (Figs 2.2c, f). The plant regeneration frequency from embryogenic calli pieces varied greatly among the explants, and even from the same explant origin at different times depending on the age of the callus. The frequency of regeneration was a maximum of 20% (expressed in terms of the percentage of calli regenerating plants) with approx. 2% of the regenerated plants being albinos. Regeneration was dependent on the explant origin of the callus.

Embryogenic callus production, and subsequent plant regeneration, were found to be dependent on the age of seedings grown in vitro for leaf-base meristem culture, on the age of immature embryos after anthesis for immature embryo culture, on the scutellar surface orientation of mature or immature embryos during callus culture initiation and on the age of callus (Table 2B). The regenerated plants were maintained and micropropagated at 4 week intervals.

Fig 2.2 Somatic embryo formation and regeneration of plants from embryogenic calli originated from different explants of *O. sativa* (cv. Taipei 309)

a) Differentiation of a somatic embryo (em) on the surface of embryogenic callus derived from mature seed-scutellum (MSS) after 2-3 weeks of culture on MSKN regeneration medium (x 4.4).

b) Further development of the somatic embryo, differentiating into two defined bipolar structures, a shoot (s) and a root (r) after 4 weeks of culture on the same medium (x 4.4).

c) Young plants with a number of green shoots growing on MSB2 medium which were regenerated from embryogenic calli, developed from mature seed -scutellum (MSS) (x 3.8).

d) Differentiation of a few somatic embryos with distinct bipolar structures, shoots (s) and roots (r) on the surface of embryogenic calli derived from immature seed-embryo after 4 weeks of culture on regeneration medium (MSKN) (x 2.3).

e) Somatic embryos differentiating into roots (r) and shoots (s) after 4 weeks of culture on the same regeneration medium (x 2.5).

f) Young green plants with a number of shoots growing on MSB2 medium which were regenerated from embryogenic callus, developed from immature seed-embryo (ISE) (x 3.8).

g) Most of the calli, originated from leaf-base meristems, differentiated with the production of only roots and a few green shoots (x 2.3).

h) A somatic embryo growing on the regeneration medium with the production of a root (r) and a shoot (s) after 4 weeks of culture (x 3.2).

i) Cell-clusters from the embryogenic cell suspension cultures (originated from MSS, LBM or ISE) without the production of any root or shoot on the regeneration medium after 5 weeks of culture (x 2.5).

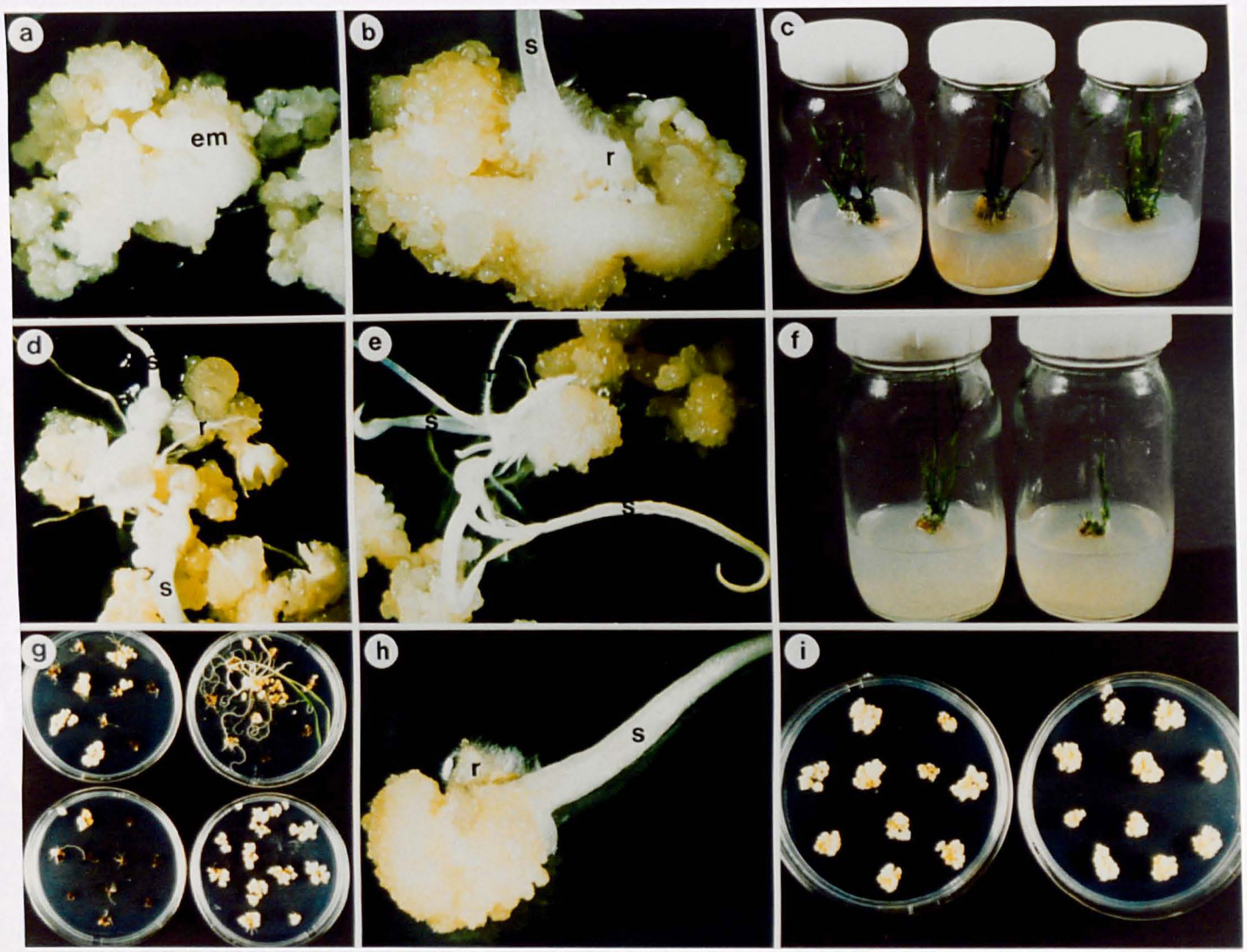

a
em
b
s
r
c
d
s
r
s
e
s
s
f
g
h
r
s
i

Table 2B. Effects of embryo age, seedling age, callus age and scutellar surface orientation on callus growth, organformation and plant regeneration (T309) using LS 2.5 and MSKN media (see Appendices 1.3 & 1.7)

Explant source	Days after anthesis or days after seed sown *in vitro*	Scutellar surface down				Scutellar surface up			
		Callus fresh weight (mg)[a]	Callus cultured on regeneration medium			Callus fresh weight (mg)[a]	Callus cultured on regeneration medium		
			Total no.	with root	with shoot		Total no.	with root	with shoot
Immature embryos[c]	7	97.3 ± 27.2	100	80(80)[b]	20(20)[b]	125.0 ± 40.3	100	67(67)[b]	33(33)[b]
	10	91.6 ± 21.9	100	5(5)	0	114.4 ± 33.5	100	72(72)	0
	13	87.1 ± 14.1	100	15(15)	0	104.2 ± 34.8	100	78(78)	0
Mature embryos[c]	90	105.2 ± 29.5	100	26(26)	0	129.5 ± 37.5	100	77(77)	23(23)
	110	96.2 ± 24.5	100	51(51)	0	118.1 ± 37.9	100	80(80)	20(20)
	120	94.7 ± 32.1	100	42(42)	0	113.7 ± 20.5	100	83(83)	17(17)
Leaf-base meristems	7	86.1 ± 14.0	100	95(95)	5(5)	105.2 ± 34.8	100	97(97)	3(3)
	10	80.3 ± 33.1	100	100(100)	0	102.1 ± 33.3	100	0	0
	14	72.3 ± 20.0	100	100(100)	0	90.4 ± 16.0	100	0	0

[a]Each mean and its standard deviation were calculated from 20 samples of 4-week-old cultures.

[b]The number in parenthesis is percentage of total callus number.

[c]Refers to those embryos which had germinated.

2.4 Summary and conclusions I

The aim of the work described in Chapter 2 (Part I) was to develop and to establish an efficient, embryogenic callus culture and plant regeneration system from different explants of the *Japonica* rice cultivar Taipei 309 (T309) using the standard Nottingham method. This study was also a pre-requisite for investigating plant regeneration from cell suspension-derived protoplast cultures using different explants as described in Part II of this Chapter. Significant variation was observed in callus production, induction efficiency, and plant regeneration capability from embryogenic callus cultures of different explant origin. It was observed that both the amount of callus production and plant regeneration were directly correlated with the age after anthesis of immature embryos; these characteristics were also correlated with the age of the seedlings used for leaf-base meristem culture. Particularly important was the scutellar surface orientation of mature or immature embryos during callus culture initiation, and callus age for plant regeneration (Table 2A & 2B).

There have been many reports on rice embryogenic callus production from explants of various origin. It is easier to induce callus from various explants of *Japonica* rices as compared with those from *Indica* rices (Datta et al. 1990a; Biswas and Zapata 1992). Although the callus induction frequency and the amount of callus production were not significantly different between mature and immature embryos, immature embryos were found to be more responsive in this study and produced a higher percentage of embryogenic callus compared with other explants (Table 2A). However, for *Indica* rices, immature embryos have been recognized as an excellent source for embryogenic callus production and subsequent plant regeneration from callus or from protoplasts in some laboratories during recent years (Lee et al. 1989, 1990; Datta et al. 1992; Biswas and Zapata 1992). It was also found in the present study that immature embryos, producing more embryogenic callus in terms of F.W., were not readily available; they were also rather difficult to culture at the initial stage compared with mature embryos of *Japonica* rice (T309). It has been reported that embryogenesis and fertile plant regeneration from competent immature microspores of both *Indica* and *Japonica* rices can be achieved (Datta et al. 1990a). A simpler, faster and more efficient procedure for establishment of cell suspension cultures from embryogenic micro-calli of immature

microspores of both *Japonica* and *Indica* rices has been reported (Datta et al. 1990b, c).

Embryogenic callus formation in the Gramineae, other than rice, has been achieved so far only from immature embryos or young developing inflorescences and leaves (Vasil 1983; Botti and Vasil 1983), and not from mature embryos or other plant organs. It has also been found that the scutellum from mature embryos of wheat showed no sign of callus development and quickly became necrotic (Botti and Vasil 1983). The regeneration of green plants from protoplasts isolated from regenerable, embryogenic cell suspension cultures has been achieved in wheat (Vasil et al. 1990) and maize (Shillito et al. 1989), and it has been established that immature embryo-derived compact callus is the most suitable starting material. In another species like barley, immature embryos were also found to be more suitable for the induction of embryogenic callus and subsequent establishment of regenerable cell suspension cultures (Lührs and Lörz 1987, 1988), but only albino plantlets or sterile green plants were regenerated from protoplasts.

Alternatively, it has been shown that microspore-derived tissues from anther culture are an attractive and suitable source for rapid initiation and efficient production of morphogenic suspension cultures. Rapid suspension culture establishment has minimized the loss of barley regenerative capability, and fertile green plants have been regenerated (Jähne et al. 1991a, b).

Part II : Plant regeneration from cell suspension-derived protoplast cultures using different explants

2.5 Introduction II

2.5.1 Regeneration of plants from protoplasts isolated from cell suspension cultures of different explant origin (T309)

Plant regeneration from protoplasts has long been considered as an important in vitro tool for the improvement of crop plants. However, the use of protoplasts depends to a considerable extent on having an efficient and reliable method of plant regeneration. The cereals, which were once considered to be recalcitrant, have recently yielded much

success. The isolation of rice protoplasts, suitable for subsequent culture and plant regeneration has been achieved only from calli or embryogenic cell suspensions (Abdullah et al. 1986; Biswas and Zapata 1990). Embryogenic cell suspensions, from *Japonica*, *Indica* and wild rices, have proved to be an excellent source of regenerable protoplasts (Bajaj 1991; Baset et al. 1991), which can be used in selection experiments (Bright 1985). Their use allows genetic manipulations at the single-cell level, such as gene transfer, and somatic hybridization and cybridization (Schieder and Köhn 1986; Lörz 1987). This has enabled somatic hybridization (Kumar and Cocking 1987; Toriyama and Hinata 1988b), somaclonal variation (Abdullah et al. 1989; Kawata et al. 1992), and genetic transformation, mainly in *Japonica* rices (Shimamoto et al. 1989; Hayashimoto et al. 1990). Recently, there have been several reports of the regeneration of green rice plants, somatic hybrid and transgenic rice plants from protoplasts, both of *Japonica* and *Indica* varieties (Tables 2C & 2D). Use was made of fast-growing young cell suspension cultures.

There are still only a few reports on plant regeneration from protoplasts of *Indica* rices (Lee et al. 1989; Datta et al. 1990b, c; Biswas and Zapata 1991a, b). The slow progress in protoplast culture of *Indica* rices is related to difficulty in the establishment of suitable embryogenic cell suspension cultures (Kyozuka et al. 1988; Wang et al. 1989). Recently, this problem is beginning to be resolved (Koetje et al. 1989; Datta et al. 1990c; Biswas and Zapata 1991b).

Sustained protoplast division and plant regeneration still seems to be limited to a small number of genotypes both for *Japonica*, *Indica* and wild *Oryza* species (Biswas and Zapata 1990, 1992; Datta et al. 1990b; Baset et al. 1991). The importance of the selection of suitable genotypes has been emphasized; it is one of the crucial factors for the success of plant regeneration from protoplasts (Yamada et al. 1986). Embryogenic callus from various explant origin has been successfully used for cell suspension initiation since 1981. Explants have included rice leaves (Wernicke et al. 1981), rice roots (Abe and Futsuhara 1984), immature embryos (Lai and Liu 1982; Fujimura et al. 1985), mature seed embryos (Abdullah et al. 1986; Kyozuka et al 1987; Finch et al. 1991), anther and shed pollen (Toriyama et al. 1986; Datta et al. 1990a; Biswas and Zapata 1991a), young inflorescences (Toriyama and Hinata 1985b; Biswas and Zapata 1990), leaf-base meristems (Abdullah et al. 1986; Finch et al. 1991) and

micropropagule segments for wild rices (Baset et al. 1991). However, the ability to produce and select suitable embryogenic callus from such explants is essential (Finch et al. 1991). Also, the ability to establish and select finely-divided, embryogenic cell suspension cultures, which consisted of densely-cytoplasmic, isodiametric cells from selected embryogenic callus of different explants, is important.

In the present study, the *Japonica* rice variety Taipei 309 was chosen as working material because it was easy to grow in the glasshouse to obtain immature embryo explants within a limited time. It was also easy to induce embryogenic callus from mature seed-scutellum and leaf-base meristems within a short time. During the study period, there were no reports of sustained division of rice protoplasts isolated directly from meristematic tissues of rice plants (Vasil et al. 1990). Therefore, research emphasis was placed on the use of embryogenic cell suspension cultures to obtain a high yield of protoplasts, capable of dividing to form large numbers of colonies, and regenerating green plants at high frequencies. Subsequently, a simple method for the regeneration of plants from mesophyll protoplasts isolated from leaf-bases and sheaths of rice seedlings has been reported (Gupta and Pattanayak 1993). It has been generally observed that the regenerability of cereal suspensions including those from the *Japonica* rice variety T309, decreases with the age of the suspension cultures (Shillito et al. 1989; Lührs and Lörz 1988; Jähne et al. 1991a). Therefore, attempts were made to develop methods for rapid establishment of suspension cultures. An alternative approach has been taken to resuspend protoplast-derived cell colonies in liquid AA2 medium (see Appendix A1.5) to retain regenerability (Finch 1991).

The aims of the work described in Part II of this Chapter are :

- to investigate the response of various explants for the establishment of embryogenic cell suspension cultures of the Japonica rice cultivar Taipei 309,
- to determine the plating efficiencies, and the plant regeneration frequencies from protoplasts, by comparing the use of different explants for the initiation of fast-growing, embryogenic cell suspensions.

Furthermore, the effect of the age of the cell suspensions on the above mentioned factors was also investigated.

Table 2C. Rice plant regeneration from cell suspension-derived protoplasts of different explant origin

Rice variety	Explant source	(%) plant regeneration	Reference
Nipponbare	ISE and MSS callus	5-20%	Fujimura et al. 1985
Sasanishiki			
A-58 MS	MSS callus	1%	Yamada et al. 1986
Taipei 309	MSS and LBM callus	10-20%	Abdullah et al. 1986
Fujisaka 5			
Yamahoushi	Anther callus (Panicle culture)	12-24%	Toriyama et al. 1986
Nipponbare	ISE and MSS callus	17-50%	Kyozuka et al. 1987
Iwaimochio			
Norin 14			
Fujisaka 5			
Koshihikari			
Chinsurah Boro II	MSS callus	2-34%	Kyozuka et al. 1988
IRAT 109			
Cyokoto			
IR 54	ISE callus	Up to 20%	Lee et al. 1989
Nipponbare	MSS callus	6-23%	Masuda et al. 1989
Akitakomachi			
Blue Bonnet			
Pantiange-Heigu			
Chinsurah Boro II	Anther callus	Up to 10%	Datta et al. 1990a
IR 43	MSS and ISE callus	4-6%	Biswas & Zapata 1991
***Oryza rufipogon* Griff**	MSB callus	10-15%	Baset et al. 1991
IR 58	MSS callus	Up to 56.8%	Torrizo & Zapata 1992
IR 72	MSS callus	Up to 43.1%	Datta et al. 1992

ISE = Immature seed-embryos; LBM = Leaf-base meristems; MSB = Micropropagated shoot-bases; MSS = Mature seed-scutellum.

Table 2D. Somatic hybrid callus or somatic hybrid plant regeneration from rice protoplast fusion

Plant species fused	Fusion procedure[a]	Fusion product	Reference
O. sativa and *Pisum sativum*	PEG	Cell colony	Bajaj 1983b
O. sativa and *Glycine max*	PEG	Callus	Niizeki et al. 1985
O. sativa and *Daucas carota*	PEG	Cell line	Sala et al. 1985
O. sativa and. *Echinochloa oryzicola* (barnyard grass)	EF	Somatic hybrid plants	Terada et al. 1987
O. sativa and *O. eichingeri* *O. sativa* and *O. officinalis* *O. sativa* and *O. perrieri* *O. sativa* and *O. brachyantha*	EF	Somatic hybrid plants	Hayashi et al. 1988
CMS rice and fertile rice protoplasts	EF	Somatic hybrid callus	Z-Q Yang et al. 1988
Yamahoushi and Murasakidaikoka	EF	Somatic hybrid plants	Toriyama & Hinata 1988
Chinsurah Boro II (CMS) and Nipponbare (Normal)	EF	Somatic cybrid plants	Kyozuka et al. 1989
O. sativa (*Japonica*) and *P. coarctata*	EF	Somatic hybrid callus	Finch et al. 1990
O. sativa (*Japonica*) and IR 54 (*Indica*)	EF	Somatic hybrid plants	Slamet I. H. 1991
O. rufipogon and *O. sativa* (*Japonica*)	EF	Putative somatic hybrid plants	Baset A. 1992
CMS MTC-9A and Norin (mutant)	EF	Somatic cybrid plants	Akagi et al. 1989

Later, variation in the ploidy level of the regenerated protoclones arising from these various explants was determined using flow cytometry (Chapter 4), and changes in chromosome number were determined by chromosome counting of root-tip squash preparations (Chapter 5).

2.6 Materials and Methods II

2.6.1 Source of working materials

Friable, embryogenic calli obtained from different explant origin, which were used for plant regeneration from callus culture experiments (Chapter 2, Part-I), were also used for the initiation of cell suspension cultures for conducting further experiments on protoplast isolation, culture and plant regeneration.

2.6.2 Initiation of embryogenic cell suspension cultures using calli from different explants

There is no substitute for practical experience in the critical and difficult procedures involved in rice cell suspension culture initiation and maintenance. The initiation procedures were identical for embryogenic callus from all explant sources. Therefore, the embryogenic callus of different explant origins was carefully selected for the initiation of suspension cultures, using a stereo-dissecting microscope. It was necessary to tease with forceps, to separate embryogenic, globular tissues from compact callus; cutting was avoided because it caused browning of the newly initiated suspension cultures.

Suspension cultures were initiated from friable, globular, embryogenic callus, which was approximately 2-3 months old. About 1.0-1.5 g F.W. callus was transferred into a 100 ml conical flask containing 20 ml of liquid AA2 medium (see Appendix A1.5). The cultures were incubated and shaken on a rotary platform shaker with a 1 cm throw at 120 rpm in the dark at 27°C ± 1°C. During the initial stage of initiation of the suspension cultures, 80% of the culture medium was replaced with fresh medium (AA2) at 3-4 day intervals, avoiding any reduction of cell density. When small cell aggregates were produced (5-6 weeks from initiation), 7 ml aliquot of cell suspension (1 ml pcv + 6 ml old medium from the existing culture) was subcultured into 21 ml fresh AA2 medium in a 100 ml conical flask, using a wide-bore (2.5 mm) plastic pipette; the freshly subcultured suspensions were maintained as before. The population of small, cytoplasmically dense, embryogenic cells was thus enriched by subculturing every week using 10 ml sterile pipettes. This subculture procedure was subsequently carried out throughout the entire experimental period. Growth rates of the new cell suspension cultures were determined using a 10 ml sterile plastic graduated pipette

(Sterilin). Depending on the growth rate of embryogenic, cytoplasmically dense, opaque cell clusters, normally suspension cultures became suitable for protoplast isolation after 3-4 months.

2.6.3 Maintenance and growth rate determination of different established embryogenic cell suspension cultures

The established embryogenic cell suspension cultures (3-4 months old) were maintained in their finely dividing state by periodic passage through a 500 μm mesh size nylon sieve and by weekly subculture. The most successful method of subculture was to divide the cell culture into sterile flasks at a 1:3, inoculum : medium dilution ratio. Each 100 ml culture flask should contain about 5-6 ml of old medium and approx. 1.5 ml of cells, plus 20 ml fresh AA2 medium (the volumes were doubled for 250 ml flasks).

The growth characteristics of the established cell suspension cultures were determined in order to select and asses the most appropriate time for protoplast isolation. For growth rate determination, suspension cultures were grown in specially designed 250 ml flasks with a graduated 10 ml side arm. The settled cell volume (scv) of 10 ml of suspension culture was measured daily for a two week period. The growth rate of the suspension culture was expressed as growth index, which was calculated as follows :

$$\text{Growth Index (GI)} = \frac{\text{final settled cell volume (scv) - initial (scv)}}{\text{initial settled cell volume}}$$

2.6.4 Protoplast isolation by enzymatic digestion of cell suspension cultures and determination of protoplast yield and viability

Protoplasts were isolated from suitable established cell suspension cultures in their exponential growth phase, as determined by microscopic observation, 4-5 days after subculture. The cell suspension cultures were filtered through a 500 μm mesh size nylon sieve into a 14 cm plastic Petri dish to obtain small cell colonies suitable for enzymatic digestion. The liquid medium was carefully removed from the dish using a sterile Pasteur pipette or a sterile 10 ml plastic pipette. Two types of incubation procedures were followed for enzymatic digestion of the cell colonies and protoplast release.

Overnight incubation : About 1.0 g F.W. of the cell clusters was resuspended and incubated in 50 ml enzyme mixture 'E101' [10 ml of enzyme for each gram F.W. of cells diluted in 40 ml of CPW13M medium (see Appendix A1.2), i.e. 1:4 ratio or 1:3 ratio depending on the age of the cell suspension cultures]. The enzyme solution was 1.0% (w/v) Cellulase RS (Yakult Honsa Co. Ltd., Japan), 0.1% (w/v) Pectolyase Y23 (Seishin Pharmaceutical Co. Ltd., Japan) and 5 mM MES (Sigma Chemical Co. Ltd., UK), dissolved in CPW salts solution (Frearson et al. 1973) containing 13% (w/v) mannitol (CPW13M) at pH 5.8 (see Appendix A1.1). The Petri dish was sealed with Nescofilm and incubated on a horizontal rotary shaker in the dark at 27°C ± 1°C with gentle shaking for 12-15 h, followed by a 1 h stationary incubation period. Sometimes, plasmolysis of the cell clusters in CPW13M medium was helpful for isolation, before adding enzyme solution for overnight incubation (20 ml/g of tissue, for 1 h).

Day incubation : About 1.0 g F.W. of the cell clusters was incubated in 20 ml of enzyme solution (full strength) for 2-3 h (depending on the age of cell suspensions) in the dark at 27°C ± 1°C on a horizontal rotary shaker with gentle shaking (30 rpm, 2 cm throw), followed by a 1-2 h stationary incubation period.

After incubation and cell digestion, the enzyme-cell mixture was examined for protoplast release using an inverted microscope (Nikon, model 'Diaphot TMD' or similar). After maximum release, the mixture was filtered through a series of nylon sieves of 64, 45 and 30 μm pore sizes, arranged in a stack on a sterile 14 cm Petri dish, to remove undigested cell clusters and large spontaneous fusion bodies. The protoplast-enzyme mixture was gently pipetted into 16 ml round-bottomed glass centrifuge tubes (Corning Ltd., UK), using a sterile Pasteur pipette, and pelleted by centrifugation at 700 rpm for 5 min. in a bench centrifuge. After centrifugation, the enzyme supernatant was removed with a Pasteur pipette and the protoplasts were washed three times with CPW13M, pelleted and resuspended each time, and finally resuspended in 10 ml of filter sterilised KPR protoplast culture medium (Kao and Michayluk 1975, modified by Thompson et al. 1986) (see Appendices A1.4 & A1.7). The protoplasts were counted using a modified Fuchs Rosenthal double chamber haemocytometer of 0.2 mm depth (Weber Scientific Intern. Ltd., Teddington UK).

Protoplast yield and viability : Protoplast yield was calculated after every isolation and the data was expressed as protoplast number per g F.W. of cells (ppts/g F.W. cells). Protoplast viability (%) was determined by using the fluorescein diacetate (FDA) staining method (Widholm 1972). One drop (or 10 µl) of a stock solution of FDA (5 mg ml^{-1}) was added to 10 ml of CPW13M. An aliquot of this solution was mixed with an equal volume from a dense protoplast suspension. After 5 min. incubation at room temperature, the protoplasts were observed using fluorescence microscopy (Nikon, model 'Diaphot TMD' inverted microscope with fluorescence attachment 'EF'). A high pressure mercury vapour lamp (HBO 100 W/2), was used in combination with a B1 filter block to provide blue illumination of the sample. Viable protoplasts labelled with FDA exhibited a green-yellow fluorescence.

Percent viability was determined as follows :

$$\% \text{ protoplast viability} = \frac{\text{number of fluorescing protoplasts}}{\text{total number of protoplasts}} \times 100$$

2.6.5 Protoplast culture and assessment of protoplast plating efficiency in culture following the standard method

Protoplast culture (standard method) : Rice protoplasts were subjected to a heat shock treatment prior to culture to increase their plating efficiencies. Freshly isolated protoplasts, after thorough washing in CPW13M medium, were resuspended in KPR liquid medium at a density of 5 x 10^5 ml^{-1} in a glass centrifuge tube. The centrifuge tube containing the protoplast suspension was incubated by immersion in a 45°C water bath, and very gently agitated by hand for 5 min., followed by immediate plunging into crushed ice for 10 sec. (Thompson et al. 1986). The heat shocked protoplasts were centrifuged and washed twice, as before, in KPR medium, then resuspended in fresh KPR medium and their final numbers and concentration were determined using a haemocytometer.

Protoplasts were finally pelleted and resuspended in previously prepared molten KPR medium containing 1.2% (w/v) sterile Sea Plaque agarose (FMC Bio Products, Rockland, ME, USA) to give a final plating density of 3.5 x 10^5 ml^{-1}. The protoplast culture medium was prepared by mixing equal volumes of double strength KPR liquid

medium and molten 2.4% (w/v) Sea Plaque agarose and allowing the mixture to cool to 40°C before addition of the protoplasts. Before solidification, the molten KPR-agarose medium containing protoplasts was dispensed into 3.5 cm sterile plastic Petri dishes (Nunc, Denmark) as a 1.5 ml agarose layer. The dishes were sealed with Nescofilm and incubated in the dark at 27°C ± 1°C . At least 4-5 replicates of culture dishes were maintained for each cell suspension for each experiment. After 7-14 days of culture, when microcolonies of about 16-20 cells were visible microscopically, the agarose layer in each dish was cut into four segments, which were transferred to a 5 cm plastic Petri dish containing 3 ml liquid KPR medium (single strength). The dishes were incubated for a further period of 2-3 weeks in the dark at 27°C ± 1°C, with replacement of fresh KPR medium every week. After that time, cell colonies 1.0-1.5 mm in diameter were visible and final protoplast plating efficiencies were calculated.

Assessment of protoplast plating efficiency in culture : The commencement of the protoplast division and formation of micro-calli in the agarose culture medium were examined every 3-4 days using an inverted microscope. The total number of dividing protoplasts at day 14, and the number of macroscopically visible colonies (>1.0 mm) at day 28, in each 3.5 cm Petri dish (agarose layer culture), were counted from randomly chosen areas using a microscope and recorded. For each cell suspension line, data were recorded from at least 3 experiments, each one with 5 replicates. The percentages of the protoplast plating efficiencies were calculated following the standard formula :

$$\text{Plating efficiency (\%) at day 14} = \frac{\text{total number of dividing protoplasts}}{\text{total number of protoplasts plated}} \times 100$$

$$\text{Plating efficiency (\%) at day 28} = \frac{\text{total number of visible macroscopic calli}}{\text{total number of protoplasts plated}} \times 100$$

2.6.6 Re-initiation of cell suspension cultures using protoplast-derived micro-calli [obtained from mature seed-scutellum-derived cell suspension protoplasts]

Protoplast-derived embryogenic micro-calli from previous experiments (see section no. 2.6.5), originating from mature seed-scutellum-derived cell suspension protoplasts, were used for re-initiation of new suspension culture. Each agarose layer containing micro-calli at 14 days of culture, was cut into four segments from each Petri dish and

transferred into 100 ml conical flasks, each containing 10 ml AA2 liquid medium. At least 3 flasks of re-suspension cultures were initiated, maintained as above (see section no. 2.6.3). Eighty per cent of the liquid medium was replaced at 5-day intervals, using a wide-bore (2.5 mm) plastic sterile pipette. During each subculture, the agarose segments were broken into smaller pieces to release micro-calli into the liquid medium.

2.6.7 Plant regeneration from micro-calli obtained from different cell suspension derived protoplast cultures

Protoplast-derived micro-calli (>1.0 mm in diameter), obtained from different cell suspension-derived protoplast cultures, were selected and transferred onto a pre-plated agarose-solidified regeneration medium MSKN (see Appendix 1.7). This medium was prepared by mixing double strength liquid MSKN medium and molten Sigma Agarose Type 1 solution to give a final concentration of 0.4% (w/v) Sigma Agarose, and dispensed into 25-well plastic Sterilin dishes, 3 ml per well (Sterilin Ltd., Feltham, Middlesex, UK). Three to four micro-calli were placed in each well of the dish, the latter sealed with Nescofilm, and incubated in the dark at 27°C ± 1°C. The development of somatic embryos usually started on the surface of the callus, 4-6 days after transfer to MSKN medium. The germination of these embryos began after a further 6-8 days incubation in the dark, depending on the growth vigour. A scutellar notch was normally visible microscopically after 2 weeks. This developed into a defined radicle and coleoptile after about 4 weeks. Sometimes, rhizogenesis was the only sign of differentiation. Approximately 1-2 cm long emerging shoot-systems were transferred to MSBP2 medium (1-2 shoot-systems per 6 oz jar containing 40 ml medium) and incubated for further development [see section no. 2.2.4 (Part I)]. After one month, regenerated green plants were transferred to MSN1.5 medium and incubated similarly for healthy root production.

The plant regeneration frequency was calculated as follows :

$$\text{Plant regeneration frequency (\%)} = \frac{\text{no. of colonies which regenerated plants}}{\text{total number of colonies plated}} \times 100$$

2.6.8 Maintenance of regenerated green plants from different sources for profuse healthy root production to determine the ploidy level by flow cytometry and chromosome counting

The shoots of regenerated green plants (6 to 7 cm tall) obtained from various cell suspension-derived protoplast cultures of different explant origin were micropropagated, first in MSBP2 and then in MSN1.5 media. These micropropagated plants also produced healthy green shoots and white, healthy roots. Five to six successive subcultures (one month intervals) were needed to establish adequate growth and to maintain plants for further experimental use.

2.6.9 Transfer of regenerated green plants obtained from cell suspension-derived protoplast culture (initially originated from MSS callus) to the glasshouse for the measurement of different agronomic traits

Regenerated green plants with extensive root development were transferred to the glasshouse. Fisons Levington M3 soil-less compost (J. Bentley Ltd., South Humberside, UK) was thoroughly mixed with Perlite (Silvaperl Ltd., Gainsborough, Lincs. UK) in the ratio of 12:1 (v/v), and 3" plastic pots were half-filled with the mixture. Regenerated and established green rice plants (10-12 cm in height), grown in MSN1.5 medium were removed from jars, the roots washed gently to remove agarose, and one plant was placed in the centre of each pot. The pots were filled with compost-perlite mixture and the mixture firmed around each plant. The potted plants were placed into small propagator (46cm x 27cm x 8cm) trays (8 pots / tray) and the soil was kept just moist with freshly prepared nutrient solution (5 ml of 'Maxicrop plus sequestered iron' organic feed in 4.5 L of tap water; Maxicrop Ltd., Tonbridge, UK). The plants were covered with a propagator hood (46cm x 27cm x 15cm). After closing the vents, the trays with plants were kept in a growth-room and maintained in a 12 h day : 12 h night cycle at 28°C ± 1°C, with a 70% relative humidity.

The plants were observed daily and fed by gently sprinkling fresh nutrient solution over the plants to keep them moist. After 2-3 days of transfer, the vents of the propagator hood were progressively opened and as the plants showed more growth the ventilation was increased. After 1-2 weeks, the vents were fully opened and after a further one week, the propagator hood was removed and the plants were fed by sprinkling with

fresh nutrient solution twice a day for the next 2 weeks. As soon as the plants started to grow vigorously and actively, root growth was checked either by tapping the intact root-ball from the pot or looking for roots protruding through the bottom of the pot. When the plants were ready to be potted on, they were transferred into 3" pots containing 1 part of Perlite to 2 parts of rice compost (1 part of Fisons M3 soil-less compost to 1 part of John Innes no. 3 compost). They were fed again with fresh nutrient solution and placed in the glasshouse in large (100cm x 39cm x 5cm) trays. During this period, particularly in summer, they were fed 2-3 times daily until they recommenced growth.

Once the plants started growing well after about a further 2 weeks, they were transferred into 6" pots containing rice compost, fed by sprinkling once with nutrient solution and subsequently with water. The potted plants were placed in large (100cm x 39cm x 5cm) trays; the temperature and humidity was maintained similar to that in the growth-room. As the plants started showing healthy, vigorous growth by producing tillers, the trays were filled with water to a depth of 1 cm, and the water level was raised every week to fill the trays completely after about 4 weeks. Finally, *Azolla pinnata*, a fern, was spread on the surface of the water to multiply and to cover the whole surface to control algal growth.

2.7 Results and Discussion II

2.7.1 Initiation and establishment of cell suspension cultures using standard medium from calli of different explant origin

White, translucent, dry, globular and friable embryogenic callus was found to be the most suitable inoculum from all explant sources for the initiation of cell suspension cultures. Contamination with rooty callus, particularly from leaf-base meristem origin (LBM), affected the type of embryogenic cell suspension initiation and establishment. Because the cells from rooty callus grew faster than embryogenic callus, obtaining desirable callus from leaf-base meristems required 3-4 subcultures, at monthy intervals from induction, for successful cell suspension initiation. However, compared to leaf-base meristems, rapid production of embryogenic callus occurred from mature seed-scutellum (MSS) and immature seed-embryos (ISE). It was possible to transfer callus to liquid medium for cell suspension initiation, within 2 to 3 months after induction.

LBM-originated cell suspension cultures were able to retain their regeneration capability for longer than those of MSS and ISE-origin. The appearance of these suspension cultures looked embryogenic and greenish-yellow in colour even after six months of culture age. A gradual reduction and subsequent loss in regeneration capability of protoplast-derived micro-calli, obtained from MSS and ISE-origin cell suspension cultures was observed between 10-12 months from initiation.

The advantage of using friable, embryogenic callus was to dissociate readily the original large cell-clusters into smaller clusters and to release both elongated and embryogenic cells into the liquid medium within the first 3 to 4 weeks of initiation. For the successful establishment of suitable, embryogenic cell suspension cultures, the essential key factors were careful selection of friable, dry, embryogenic callus, and the regular replacement of 80% of the liquid medium for the first 5-6 weeks from initiation. During this initial growth period, most of the cell-clusters or cells, which were thick-walled and vacuolated, dispersed into the liquid culture medium. Usually the small cell-clusters, consisting of densely cytoplasmic cells, were actively dividing, and the number of these dividing cell-clusters increased gradually as the cell suspensions aged. Five to 6 weeks after initiation, subculture in the ratio of 1:3 [7 ml old suspension of mostly small cell-clusters (1 ml pcv + 6 ml old existing medium) added to 21 ml of fresh medium] was beneficial for the production of finely divided cell suspension cultures, without any need for sieving. Later, it was noted that subculture at 7 day intervals was enough to produce suitable, embryogenic suspension cultures consisting of large numbers of small cell-clusters (Fig 2.3a, c).

To establish embryogenic cell suspension cultures from MSS and ISE explants, a similar time was required when friable, embryogenic calli were used. It was noted that for the LBM explant, a longer time was required to establish the cell suspension cultures, because the LBM-derived calli usually possessed a larger number of root-primordia than those derived from MSS and ISE explants. The cell suspension cultures, composed of fast and actively dividing cell-clusters, were ready for protoplast isolation 3 to 4 months after initiation. The origin of the callus generally affected the initiation and establishment of the suspension cultures (Table 2E). Regular microscopic observation of suspension cultures was useful to determine the structure and size of the cell-clusters, which was very important for successful protoplast isolation. An optimal

yield of protoplasts was achieved from loosely-packed cell-clusters containing at least 30-40% peripheral cells protruding from the cell mass. Generally, 3 to 4-month-old cell suspension cultures were composed of compact cell-clusters. Young cell suspension cultures (3 to 4-month-old) usually produced very low yields of protoplasts, with the production of large numbers of spontaneous fusion bodies. Protoplast isolation could be started from 3 to 4-month-old suspension cultures, but better results, with higher yields, higher plating efficiencies and increased plant regeneration frequencies were obtained from 5 to 6-month-old suspension cultures. Optimal results were obtained for protoplast isolation when the cell suspensions were used at their exponential growth phase (4 to 5 days after subculture). The time required to double the pcv in the suspension cultures varied according to explant source, but was usually between 2 and 4 days for an established, optimal culture.

2.7.2 Maintenance and growth-rate determination of the established, cell suspension cultures of different explant origin

The maintenance of regenerable cell suspension lines from any particular explant origin, needed careful management, because any loss in the culture system was very difficult to overcome. It was noted that the correct dilution ratio, during each regular subculture, was critical to avoid cell vacuolation and a poor yield of protoplasts. However, poor culture conditions (unfavourable temperature and non-standard shaker speed) affected cell growth of the suspension cultures, producing transparent cell-clusters with low cytoplasmic density. These suspension cultures were often fast-growing and yielded a large number of protoplasts with poor regenerative ability. Re-selection by suitable sieving or subculturing into AA1 medium (AA medium containing 1 mgl^{-1} 2,4-D) for 2 to 4 passages, helped to enrich the proportion of cytoplasmically dense, embryogenic cell-clusters (Abdullah et al. 1986). As a result of re-selection, rapid proliferation of embryogenic cell-clusters with an increase in cytoplasmic density occurred showing a rejuvenation effect. An alternative method of re-selection was to re-initiate cell suspension cultures using protoplast-derived micro-calli, when the original suspension culture was at its optimal culture age. This re-initiation procedure helped to save time from the lengthy process of callus induction, subculture and selection. It also helped to achieve a good regeneration frequency, and to extend the

effective life of suspension cultures, but they were not as good as the original suspension cultures.

However, when the established cell suspension cultures were composed of thin-walled, actively dividing, cytoplasmically dense, isodiametric small cell-clusters, rapid increase in cell volume was observed during their growth period from day 2 to day 7 (Fig A2.1 & A2.2). It was very important to carry on subculturing regularly to keep the suspension cultures in their exponential growth phase, and to maintain their embryogenic nature. Subculture at day 7 was found to be the most suitable time for their maintenance. It was also observed that the amount of pcv, used during subculture, was crucial for satisfactory growth. The use of less or more than the optimal amount of pcv (1.0 to 1.5 ml / 100 ml conical flask), resulted in poor growth of cells causing degeneration and browning of the cell suspension cultures.

In the present study, maintenance of the established cell suspension cultures of different explant origin and culture age, by two different individuals, affected the growth rate of cells per day (Fig A2.1 & A2.2), the plating efficiencies and the regeneration frequencies obtained from isolated and cultured protoplasts.

2.7.3 Protoplast isolation from different types of established cell suspension cultures of different explant origin

Protoplasts were isolated from different types of cell suspension cultures of different explant origin, maintained by two workers. Almost all the experiments were carried out using the overnight incubation method (see section 3.6.4). The overnight incubation, in diluted enzyme mixture using CPW13M medium, always gave higher protoplast yields and a lower frequency of spontaneous fusion (Table 2F). Usually the average yield of protoplasts, isolated from cell suspension cultures of various explant origin, at their optimal, exponential growth phase varied from 3×10^6 to 1×10^7 g^{-1} F.W. of cells. The average yield of protoplasts from few cell suspension cultures of various explant origin is presented in Table 2G. Generally, freshly isolated protoplasts from suspension cultures of optimal age (5 to 6 month-old), exhibited dense cytoplasm and had an average size, ranging from 10 to 30 µm (Fig 2.3b, d). The average viability ranged from 80 to 90%. The size of the protoplasts, when isolated from suspension cultures of various explant origin, did not differ significantly. The culture age of the

cell suspension cultures greatly influenced the yield of protoplasts. Cultures less than 5 months age from callus induction were not suitable for protoplast isolation. Compared to MSS and ISE-derived suspension cultures, it took a longer time (6 to 7 months from callus induction) for LBM-derived suspension cultures to reach an optimal age for efficient protoplast isolation.

Generally, a higher protoplast yield was obtained when the suspension cultures aged irrespective of explant origin (Table 2G). The time duration between subculture and protoplast isolation was positively correlated with both protoplast yield and the frequency of spontaneous fusion. Protoplast yield decreased markedly when suspension cultures were used within 3 days of subculture, or 7 days after subculture. Efficient and successful protoplast isolation was achieved when suspension cultures were used 4 to 5 days after subculture. Protoplast isolation within 3 days of subculture also resulted in a high frequency of spontaneous fusion (Table 2F & 2G).

2.7.4 Culture of rice protoplasts in semi-solid KPR-agarose medium

Cultured rice protoplasts began to swell during the first 2 to 3 days of culture, and cell wall regeneration commenced. Normally, the first division was observed within 5 to 7 days of culture (Fig 2.3e). Micro-colonies of about 20 to 25 cells or more were formed after 2 weeks of culture (Fig 2.3f, g). At this stage, transfer of agarose segments to liquid KPR medium was essential for continuous growth and nourishment of the micro-colonies. Depending on the adequate growth of the micro-colonies, the time for transferring them to liquid medium (between 7 to 12 days of culture) was found to be the most favourable. Very few macroscopic micro-calli were visible after 4 weeks, when the micro-colonies were transferred into liquid medium within 7 days or after 15 days of culture (4 to 8 or 32 to 40 cell stage) respectively. Generally, micro-calli reached a size of between 0.5 to 1.0 mm in diameter after 14 to 18 days of transfer to liquid medium (Fig 2.4a, d). At this stage of growth, it was important to transfer the micro-calli onto regeneration medium as soon as possible to achieve a high frequency plant regeneration. It was also found that a long period of culture of the micro-calli in liquid KPR medium had an adverse effect, causing browning and degeneration.

The age of the cell suspension cultures, from which protoplasts were isolated, greatly influenced plating efficiencies. Usually, protoplasts from older cell suspension cultures

Fig 2.3 Protoplast isolation from cell suspension culture and division of protoplast in *O. sativa* (cv. Taipei 309)

a) Loosely packed cell-clusters of embryogenic cell suspension culture (MSS-origin) after 3-month of transfer to AA2 liquid medium, showing the presence of actively dividing, densely cytoplasmic, embryogenic cells (x 148).

b) Freshly isolated cell suspension-derived protoplasts (MSS-origin) (x 72).

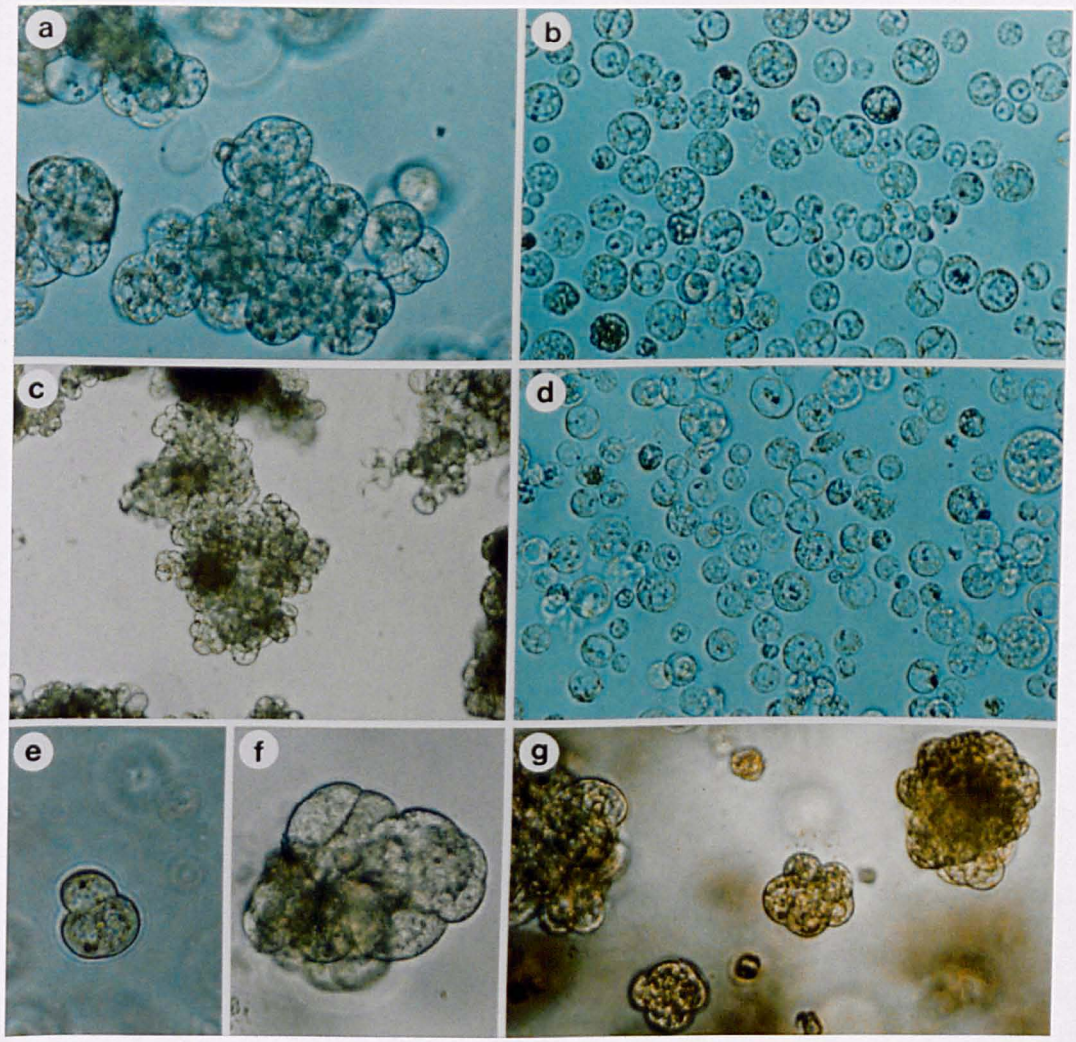
c) Loosely packed cell-clusters of embryogenic cell suspension culture (LBM-origin) after 4-month of transfer to AA2 liquid medium, showing the presence of actively dividing, densely cytoplasmic, embryogenic cells (x 76).

d) Freshly isolated cell suspension-derived protoplasts (LBM-origin) (x 50).

e) First division of a protoplast after 3-5 days of culture on agarose solidified KPR medium (x 80).

f) A protoplast-derived microcolony (LBM-origin) after 10-12 days of culture on agarose solidified KPR medium (x 156).

g) Three stages of growth of protoplast-derived microcolonies (MSS-origin) in the same culture, showing 4-celled, 8-celled and multicellular colonies after 14-16 days of culture on KPR-agarose medium (x 152).


a
b
c
d
e
f
g

produced higher plating efficiencies than those from younger suspension cultures (Figs 2.4a, d). Protoplast plating density also had a direct effect on protoplast plating efficiency. Maximum plating efficiencies were obtained when protoplasts were cultured at a density of between 3.5 x 10^5 and 4.0 x 10^5. Negligible growth of micro-calli was observed when the protoplast plating density was lower than 3.0 x 10^5. Generally, plating efficiencies increased with increasing protoplast density. However, it was also observed that protoplasts, cultured at a density higher than 5.0 x 10^5, resulted in a decreased division frequency and rapid browning in liquid KPR medium. The cell suspension cultures of different explant origin had no significant effect on protoplast plating efficiencies, when suitable callus was used for cell suspension initiation (Table 2H).

2.7.5 Plant regeneration from protoplast-derived micro-calli

After placing protoplast-derived embryogenic micro-calli on regeneration medium, the first sign of differentiation was indicated either by somatic embryogenesis or rhizogenesis. This observation was not affected by the explant origin of different cell suspension cultures. Most frequently, white embryoid-like structures developed on the surface of the micro-calli, after 7 to 10 days of culture (Fig 2.4b). Later, after about a further week of incubation in the dark (Fig 2.4b, e), an organised and defined bipolar structure was formed consisting of a coleoptile and a radicle. Depending upon the explant source and the culture age, plantlets were developed from this organised structure at maximum frequencies ranging from 40 to 45%, within 3 to 4 weeks. At this stage, it was essential to transfer them to the light for further development. The plantlets became green within 2 days of transfer to the light (Fig 2.4c, f). They were subsequently transferred to soil (Fig 6.1b, e) when they were tall enough (10-12 cm). For successful transfer and growth to mature of fertile plants (Figs 2.4h, i), it was also essential to check for healthy shoot and abundant root growth before transfer.

The plant regeneration frequencies from protoplast-derived micro-calli varied for different cell suspension cultures of different explant origin when the culture age was similar. They also varied for each cell suspension culture of the same explant origin, when the culture age was different. An optimal frequency of plant regeneration was obtained over a period of 5 to 6 months; it then gradually declined (Table 2I).

Fig 2.4 The regeneration process of protoplast-derived plants of *O. sativa* (cv. Taipei 309)

a) Protoplast-derived micro-calli growing on the surface of KPR-agarose sections after 3-4 weeks of culture, consisted of compact and loose calli; demonstrating variation in plating efficiencies obtained from different cell suspension cultures (MSS-origin) (x 3.6).

b) Differentiation of somatic embryos with multiple shoots and roots on the surface of protoplast-derived calli (MSS-origin) after 2-3 weeks of culture on MSKN regeneration medium under dark condition at 27°C ± 1°C (x 3.8).

c) A somatic embryo derived from protoplast culture (MSS-origin), with the production of a number of green shoots(s) after 4-5 weeks of culture on MSKN regeneration medium under white, fluorescent light (1000 lux) in the growth room at 25 °C ± 1°C (x 3.8).

d) Protoplast-derived micro-calli growing on the surface of KPR-agarose sections after 3-4 weeks of culture, demonstrating variation in plating efficiencies obtained from different cell suspension cultures (LBM-origin) (x 3.7).

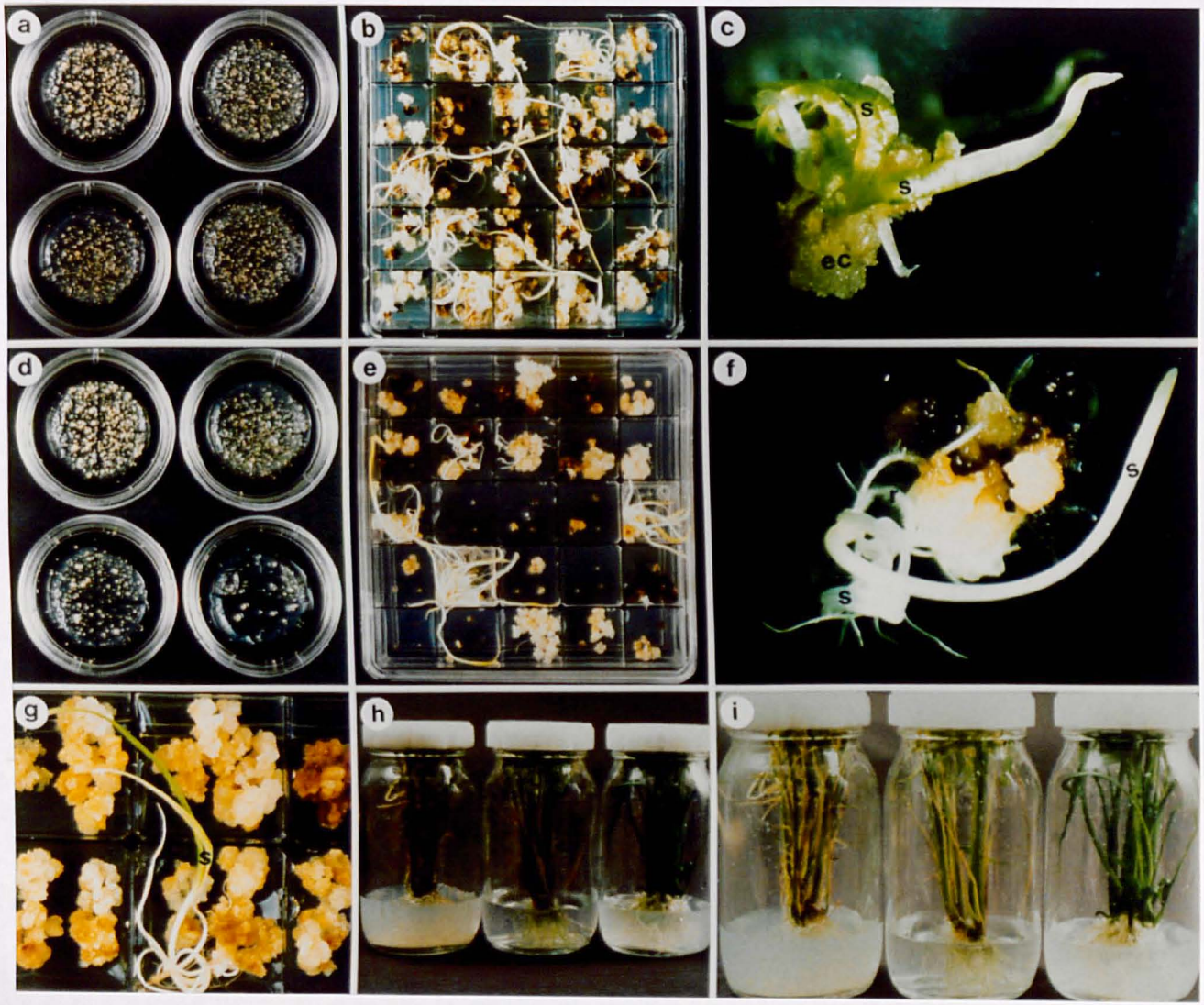
e) Differentiation of somatic embryos with multiple shoots and roots on the surface of protoplast-derived calli (LBM-origin) after 2-3 weeks of culture on MSKN regeneration medium under dark condition at 27°C ± 1°C (x 3.7).

f) A somatic embryo derived from protoplast culture (LBM-origin), with the production of a number of shoots(s) and roots(r) after 4-5 weeks of culture on MSKN regeneration medium under the same culture conditions as in (c) (x 3.6).

g) A single protoplast-derived plant (LBM-origin) with roots and green shoots(s) after 4-5 weeks of culture on regeneration medium in the dark and a week of subsequent transfer to light (1000 lux) in the growth room at 25 °C ± 1°C (x 3.6).

h) Growth of the regenerated plants from protoplasts and production of multiple shoots and roots in MS0 medium (left), MSN1.5 liquid medium (middle) and MSN1.5 semisolid medium (right) after 4 weeks of culture under the same culture conditions as in (c) (x 3.8).

i) The growth of regenerated plants described in (h) after 7 weeks of culture, showing good growth in MSN1.5 semisolid medium (x 5.0).


a
b
c
s
s
ec
d
e
f
s
s
g
s
h
i

Protoplasts isolated from cell suspension cultures, aged between 5 to 6 months, initiated from MSS-derived callus produced the highest plant regeneration frequency. Protoplasts from ISE-derived suspension cultures produced a lower regeneration frequency. When the original suspension culture (MSS-55) was at its optimal culture age, the initiation of a re-suspension line (MSS-55R1) from protoplast-derived micro-calli was very useful. It extended the effective life of the original suspension culture, resulting in a rejuvenation effect and also resulted in satisfactory plant regeneration frequency. Following this procedure, maintenance of protoplast regeneration potential was possible for several months. Compared to the original suspension line, the re-suspension line showed a lowered regeneration potential.

2.7.6 Successful transfer of regenerated green plants (MSS-origin) to the glasshouse for growth to maturity and measurements of different agronomic traits

Healthy, regenerated green plants from protoplasts which had been isolated from three cell suspension cultures (MSS-origin), were successfully transferred to the glasshouse following the modified, standard procedure (see section 2.6.9). After careful maintenance for 12 months, it was possible to grow some of these plants (about 20%) to maturity for the measurement of agronomic traits. During this experimental period, it was generally observed that most of the protoplast-derived plants were highly sensitive to the glasshouse conditions, and the mortality percentage was very high (about 80%). It did not happen to the seed grown plants, all of them survived with 80-90% fertility.

2.8 Summary and Conclusions II

The basic objective of this part of the work was to produce and to establish embryogenic cell suspension cultures from various explant origins, and to develop an efficient, reproducible protoplast-to-plant regeneration system for Japonica rice cultivar Taipei 309. In addition to the influence of the explant origin on embryogenic callus production and subsequent initiation of cell suspension cultures, the selection of suitable callus was of major importance for the establishment of cell suspensions. The best cell suspensions were established from young callus material (2 to 3 months after callus induction, ISE-derived), and from calli which had been subcultured 3-4 times

(LBM and MSS-derived). Subsequently, the optimum growth of cell suspensions required selection of cells capable of further growth under specific growth conditions (liquid medium, growthroom culture conditions).

To obtain fertile green plant regeneration at a high frequency from rice protoplasts, the ability to produce and to regularly monitor embryogenic suspension cultures is very important. Adequate control of the duration of enzyme treatment required for the production of viable protoplasts was necessary to ensure satisfactory plating efficiencies and plant regeneration frequencies, and a lower level of spontaneous fusion. Careful and regular monitoring of the suspension cultures, making changes in the subculture procedures (re-selection), checking shaker speed and the temperature at which the suspensions were maintained, and selection of the suitable portions of the suspension after it had been allowed to settle momentarily (Vasil and Vasil 1984), were also required. Careful and delicate handling of the protoplasts, maintenance of optimal plating density, even distribution of protoplasts during culture and plating, the use of agarose medium at room temperature, and the use of an exact final concentration of agarose (1.2%), were all further critical requirements to obtain optimum plating efficiencies.

It is now well recognised that rice genotype is one of the important factors for the establishment of embryogenic suspension cultures (Abdullah 1987). In this present study, the Japonica rice variety Taipei 309 was chosen because it was easy to grow in the glasshouse to obtain ISE explant within a short time. It was also easy to induce embryogenic callus from MSS and LBM explants. During the study period, sustained division of rice protoplasts isolated directly from mesophyll tissues of rice plants have not been reported (Vasil et al. 1990). In this study, the use of cell suspension cultures for protoplast isolation was chosen for easy production of high yields, with a high percentage of viable protoplasts.

After achieving successful establishment of embryogenic cell suspension cultures from different explant origins, an in depth study of protoplast culture and plant regeneration from protoplast-derived micro-calli of different cell suspension cultures of various explant origins was undertaken. The optimum production of protoplasts, micro-calli formation from protoplasts in culture, and green rice plant regeneration from micro-

calli derived from protoplasts was compared among the different cell suspensions of the same explant origin. Concomitantly, the optimum production efficiency was also compared among the cell suspension cultures of different explant origins. Other important observations were the variation in protoplast yields, plating efficiencies, plant regeneration frequencies, and production of spontaneous fusion bodies from the suspension cultures maintained by two different workers using the same callus source. Later, these regenerated green plants obtained from callus cultures (Chapter 2, Part I) and cell suspension-derived protoplasts culture of various explant origin (Chapter 2, Part II), were used to determine the ploidy levels by flow cytometry (Chapter 4) and chromosome counting from root-tip squash preparations (Chapter 5).

Results from various experiments, conducted every month using cell suspension cultures of the same and different explant origins, maintained by two different workers (worker 1 & worker 2), were recorded. It was generally observed that within the same culture age, there was no significant difference in protoplast yields, protoplast viability and frequency of spontaneous fusion, even after maintenance by two different individuals. However, marked variation was observed in plating efficiencies and plant regeneration frequencies, within the same culture age of the different cell suspension cultures, and also among the different culture ages of the same cell suspension culture.

Similar variations in results were obtained when protoplasts were isolated from the cell suspension cultures of same explant origin or different explant origins and maintained by two different workers. Initially, a variation in the time (one month) required for embryogenic suspension culture establishment for satisfactory protoplast isolation by two different workers was also noticed. It was normally observed that each cell suspension line, either derived from the same explant source of the same callus age or from the different explant sources of the same callus age, differed significantly. In both cases, a particular period of time was required to produce optimal plating efficiencies and plant regeneration frequencies. However, for each cell suspension culture, a specific period of time was needed to reach its exponential growth phase and to produce maximum plating efficiencies and plant regeneration frequencies. Variable results were also noted between two cell lines, when initiated from the same callus source (MSS) of the same age, maintained by the same worker or by two different

workers. Sometimes, a non-regenerable cell line was produced from the same callus source of the same callus age, irrespective of worker's variation.

Comparing three different explants for embryogenic callus production, suspension culture initiation and establishment, the shortest time period was required for ISE explants (2 to 3 months). The longest time period required was for LBM explants (5 to 6 months), because the LBM-derived calli usually possessed a larger number of root-primordia than those derived from MSS and ISE explants. Similar results were obtained for protoplast isolation, optimal yield production, micro-calli formation and plant regeneration (section 2.7). On the other hand, retention of regeneration capability for LBM-derived cell suspension cultures was longer than those of MSS and ISE-derived suspension cultures. For rapid dissociation of large cell-clusters into small cell-clusters, the use of friable, embryogenic calli and regular replacement of 80% of the liquid culture medium after every 2 to 3 days for the first 5 to 6 weeks, were essential. It was also observed that the amount of pcv used during subculture was crucial for satisfactory growth of cells (section 2.7.2). Protoplasts could be isolated started from 3 to 4-month-old suspension cultures of different explant origins, but better results were obtained from 5 to 6-month-old suspension cultures of MSS and ISE origin and 6 to 7-month-old suspension cultures of LBM origin.

However, younger suspension cultures from every explant origin (3 to 4-month-old), usually produced very low yields of protoplasts with a high frequency of spontaneous fusion. Similar results were obtained, when the time duration between subculture and protoplast isolation was less than optimal, or more than optimal. The production of large number of spontaneous fusion bodies is undesirable for somatic hybridisation and genetic transformation studies.

Overnight incubation in diluted enzyme mixture with CPW13M was preferred, because it always gave higher protoplast yields and a lower frequency of spontaneous fusion. The culture age of the suspension cultures, irrespective of explant origin, greatly influenced the yields of protoplasts, plating efficiencies and plant regeneration frequencies. The most favourable time for transferring protoplast-derived micro-calli to liquid KPR medium varied between 7 to 12 days of culture. When the micro-calli reached a size between 0.5-1.0 mm in diameter, it was important to transfer them onto

regeneration medium as soon as possible to achieve a high frequency plant regeneration (section 2.7.4). Delay in transfer caused albino plant regeneration. The protoplast plating density also had a direct effect on protoplast plating efficiencies, and subsequent plant regeneration frequencies. Protoplasts cultured at a lower or higher plating density than the optimal range (between 3.5×10^5 and 4.0×10^5) resulted in a decreased division frequency and rapid browning in liquid KPR medium. Generally, it has been found in this laboratory that a high plating efficiency was obtained when protoplasts were subjected to heat-shock treatment before culture. Previously, similar results have been reported in the *Japonica* rice cultivar T309 (Zhang 1990). The selection of compact protoplast-derived micro-calli was also beneficial, enabling them to be transferred into regeneration medium with subsequent high plant regeneration frequencies. In this study, a maximum frequency ranging from 40-45% plant regeneration was achieved from one MSS-derived cell suspension culture when it was at its exponential growth phase (section 2.7.5). The number of regenerable cell lines derived both from MSS and LBM explants, also varied when these were maintained by two different workers.

The protoplast regeneration capacity of the rice cell suspension cultures declined gradually, as has been generally observed for cereal suspensions (Vasil and Vasil 1982; Jähne et al. 1991a). Regeneration can be partially or completely lost (Vasil and Vasil 1987; Kamo et al. 1987). In the case of ISE-derived suspension cultures, optimum regeneration frequencies from protoplast-derived micro-calli were achieved from cell lines aged between 5 to 7 months. However, this varied for cell lines aged between 6 to 9 months, when the suspension cultures were derived from MSS and LBM explants. Although plants could be regenerated from protoplasts isolated from older cultures, the plant regeneration frequency was reduced significantly, and subsequently regeneration capability was lost (Table 2H). It was also observed that re-selection by suitable sieving or subculturing into AA1 medium (section 2.7.2) for 2 to 4 passages was very helpful to minimise this tendency. An alternative method of re-selection by re-initiating new cell suspension cultures, using protoplast-derived micro-calli, was also helpful for plant regeneration. The re-suspension line (55R1) was capable of yielding good, viable protoplasts which could be regenerated into plants (Table 2I). It

is likely that the re-selection procedure selected those cells which had the potential to produce regenerable protoplasts capable of regenerating plants.

Another important step is the storage of protoplasts through immobilization and cryopreservation. Such regenerable cell suspension-derived protoplast cultures also provide the potential for the production of somatic hybrids and transgenic rice plants, if stable transformation of protoplasts and efficient plant regeneration can be combined (Jähne et al. 1991a). To achieve the goal, some of the newer techniques, such as flow cytometry, electrofusion and electroporation are now ready for use for somatic hybridization, somaclonal variation and genetic manipulation, especially through direct gene transfer (Bajaj 1989c). The plant regeneration potential from cell suspension-derived protoplasts is a dominant factor, and this can be used as a selectable marker in protoplast fusion studies to transfer desirable traits from wild species to cultivated species.

Table 2E. Effect of origin of callus on cell suspension culture initiation, establishment and maintenance of regenerability (T309)

Callus source	Number of cell suspensions		
	Initiated	Established	Capable of plant regeneration from protoplasts
Immature seed-embryos (ISE)	4	3	2
Mature seed-scutellum (MSS)	8	8	3
Leaf-base meristems (LBM)	8	8	5

AA2 liquid medium was used for initiation and maintenance of all cell suspension cultures.

Table 2F. Influence of incubation procedures on protoplast yield, viability and spontaneous fusion body formation (T309)

Incubation procedure	Protoplast yield[1] (x 10^6 ppts/g cells)	Protoplasts		Spontaneous fusion body[1] (%)
		Viability[1] (%)	Diameter[1] (µm)	
Day Incubation	9.2 ± 2.3 (LBM)	92.0 ± 2.1	16.5 ± 1.4	5.7 ± 2.34
	8.5 ± 1.9 (MSS)	93.0 ± 2.2	17.1 ± 1.3	6.2 ± 2.43
Overnight incubation	11.1 ± 3.1 (LBM)	75.0 ± 5.1	12.5 ± 1.5	0.9 ± 0.33
	12.3 ± 3.5 (MSS)	79.0 ± 4.9	13.2 ± 1.6	0.8 ± 0.27

[1] Data represent the means ± standard error based on three replicate experiments. A leaf-base meristem-derived (LBN2 b) and a mature seed-scutellum-derived (MSN1 b) cell suspension culture were used (LBN2 b, 41-weeks-old and MSN1 b 42-weeks-old).

Table 2G. Effect of cell suspension culture age on average yields of protoplasts and spontaneous fusion body formation (T309)

Suspension lines	Age of the suspension lines (months)	Protoplast yields[1] (x 10^6) /g F.W. cells	Spontaneous fusion body[1] (%)
MSN1b (N)[2]	2	0.76 ± 0.03	10.47 ± 2.45
	3	1.97 ± 0.03	10.12 ± 2.37
	4	3.21 ± 0.18	9.47 ± 2.22
	5	4.45 ± 0.08	8.29 ± 2.01
	6	5.47 ± 0.07	7.23 ± 1.83
	8	6.96 ± 0.11	5.07 ± 1.23
MSN1 (J)[2]	2	0.45 ± 0.10	11.02 ± 2.69
	3	0.67 ± 0.09	10.59 ± 2.47
	4	1.93 ± 0.09	10.38 ± 2.39
	5	2.93 ± 0.06	10.01 ± 2.23
	6	3.01 ± 0.14	9.39 ± 2.03
	8	4.43 ± 0.14	8.25 ± 1.97
LBN2b (N)[3]	2	1.25 ± 0.07	11.14 ± 2.67
	3	1.53 ± 0.10	11.01 ± 2.43
	4	2.68 ± 0.09	10.76 ± 2.39
	5	3.25 ± 0.09	10.47 ± 2.01
	6	4.75 ± 0.07	6.23 ± 1.78
	8	5.99 ± 0.14	3.27 ± 0.77
LBN2 (J)[3]	2	0.96 ± 0.06	11.47 ± 2.71
	3	1.10 ± 0.10	11.37 ± 2.67
	4	1.23 ± 0.09	11.02 ± 2.43
	5	2.79 ± 0.13	10.88 ± 2.32
	6	3.36 ± 0.15	8.01 ± 1.97
	8	4.47 ± 0.14	6.22 ± 1.69

[1] Data represent the means ± standard error based on 3 replicate experiments.

[2] MSN1b (N) - Suspension culture initiated by worker no. 1 from MSS-derived callus.

[3] LBN2b (N) - Suspension culture initiated by worker no. 1 from LBM-derived callus.

[2] MSN1 (J) - " " " " " 2 MSS-derived callus.

[3] LBN2 (J) - " " " " 2 LBM-derived callus.

Table 2H. Protoplast plating efficiencies and plant regeneration frequencies from cell suspension cultures of different age and different explant origin (T309)

Suspension lines	Age of the suspension lines (months)	Plating efficiencies[1] (%)		Average no. of shoots per callus[1]	Plant regeneration frequency[1] (%)
		at 14 d	at 28 d		
MSN1b(N) (MSS-derived)	3	2.25	0.08	3.33	45.0
	4	6.33	0.14	3.12	49.9
	5	9.41	0.38	3.25	37.1
	6	13.77	0.63	3.28	31.5
	7	18.60	0.81	3.11	23.7
	8	22.32	0.83	3.27	15.3
	9	23.45	0.96	3.03	9.9
MSN1(J) (MSS-derived)	3	1.97	0.05	2.02	15.7
	4	5.51	0.11	1.96	18.2
	5	8.37	0.29	2.01	13.4
	6	12.91	0.55	2.00	9.9
	7	16.44	0.78	1.86	8.5
	8	19.32	0.79	2.01	7.3
	9	20.67	0.87	1.93	6.1
LBN2b (N) (LBM-derived)	4	6.21	0.12	1.01	3.5
	5	7.99	0.21	1.00	6.2
	6	12.27	0.45	1.02	14.2
	7	14.53	0.91	1.03	11.3
	8	13.37	0.88	1.10	8.7
	9	14.82	0.97	1.23	6.5
LBN2 (J) (LBM-derived)	4	5.11	0.09	1.00	2.2
	5	6.03	0.15	1.01	4.7
	6	10.21	0.38	1.02	13.9
	7	12.83	0.82	1.05	10.7
	8	11.41	0.74	1.01	6.1
	9	12.71	0.87	1.03	4.2

[1] Data represent the means based on 3 replicate experiments conducted every month. In each experiment, a minimum of 100 protoplast-derived micro-calli were transferred to regeneratiom medium.

Table 2I. Protoplast plating efficiencies and plant regeneration frequencies from protoplasts isolated from the original suspension culture and from re-suspension culture

Suspension lines	Age of the suspension lines (weeks)	Plating efficiencies[1] (%)		Average no. of shoots per callus[1]	Plant regeneration frequency[1] (%)
		at 14 d	at 28 d		
MSS-55 (MSS-derived)[2]	5	0.67	0.23	1.05	12.5
	8	1.10	0.61	1.27	16.7
	12	2.22	0.73	1.53	41.2
	16	3.10	0.93	2.11	23.3
	20	3.57	1.13	2.71	24.5
MSS-55R1 (PMC-derived)[3]	20	3.10	1.09	1.01	14.3
	24	3.22	1.23	1.05	15.7
	28	3.67	0.98	1.25	15.6
	32	3.51	0.87	1.33	17.3
	36	2.73	0.63	1.00	19.9
	40	2.23	0.51	1.02	18.1

[1] Data represent the means based on 3 replicate experiments.

[2] Mature seed-scutellum callus derived cell suspension culture.

[3] Protoplast micro-calli derived re-suspension culture.

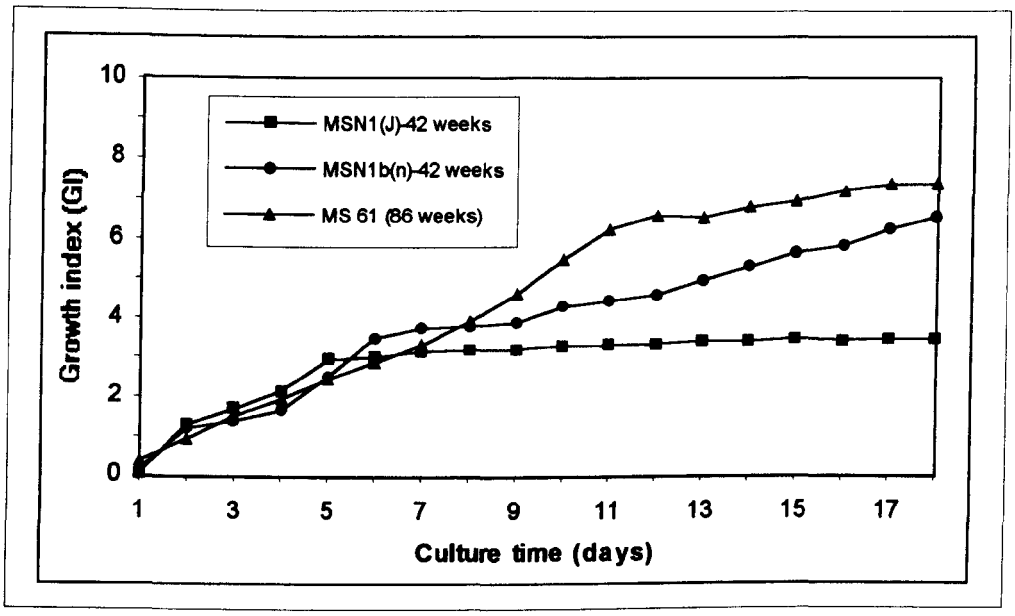


Fig-A2.1. Growth of established cell suspension cultures of T309 (MSS-derived). Suspension cultures were subcultured and growth index was measured, starting from the first day of subculture. Data were calculated from three experiments and the means plotted.

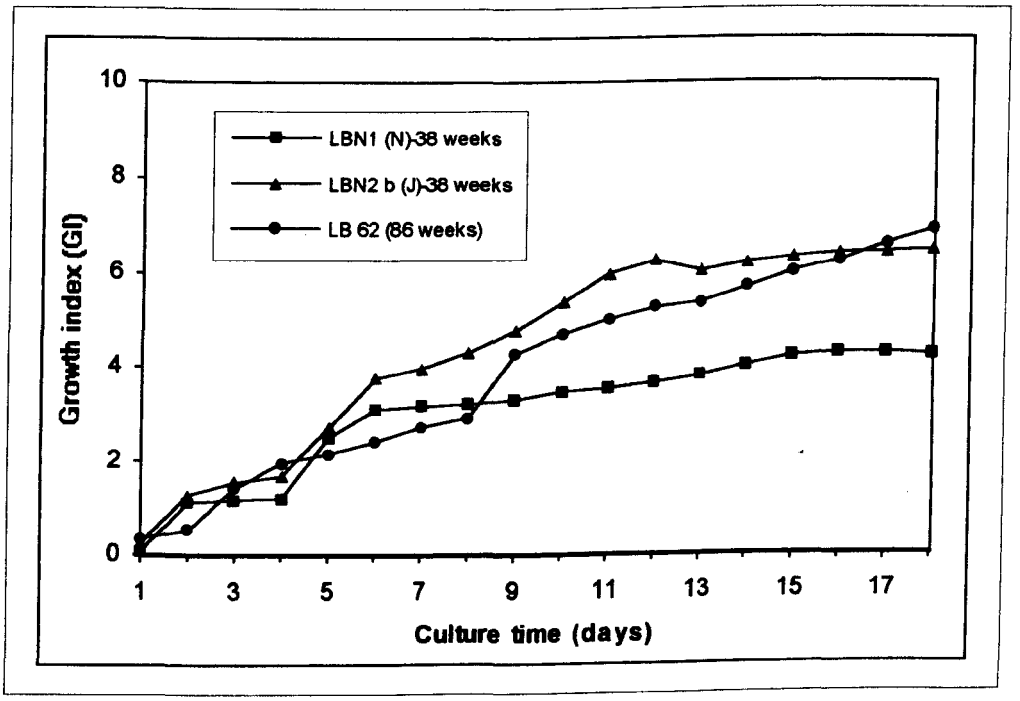


Fig-A2.2. Growth of established cell suspension cultures of T309 (LBM-derived). Suspension cultures were subcultured and growth index was measured, starting from the first day of subculture. Data were calculated from three experiments and the means plotted.

Chapter 3

In vitro production of haploid and diploid rice plants through anther float and microspore culture

Part I: Haploid and diploid green plant regeneration from anther callus and microspore callus cultures

3.1 Introduction I

In flowering plants, such as *Oryza sativa*, the flower contains specialized structures, the anthers and the pistil, or gynoecium, in which the male and the female gametophytes, (pollen grains and embryo sacs respectively), are formed. The male gametophyte completes its early development within the anther (Mascarenhas 1989). The term 'haploid' refers to those plants which possess the gametophytic number of chromosomes in their sporophytes. Haploids can be regenerated from both male and female gametophytic cells through the culture of anthers, microspores, ovaries and ovules. A positive *in vitro* response leads to the development of embryos and/or callus, from which green plants can be regenerated (Keller et al. 1987).

Major advances have already been achieved in rice improvement through conventional breeding methods. *In vitro* production of haploid and diploid green rice plants through the culture of anthers or microspores and protoplasts of anther or microspore callus-derived cell suspensions, has important applications in rice breeding. This novel, innovative approach can be attained by spontaneous doubling of the chromosome complements of haploid material, which is a much quicker method of inducing homozygosity and stability in the plant genome, compared to the conventional breeding approach. For introducing desirable traits, the latter approach is considered to be stable by the end of the F5 or F6 generations. Haploids, with their unique genomic constitution as a result of meiotic recombination, have potential for accelerating or shortening the time required for the development of homozygous new improved rice varieties (Zapata et al. 1983). Moreover, haploids express recessive genes, which become fixed when the chromosome complements are doubled. By utilizing *in vitro* haploid production, the normal gametophytic development of both the microspore and

the megaspore of rice can be diverted to a sporophytic pathway. The use of anther or microspore culture to induce sporophytic development of microspores has become more important than the culture of ovaries or ovules, because the latter generally exhibited a very low response when applied to rice breeding. However, the culture of ovaries and ovules is of great value in the production of male sterile plants (Lynch et al. 1991).

Following the extensive reviews on haploids (Kimber and Riley 1963; Magoon and Khanna 1963), a tremendous amount of interest was generated in this area. The first report of haploid plant regeneration from *Datura innoxia* anthers was published by Guha and Maheshwari in 1964. This work greatly stimulated interest in the induction of androgenetic haploids. Since then, intensive studies of the *in vitro* culture of gametophytic cells with the aim of producing haploid plants have been reported in 171 species, including some hybrids, belonging to some 60 genera and 26 families of angiosperms, from many countries of the world (Maheshwari et al. 1982; Baenziger and Schaeffer 1983). During the last two decades, the progress on haploid production has led to extensive studies on the utilization of haploidy in plant breeding (Chu 1982). *In vitro*-produced haploids have been incorporated into the breeding programs of many agricultural crops, including rice, wheat, potato, barley, maize, asparagus, sunflower, brassicas, and tobacco. Among these, rice and wheat are the best examples in which a number of improved varieties have been released. In both rice and wheat, the breeding cycle can be shortened by three or four generations when the pollen haploid breeding method is used instead of conventional cross-breeding (Bajaj 1990). Examples are the release of the rice varieties Zhonghua Nos. 8, 9, 10, and 11, Guan 18 and the wheat varieties Jinghua 1 and Florin (Zhu and Pan 1990; Loo and Xu 1991). In 1976, rice varieties derived from haploid breeding were released for commercial production in China (Yin et al. 1976). Over 100 new rice varieties and lines have been developed through anther culture breeding for earliness, yield, resistance to diseases, and grain quality (Zhang and Chu 1986; Shen et al. 1983; Hu 1985). These are currently growing on 250,000 hectares in China (Oono 1988).

Various *in vitro* methods for the induction of haploids in 26 agricultural crops and trees have been reported (Bajaj 1990). Emphasis has been given to field trials of *in vitro*-produced haploids and pure lines, and the significance of these plants in crop

improvement and the early release of cultivars. Ever since the first production of haploid plants of rice through anther culture (Niizeki and Oono 1968), *in vitro* production of haploid rices and their utilization has generated much enthusiasm for the improvement and early release of rice varieties. The theoretical basis for the utilization of anther-derived rice plants for breeding has also been established. In fact, rice has become one of the few crops in which anther culture can be readily applied to breeding programmes (Chen et al. 1983, 1991).

The first event in androgenesis is a shift of the microspores from gametophytic to sporophytic development. After this induction phase, unlike *Datura* and *Nicotiana* where the microspores develop into plants directly via embryogenesis (Guha and Maheshwari 1966; Nitsch and Nitsch 1969), androgenesis in rice usually has an intermediate step of callus formation (Niizeki and Oono 1968; Chen et al. 1991). In rice, normally organogenesis or embryogenesis of the callus gives rise to plants. Subsequent studies have shown that the development of microspores into fertile plants depends on many limiting environmental factors. These factors include the growth conditions or physiological status at the time of anther excision and the age of the donor plants (Lupotto 1982; Huang et al. 1985; Lee et al. 1988; Raina et al. 1987), the genotype of the donor plants (Mikami and Kinoshita 1988; Powell 1988a; Loo and Xu 1991), the inductive pretreatments (Genovesi and Magill 1979; Zapata et al. 1983, 1985; Pulver and Jennings 1986; Zapata and Torrizo 1986), and the stage of anther or pollen development at the time of anther excision and culture (Chen 1977; Dunwell 1985; Gupta and Borthakur 1987). Other factors include the nature of the callus induction and regeneration media (Zapata et al. 1983; Yeh and Tsay 1988; Powell 1990; Chen et al. 1991), the general culture conditions (Guha-Mukherjee 1973; Niizeki and Oono 1971; Chen et al. 1983, 1991), the anther orientation on the surface of the semi-solid medium (Mercy and Zapata 1987; Powell 1988), the use of float anther culture on shallow layers of liquid medium (Zapata 1985; Chen 1986a), the effect of the anther wall (Kohlenbach et al. 1978; Tsay et al. 1986), the age and quality of the anther or microspore callus (Wang et al. 1974; Chen et al. 1984, 1986) and the extent of albinism in the rice anther cultures (Chen and Wu 1983; Chen et al. 1991; Loo and Xu 1991).

The development of callus and plants from dividing microspores isolated after 3-4 days of anther pre-culture was first achieved in the early eighties (Chen et al. 1982b). The importance of isolated microspore culture as an experimental system for basic genetic studies and crop breeding has been recognized (Dunwell 1985)., The microspore provides an effective cell selection system because it is both a single cell and a haploid. Microspores are also attractive for microinjection. Direct injection of DNA into microspores is generally of interest for crop improvement (Gunn and Day 1986); transgenic rapeseed plants have been obtained from microspore-derived embryos after microinjection of foreign genes (Neuhaus et al. 1987). There has also been success using isolated rice microspores. Microspore-derived plants have been produced from three *Japonica* varieties, namely Sasanishiki, Nipponbare, and Reimei (Jia et al. 1987). At IRRI, the requirement for anther pre-culture has been overcome, and plant regeneration from callus derived from isolated microspores has been obtained from the anther culture-responsive *Japonica* variety, Taipei 309. Transformation of microspores may be even more advantageous than using somatic cells, since the inserted gene can readily be duplicated by chromosome doubling, thus eliminating segregation in future generations (Cho and Zapata 1988). Until 1989, there were no reports of isolated microspore embryogenesis, on plant regeneration, in *Indica* rices. In 1990, a system of culture of shed microspore involving embryogenesis and fertile plant regeneration from competent microspores of both *Indica* type II and *Japonica* rices was reported (Datta et al. 1990). Subsequently, this type of work was extended to the true *Indica* type I variety, IR 43, and green plants were regenerated (Zapata et al. 1991). Since then, efforts have been made to enhance the efficiency of plant regeneration. The list of species or subspecies and their hybrids obtained by excised anther or microspore culture and the basal media used are presented in Table 3A and Table 3B.

The aims of the work described in the part I of this Chapter were :

- to establish feasible and reproducible systems of anther float and isolated microspore culture, embryogenesis and plant regeneration from competent microspores of the *Japonica* rice cultivar T309,
- to compare the variation in callus formation and haploid-diploid plant regeneration frequencies from anther or microspore-derived calli or embryoids.

Later, variation in the ploidy level of the regenerated green plants derived from anther float culture was determined by flow cytometry (Chapter 4). Changes in chromosome number were determined by chromosome counting from root-tip squash preparations (Chapter 5).

Table 3A. List of *Oryza* species or subspecies and their hybrids in which haploid callus (C), embryos (E) or plants (P), have been obtained by the culture of excised anthers or microspores [b]

***Oryza* species or subspecies and their hybrids**	**Mode of development**	**References**
***Oryza sativa* L.**	C,E, P	Niizeki (1968) Niizeki and Oono (1968, 1971) Nishi and Mitsuoka (1969) Guha-Mukherjee (1973) Wang et al. (1974, 1987) Chen C. C. (1977) Genovesi and Magill (1979) Chen and Chen (1980) Bajaj (1980, 1984) Chaleff and Stolarz (1981) Chang and Hong-Yuan (1981) Zapata et al. (1981, 1982, 1983) Liang et al. (1982) Chen et al. (1982a, b, 1986, 1991) Tsay et al. (1982, 1986a, b) Chen Y (1983, 1986) Schaeffer et al. (1984) Mercy et al. (1984) Chu et al. (1985) Mia et al. (1985) Reddy et al. (1985) Nam and Heszky (1986) Torrizo and Zapata (1986) Guiderdoni (1986b, 1991) Raina et al. (1987) Gupta and Borthakur (1987) Mercy and Zapata (1987) Cho and Zapata (1988) Kim and Raghavan (1988) Datta et al. (1990) Zhu and Pan (1990) Bajaj (1990) Guiderdoni et al. (1991) Loo and Xu (1991 Woo and Huang (1980)
***O. glaberrima* Steud**		

Table 3A continued

Oryza species or subspecies and their hybrids	Mode of development	References
O. nivara	C, E, P	D. T. Cai (1984)
(IRRI ACC 100195)		
O. perennis		C. T. Cai (1984)[a]
(IRRI ACC 103848)		
O. rufipogon		D. T. Cai (1984)[a]
(IRRI ACC 102158		
O. sativa* f. *spontanea		D.T. Cai (1984)[a], Wu et al. (1987)
O. sativa		Inst. of Genetics, Academia Sinica (1972); Inst. of Botany,
subsp. *Keng (japonica)*		Academia Sinica (1972)
O. sativa		Lab. of Genetics, Guangdong
subsp. *Hsien (indica)*		Inst. of Botany (1976)
Hsien x Keng hybrid		Woo and Tung (1972), T. H. Hsu (1978)
Three lines and their hybrid		
Male sterile line		Ling et al. (1978), Ge et al. (1985a, b)
Maintainer line		Wei et al. (1983), Ge et al. (1985a, b)
Restorer line		Wei et al. (1983), Ge et al. (1985a, b
Hybrid rice		Ling et al. (1978), Yang et al.(1978), Zhou and Cheng (1983)
Hubei photosensitive genic male-sterile rice (HPGMR)		D. T. Cai et al. (1988)
O. sativa* x *O. glaberrima		Woo and Huang (1980)
O. sativa* x *O. perennis		Woo et al. (1978)
O. sativa* x O. *spontanea		Woo and Huang (1980)

[a] Only albino microspore-derived plants were obtained.

[b] Adapted the table from Bajaj (1990) and Loo and Xu (1991).

Table 3B. Composition of some of the basal media commonly used for the culture of excised anthers

Constinuents (mg l^{-1})	Media				
	White (1943)	Murashige and Skoog (1962)	Gamborg (1968)	Nitsch and Nitsch (1969)	N6 Chu (1978)
Macronutrients					
$Ca(NO_3)_2.4H_2O$	288	-	-	-	-
KNO_3	80	1900	2500	950	2830
NH_4NO_3	-	1650	-	720	-
KCl	65	-	-	-	-
KH_2PO_4	-	170	-	68	400
$NaH_2PO_4.4H_2O$	19	-	150	-	-
$CaCl_2.2H_2O$	-	440	150	166	166
$MgSO_4.7H_2O$	737	370	250	185	185
Na_2SO_4	200	-	-	-	-
$(NH_4)_2SO_4$	-	-	134	-	463
Micronutrients					
$Fe_2(SO_4)_3$	2.5	-	-	-	-
$FeSO_4.7H_2O$	-	27.8	-	27.8	27.8
Na_2-EDTA	-	37.3	-	37.3	37.3
Na-Fe-EDTA	-	-	43.0	-	-
$MnSO_4.4H_2O$	6.7	22.3	-	25.0	4.4
$MnSO_4.H_2O$	-	-	10.0	-	-
H_3BO_3	1.5	6.2	3.0	10.0	1.6
$ZnSO_4.7H_2O$	2.2	8.6	2.0	10.0	1.5
KI	0.75	0.83	0.75	-	0.8
$Na_2MoO_4.2H_2O$	-	0.25	0.25	0.25	0.25
$CuSO_4.5H_2O$	-	0.025	0.025	0.025	0.025
$CoCl_2.6H_2O$	-	0.025	0.025	-	0.025
Organic nutrients					
Biotin	-	-	-	0.05	-
Glycine	3.0	2.0	-	2.0	2.0
Inositol	-	100.0	160.0	100.0	-
Amine acetic acid	-	-	-	-	2.0
Nicotinic acid	0.5	0.5	1.0	5.0	0.5
Thiamine-HCL	0.1	0.1	10.0	0.5	1.0
Folic acid	-	-	-	5.0	-
Kinetin	-	-	1.0	-	-
BAP	-	-	0.5	-	-
NAA	-	-	0.5-1.0	-	-
2, 4-D	-	-	1.0	-	-
Sucrose	20000	30000	30000	20000	50000
pH	5.8	5.8	5.6	5.8	5.8

3.2 Materials and Methods I

3.2.1 Source of working materials

Plant materials :

The *Japonica* rice cultivar Taipei 309 (Acc. no. 425761, supplied by Int. Rice Germplasm Center, IRRI, Philippines) was used for both anther float and microspore culture experiments. All seed-derived donor plants were grown in the glasshouse under a 16 h photoperiod (195 $\mu E\ m^{-2}\ s^{-1}$, daylight fluorescent tubes, Coolight, Thorn EMI Ltd, UK) at approximately 70-80% relative humidity during the 1989-90 summer seasons. Each year, donor plants were grown for six successive plantings at 3-week intervals to obtain the anther explants regularly in batches at their booting stage.

Panicle collection and sterilization of boots :

Young panicles were collected from the boots at the stage when the auricle distance of the flag leaf to that of the subtending (first) leaf was from 5 to 8 cm (Fig 3.2c). The samples were then cleaned with 70% ethanol and wrapped in polyethylene bags and subjected to cold pretreatment at 6-10°C for 8-13 days. Following the cold shock, panicles were excised after cleaning the boots with 70% ethanol and removing the enveloping leaf sheaths. A portion of each panicle was selected and cut out after determining the developmental stage of microspores, surface sterilized first with 70% ethanol for a few seconds, and then in 10% (v/v) 'Domestos' (Lever Bros. Ltd., Kingston-upon-Thames, UK) for 15-20 min, with frequent agitation. Finally, panicles were washed 5-6 times with sterile distilled water.

Microscopic staging of microspores from anther squash preparations :

Before culturing intact anthers or isolated microspores, the developmental stage of the microspores was determined using iron alum-haematoxylin staining of the microspores in anther squash preparations.

Preparation of iron alum-haematoxylin stain for staining microspores :

A stock solution of 4% (w/v) haematoxylin in 45% (v/v) acetic acid was prepared and 1 g of iron alum was added to this stock. The solution was allowed to stand for 24 h. To 10 ml of this stock solution, 4 g of chloral hydrate was added and after complete

dissolution it was used for staining microspores (Chang et al. 1978; Gupta and Borthakur 1987).

Preparation of microspores for determining the developmental stage :

Since spikelets in each individual panicle contain anthers bearing microspores at different stages of development, five spikelets were collected randomly from five different places starting from the top to the bottom of the panicle. Anthers were squashed for each spikelet in a drop of staining solution on a clean slide under a coverglass. After 2-3 min, the slide was observed using an inverted microscope (Nikon, model Diaphot TMD). The portion of the panicle bearing spikelets at the uni- to mid-uninucleate stage was extracted.

3.2.2 Preparation of selective liquid and semisolid media

Callus induction media for anther culture :

Both semisolid and liquid media were used for inducing callus from anthers. Three different media (E10, E24, and J19) (Zapata et al. 1982b, 1983; Mercy and Zapata 1987; Cho and Zapata 1988) were prepared using modified Gamborg's B5 medium (Gamborg et al. 1968), which contains 26.72 mM total nitrogen compared to 60 mM nitrogen in MS medium (Murashige and Skoog 1962) and 35 mM nitrogen in N6 (Chu et al. 1975). Each medium was freshly prepared in liquid and semisolid forms, both of which were supplemented with vitamins, hormones and cytokinins (see Appendices 1.6 &1.7). Semisolid media were gelled with 0.4% (w/v) agarose (Sigma Type I).

Callus induction media for isolated microspore culture :

Modified Gamborg's B5 liquid medium (E10) was used for anther preculture to isolate microspores mechanically (Mercy and Zapata 1987; Cho and Zapata 1988). After isolation of microspores, the media used to initiate callus were : E10 liquid medium, E10A (with 1 mM alanine), E10G (with 1 mM glutamine), and E10P (with 1 mM proline) (see Appendices A1.6 & 1.7).

Plant regeneration media for regenerating anther calli or embryoids :

Both liquid and semisolid media were also used for regenerating plants from anther calli or embryoids. Four different media (J19, $J19Y_1$, N19 and $N19Y_1$) were tried, liquid media for proliferating calli or embryoids and semisolid media for shoot-root

induction. J19 and $J19Y_1$ media were prepared using modified Gamborg's B5 medium, N19 and $N19Y_1$ media were prepared using MS basal medium. Both $J19Y_1$ and $N19Y_1$ media were further supplemented with yeast extract (10g l^{-1}) (Zapata et al. 1983) (see Appendix A1.7).

Media for plant regeneration from isolated microspore calli or embryoids :

For proliferation of vigorously growing calli or embryoids obtained from isolated microspore culture, modified MS liquid medium (MSA20) was used. For further growth and induction of shoots and roots, semisolid MS medium (MSYo) was used lacking growth regulators (see Appendices A1.7).

3.2.3 Preculture and culture of anthers at their correct developmental stage

Anther excision and culture :

At least 30 sterilized spikelets containing microspores, at the uni- to mid-uninucleate stage, were dissected out aseptically. About 100 anthers, in a group of 6 from each spikelet, were inoculated on the surface of 5 ml liquid media or 10 ml semisolid media in 5 cm disposable plastic Petri dishes. Replenishment of the liquid cultures was carried out with 5 ml of the same media, at 2 week intervals. Care was taken to position anthers on their edges, i.e. with one anther lobe in contact with the semisolid medium, rather than positioned flat (with both lobes touching the medium) (Mercy and Zapata 1987). Two to three weeks later the anthers were exposed to light and incubated for a further one or two week under continuous cool, white, fluorescent light (1000 lux) at 27°C ± 1°C. Few successive experiments were conducted and each experiment was replicated 4 times.

3.2.4 Isolation of microspores (mechanical) after preculture of anthers in selective medium

Microspore isolation (mechanical) and culture :

After 7 days of preculture of anthers in the dark at 27°C ± 1°C, the anthers were transferred from four Petri dishes to a 50 ml Erlenmeyer flask with a magnetic stirrer containing 15-20 ml E10 liquid medium. The liquid medium containing the anthers was stirred for 30-40 min to release the microspores. The extract was then filtered through a nylon sieve (100 µm pore size) and centrifuged at 100 x g for 5 min. The pellet

containing the microspores was resuspended in fresh E10 liquid medium and washed three times to remove the debris. Finally, the microspores were resuspended in a known volume of fresh E10 liquid medium and their final numbers and yield per ml were determined using a modified Fuch's Rosenthal double chamber haemocytometer of 0.2 mm depth (Weber Scientific Intern. Ltd., Teddington, UK). The microspores were then divided into four centrifuge tubes, pelleted by centrifugation and resuspended in the same volume of four different media (E10, E10A, E10G, E10P), at a density of 4.0 x 10^3. Each specific medium containing microspores was dispensed into disposable sterile 'Nunc' plastic Petri dishes (3.5 cm in diameter) as a shallow layer in each dish (1 ml per dish) and replicated three times. The dishes were sealed with Nescofilm and incubated in the dark at 27°C ± 1°C for callus initiation. Replenishment of the cultures was done with the same media (1 ml aliquot) by replacing an equal amount of media at 2 week intervals. The frequency of division was calculated as the percentage of dividing microspores after 0, 5, 10, 15, 20 and 25 days in culture. The plating efficiency was calculated as the percentage of plated microspores forming microscopic calli (approx. 1-2 mm in diameter) 30-35 days after inoculation of microspores.

3.2.5 Harvesting of anther-derived micro and macro calli for direct or indirect plant regeneration

About 2 weeks after transfer of the dishes to constant light conditions, both single (1-2 mm in diameter) and multiple calli (about 2-4 mm in diameter) were formed in a single dish for both liquid and semisolid media. Some of the vigorously growing, highly embryogenic calli produced multiple green shoots directly in the induction medium. They were then transferred to semisolid N19 regeneration medium for further development and root production. Other vigorously growing embryogenic single or multiple calli (about 1-3 mm in diameter) were randomly chosen every 3-4 days and cultured on M-shaped sterile filter paper bridges in test tubes. The bridges were immersed in four different media (modified Gamborg's B5 or modified MS liquid, with or without yeast extract). The test tubes were sealed and incubated for three to four weeks at 27°C ± 1°C under continuous cool, white, fluorescent light (1000 lux). The growing embryogenic calli with shoot primordia were then transferred to four different semisolid media (N19, $N19Y_1$, J19, $J19Y_1$) in 6 oz powder round glass jars and kept

under continuous light at the previous temperature. After further development and production of green or albino shoots, the plant regeneration frequency was calculated as the percentage of plated calli producing green or albino plantlets.

Harvesting of isolated microspore-derived calli or embryoids for plant regeneration :

After 4-5 weeks of transfer of the dishes to dark conditions, a few of the vigorously growing embryogenic calli or embryoids were harvested and placed on M-shaped sterile filter paper bridges in test tubes dipped in modified MS liquid medium (MSA20). The test tubes were sealed and incubated for four to five weeks at 27°C ± 1°C under continuous cool, white, fluorescent light (1000 lux). The proliferating calli or embryoids were then transferred to semisolid MS medium (MSY_o) and maintained under the same conditions in order to induce the production of green or albino shoots or plantlets. Regeneration frequency was calculated as the percentage of plated microspore calli or embryoids forming green or albino shoots or plantlets.

3.2.6 Maintenance and bulking up the amount of anther calli for further experimental use

Vigorously growing anther culture-derived embryogenic calli were selected up from the liquid medium and subcultured onto fresh semisolid LS medium (Linsmaier and Skoog 1965) supplemented with 2.5 mgl^{-1} 2,4-D and E24 media at 4-5 weeks intervals. Likewise, embryogenic calli were collected from six successive experiments and subcultured onto fresh media. Three cycles of subculture were necessary to make them friable and suitable for the initiation of cell suspension cultures. It was also necessary to increase the amount of initially growing compact embryogenic calli and to make them friable.

3.2.7 Micropropagation and maintenance of regenerated green plants

The multiple green shoot systems (5-7 cm tall) regenerated from anther calli were divided and micropropagated using two different semisolid media, MSB2 and N19 (see Appendix A 1.7). Four to five successive subcultures (one month interval) of these micropropagated plants were required to establish adequate, healthy shoots and vigorous, white, functional root growth. Later, these plants were used for

determination of ploidy levels by flow cytometry and chromosome counts of root-tip squash preparations.

3.3 Results and Discussion I

3.3.1 Callus production: comparison of isolated microspores and intact anthers in terms of plating efficiency

Microspore isolation, culture and division frequency :

Isolation of microspores was done successfully in several successive experiments after preculturing cold-shocked anthers at their proper developmental stages at 8-10°C for 7-10 days. Two types of microspores were obtained after isolation. One type consisted of large microspores with thin, light pink-coloured cell walls; the other type consisted of microspores of smaller size with thick cell walls. The majority of the population consisted mostly of the smaller microspores but only the large microspores divided. (after 10-15 days in culture, Fig 3.1b). Within 20-25 days of culture, the cell division rate increased and some multicellular microspores were formed in each plate containing E10P liquid medium (Figs 3.1b, c). Some of the embryogenic microspores showed a certain degree of plasmolysis at the beginning, but later they divided normally like the ones which did not plasmolyse. Eventually, ruptures appeared in the exines of the microspores and unorganized micro-calli were formed (Figs 3.1d, e).

The effect of four different induction media, one without amino acid and the other three containing amino acids (alanine, glutamine and proline respectively; 1mM each), on the division frequency of microspores was observed after 5, 10, 15, 20, and 25 days in culture (Table 3G).After 10-15 days in culture, the growth of the microspores was stimulated and the division frequency was higher when proline or glutamine were added to the medium. The highest division frequency at 10-15 days was observed with the microspores cultured in E10P liquid medium. After 10 days of culture, the microspore division frequency remained unchanged at the same level in E10 and E10A media, but the microspores cultured in E10G and E10P media were still dividing. However, they were dividing after 10 days, but usually a very slow rate in division frequency was noticed after 15-20 days, even in the media supplemented with glutamine or proline. The highest microspore division frequency was obtained in E10P medium compared to E10G medium (Table 3G). There was no significant difference in

Fig 3.1 Isolation of microspores (pollen grains) from pre-cultured anthers and microcolony formation of *O. sativa* (cv. Taipei 309) in liquid medium

a) Freshly isolated microspores, following mechanical isolation procedure, from pre-cultured anthers after 7-10 days of culture in liquid medium (x 74)

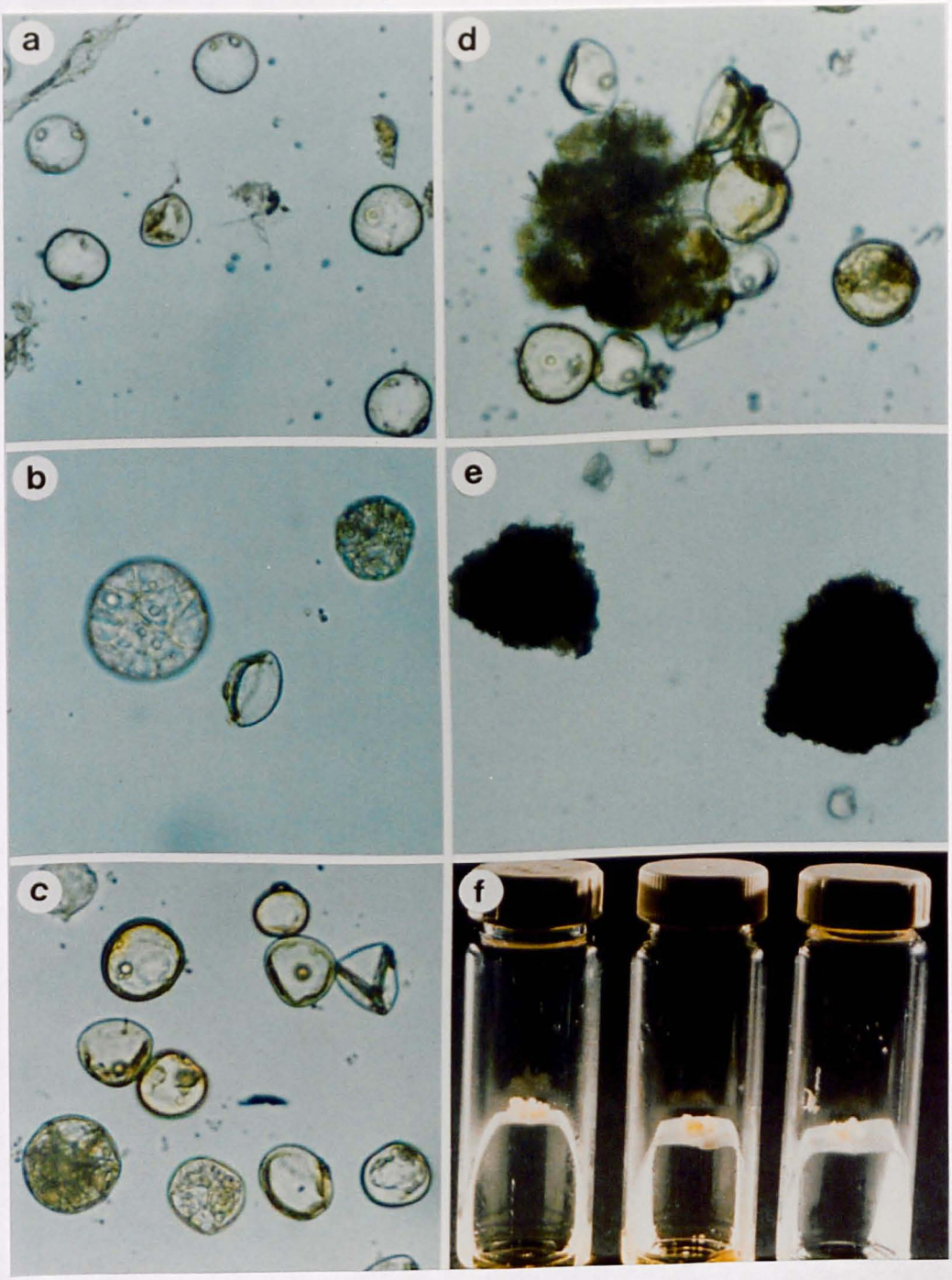
b) Division of microspores after 5 weeks of culture in shallow layer of liquid medium containing proline (E10P) and formation of multicellular microspores (x 160)

c) A mixed population of unicellular and multicellular microspores after 5-6 weeks of culture in shallow layer of liquid medium (x 152).

d) The release of microspore-derived micro-callus into the liquid medium after rupturing the exine wall of the microspore and some non-dividing microspores (x 140).

e) Two compact, densely cytoplasmic microspore-derived micro-calli floating on the surface of the liquid medium (x 144).

f) Growth of micro-calli derived from isolated microspores on top of the filter paper-bridge soaked in liquid regeneration medium (N19L), after 4 weeks of transfer under cool, white, fluorescent light (1000 lux) at 27°C ± 1°C (x 3.5).


a
d
b
e
c
f

division frequency or percentage of microspore division between E10P and E10G and between E10 and E10A.

Anther float culture, callus formation and plating efficiency :

After exposing the anthers for 15-20 days to light followed by 15-20 days dark incubation in either semisolid or liquid medium, the anther sacs split longitudinally and single or mutiple calli or proembryo like structures were visible (Fig 3.2f). For floated anther cultures, microspores were shed into the liquid medium within 7-10 days. After 2-3 weeks of incubation in the light followed by 2 week darkness, microspore derived calli or proembryos floated freely on the surface of the culture media (Figs 3.3a, b). Sometimes, differentiation of some calli or proembryos was observed directly in the liquid E10 and E24 induction media, 15-20 days after transfer of cultured anthers from the dark to continuous light (Fig 3.3d, e, f). Striking differences were observed in the response of floated anthers cultured in liquid media, compared to those cultured in semisolid media (Table 3C). It was possible to recover more than 500 micro-calli from 112 plated anthers in E10 liquid medium and the frequency of callus production was 97.3% compared to 37.4%, when the same medium was solidified with agarose (Sigma Type I). Although the frequency of callus production in E24 liquid medium was not significantly higher than that in E24 semisolid, nearly 3000 micro-calli or proembryos were recovered from 631 anthers plated. The average number of micro-calli produced per callusing anther was two fold higher and the callus yield was more than five fold higher in liquid E24 compared to semisolid E24 (Table 3D).

It was noticed that addition of 2,4-D in combination with NAA to the basal medium (E24) resulted in significant increases in callus formation. In medium E10 supplemented with BAP, IAA and 2,4-D, without NAA or kinetin, the lowest number of embryogenic micro-calli or proembryos was recovered in both liquid or semisolid media, although the frequency of anthers which produced callus was the highest in liquid medium (97.3%). Therefore, it is clear that both synthetic auxins (2,4-D and NAA) are equally effective in inducing embryogenic callus formation and supporting callus growth. Most of the time, direct differentiation was observed in E10 medium, because of the presence of native auxin IAA which stimulated embryogenesis.

Fig 3.2 Anther float culture: collection of boots (tillers), selection of spikelets, culture of excised anthers and formation of embryogenic micro-and macro-calli using cultivar Taipei 309

a) A healthy rice plant at booting stage, ready for the collection of boots (tillers) to do anther float culture or preculture of anthers for the isolation of microspores (x 4.0).

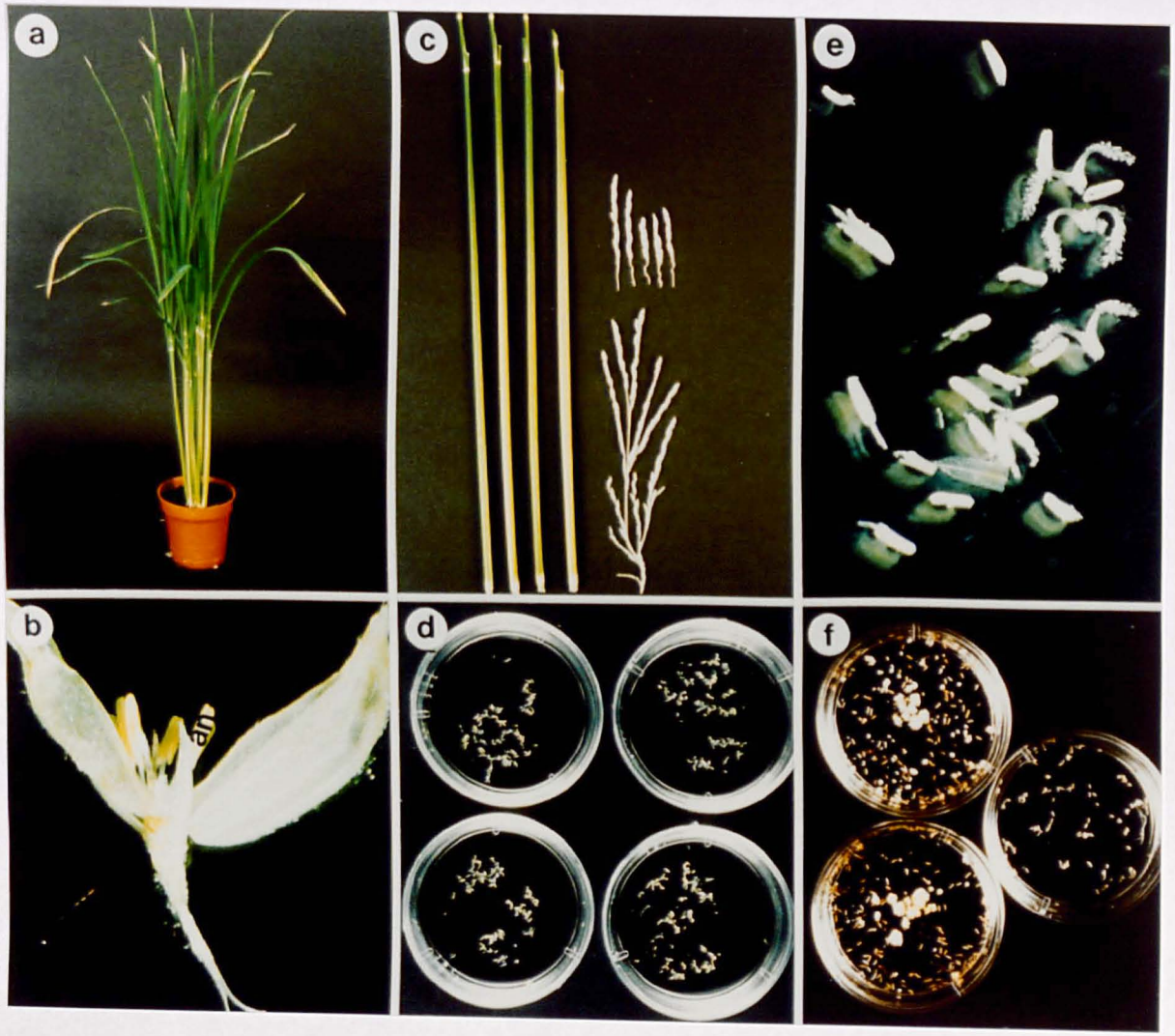
b) An immature open rice flower with palea on the left, lemma on the right and six immature anthers in the middle (x 3.3).

c) Young boots (tillers) enclosing immature panicles, collected when the auricle distance of the flag leaf to that of the subtending (first) leaf was from 5 to 8 cm, ready for cold pretreatment (left). An immature panicle dissected out from the boot and some selected immature spikelets for dissection and collection of anthers (right) (x 3.6).

d) Culture of sterilised, excised anthers (dissected out from the spikelets) on the surface of liquid induction media for inducing micro-callus or for the isolation of microspores for 10-14 days under dark condition at 27°C ± 1°C (x 3.8).

e) The floating anthers (with higher magnification) on the surface of the liquid induction medium (E 24) after one week of culture under dark condition at 27°C ± 1°C (x 3.4).

f) Formation of both micro- and macro-calli (in the middle) from cultured anthers on the surface of the liquid medium after 3 weeks of transfer under cool, white, fluorescent lights (1000 lux) at 27°C ± 1°C (x 3.8).


a
c
e
b
d
f

Finally, intact anther culture (anther float culture) is more efficient in terms of division frequency, embryogenic callus production and green plant regeneration than culture of mechanically isolated microspores for haploid/diploid rice production.

3.3.2 Direct and indirect plant regeneration from anther and microspore-derived and macro calli or embryoids

Plant regeneration efficiency from isolated microspore culture

Micro-calli formation from isolated microspores in four different media is shown in Table 3H. In E10 and E10A media, the plating efficiency was significantly lower than in the other two media E10G and E10P at 35 days after culture. The highest plating efficiency was obtained from microspores cultured in the medium containing proline, but there was no significant difference between proline and glutamine. Although micro-calli were obtained from the four different media tested, the size and the number of micro-calli were unexpectedly small when transferred to liquid regeneration medium. Those micro-calli <1mm diameter, after transferred to MSA20 liquid regeneration medium for proliferation, became brown, necrotic and eventually died within a few days. Only those micro-calli >1mm proliferated further upon transfer to MSA20 medium (Fig 3.1f); when transferred onto semisolid MSYo regeneration medium, rhizogenesis occurred from some of these micro-calli. There was no green or albino shoot production, even from the proliferated micro-calli obtained from E10G and E10P. One of the reasons could be that long term culture of the isolated microspores in the liquid induction media or mechanical isolation of the microspores affected regeneration capability.

Plant regeneration efficiency from intact immature anther cultures

Four different regeneration media, liquid and semisolid forms were used for regeneration of the embryogenic micro-and macro-calli (obtained from anther cultures in either liquid or semisolid E24, E10 and J19 callus induction media, Table 3E). Some of the single or multiple calli in E24 and most of the calli in E10 liquid media developed directly into proembryos with shoot and root primordia (Figs 3.3d, e, f). Upon transfer to semisolid N19 medium, they produced numerous multiple shoots ranging in number from 22-70, with profuse development of roots (Fig 3.3g). Other micro-calli, after proliferation on the top of a filter paper bridge wetted with liquid

Fig 3.3 Formation of embryogenic micro- and macro-calli, direct and indirect embryoid formation and regeneration of plants from anther float culture of *O. sativa*

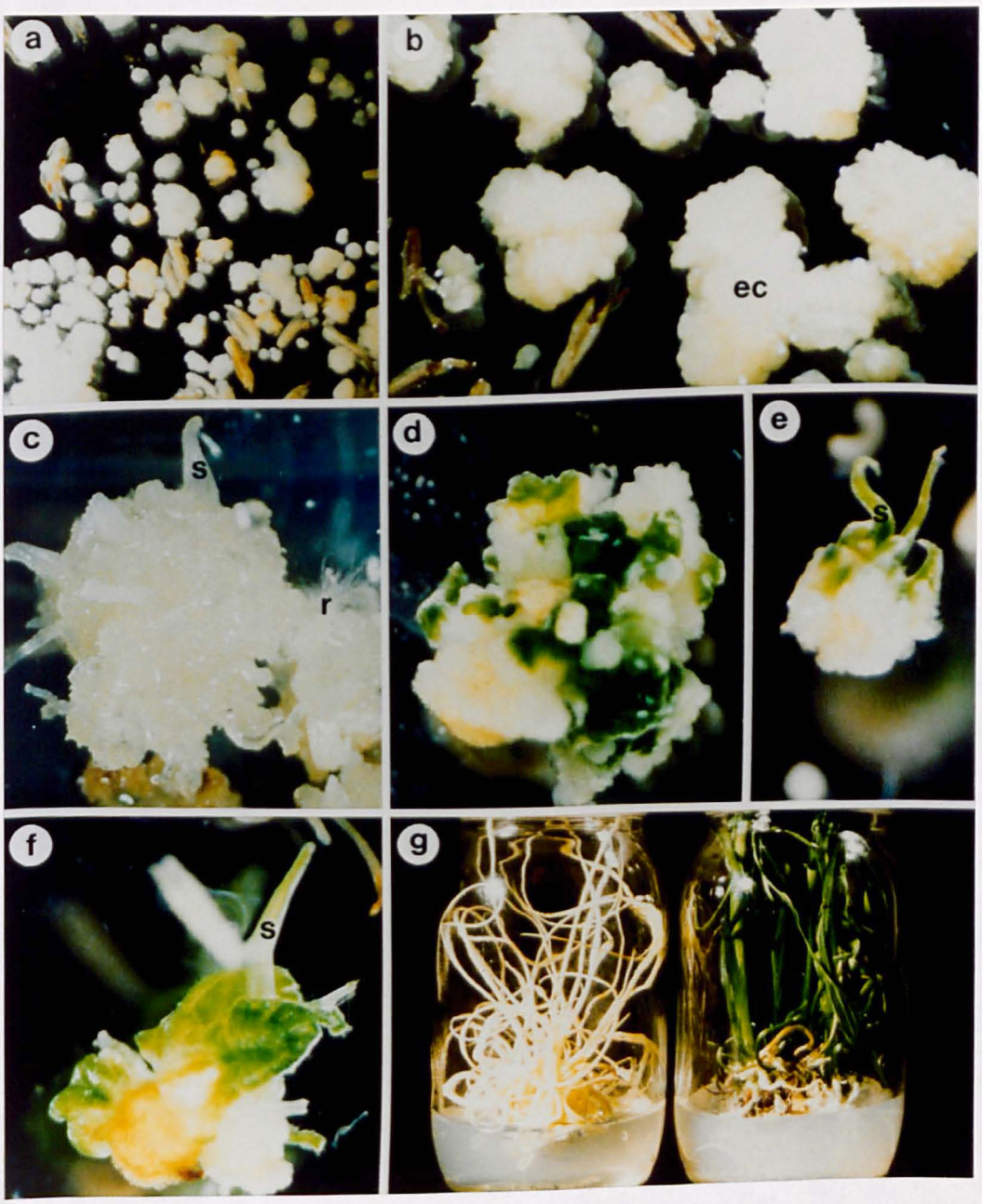
a) Numerous micro- and macro-calli formation from cultured rice anthers, floating on the surface of the liquid induction medium (E24), after 2 weeks of culture in the dark and a further 3 weeks of culture under white, fluorescent light (1000 lux) at 27°C ± 1°C (x 2.0).

b) Some embryogenic macro-calli with the initiation of embryoids in the liquid induction medium (E10) after 4-5 weeks of culture under the culture conditions as in (a) (x 3.8)

c) Differentiation of embryoids, obtained from anther-derived microspores, with the production of shoots (s) and roots (r) on the surface after 4-5 weeks of culture on "semisolid regeneration medium" (N19S) under cool, white, fluorescent light (1000 lux) at 27°C ± 1°C (x 4.0).

d-f) Differentiation of an embryoid, obtained from microspore (anther float culture), with the 'direct' formation of green shoots (s) on the surface after 4(d)/5(e)/6(f) weeks of culture in 'liquid induction medium (E10)' under the culture conditions as in (c) (x 3.7; x 3.6; x 3.8).

g) Young green and albino plants, obtained from anther-derived embryoids, growing on semisolid (N19S) regeneration medium for 4-5 weeks under the culture conditions as in (c) (x 3.7).


a
b
ec
c
s
r
d
e
s
f
s
g

regeneration media (N19L or J19L), produced only green clones (9.2% to 30.2%) with numerous multiple shoots (Table 3E). The calli obtained from three induction media, when transferred to ($N19Y_1S$) semisolid medium after proliferation in liquid ($N19Y_1L$) medium, did not produce any shoots or roots, and became necrotic and died. About ten per cent plant regeneration was obtained in $J19Y_1S$ medium, only when they were induced in E24 and proliferated in $J19Y_1L$ media. A total of 306 green and albino plants were regenerated from at least 10 separate experiments conducted for each individual callus induction medium. Among 306 plants, only 29 plants were albino (9.5%) and 277 plants were green (90.5%). Of the 277 green plants, 225 were haploids (81.2%) and 52 were spontaneous diploids (18.8%) (Table 3F). It was observed that micro-calli which formed in the presence of NAA were more capable of plant regeneration than those formed in its absence. In the present study, the highest number of plants (236) were regenerated from those micro calli derived from E24 medium supplemented with both NAA and 2,4-D (Table 3E). Although the maximum number of regenerating calli per 1000 anthers plated (286) and 82.1% plant regeneration were observed from calli induced in E10 medium, the number of plants regenerated was remarkably reduced than those induced in E24 medium (Table 3F).

3.3.3 Bulking up of embryogenic calli and successful micropropagation of regenerated green plants (haploids and diploids) for ploidy determination

Bulking up the embryogenic calli derived from anther float culture

For bulking up the amount of embryogenic calli obtained from anther float culture in liquid E24 medium, two different semisolid callus induction media E24 and LS2.5 (Linsmaier and Skoog 1965) were used. E24 (modified Gamborg's B5) medium is generally used for inducing callus from immature rice anthers whereas LS2.5 medium is generally used for inducing and bulking up the amount of embryogenic calli. It was observed from at least 5 experiments that on LS2.5 medium, the amount of embryogenic calli was increased upto 3-4 fold, each time compared to the initial amount plated, producing friable, smooth-textured calli suitable for initiation and establishment of embryogenic cell suspension cultures. However, on E24 medium, the amount of calli was not increased as expected. Rather, from the initially plated calli,

non-embryogenic and compact masses of calli were produced, which were not suitable for cell suspension initiation.

Micropropagation and maintenance of anther-derived haploid and diploid green rice plants for ploidy determination

The multiple green shoots (clones) derived from anther float cultures were separated into individual plantlets, micropropagated and maintained in three different shoot and root producing semisolid media, MSBP2, MSN1.5 and N19 for healthy growth. It was possible to micropropagate and to maintain them easily with 100% recovery first in N19 and then in MSN1.5 semisolid media, however, it was not possible to maintain them in MSBP2 semisolid medium, which is commonly used for micropropagation and maintenance of wild rice species. The development of new adventitious shoots and roots was also noticed from each individual green plantlet or clone. The percentage of mortality was very low. These green plants or clones were established after sometimes, producing healthy shoots and white, functional roots. It was interesting to note that some of the individual plantlets, when transferred to MSBP2 semisolid medium, 3-4 weeks after regeneration, degenerated and became necrotic, but immediately after transfer to N19 semi-solid medium they recovered rapidly producing adequate healthy shoots and roots.

Table 3C. *In vitro* responses of the excised anthers of *Oryza sativa* (cultivar Taipei 309) cultured on semisolid and liquid media

Callus induction medium	Anther responses[c]						
	Cultured anthers No.	Callus or proembryo formation					
		Single		Multiple		Total	
		No.	(%)	No.	(%)	No.	(%)
E 24 L	631	124	19.7	308	48.8	432	68.5[ab]
E 24 S	153	23	9.1	41	16.2	64	25.3[b]
E 10 L	112	55	49.1	225	200.9	280	250.0[a]
E 10 S	150	19	12.7	37	24.7	56	37.4[b]
J 19 L	752	0	0.0	240	37.1	240	37.1[b]
J 19 S	710	23	3.2	134	18.9	157	22.1[b]

[c] Based on average results of at least 10 experiments under optimal conditions, each one replicated 4 times.

[d] Treatment means followed with the same letter within the column are not significantly different at the 1% level by 'Duncan's Multiple Range Test (DMRT)'.

Table 3D. *In vitro* responses of excised anthers of the *Japonica* rice cultivar (T309) in terms of frequency of callusing and callus yield, cultured on liquid and semi-solid media[a]

Callus induction medium	Anthers plated (A)	Callusing anthers (B)	Frequency of callusing anthers (B/A x 100)	Subculturd micro-calli or proem-bryos (C)	Average no. of calli pro-duced per callusing anthers (C/B)	Callus yield (C/A x 100)
E 24 L	631	432	68.5	2981	6.9	472
E 24 S	253	64	25.3	207	3.2	82
E 10 L	112	109	97.3	517	4.7	462
E 10 S	150	56	37.4	153	2.7	102
J 19 L	752	240	37.1	727	3.0	97
J 19 S	710	157	22.1	269	1.7	38

[a] Based on average results of at least 10 individual experiments under optimal conditions, each one replicated 4 times.

Table 3E. Plant regeneration from anther calli or proembryos onto different semi-solid or via liquid to semi-solid regeneration medium (T309)[a]

Callus induction medium	Regeneration medium	Anthers plated (A)	Subcultured micro-calli or proem-bryos (C)	Regenera-ting calli or proembryos (D)	No. of regenerating calli per 1000 anthers plated (D/A x 100)	Callus regeneration frequency (D/C x 100)
	N 19S		156	52	82	33.3
E 24 L	**N 19L-N 19S**	631	431	130	206	30.2
E 24 S	**J 19L-J 19S**	253	129	39	154	30.2
	J19Y$_1$L-J19Y$_1$S		148	15	59	10.1
E 10 L	**N 19S**	112	39	32	286	82.1
E 10 S	**J 19L-J 19S**	150	47	11	73	23.4
J 19 L	**N 19L- N 19S**	752	56	13	17	23.2
J 19 S	**J 19L-J 19S**	710	76	7	10	9.2

[a]Based on the average results of at least 10 experiments under optimal conditions. Each one replicated 4 times.

Table 3F. Anther calli or proembryo-derived plant regeneration after direct transfer of calli or proembryos onto semi-solid or via liquid to semi-solid regeneration medium (T309)

Callus induction medium	Regenerated medium	Plated calli or proembryos	Plantlets regenerated			
			Green		Albino	
			No.	%	No.	%
E 24 L E 24 S	N 19S	156	52 (2n)	33.3	-	-
	N 19L-N 19S	431	108 (n)	25.0	22	20.0
	N $19Y_1$L-N $19Y_1$S	152	-	-	-	-
	J 19L-J 19S	129	39 (n)	30.2	-	-
	J $19Y_1$L-J $19Y_1$S	148	15 (n)	10.1	-	-
E 10 L E 10 S	N 19S	39	32 (n)	82.1	7	17.9
	N 19L-N 19S	78	-	-	-	-
	N $19Y_1$L-N $19Y_1$S	63	-	-	-	-
	J 19L-J 19S	47	11 (n)	23.4	-	-
	J $19Y_1$L-J $19Y_1$S	61	-	-	-	-
J 19 L J 19 S	N 19S	51	-	-	-	-
	N 19L-N 19S	56	13 (n)	23.2	-	-
	N $19Y_1$L-N $19Y_1$S	43	-	-	-	-
	J 19L-J 19S	76	7 (n)	9.2	-	-
	J $19Y_1$L-J $19Y_1$S	62	-	-	-	-

(n) = Haploids

(2n) = Spontaneous diploids

Table 3G. The effect of induction media containing various amino acids on the division frequency of isolated microspores at different durations in culture (T309)[a]

Callus induction medium	Isolated microspore division frequency (%)									
	Day 5		Day 10		Day 15		Day 20		Day 25	
	Df %	SE ±	Df %	SE ±	Df %	SE ±	Df %	SE ±	Df %	SE ±
E10	0.08	0.02	0.09	0.03	0.10	0.03	0.12	0.02	0.14	0.02
E10+1mMA	0.08	0.01	0.07	0.02	0.16	0.02	0.19	0.04	0.23	0.05
E10+1mMG	1.16	0.08	1.23	0.04	1.44	0.08	1.77	0.11	2.73	0.17
E10+1mMP	2.13	0.13	2.36	0.05	2.68	0.10	2.85	0.12	3.15	0.13

1mMA - 1mM alanine (E10A), 1mMMG - 1mM glutamine (E10G), 1mMP - 1mM proline (E10P)

[a] Based on average results of at least 10 individual experiments. Each one replicated 3 times.

Table 3H. Plating efficiency and plant regeneration in isolated microspore cultures from precultured anthers of Taipei 309

Callus induction medium	Plating efficiency (%), 35d after culturing[c]	Micro-calli plated on regeneration medium (No.)	Plant regeneration efficiency (%)	
			Green	Albino
E10	0.09 a	13	-	-
E10 + 1mM alanine	0.13 a	5	-	-
E10 + 1mM glutamine	2.79 b	17	-	-
E10 + 1mM proline	3.15 b	45	-	-

[c]Treatment means followed by a common small letter within the column are not significantly different at the 5% level by 'Duncan's Multiple Range Test (DMRT)'.

3.4 Summary and conclusions I

The basic aim of this part of the work discussed in this Chapter was to establish an efficient, embryogenic callus culture and plant regeneration system from excised anthers and mechanically isolated microspores of precultured anthers, using both liquid and semisolid media. The *Japonica* rice cultivar Taipei 309 was used for this particular study, because it was easy to grow healthy, anther-donor plants in the glasshouse, which is the most important factor for successful culture establishment and plant regeneration. This investigation was also a pre-requisite for the study of green plant regeneration from cell suspension-derived protoplast cultures using anther calli as a source in Part II of this Chapter. The variation in callus or proembryo formation, frequency of callusing anthers, division frequency, induction or plating efficiency and plant regeneration capability were found to be highly significant from embryogenic callus or proembryo cultures of two different explant origins. Among the various experiments conducted at definite intervals, it was also observed that anthers collected and cultured at the beginning of the flowering period were more productive than those harvested at the end of this period. As with previous results (Chen and Tsay 1984), the frequency of callus formation was significantly lower in anthers collected from the tertiary tillers than in those from the main culms and primary and secondary tillers. In the present study, anthers collected from the plants supplied with proper nutrients and maintained in a vigorous and healthy state under optimum conditions, were very responsive for embryogenic callus formation and green plant regeneration.

Remarkable variation was noticed in the frequency of callusing anthers, the average number of micro-calli produced per callusing anther and callus yield, when anthers were cultured in two different states (liquid and semisolid) of the same callus induction medium (Table D). The variation in frequency of callusing anthers was insignificant between J19 liquid and semisolid media. This might be because of the absence of the synthetic auxin 2,4-D. A 50% reduction in average number of calli produced per callusing anther and callus yield was obtained. Among the three callus induction media tested in this study, the highest percentage of haploid green plant regeneration was observed from direct proembryos which originated in liquid E10 medium, followed by spontaneous diploid green plant regeneration also from direct proembryos which

originated in E24 liquid medium. In total, the maximum number of green plants (214) was produced from embryogenic calli and proembryos which originated in liquid and semisolid E24 medium, because of the presence of both synthetic auxins, NAA, 2,4-D and the cytokinin BAP. According to the previous workers' report (Huang et al. 1986), the two synthetic auxins are about equally effective in inducing callus formation and in supporting callus growth, but callus formed in the presence of only 2,4-D is less capable of plant regeneration than that formed on medium with NAA. Observations in the present study also confirm the previous workers' report. Still, there are some controversial points, arising about the use of either liquid or semisolid callus induction medium for excised anthers, which need to be confirmed. As with previous results reported earlier (Zapata et al. 1983; Guiderdoni et al. 1990, 1991), we have confirmed that compared to semisolid medium, the possibility of contamination in the liquid medium was higher. It allows only one transfer of calli or proembryos of different sizes to the regeneration medium. It did not allow determination of the rate of callogenetic anther and also individual monitoring of the anthers when the liquid medium was used.

It was found that for the culture of excised anthers, the nutritional requirements are much simpler than those of isolated microspores. Obviously, for the culture of mechanically isolated microspores, there are certain factors responsible for the induction of androgenesis. These factors, which might have been provided by the somatic tissues of the anther wall, are missing and need to be provided through the specific medium. A few years ago, Cho and Zapata (1988) reported that the culture of isolated rice microspores requires higher amounts of nitrogen in the form of amino acids like proline and glutamine. This was certainly the case in tobacco (Nitsch 1974; Reinert et al. 1975) and the stimulatory effect of these two amino acids in the development of isolated rice microspores was also evident in the previous study. It was observed that the addition of proline and glutamine was required in the basal medium, suggesting that these are utilized in the development of isolated rice microspores. Similar results were obtained in the present study (Chapter 3, Part I). It has been indicated by several researchers that different species or materials, different genotypes within the same species and different explants from the same genotype vary in their requirements for nitrogen sources in the medium. Emphasis has also been given to several important conditions / factors involved for the successful embryogenesis and

regeneration of fertile rice plants from isolated or shed microspores (Datta et al. 1990a; Zapata et al. 1991).

Mechanical isolation of cereal or rice microspores has not yet proved to be as efficient as shed microspore culture (Datta et al.1990a), which was also true in the present study. Although it is difficult to monitor the anthers individually in the liquid medium, microspores from anther shed cultures offer a more efficient system to regenerate a random sample from the microspore populations than does anther culture onto semisolid medium. In the early 1980s, it was demonstrated from a systematic study, that the shed microspores of rice produced much higher-yielding microspore cultures as compared to mechanically isolated microspores (Y. Chen et al. 1980, 1981). The fact appears to be positively related to the effect of the anther wall, i.e., anther factor(s) or conditioning factor(s) released from the somatic tissues of anthers in the liquid culture (Sunderland 1983; Xu and Huang 1984). The development of green plants through shed microspore embryogenesis is most advantageous and desirable, because early selection of microspore populations is possible when the procedure starts from single microspores. This procedure also helps to establish embryogenic cell lines from the poorly responding *Japonica* and *Indica* rice cultivars to make them amenable to protoplast culture and further genetic manipulation. With this callus induction and subsequent regeneration procedures, uncontrolled callus formation can be avoided and it might be possible to reduce the frequency of unwanted somaclonal variants (Datta et al. 1990a). It might ~~probably~~ open the pathway to regenerate a high frequency of green plants with desirable crop quality.

Although much success has been achieved leading to the release of commercial rice and wheat varieties in China, through the culture of isolated or shed microspores, there are still some problems to be solved. For example, the frequency of plant regeneration and also green plant regeneration is much lower, and albino plant regeneration frequency is higher than from excised anther culture. However, isolated or shed microspore culture, at least, provides a useful experimental system to study various problems involving the effect of the anther wall, and the nutritional requirement of cultured microspores. It is therefore obvious that the difference in anther or microspore culture ability and plant regeneration is under genetic control (Loo and Xu 1991; Wang and Iwata 1991).

At the time of anther excision, microspore isolation and culture, it was found that the developmental stage of microspores is important and was a critical factor affecting androgenesis. In addition, the ability of calli or embryos to regenerate plants and the ratio of green to albino plants regenerated were also related to the stage of microspore development. Generally, in rice, the maximum response was found to shift from the mid-uninucleate to an early-uninucleate stage when sucrose concentration of the culture medium was increased. Cytological examination of the microspores was the most reliable method for the identification of the anther stage, but this procedure was tedious and time-consuming. Because of that, other criteria were also used for identification. These were, the distance between the ligules of the flag leaf and the next lower leaf (Chaleff and Stolarz 1981), the colour and size of the spikelets and anthers (C.C. Chen and C.M. Chen 1979) and the texture of the spikelets.

As indicated by J.J. Chen et al. (1986), it was possible to confirm that rice calli induced at an early stage, between 30-50 days after anther inoculation, had a high level of differentiation of green plants. Microspore callus of rice loses its morphogenetic potential very rapidly during culture. In this respect frequent transfer to fresh medium is essential. Another advantage of frequent transfer was, during callus proliferation stage the chances for change in ploidy level could be minimized so that more haploid and few diploid, polyploid and aneuploid plants can be regenerated (J.J. Chen et al. 1984).

Among many other research workers throughout the world (Chung 1987; Guiderdoni et al. 1991), it is an agreed and established fact that anther culture or isolated microspore culture can be used as a powerful tool for the large-scale production of true breeding materials. These can compete with elite lines after selection and eventually be released as varieties, like IRAT156 and Zhonghua Nos. 8, 9, 10 and 11 (Li MF et al. 1988). It has also been confirmed that anther and isolated microspore cultures are interesting tools for more basic genetic studies.

Part II: Green plant regeneration from cell suspension-derived protoplast cultures using anther calli as a source

3.5 Introduction II

Karyotype instability is an inherent problem of *in vitro* cultures in general, because most of the cultures consist of a heterogeneous mass of cells in different states of differentiation and dedifferentiation. Haploid cell cultures exhibit similar types of instability, even after possible initiation from a uniform population of uninucleate haploid microspores or haploid protoplasts. They can provide a unique opportunity to study the factors leading to variability, because of their origin from single haploid cells. There is also a great importance of haploid cell cultures for advances in somatic fusion and gene transfer into defined plant cells, since *in vitro* cultures and subsequent plant regeneration are usually necessary (Ziauddin and Kasha 1990). However, induced variability might interfere with controlled genetic manipulation (Lörz and Brown 1986).

The development of haploid cell or haploid protoplast cultures is also of particular interest for mutation and selection and in studies of the events occurring in cell cultures. As most of the mutations are recessive, the haploid system with one set of chromosomes, makes their detection easier and direct since they are not concealed by another gene copy. However, this advantage is lost when additional chromosomes are generated or when rearrangement causes duplications. It is also a well established fact that haploid cell suspension lines have a greater tendency to increase in ploidy level to diploid than diploid cell lines to tetraploid (Sacristan 1971; Novak and Vsykot 1975). This tendency for cells in culture to undergo natural duplication is a major obstacle to the maintenance of haploid cells in culture. The haploid cell cultures may differ little from diploid cell cultures with respect to the types and mechanisms of genetic variation. However, the variability may be expressed more directly in haploid cell cultures, which makes it easier to find out possible causes and mechanisms (Ziauddin and Kasha 1990).

Haploids are of great importance in plant breeding, and they can be produced directly from cultured anthers, microspores, megaspores, and haploid cells (Bajaj 1983 b), or immature embryos from certain wide crosses undergoing chromosome elimination

(Kasha 1974). They may also be induced from explants of tissues from haploid plants. The culture and potentials of haploid protoplasts have been well emphasized in the early 1980s (Bajaj 1983 b). The other advantage of haploid cells is that the chromosomes can be easily doubled to obtain homozygous diploids.

By using agar-plating techniques and suspension cultures, isolated haploid protoplasts, microspores, megaspores and cells can be handled like microbes, and can be treated with various mutagenic chemicals and irradiations to produce mutants. The protoplast culture system has various advantages and similarities with the micro-organisms. Protoplasts can be plated and exposed to various mutagens in large numbers (10^6/ml), and each protoplast is potentially capable of regenerating to a plant (Bajaj 1990). In earlier experiments, haploid callus cultures were used for the induction of mutations (Binding et al. 1970 and Carlson 1970). The mutants obtained in this way were leaky auxotrophs and perhaps this was due to the amphidiploid nature of *N. tabacum*. Later, realising the limitations of the use of callus cells, haploid protoplasts were used to obtain methionine and sulfoximine-resistant calli, which regenerated into plants after diploidization (Carlson 1973a, b).

In 1980s, a number of reports were published on the use of haploid protoplasts for the induction of mutations of various types from different plant species (Marton et al. 1982; Shimamoto and King 1983; Steffen and Schieder 1984; Negrutiu et al. 1985; King 1986). Haploid and diploid plant regeneration was achieved from protoplasts isolated from anther calli-derived cell suspensions and a viable system for the culture of totipotent haploid protoplasts in rice was reported (Toriyama et al. 1986). Transgenic rice plants were regenerated after direct gene transfer into protoplasts isolated from anther-derived cell suspensions of *Japonica* rice cultivar 'Yamahoushi' (Toriyama et al. 1988a). In the same year, fusion between protoplasts from haploid cytoplasmic atrazine resistant (CATR) and haploid cytoplasmic male sterile (CMS) *Brassica napus* plants was employed to produce a diploid (CMS/CATR) cybrid (Chuong et al. 1988)

In 1990s, from microspore-derived calli and proembroyos, a long term embryogenic suspension cultures were established and maintained for more than a year. Later, these cultures were used for protoplast isolation and plant regeneration for both *Indica* and

Japonica varieties. This year, fertile *Indica* rice plants were regenerated from protoplasts isolated from microspore-derived cell suspensions and genetically engineered fertile *Indica* rice plants were also recovered from haploid protoplasts using the same variety (Datta et al. 1990a, b, c). In the same and the following year at IRRI, plant regeneration were obtained from young inflorescence-derived suspension cultures of a *Japonica* rice cultivar (Taipei 177) as well as plants were recovered through morphogenesis in anther-derived cell suspension protoplasts of *Indica* rices (Tetep) (Ghosh Biswas and Zapata 1991a, b).

In 1992, it was possible to regenerate haploid to pentaploid plants from haploid cell suspension-derived protoplast cultures of the Mediterranean high-yielding *Japonica* rice cultiver Miara (Guiderdoni and Chaïr 1992). A detailed summary of the work done on the isolation, fusion and culture of haploid protoplasts is shown in Table 3I.

Table 3I. Summary of the work done on the isolation, fusion and culture of haploid protoplasts (adapted from Bajaj 1991)

Plant species	Observations	References
Mesophyll protoplasts		
Atropa belladonna	Regeneration of haploid, diploid, polyploid and aneuploid plants.	Bajaj et al. 1978
Brassica napus	Haploid plants from mesophyll-derived callus, retain haploidy.	Thomas et al. 1976
Brassica napus	Somatic transfer of cytoplasmic traits by haploid protoplast fusion.	Chuong et al. 1988
Datura innoxia	Only 7.7% regenerants were haploid.	Schieder 1975, 1976
	The X-irradiated (1 krad) protoplasts yielded 10 strains with altered pigments.	
Datura innoxia	Protoplasts treated with NG (10 µg /ml) showed 50% survival, pigments were altered, mutation frequency 2 x 10^{-4}.	Krumbiegel 1979
Hyoscyamus muticus	Autotrophic and temperature-sensitive clones from protoplasts subjected to N-methyl-N-nitro-N-nitrosoguanidine.	Gebhard et al. 1981
Hyoscyamus muticus	Chlorate-resistant clones.	Straus et al. 1981
Petunia hybrida	Regeneration of haploid and diploid plants.	Binding 1974a, b
Nicotiana alata	Regenerated complete plants.	Bourgin and Missonier 1978
N. plumbaginifolia	Improved conditions for the large scale culture and the induction of mutations.	Negrutiu 1981
Nicotiana sylvestris	Haploid and diploid plants.	Bourgin et al. 1976
	In contrast to *N. tabacum* no effect of feeder layer on division was observed.	Cella and Galun 1980
Nicotiana tabacum	Complete haploid and diploid plants.	Ohyama and Nitsch 1972; Bajaj 1972
	Methionine sulfoximine-resistant calli obtained were diploidized. Two out of five plants regenerated contained up to five times the amount of free methionine	Carlson 1973a, b
	X-irradiated protoplasts showed LD_{50} of 0.75 krad.	Galun and Raveh 1975
	Valine-induced inhibition of protoplasts was reversed by isoleucine.	Bourgin 1976
	Valine-resistant plants from protoplasts subjected to UV and plated on valine.	Bourgin 1978

Table 3I continued.....

Plant species	Observations	Reference
Nicotiana tabacum	Studied nutritional requirements of protoplast-derived haploid cells grown at low cell density in liquid media.	Caboche 1980
	Selection of nitrate reductase-deficient cell lines by their resistance to 40mm chlorate. The plants were regenerated only in clones which contained residual NR activity.	Marton et al. 1982
Nicotiana tabacum + *N. glutinosa*	Production of fertile gametosomatic hybrid plants by protoplast fusion.	Pirrie and Power 1986
Solanum tuberosum	Plants showing stability of the karyotype.	Binding et al. 1978
	Plants from protoplasts bleached with a herbicide (SAN 6706).	Uhrig 1981
Callus protoplasts		
Datura innoxia	10% callus cells were haploids. No haploid plants.	Furner et al. 1978
Oryza sativa	Fusion with pea protoplasts, survived at -196°C.	Bajaj 1983c
Triticum aestivum		
Pollen tetrad protoplasts		
Atropa belladonna	Tetrads treated with 0.5-1% helicase released 100% protoplasts. Occasional division.	Bhojwani & Cocking 1972
Avena sativa		Bajaj and Cocking 1972
Datura innoxia		Rajasekhar 1973
Nicotiana tabacum		Bajaj 1974, 1975
Petunia hybrida		Bajaj et al. 1975
Triticum aestivum		Babbar and Gupta 1979
Pollen protoplasts		
Atropa belladonna	Maturing pollen treated with a mixture of cellulase, macerozyme, rhozyme, and helicase released protoplasts. Frequent fusion, occasional division.	Bajaj 1975
Avena sativa		Bajaj and Davey 1974
Nicotiana tabacum		Bajaj et al. 1975
Petunia hybrida		
Triticum aestivum		

3.6 Materials and Methods II

3.6.1 Source of working materials

Friable, embryogenic calli, whitish in colour produced from excised anther origin, were used for plant regeneration in callus culture experiments (Chapter 3, Part I). These calli were also used for the initiation of embryogenic cell suspension cultures for conducting further experiments on haploid protoplast isolation, culture and plant regeneration (Chapter 3, Part II). This experiment was conducted once in 1990 and again in 1991.

3.6.2 Initiation and maintenance of embryogenic cell suspension cultures using micro- and macro-calli or embryoids from excised intact anthers

Suspension cultures were initiated from friable and globular embryogenic callus, that was obtained from anther float culture and maintained on semisolid LS 2.5 medium for several passages. For multiplication and bulking up the amount of friable calli, several successive subcultures were necessary. Approximately 0.2 g (F.W.) of callus was inoculated into each well of 6-well flat-bottomed Sterilin plastic dish containing 10 ml AA1 liquid medium (Toriyama and Hinata 1986) (Appendices A1.5 & A1.7) for one set and AA2 liquid medium (Müller and Grafe 1978) for the other set. The cultures were incubated on a gyratory platform shaker at 120 rpm under diffuse light (1000 lux) at 27°C ± 1°C. Eighty percent of the culture medium was replaced by fresh AA1 or AA2 medium at 4 day intervals. When small cell aggregates were produced (5-6 weeks from initiation), a 7 ml aliquot of cell suspension (1 ml pcv, and 6 ml old medium from the existing culture in each well) was subcultured into 21 ml fresh AA1 or AA2 medium in 100 ml conical flasks, using a wide-bore plastic pipette. The freshly subcultured suspensions were maintained as before. The subculture procedure was subsequently carried out at weekly intervals. Following this procedure 12 cell suspension lines were initiated using both AA1 or AA2 culture medium separately.

3.6.3 Establishment of different anther calli-derived cell suspension cultures

The population of small, cytoplasmically dense, isodiametric embryogenic cells was thus enriched by subculturing every week using 10 ml sterile plastic pipettes, when AA2 liquid culture medium was used. However, in AA1 liquid culture medium, the

calli proliferated, the packed cell volume (pcv) increased and later, after one month, they became brown and compact, not friable. This could have happened because of genotypic difference. This subculture procedure was subsequently carried out until fast growing embryogenic cell suspensions (ECS) were established. The established anther calli-derived cell suspension cultures were maintained as described in section 2.6.3 (Chapter 2, Part II). Depending on the growth rate of embryogenic, cytoplasmically dense, opaque and friable cell clusters, cell suspension cultures normally became suitable and ready for protoplast isolation after 3-4 months.

3.6.4 Protoplast isolation by enzymatic digestion of cells from embryogenic cell suspension cultures and determination of protoplast yield and viability

Protoplasts were isolated from suitable, established and actively dividing cell suspension cultures in their exponential growth phase, as determined by regular microscopic observation, 4-5 days after subculture. The rest of the procedures for enzymatic digestion, isolation, protoplast yield and viability determination were followed as described in the section 2.6.4 (Chapter 2, Part II).

3.6.5 Protoplast culture and assessment of protoplast plating efficiency in culture following the standard method

The purified and thoroughly washed protoplasts were divided into two parts, and one part after resuspending in liquid KPR medium was subjected to heat shock following the standard procedure as mentioned in section 2.6.5. The heat-shocked and non heat-shocked protoplasts were centrifuged again and washed twice, then resuspended in known volume of fresh KPR medium to determine their final numbers and yield to adjust the exact plating density ($3.5x10^5$ ml^{-1}) before culturing them in protoplast culture medium (section 2.6.5). The protoplasts were cultured and maintained following the standard method (section 2.6.5). The total number of dividing protoplasts at day 14, and of macroscopic visible colonies were counted at day 28 to determine the percentages of protoplast plating efficiencies as described in section 2.6.5.

3.6.6 Plant regeneration from protoplast-derived micro calli using different cell suspension lines

Protoplast-derived micro-calli which were between 1 and 2 mm in diameter, were transferred individually to regeneration medium (MSKN, Appendices A1.3 & A1.7) following the procedures described in section 2.6.7. Also, the standard formula was used to calculate the plant regeneration frequency after about 4 weeks. Morphogenesis of the transferred calli was frequently examined using a stereo-microscope. The differentiating colonies with approximately 2 cm long shoots, were transferred to MSB2 medium (Appendices A1.3 & A1.7) for 2 weeks for further development.

3.6.7 Maintenance of regenerated green plants from two different anther calli-derived lines for sufficient root production and ploidy determination

The shoot systems of regenerated green plants (5-7 cm tall) produced from protoplasts of two different anther calli-derived cell suspension cultures were first transferred to MSB2 medium for more healthy shoot growth. They were further transferred to MSN1.5 (Appendices A1.3 & A1.7) medium for healthy, white sufficient root growth. In both cases, plant cultures were maintained at 24°C ± 1°C under the light intensity (16h day length, 1000 lux) for further development. Six successive subcultures (one month intervals) were needed to establish their adequate growth and to maintain these plants for ploidy determination by flow cytometry and chromosome analysis from root-tip squash preparations, which will be discussed in detail in Chapter 4.

3.7 Results and Discussions II

3.7.1 Initiation and establishment of cell suspension cultures using standard culture medium from calli originated from intact anther explant

Cell suspension culture initiation from white, dry, globular and friable embryogenic callus originated from intact anther explant was found to be as suitable as from other explant sources. However, compared to other explants, rapid proliferation of calli occurred from primary callus after 2-3 subcultures onto LS 2.5 medium producing suitable, friable callus type. Inoculation of these calli to liquid AA1 and AA2 media for cell suspension initiation was possible within 2-3 months after callus induction. In AA1 medium, the amount of calli increased in volume in terms of pcv, later they became

compact and brown and produced numerous elongated and vacuolated cells, which were not suitable for totipotent protoplast isolation.

Careful selection of friable, dry, embryogenic callus and regular replacement of 80% of the liquid culture medium for the first 5-6 weeks from initiation, were the essential factors for the successful establishment of embryogenic cell suspension cultures. Most of the cell-clusters or cells, dissociated into the liquid culture medium, were thick-walled and vacuolated during the initial growth period. Normally, the small cell-clusters consisted of densely cytoplasmic cells, which were actively dividing and their number increased gradually when AA2 liquid culture medium was used. However, at the initial stage of the suspension culture initiation, the main problem was browning possibly because of rapid growth of cells. For the recovery of such browning problem and successful establishment of embryogenic suspension cultures, regular replacement of 80% of the existing medium every 4-5 days for 6 weeks, division of suspensions into more than one flask and use of AA2 liquid medium were beneficial.

After 5-6 weeks of suspension culture initiation, subculture in the ratio of 1:3 (1 ml pcv + 6 ml old existing medium, added to 21 ml of fresh medium) was also beneficial for producing finely divided cell suspension cultures. Later, it was found that subculture at 7 day intervals produced suitable suspension cultures, composed of both embryogenic and densely cytoplasmic cells at about the 16-20 cell stage (Fig 3.4a). Protoplast isolation could be started from 3 to 4-month-old suspension cultures, but better results were obtained from 5 to 6-month-old cultures. Always optimal results were obtained for protoplast isolation when the cell suspensions were used at their exponential growth phase (4 to 5 days after subculture). Normally, established cell suspension cultures were composed of small clusters of isodiametric cells, which had dense cytoplasm and were capable of dividing actively (Fig 3.4b). The time required to double the pcv in an established, optimal culture was between 3 and 5 days. A weekly subculture cycle was found useful for maintaining the suspension cultures in their embryogenic nature. It was only possible to maintain 12 different suspension lines using AA2 liquid medium, probably because of their initiation and maintenance in the same medium. A comparison of increase in settled cell volume (scv) between MSS-derived and AC-derived established, embryogenic cell suspension cultures is shown in Fig A3.1.

3.7.2 Protoplast isolation from different lines of cell suspension cultures originated from anther explants and assessment of viability and yield

Totipotent protoplasts were isolated from 12 different anther calli-derived cell suspension cultures, for the first time when they were 3-months old. Among them, 6 were found to be a suitable source for an abundant release of totipotent protoplasts. All the experiments were carried out following the overnight incubation method (section 2.6.4). The overnight incubation, in diluted enzyme mixture, always produced higher protoplast yields and a lower frequency of spontaneous fusion (Table 2F, Chapter 2, Part II). Similar results were also obtained with AC-derived cell suspensions. Usually, the average yield of protoplasts, isolated from different cell suspension cultures of anther calli origin, at their exponential growth phase did not vary significantly (g^{-1} F.W.) of cells. The average yield of protoplasts and spontaneous fusion body formation from a few anther-calli-derived and one MSS-derived cell suspension cultures are shown in Table 3J. There was no significant variation between MSS and AC-derived suspension cultures. Freshly isolated protoplasts after 5 or 6 day of subculture, from different suspension cultures of optimal age (5-6 months old), were densely cytoplasmic and had an average size ranging from 10 to 30 μm (Fig 3.4c).

The culture age of the AC-derived like MSS and LBM-derived suspension cultures also greatly influenced the yield of protoplasts (Table 3J). Cultures less than 5 months in age from callus initiation, were not suitable for protoplast isolation. The time duration between protoplast isolation and subculture always affected both protoplast yield and the frequency of spontaneous fusion irrespective of explant origin. When suspension cultures were used for protoplast isolation within 3-4 days of subculture, or 7 days after subculture, the protoplast yield decreased markedly and also resulted in a high frequency of spontaneous fusion. For obtaining a higher protoplast yield and efficient protoplast isolation, it was confirmed that anther calli-derived suspension cultures should be used 4 to 5 days after subculture. Also these suspension cultures had to be maintained for at least 2 months in liquid AA2 medium. Successful protoplast isolation was not possible from suspension cultures, which had been maintained less than 2 months in liquid medium after initiation. The viability of the freshly isolated protoplasts, assessed after FDA staining was 80-90%.

3.7.3 Culture of anther-calli origin cell suspension protoplasts in semi-solid KPR-Agarose medium

The first division of cultured protoplasts commenced normally within 3-5 days in KPR-agarose solidified medium (Fig 3.4d), but sometimes it was observed within 5-7 days of culture. Subsequent divisions also took place rapidly and microcolonies of about 20 to 25 cells or more were formed after 12-14 days of culture (Figs 3.4e, f). At this particular stage, it was essential to transfer agarose segments to liquid KPR medium for continuous growth and nourishment of the microcolonies. Depending on the adequate growth of the microcolonies, the time for transferring agarose sections to liquid medium (between 10-12 days after culture in this case) was found to be the most suitable. Usually, micro-calli reached a size of between 0.5 to 1.0 mm in diameter and became visible on the surface of the agarose sections after about 14 to 18 days of transfer to liquid medium (Fig 3.4g). At this stage of growth, it was also important to transfer micro-calli onto regeneration medium as soon as possible to achieve a high plant regeneration frequency. There was an adverse effect on prolonged culture of the micro-calli in liquid KPR medium, causing browning and loss of regeneration capability.

Protoplast plating efficiencies were greatly influenced by the age of the cell suspension cultures and the protoplast plating density (Tables 3K & 3L). Normally, protoplasts from older suspension cultures produced higher plating efficiencies than those from younger suspension cultures (Fig 3.4g, left). Protoplast plating density had also a direct effect on protoplast plating efficiencies. Similar results were obtained irrespective of explant origin of the cell suspension cultures. However, the cell suspension cultures of different explants origin including anther explant had significant variation on protoplast plating efficiencies and plant regeneration frequencies, although suitable embryogenic callus was used from the same batch for the initiation of cell suspension cultures (Table 3K).

3.7.4 Plant regeneration from protoplast-derived micro-calli

After transferring protoplast-derived embryogenic micro-calli, obtained from different cell suspension cultures of anther calli origin, they grew faster on the regeneration medium (MSKN) than in KPR liquid medium. Micro-calli formation was obtained

Fig 3.4 Cell suspension initiation from anther-derived callus, protoplast isolation and culture using cultivar Taipei 309

a) Early growth stages of cell suspension cultures (5-6 weeks from initiation), initiated from anther-derived embryogenic calli in liquid AA2 (left) and AA1 (right) media, showing growth of small cell-clusters in AA2 liquid medium under cool, white, fluorescent light (1000 lux) at 25°C ± 1°C (x 4.0).

b) Loosely packed, densely cytoplasmic and actively dividing cell-clusters of an AC-derived suspension after 2 months of culture from initiation in liquid AA2 medium (x 78).

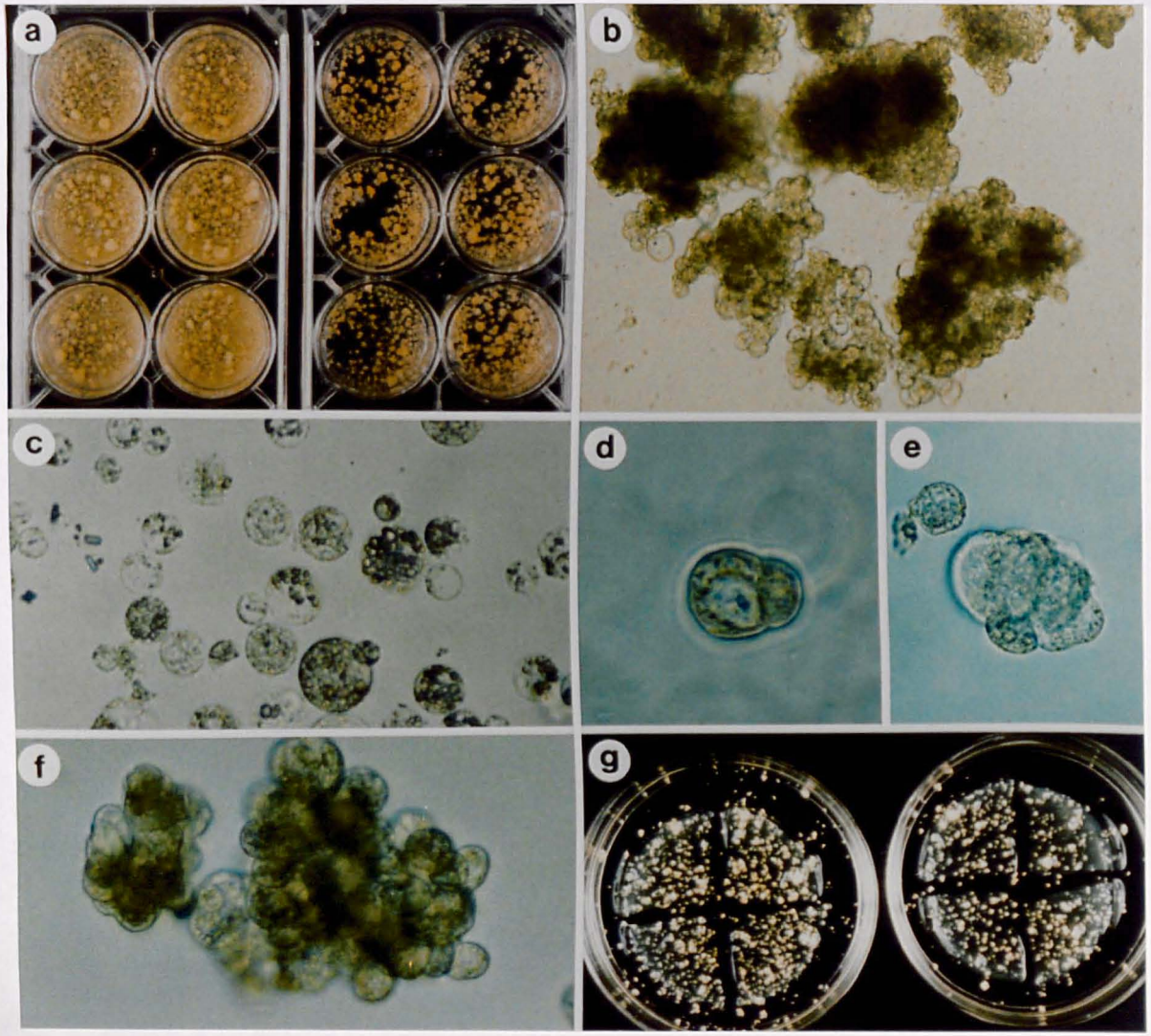
c) Freshly isolated cell suspension derived protoplasts (anther-calli origin) (x 72).

d) First division of protoplast after 3-5 days of culture on agarose solidified KPR medium (x 96).

e) A protoplast-derived microcolony (AC-origin) after 6-8 days of culture in the dark on agarose solidified KPR medium (x 72).

f) Protoplast-derived microcolonies (AC-origin) after 14-16 days of culture in the dark on agarose solidified KPR medium (x 76).

g) Protoplast-derived micro-calli growing on the surface of KPR-agarose sections after 3-4 weeks of culture from isolation, consisted of both compact and loose calli; showing variation in plating efficiencies from two different suspension cultures (AC-origin) (x 3.6).


a
b
c
d
e
f
g

from 6 different cell suspension lines, and plant regeneration from 2 different cell lines. The micro-calli doubled in size within the first 5 days of culture. The first indication of differentiation was observed either by somatic embryogenesis or rhizogenesis and most frequently white embryoid-like structures developed on the surface of the micro-calli, after 7 to 10 days of culture in the dark. These embryoid-like structures germinated to form well defined coleoptiles and sometimes radicles after about a further week of incubation in the dark at 27°C ± 1°C (Fig 3.5a). At that stage it was essential to transfer the germinating calli to shoot producing medium (MSB2) for further and optimal shoot development under continuous light (1000 lux) photoperiod at 27°C ± 1°C. The plantlets became green within 2-3 days of transfer to light (Figs 3.5b-d). After 2 weeks, it was necessary again to transfer each shoot-system (4-5 cm tall) to root producing medium (MSN1.5) for healthy and white root production and further shoot elongation (Figs 3.5e, f), maintaining under the same culture conditions.

Although there was no marked variation between plating efficiencies of two Ac-derived cell suspension lines, one of them was highly regenerative (approx. 81%) when it was 3-4 months-old from culture initiation (Table 3K). All of the plants, which were regenerated from these two lines were green. Approximately 20-25% of the protoplast-derived micro calli differentiated by rhizogenesis, producing only roots. The plant regeneration frequency was the highest, when the cell suspensions were used at their embryogenic state within 3-4 months of culture age from culture initiation. After this time, the regeneration frequency gradually declined and they only regenerated roots (Table 3K). Even the suspensions originated from the same batch of anther calli, behaved differently. Some of them did not produce any microcolonies, some produced microcolonies but did not differentiate, some differentiated roots only and two differentiated both roots and shoots. There was no differentiation observed when the suspension cultures were 8 or more than 8-months-old (Table 3K), and their regeneration capability was completely lost.

3.7.5 Care and maintenance of regenerated green plants obtained from anther calli-derived cell suspension protoplasts for ploidy determination by flow cytometry and chromosome counting

The regenerated each green shoot systems (5-7 cm tall) obtained from two different

Fig 3.5 Differentiation, regeneration and growth of plants from AC-derived cell suspension protoplast culture of *O. sativa* (cv. Taipei 309)

a) Differentiation of somatic embryos with multiple shoots and roots on the surface of protoplast-derived callus (AC-origin) after 2-3 weeks of culture on MSKN regeneration medium under dark condition at 27°C ± 1°C (x 3.6).

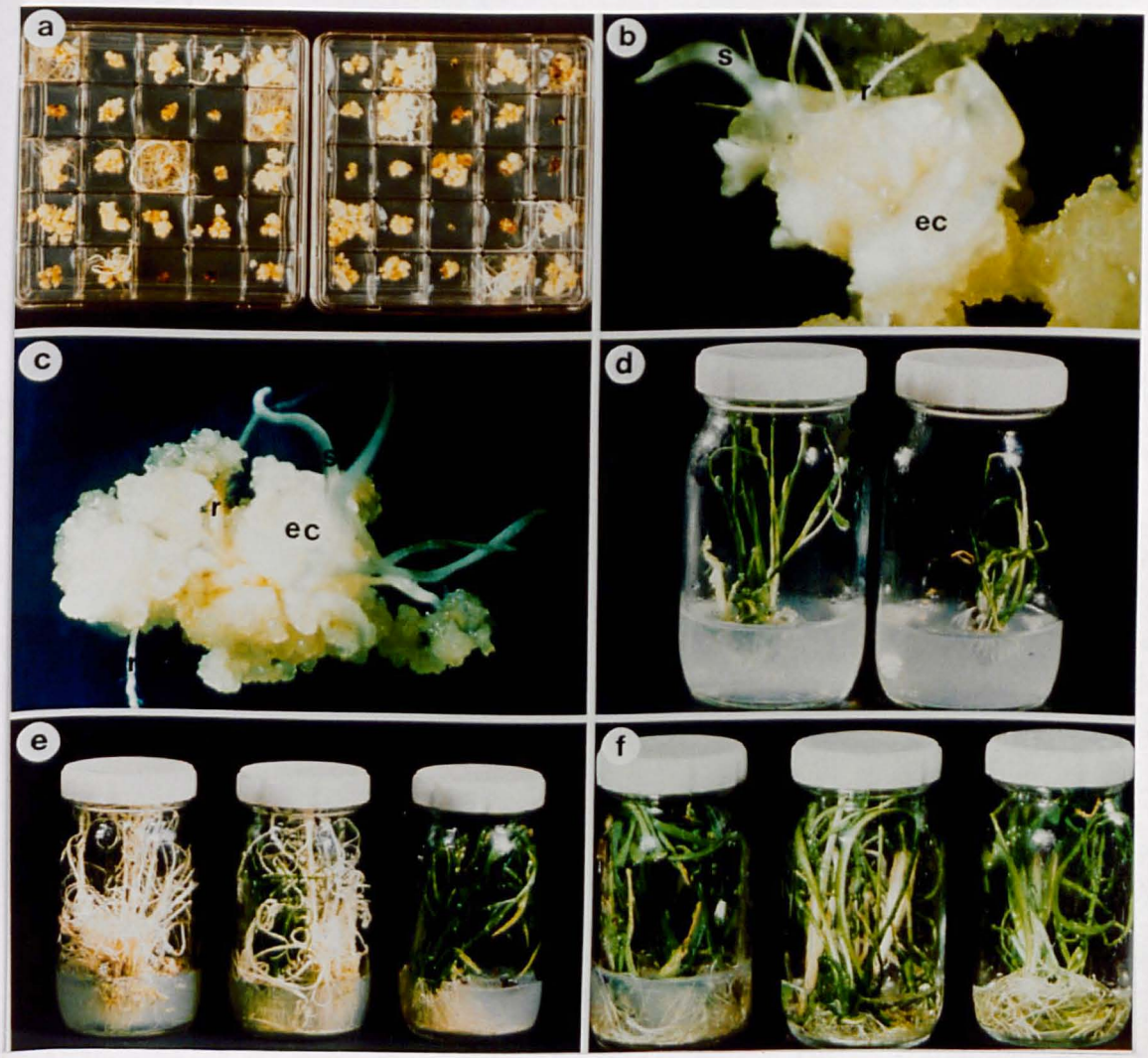
b) A somatic embryo, producing one green shoot (s) and a number of roots (r) after a week of transfer under white, fluorescent light (1000 lux) in the growth room at 25°C ± 1°C following 2-3 weeks of dark treatment in the incubator (x 3.7).

c) A somatic embryo, producing multiple green shoots (s) and one or two roots (r) after a week of transfer under the culture conditions as in (b) (x 3.7).

d) Growth and development of young regenerated plants from protoplast-derived callus (AC-origin) and production of multiple shoots and roots after 3 weeks of transfer to MSB2 medium under cool, white, fluorescent light (1000 lux) in the growth room at 25°C ± 1°C (x 4.0).

e) Regeneration of only albino plants (left), both albino and green plants (middle) and green plants only (right) from protoplast-derived calli, originated from the same AC-derived cell suspension culture, after 4-5 weeks of culture on MSB2 medium under the culture conditions as in (d) (x 3.3).

f) Growth of green protoplast-derived plants and production of numerous white, healthy roots after a further transfer to MSN1.5 semisolid (left) and liquid medium (middle & right) for 4 weeks under the culture conditions as in (d) (x 3.7).


a
b
s
r
ec
c
r
ec
d
e
f

cell suspension cultures were divided into few individual plantlets, micropropagated first in MSB2 medium and then grown in MSN1.5 medium for white and healthy root production. Following the established procedure, it was possible to grow and maintain successfully all the regenerated green plants with 100% recovery from each clone for ploidy determination after six successive subcultures (1-2 month intervals).

Table 3J. The average yield of protoplasts and spontaneous fusion body formation from few anther calli-derived and one MSS-derived cell suspension cultures (T309)

Suspension lines	Protoplast yields[1] (x 10^6) / g F.W. cells	Spontaneous fusion body (%)
MSN1b[2]	3.80 ± 0.08	8.44 ± 2.02
AC4A[3]	3.89 ± 0.34	4.39 ± 0.96
AC4C[4]	4.41 ± 0.65	4.50 ± 0.92
AC4D[5]	4.47 ± 0.79	4.21 ± 0.77
AC4E[6]	3.28 ± 0.45	5.11 ± 1.28
AC4F[7]	3.24 ± 0.09	8.81 ± 2.01

[1]Data represent the means ± standard error based on 6 individual experiments.

[2]MSN1b(N) - Suspension culture initiated from MSS-derived callus.

[3-7]AC4A-AC4F - Suspension culture initiated from anther-derived callus.

Table 3K. Protoplast plating efficiencies and plant regeneration frequencies from MSS, LBM and AC-derived cell suspensions of different culture age.

Suspension lines	Age of the suspension lines (months)	Plating efficiencies (%)		Average no. of shoots / roots per callus	Plant regeneration frequency (%)
		14d	28d		
MSN1b (N) (MSS-derived)	3	2.25	0.08	3.33(S)	45.0(S)
	4	6.33	0.14	3.12(S)	49.9(S)
	5	9.41	0.38	3.25(S)	37.1(S)
	6	13.77	0.63	3.28(S)	31.5(S)
	7	18.60	0.81	3.11(S)	23.7(S)
	8	22.32	0.83	3.27(S)	15.3(S)
LBN2b (N) (LBM-) derived	4	6.21	0.12	1.01(S)	3.5(S)
	5	7.99	0.21	1.00(S)	6.2(S)
	6	12.27	0.45	1.02(S)	14.2(S)
	7	14.53	0.91	1.03(S)	11.3(S)
	8	13.37	0.88	1.10(S)	8.7(S)
	9	14.82	0.97	1.23(S)	6.5(S)
AC4C (N) (AC-derived)	3	2.23	0.07	1.96(S)	15.9(S)
	4	5.11	0.09	1.85(S)	16.3(S)
	5	6.27	0.14	1.93(S)	14.9(S)
	6	8.37	0.19	2.05(S)	81.7(R)
	7	9.25	0.21	2.01(S)	80.0(R)
	8	10.95	0.15	0.00	91.3(R)
AC4D (N) (AC-derived)	3	1.95	0.05	1.01(S)	83.2(S)
	4	2.21	0.09	1.03(S)	84.3(S)
	5	5.13	0.05	1.04(S)	75.5(S)
	6	6.07	0.08	1.02(S)	15.1(R)
	7	7.95	0.07	2.07(S)	18.8(R)
	8	8.17	0.09	0.00	23.2(R)

[1]Data represent the means based on 3 replicate experiments conducted every month, for six consecutive months.

In each experiment the visible embryogenic protoplast-derived micro-calli (1-2 mm in size) were transferred to regeneration medium.

Table 3L. The effect of protoplast plating density on protoplast plating efficiency from AC-derived cell suspension compared to MSS-derived cell suspension cultures (T309)

Suspension lines	Protoplast plating density (x 10^6)	Protoplast plating efficiencies[1] (%) at 14d
MSN1b(N) (MSS-derived)	1.0 x 10^6	24.95
	2.0 x 10^6	22.33
	3.0 x 10^6	19.17
	3.5 x 10^6	16.21
	4.0 x 10^6	12.41
	5.0 x 10^6	9.34
	6.0 x 10^6	6.12
AC4C(N) (AC-derived)	1.0 x 10^6	10.85
	2.0 x 10^6	9.37
	3.0 x 10^6	8.53
	3.5 x 10^6	7.02
	4.0 x 10^6	6.43
	5.0 x 10^6	5.23
	6.0 x 10^6	2.34

[1]Protoplasts were isolated from a MSS-derived and a AC-derived suspension cultures; protoplast plating efficiencies were recorded at day 14. Data were collected from three individual experiments and for each treatment three replicates were used. Data shown here are the average plating efficiencies for each treatment.

3.8 Summary and conclusions II

The main objective of the work described in this Part of Chapter 3 was to initiate and establish a suitable and reproducible, embryogenic cell suspension cultures from anther calli explant, to regenerate and recover plants from cell suspension-derived protoplasts using the *Japonica* rice variety Taipei 309. The same standard procedure discussed in Chapter 2, Part II was followed to conduct all the experiments. Among the three liquid, callus-inducing media (E24, E10 and J19), it was much easier to initiate and establish embryogenic cell suspensions from calli produced in E24 medium, which consisted of fine cell clusters and slightly greenish in colour within 2 months. In the middle, it was essential to bulk the amount of friable, globular and translucent calli in

LS 2.5 semi-solid medium for successful establishment of the cell suspension lines. In comparison to two suspension culture media (AA1 and AA2) for suspension initiation and maintenance, it was observed that in AA1 the increase in amount of cells was more for each line than in AA2. But the cell-clusters in AA2 were finer and smaller in size, greenish in colour as compared to those in AA1, where a mixture of both large and fine clusters were obtained. After one and a half months, the suspensions became brownish in colour even after regular weekly subculture in AA1, unsuitable for protoplast isolation and lost their embryogenic nature. Therefore, the selection of friable, embryogenic callus and the use of selective culture media for callus production, suspension initiation and maintenance, protoplast division and microcolony formation and plant regeneration are crucial and important factors to establish protoplast-to-plant regeneration systems.

The culture conditions or the culture environment, especially temperature and light intensity, had an important role for anther calli-derived suspension culture establishment and maintenance. It was essential to maintain them under cool, fluorescent, diffuse light (1000 lux), at 25°C ± 1°C on a gyratory shaker at 100 rpm. The whole set of cell suspension cultures, maintained in the dark at 28°C ± 1°C at a shaker speed 110-120 rpm, became brown and the cells lost their embryogenic nature for protoplast isolation. Compared to the cell suspensions originated from other explants like MSS, ISE and LBM, the regenerative ability of the anther calli-derived cell suspensions was low. Although the protoplast yield was high for two embryogenic cell suspensions (AC4C and AC4D), the percent protoplast viability, the plating efficiencies and the number of colony formation ml^{-1} were very low in the present study. Therefore, the availability of high percentage of viable protoplasts, with a high division frequency is the most critical factor to achieve high frequency plant regeneration. The microcolonies obtained from AC-derived cell suspension protoplasts were also soft and friable, not compact and 20-25% differentiated by rhizogenesis only. All the regenerated plants from protoplasts were green. Plant regeneration from protoplasts was obtained only when the AC-derived cell suspensions were more than 7 months old from callus initiation, whereas it was achieved within 5-6 months from cell suspension protoplasts of other explant origin. In case of other explants, the plant regeneration frequency declined gradually with the age of cell suspension, whereas

differentiation by embryogenesis was lost after 9 months from callus initiation in AC-derived cell suspensions.

If the main problems of low microspore division frequency and plant regeneration efficiency can be overcome by manipulating the culture conditions and the culture procedures, many scientists throughout the world think that anther culture and shed microspore culture can be powerful tools applied to large-scale production of true breeding materials (Zapata et al. 1983; Chung 1987; Guiderdoni et al.1991). Anther culture has also been confirmed to be an interesting tool for more basic genetic studies. In the past 20 years, the techniques of rice anther and microspore cultures have been much developed and improved, these techniques have also been well integrated into the rice breeding system in some institutions in China (Loo and Xu 1991). After the first success of haploid and diploid plant regeneration from protoplasts that were isolated from cell suspensions of anther callus in rice (Toriyama and Hinata 1985a, b; Toriyama et al. 1986), the future prospects and the importance of haploids and double haploids in cereal breeding has been emphasized (Snape and Simpson 1986; Snape 1989).

Haploid cell or protoplast cultures offer advantages over diploid and polyploid cultures for the study of karyotype instability of cultures. These advantages are that a large population of uniform, uninucleate microspores for initiation of cell cultures, immediate expression of recessive genes or mutation, and fewer chromosomes to analyze and distinguish (Ziauddin and Kasha 1990). The use of haploid cell or protoplast systems for the induction of mutants that are resistant to pests, pathotoxins, herbicides, and tolerant to various salts and environmental stresses, would be the most rewarding investigations for plant improvement in near future (Bajaj 1991). After the achievement of successful plant regeneration from anther-derived (haploid) cell suspension protoplasts of *Japonica* rices in 1980s, work has been extended to different laboratories throughout the world to achieve plant regeneration from both *Japonica* and *Indica* rices, and from other cereal like barley using haploid cell suspension protoplasts.

Following the production of transgenic rice plants after direct gene transfer into haploid cell suspension protoplasts of *Japonica* rice (Toriyama et al. 1988; Biswas and

Zapata 1990a), it was possible to recover non-transformed and transformed 'true-*Indica*' and '*Indica*-type' rice plants. Transmission of the transgene (foreign gene) to the progeny using haploid protoplast culture system was also possible (Datta et al. 1990b, c; Biswas and Zapata 1991a, b). In 1991, Plant regeneration from anther-derived embryogenic cell suspension protoplasts of barley (*Hordeum vulgare* L.) was also obtained and the stable transformation of barley protoplasts has recently been reported (Jähne et al. 1991a, b; Lazzeri et al. 1991). As co-transformation has been established as an efficient procedure for the recovery of transgenic plants for non-selectable genes (Schocher et al. 1986), it should now be feasible to produce transgenic *Indica* rice plants, for agronomically more important genes. As for example, the genes conferring resistance to insect pests or diseases, as available from the *Bacillus thuringiensis* system (Delanny et al. 1989). Transgenic cereals (rice, maize) have so far been recovered exclusively by direct gene transfer applied to protoplasts. This method is not applicable to all varieties of all plant species. Therefore, the challenge is to develop a routine gene transfer method for the achievement of integrative transformation with rice, other cereals and important crop plants, or with any given plant species and variety (Potrykus 1990). Perhaps, microspore-derived embryogenic cell suspensions are well suited for reproducible production of transgenic colonies and plants. Since the starting material for protoplast isolation and transformation is a microspore-derived cell suspension which is haploid at the time of use, homozygous fertile primary transgenic plants could possibly be recovered.

Finally, work needs to be carried out on pollen protoplasts. No substantial progress has been made since the early work (Bajaj and Davey 1974; Bajaj et al. 1975). Pollen protoplasts, being natural haploids, would be ideal for DNA uptake and genetic transformation. Triploid gametosomatic hybrid plants were recovered by fusing pollen tetrad protoplasts (haploid) with mesophyll or cell suspension protoplasts (diploid). The plants were fertile and set seed in the case of *Nicotiana and Petunia* species (Pirrie and Power 1986; Lee and Power 1988). Therefore, pollen tetrad protoplasts and their utilisation in triploid gametosomatic hybrid plant production would be expected to provide a novel and alternative approach for the genetic manipulation of crop plants.

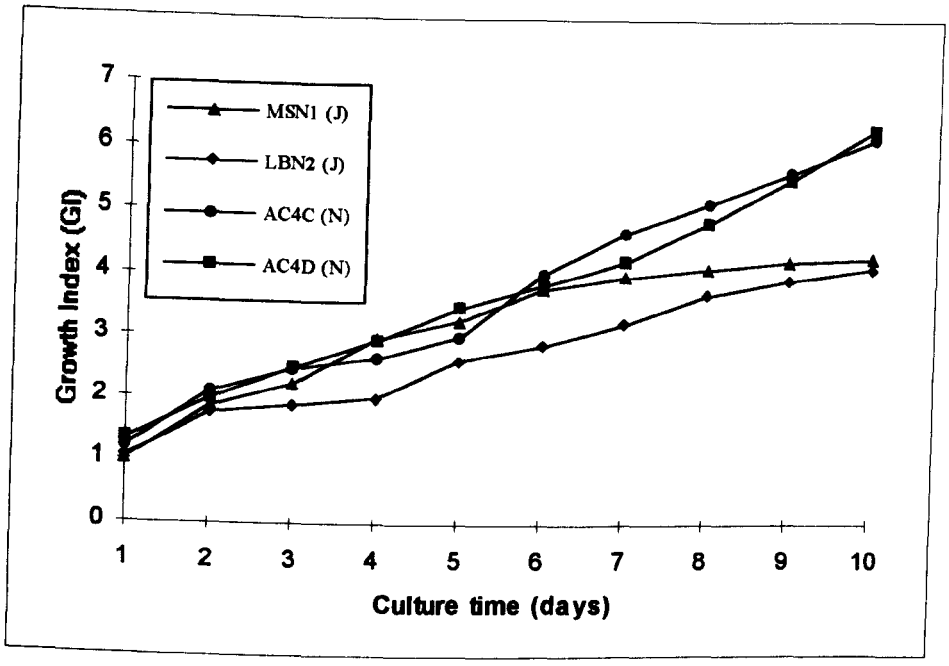


Fig A3.1. Growth rate comparison of settled cell volume (scv) per day of one MSS, one LBM and two AC-derived, established cell suspension cultures (13 weeks old). Suspension cultures were subcultured and growth index was measured, starting from the first day of subculture. Data were calculated from three experiments and the means plotted.

Chapter 4

Determination of ploidy level of regenerated plants by flow cytometry

4.1 Introduction

Considerable variation occurs in nuclear DNA content both within and among the plant species. In a number of crops, manipulation of ploidy level is an important tool for plant breeding. For example, in sugarbeet, most present varieties are triploid, originating from crosses between a male sterile diploid seed parent and a tetraploid pollinator. In wheat the hexaploid state is optimal for productivity. Ploidy level can be increased by the treatment of meristematic plant tissues with spindle toxins, for example, colchicine. But there is growing interest in the use of haploid production by the plant breeders, which can be produced by culturing gametophytic tissues in a defined medium.

Determination of ploidy levels of plant tissues is conventionally conducted by means of chromosome counting under a microscope, using meristematic tissues from individual plants. In this particular method, mitotic cells are arrested in metaphase stage, followed by DNA stained squash preparation (Dyer 1963). This work needs trained personnel and a well equipped cytology laboratory. Since only individual plants can be screened by following this method, the analysis of hundreds of plants for the presence of undesired ploidy contamination is a very time-consuming process. At least one hundred seedlings may have to be checked for one plant species and chromosome countings are rather difficult in some plant species. Alternatively, ploidy level can be determined from the size of pollen grains and also from the number of chloroplasts in the epidermal guard cells in the leaves of individual plant. When the ploidy difference is small, these procedures have a low level of accuracy (e.g. 3n vs. 4n).

Flow cytometry can be used for rapid measurement of the DNA content of interphase nuclei or chromosomes in order to study synchronization of cell cultures, cell cycle patterns, ploidy levels and karyotyping (De Laat et al. 1989). Both individual plant as well as plant populations can be used to obtain the DNA amount plotted in histograms. Flow cytometry is highly competitive in terms of simplicity and accuracy compared to the conventional chromosome counting (De Laat et al. 1987). The DNA of plants, like other DNA, can fix various fluorochromes in a manner proportional to the quantity of

DNA (Brown et al. 1986). Therefore, it is possible to examine the DNA status of the nuclei of plant tissues and cell cultures to determine the ploidy level, the kinetics of the cell cycle or other characteristics by flow cytometry (Galbraith and Shields 1982; Galbraith et al. 1983; Sharma et al. 1983; Sundberg and Glimelius 1986). During the same period, several authors have applied flow cytometry to various research areas, and documented the unique potential of flow cytometry. Their achievements in the application of flow cytometry in plants are presented in Table 4A.

In plant cell systems, a variety of flow cytometric procedures have been developed for the analysis of nuclear DNA content (De Laat et al. 1987; Dolezel et al. 1989; Ulrich et al. 1988; Galbraith et al. 1983; Hammatt et al. 1991). All these procedures involve the staining of the nuclei with specific fluorochromes, under conditions in which the amount of emission of fluorescence is linearly correlated to DNA content.

Several fluorochromes have been used for DNA staining; they can be considered as two groups. The DNA binding dyes, the Hoechst dyes, mithramycin, chromomycin A3 and DAPI (4', 6-diamidino-2-phenylindole) bind only DNA; whereas intercalating dyes, propidium iodide and ethidium bromide, stain both DNA and RNA. All the fluorochromes emit fluorescence when excited at a suitable wavelength.

For determination of nuclear DNA content from intact plant tissues, flow cytometry provides a more reliable, rapid alternative to microdensitometry and traditional chromosome counting. It is useful and highly sensitive tool for the determination of DNA content variation among the somaclones and protoclones derived from tissue and protoplast cultures of rice. The sample preparation procedure is simple, rapid and much easier than chromosome preparation technique in rice. Using this simple chopping procedure, suspensions of isolated nuclei from a large population of plants can be prepared in a very short time. The staining of nuclei is also much easier than rice chromosome staining, may be because of very low amount of DNA content in *Oryza* species compared to wheat. Since a large number of nuclei can be analysed in a short time, the chances of detecting mixoploidy are much larger than if these analyses are made by chromosome counting. There is no interference of RNA problem and the staining method works very well for rice.

Cell cycle analysis has also been used to show delayed DNA synthesis in cultured *Petunia* protoplasts when these were obtained from male-sterile plants (Bergounioux et al. 1986). The preparation of intact plant mitotic chromosomes has introduced flow karyotyping and chromosome sorting. The first success was reported with *Haplopappus gracilis*, 2n = 4, and subsequently with *Petunia hybrida* (2n = 14) and *Nicotiana plumbaginifolia* (2n = 10) (De Laat and Schel 1986; De Laat and Blaas 1984; Petit et al. 1986b).

The present study was undertaken to use the efficient and quicker method of flow cytometry for accurate detection of individual plants with different ploidy levels, i.e., haploids, diploids, tetraploids, aneuploids and polyploids, from a population of regenerated rice plants obtained through various culture systems using the cultivar Taipei 309. In a sample of plants with varying ploidy levels, the relative fluorescence intensity (RFI) of nuclei stained with propidium iodide is a function of the number of chromosome sets (x), as represented by the linear equation of RFI (Costich et al. 1993). The nuclear DNA content of each sample was compared with that of seed-grown Taipei 309 plants (1.2 pg/2C nucleus) (Bennett and Smith 1976).

The aims of the work described in this Chapter were :

- to estimate and measure the nuclear DNA content of isolated nuclei from young leaf tissues of regenerated and seed-grown plants by flow cytometry;
- to learn the quicker and efficient method for the measurement of the DNA content to determine the ploidy level.

4.2 Materials and Methods

4.2.1 Source of plants

Seedlings of Taipei 309 were grown at least for 15-20 days from seeds under standard growth room condition (12h day, 12h night; at 28°C ± 1°C) for each experiment. Their fresh young leaves were used to isolate nuclei for measuring the amount of nuclear DNA in a known standard of ploidy level (diploid, 2C). Regenerated rice plantlets obtained by anther, callus and protoplast cultures, which has been discussed in Chapter 2, Part I & II, and Chapter 3, Part I & II, were used as major working materials.

4.2.2 Collection and preparation of samples (chopping procedure)

The leaf samples were collected from the regenerated plantlets grown in vitro. Before collection and preparation of samples they were subcultured at least 3 weeks previously to get the freshly grown leaves for conducting each experiment. Four or five young leaves, approx. 6 cm long were excised from individual plantlets or seedlings. Excised leaf tissues were chilled on ice, and finely chopped (0.5 mm^2) with a scalpel blade in a glass Petri dish (9 cm) containing 2 ml of ice cold 'Chopping buffer solution' (45 mM magnesium chloride, 30 mM sodium citrate, 20 mM [3-(N-morpholino)propane-sulphonate], 1 mg/ml Triton X-100, pH 7.2, (Galbraith et al. 1983). Propidium iodide (25 µg/ml), was added to the chopping buffer before use. All reagents were from Sigma Chemical Co.

The nuclei were released by this chopping procedure, then the homogenates containing cell constituents and cellular debris, consisting of finely chopped tissue fragments, were filtered through a 30 µm nylon mesh sieve. The stained nuclei in the filtrate were collected in an Eppendorf tube, labelled with sample number and kept on ice until ready for analysis on the flow cytometer.

A small aliquot (50 µl) of 'DNA Check' fluorescent plastic beads (Coulter) was added to each sample of isolated nuclei prior to analysis, as an internal standard for a series of DNA analyses, so that differences in instrument sensitivity could easily be monitored.

4.2.3 Flow cytometry

Sample were analysed using a Coulter EPICS 541 Flow Cytometer, equipped with a 5W argon ion laser tuned to 488 nm, and a power output of 100 mW. Sheath fluid consisted of 0.9% saline and was passed through a 150 µm flow cell tip at a pressure of 7 psi. Fluorescence emissions were collected through a 490 nm band stop filter and a 515 nm long pass filter. The optimum calibration and operation of the flow cytometer was checked by analysing a known mixture of a sample of fluorescent plastic beads with varying fluorescence and/or light scatter characteristics on each day of experimentation.

The samples of isolated nuclei were analysed for DNA content by measuring fluorescence associated with propidium iodide. Histograms of log integral fluorescence

were collected for the samples used as DNA standards and for each experimental sample (10,000 particles for each sample). Histograms for all the samples were transferred to the computer system (IBM AT compatible) and analysed using the 'Peak Detect' program (V3.0) developed by N.W. Blackhall at the Department of Life Science, Nottingham. By examining these histograms, peaks corresponding to the G_o/G_1 and G_2/M phases of the cell cycle could be these peaks corresponded to the amount of DNA contained within the nuclei.

Using the 'Peak Detect' program, two ranges were defined to correspond to the standard fluorescent bead peak, and the G_o/G_1 nuclei (Fig 4.1). The mean, standard deviation and number of particles were calculated for each range. The position of the bead peak was used as an estimate of zero-offset drift (Vindelov et al. 1983) and an offset correction factor calculated. As log histograms had been obtained, this factor was added to the mean of the G_o/G_1 peaks. A difference of 100 (approx.) in the position of two peaks represents a doubling of their respective DNA contents. The logarithmic fluorescence values of the G_o/G_1 peaks were then converted automatically to mean linear fluorescence values. These values were subsequently used to calculate the DNA index for the determination of ploidy level of each sample compared to seed-grown diploid plants. The ploidy status of each plant being analysed was compared with the histograms from seed-grown and, presumably, diploid plants.

4.3 Results and Discussion

4.3.1 Successful and quicker determination of ploidy level of regenerated green haploid, diploid, aneuploid and tetraploid plants

By chopping young rice leaf material from each *in vitro* grown plant, intact interphase nuclei were released from the cut surfaces. The majority of cells in full grown material do not participate in cell divisions and stay in the G_o stage of the cell cycle. In this stage, the nuclear DNA content reflects the ploidy state of the plant. Cells which are involved in divisions start from the G_1 state and subsequently pass through S (= DNA-synthesis), G_2 (= an interphase nuclear stage with a doubled DNA content preceding the actual nuclear division) and M (= mitosis) stages. Nuclei isolated from all the samples (controlled growth room grown diploid seedlings and *in vitro* grown plantlets obtained through various culture systems) examined, emitted fluorescence after

staining with propidium iodide, which was quantitated electronically by the flow cytometer. The coefficients of variation for the G_1 peaks of nuclei prepared in this manner were similar to those obtained with larger tissue samples (Galbraith et al. 1983).

4.3.2 Interpretation of flow cytometry data

Typical sample histograms of relative fluorescence are presented in Figures 4.1a-f. Variation in mean relative fluorescence was found for all the samples in each experiment. There was considerable variation in DNA index (0.66-2.47) among the protoplast-derived plants irrespective of callus source (MSS or LBM), compared to the standard control plants (1.00). The ploidy level varied from aneuploid to pentaploid but the frequency of diploids was highest (43.3%) compared to tetraploids (31.7%) and triploids (20.0%) (Table 4C & Fig. 4.3). The frequency of aneuploid and pentaploid were negligible compared to the other three groups (Table 4B). There were variations in DNA index (0.31-1.04) among the callus-derived plants from different explants (ISE, LBM, MSS, AC) but the range of variation was not wide compared to protoplast-derived plants.

Among 26 plants analysed, which were regenerated from ISE, LBM and MSS-derived callus cultures, 1 was haploid (3.8%) and 19 were diploids (96.2%). In one set, 201 micropropagated plants derived from 5 original clones, regenerated from anther callus culture, were analysed; among these plants 149 were haploids (74.1%) and 52 were diploids (25.9%). In another set, 5 original clones derived from anther callus cultures were analysed and among these plants, all of them were haploids (100%). The DNA indices were consistent for both haploid (0.70-0.86) and diploid (0.90-1.04) plants obtained from anther callus cultures (Table 4B). It was important to note that the relative fluorescence values were still consistent even after micropropagation of 149 haploid and 52 diploid plants compared to respective seed grown control standard plants.

4.4 Summary and conclusions

The results described in this Chapter summarizes that flow cytometry provides the facility for the analysis of nuclear DNA content in a large populations of cells and hundreds of plant samples can be analysed within a short period of time. It can also be

used to study nuclear DNA content per genome, cell cycle parameters and ploidy changes (Melamed et al. 1979). In the present study, for the analysis of nuclear DNA content of young rice leaves, obtained from in vitro grown regenerated plants derived from various cultute systems, the measurement of isolated nuclei by flow cytometry was found to be very accurate and rapid. The simple chopping of young leaf material in buffer solution to isolate thousands of intact nuclei was also very rapid and convenient. The set up of the flow cytometer was rather complicated, but automatic conversion of the log. fluorescence values of the G_o/G_1 peaks to mean linear fluorescence values was done by the computer. Before conversion of log. values to linear values, it was easy to calculate an offset correction factor with a computer by using the position of the respective bead peak on each histogram. As log histograms were obtained, this correction factor was added to the mean value of the G_o/G_1 peaks. It was also easy to calculate the DNA index of each sample by using the relative fluorescence values of known standard control and test sample to determine the ploidy level. Most of the calculated DNA index values were more or less the same as expected values, so the samples having values nearest to the expected were also categorized to the nearest ploidy level.

Furthermore, it could not have been possible to determine the ploidy level of large populations of plants by using root-tip squash technique. From the experience of the author, it was found that the root-tip squash technique for preparation, staining and counting of rice chromosomes to determine ploidy level (discussed in Chapter 5) was very difficult and time consuming compared to the easy flow cytometry technique. This may be because of the very low amount of DNA content of *Oryza* species (1.2 pg) compared to wheat (34.6 pg) (Bennett and Smith 1976) and hence the small size of the chromosomes.

It was found from the results that the plants which were regenerated from callus culture of MSE or ISE-origin, all of them were diploids. The plants which were derived from immature seed-embryos, some of them were haploids and some were diploids. Seventy five percent haploids and 25% spontaneous diploid plants were regenerated through anther-derived callus culture system (Table 4C, Fig 4.2). It was interesting to note that all the plants regenerated from cell suspension-derived protoplasts, where the suspension was initiated from anther callus, were tetraploids. In

other cases, where the suspensions were initiated from MSE or ISE, gave rise to diploid, triploid and tetraploid protoclones (Table 4C, Fig 4.3).

Table 4A. Achievements in the application of flow cytometry in plants

Type of plant material	Achievements	Reference
Pollen	Detection and sorting of pollen mutants	Pinkel 1981
Protoplasts	Adaptation of commercial flow cytometric instruments for viable sorting	Harkins & Galbraith 1984; Jett & Alexander 1985; Glimelius et al. 1986; Harkins & Galbraith 1987
	Sorting & selection of viable plant heterokaryons	Afonso et al. 1985; Alexander et al. 1985
Cells, nuclei	Ploidy measure ments	Galbraith & Shields 1982; Sharma et al. 1983; Carlberg et al. 1984; Sundberg & Glimelius 1986; Sree Ramulu & Dijkhuis 1986; Hänisch ten Cate & Sree Ramulu 1987
	Cell cycle	Meadows 1982; Galbraith et al. 1983; Puite & Ten Broeke 1983; Galbraith 1984; Anderson et al. 1985; Karlsson & Vasil 1986; Bergounioux et al. 1986; Ulrich and Ulrich 1986
	Selection of cells containing specific secondary products	Brown et al. 1984, 1986
Chromosomes	Karyotyping, sorting of individual chromosomes	de Laat & Blaas 1984; Petit et al. 1986; Conia et al. 1987

The table was adapted from De Laat et al 1989b.

Table 4B. Flow cytometric estimation of the DNA content of control and *in vitro* regenerated rice plants obtained from callus and protoplast cultures using different explants of *Japonica* cultivar T309

Sample code[a]	Relative fluorescence	DNA index	Ploidy
Control(T309)	185	1.00	Diploid
LBN2 (J1)	151	0.82	Diploid
LBN2 (J3)	325	1.76	Tetraploid
LBN2 (J4)	311	1.68	,,
LBN2 (J7)	303	1.64	,,
LBN2 (J8)	328	1.77	,,
LBN2 (J15)	317	1.71	,,
LBN2 (J16)	287	1.55	Triploid
LBN2 (J18)	278	1.50	,,
LBN2 (J19)	294	1.59	,,
LBN2 (J20)	228	1.23	Diploid
LBN2 (J21)	174	0.94	,,
Control(T309)	47	1.00	Diploid

Table 4B continued.....

Sample code[a]	Relative fluorescence	DNA index	Ploidy
Control(T309)	47	1.00	Diploid
LBN2 (J2)	76	1.62	Triploid
LBN2 (J6)	71	1.51	"
LBN2 (J9)	98	2.09	Tetraploid
LBN2 (J10)	93	1.98	"
LBN2 (J11)	89	1.89	"
LBN2 (J12)	85	1.81	"
LBN2 (J13)	56	1.19	Diploid
LBN2 (J14)	91	1.94	Tetraploid
LBN2 (J17)	51	1.09	Diploid
Control(T309)	1027	1.00	Diploid
LBN4A (1)	803	0.78	"
LBN4A (2)	816	0.79	"
LBN4A (6)	1262	1.23	"
Control(T309)	185	1.00	Diploid
MSN1 (J3)	189	1.02	"
MSN1 (J4)	143	0.77	"
MSN1 (J6)	123	0.66	Aneuploid
MSN1 (J9)	128	0.69	"
MSN1B (20)	298	1.61	Triploid
MSN1B (21)	265	1.43	"
MSN1B (23)	337	1.82	Tetraploid
MSN1B (24)	296	1.60	Diploid
MSN1B (25)	334	1.81	Tetraploid
MSN1B (31)	244	1.32	Diploid
MSN1B (32)	296	1.60	Triploid
MSN1B (34)	285	1.54	"
MSN1B (37)	329	1.79	Tetraploid
Control(T309)	47	1.00	Diploid
MSN1 (J1)	98	2.09	Tetraploid
MSN1 (J2)	54	1.15	Diploid
MSN1 (J7)	62	1.32	"
MSN1 (J8)	44	0.94	"
Control(T309)	654	1.00	Diploid
MSN1B (5)	1295	1.98	Tetraploid
MSN1B (13)	1074	1.64	Triploid
MSN1B (22)	1441	2.20	Tetraploid
MSN1B (28)	753	1.15	Diploid
MSN1B (30)	1185	1.81	Tetraploid
MSN1B (33)	1208	1.85	"
MSN1B (43)	772	1.18	Diploid
MSN1B (45)	660	1.01	"
Control(T309)	676	1.00	"
MSN1B (46)	840	1.24	"
MSN1B (51)	1669	2.47	Pentaploid
MSN1B (55)	1185	1.75	Tetraploid
MSN1B (56)	911	1.35	Diploid
MSN3A (5)	710	1.05	"

Table 4B continued.....

Sample code[a]	Relative fluorescence	DNA index	Ploidy
Control(T309)	185	1.00	Diploid
MSN3A (6)	1061	1.57	Triploid
MSN3A (7)	900	1.33	Diploid
MSN3A (8)	1039	1.54	Triploid
LBCL(2)	213	1.15	Diploid
LBCL(3)	184	0.99	,,
LBCL(4)	209	1.13	,,
LBCL(5)	224	1.21	,,
JEN2 (2)	249	1.35	,,
JEN2 (4)	101	0.56	Haploid
Control(T309)	195	1.00	Diploid
MSCL (1)	187	0.96	,,
MSCL (2)	181	0.93	,,
MSCL (3)	193	0.99	,,
MSCL (4)	203	1.04	,,
MSCL (5)	212	1.09	,,
MSCL (6)	197	1.01	,,
MSCL (7)	189	0.97	,,
MSCL (8)	186	0.95	,,
MSCL (9)	215	1.10	,,
MSCL (10)	198	1.02	,,
MSCL (11)	203	1.04	,,
MSCL (12)	211	1.08	,,
MSCL (13)	207	1.06	,,
MSCL (14)	213	1.09	,,
Control(T309)	185	1.00	Diploid
AC3 (91)	105	0.57	Haploid
AC6 (91)	68	0.37	,,
AC7 (91)	58	0.31	,,
AC8 (91)	79	0.43	,,
AC15 (91)	73	0.39	,,
Control(T309)	512	1.00	Diploid
AC4A (1)	394	0.77	Haploid
AC4A (2)	395	0.77	,,
AC4A(3)	411	0.80	,,
AC4A(4)	413	0.81	,,
AC4A(5)	417	0.81	,,
AC4A(6)	419	0.82	,,
AC4A(7)	414	0.81	,,
AC4A(8)	418	0.82	,,
AC4A(9)	415	0.81	,,
AC4B(1)	403	0.79	,,
AC4B(2)	409	0.80	,,
AC4B(3)	441	0.86	,,
AC4B(4)	436	0.85	,,
AC4B(5)	434	0.85	,,
AC4B(6)	429	0.84	,,
AC4B(7)	425	0.83	,,

Table 4B continued.....

Sample code[a]	Relative fluorescence	DNA index	Ploidy
Control(T309)	512	1.00	Diploid
AC4B(8)	430	0.84	Haploid
AC4B(9)	432	0.84	,,
AC4B(10)	425	0.83	,,
AC4B(11)	438	0.85	,,
AC4B(12)	433	0.85	,,
AC4B(13)	428	0.84	,,
AC4B(14)	429	0.84	,,
AC4B(15)	428	0.84	,,
AC4C(1)	431	0.84	,,
AC4C(2)	434	0.85	,,
Control(T309)	518	1.00	Diploid
AC4C(3)	429	0.83	Haploid
AC4C(4)	427	0.82	,,
AC4C(5)	425	0.82	,,
AC4C(6)	413	0.80	,,
AC4C(7)	422	0.81	,,
AC4C(8)	420	0.81	,,
AC4C(9)	413	0.80	,,
AC4C(10)	417	0.81	,,
AC4C(11)	423	0.82	,,
AC4D(1)	403	0.78	,,
AC4D(2)	411	0.79	,,
AC4D(3)	427	0.82	,,
AC4D(4)	433	0.84	,,
AC4D(5)	433	0.84	,,
AC4D(6)	429	0.83	,,
AC4D(7)	418	0.81	,,
AC4D(8)	425	0.82	,,
AC4D(9)	422	0.81	,,
AC4D(10)	424	0.82	,,
AC4D(11)	420	0.81	,,
AC4D(12)	426	0.82	,,
AC4D(13)	426	0.82	,,
AC4D(14)	428	0.83	,,
AC4D(15)	420	0.81	,,
AC4D(16)	425	0.82	,,
AC4D(17)	420	0.81	,,
AC4D(18)	426	0.82	,,
AC4D(19)	426	0.82	,,
AC4D(20)	422	0.81	,,
AC4D(21)	426	0.82	,,
AC4D(22)	424	0.82	,,
AC4D(23)	420	0.81	,,
AC4D(24)	425	0.82	,,
AC4D(25)	412	0.80	,,
AC4D(26)	424	0.82	,,
AC4D(27)	425	0.82	,,

Table 4B continued.....

Sample code[a]	Relative fluorescence	DNA index	Ploidy
Control(T309)	518	1.00	Diploid
AC4D(28)	425	0.82	Haploid
AC4D(29)	418	0.81	"
AC4D(30)	424	0.82	"
AC4D(31)	417	0.81	"
AC4D(32)	423	0.82	"
AC4D(33)	419	0.81	"
AC4E(1)	396	0.76	"
AC4E(2)	395	0.76	"
AC4E(3)	396	0.76	"
AC4E(4)	397	0.77	"
AC4E(5)	398	0.77	"
AC4E(6)	402	0.78	"
AC4E(7)	392	0.76	"
AC4E(8)	402	0.78	"
AC4E(9)	394	0.76	"
AC4E(10)	400	0.77	"
AC4E(11)	397	0.77	"
Control(T309)	512	1.00	Diploid
AC4E(12)	426	0.83	Haploid
AC4E(13)	416	0.81	"
AC4E(14)	413	0.81	"
AC4E(15)	416	0.81	"
AC4E(16)	415	0.81	"
AC4E(17)	411	0.80	"
AC5A(1)	399	0.78	"
AC5A(2)	398	0.78	"
AC5A(3)	398	0.78	"
AC5A(4)	404	0.79	"
AC10B(1)	400	0.78	"
AC10B(2)	397	0.78	"
AC10B(3)	405	0.79	"
AC10B(4)	402	0.79	"
AC10B(6)	398	0.78	Haploid
AC10B(7)	399	0.78	"
AC10B(8)	404	0.79	"
AC10B(9)	399	0.78	"
AC10B(10)	404	0.79	"
AC10B(11)	400	0.78	"
AC10B(12)	402	0.79	"
AC10B(13)	405	0.79	"
Control(T309)	533	1.00	Diploid
AC10B(5)	429	0.80	"
AC10B(14)	416	0.78	Haploid
AC10B(15)	430	0.81	"
AC10B(16)	424	0.80	"
AC10B(17)	425	0.80	"
AC10B(18)	422	0.79	"

Table 4B continued.....

Sample code[a]	Relative fluorescence	DNA index	Ploidy
Control(T309)	533	1.00	Diploid
AC10B(19)	425	0.80	Haploid
AC10B(20)	422	0.79	,,
AC10B(21)	425	0.80	,,
AC10B(22)	417	0.78	,,
AC10B(23)	425	0.80	,,
AC10B(24)	435	0.82	,,
AC10B(25)	425	0.80	,,
AC10B(26)	420	0.79	,,
AC10B(27)	426	0.80	,,
AC10B(28)	425	0.80	,,
AC10B(29)	429	0.80	,,
AC10B(30)	428	0.80	,,
AC10B(31)	429	0.80	,,
AC10B(32)	426	0.80	,,
AC10B(33)	427	0.80	,,
AC10B(34)	431	0.81	,,
AC10B(35)	427	0.80	,,
Control(T309)	518	1.00	Diploid
AC10B(36)	425	0.82	Haploid
AC10B(37)	433	0.84	,,
AC10B(38)	432	0.83	,,
AC10B(39)	433	0.84	,,
AC10B(40)	431	0.83	,,
AC10B(41)	426	0.82	,,
AC10B(42)	416	0.80	,,
AC10B(43)	432	0.83	,,
AC10B(44)	429	0.83	,,
AC10B(45)	429	0.83	,,
AC10B(46)	425	0.82	,,
AC10B(47)	432	0.83	,,
AC10B(48)	430	0.83	,,
AC10B(49)	430	0.83	,,
AC10B(50)	426	0.82	,,
AC11A(1)	425	0.82	,,
AC11A(2)	424	0.82	,,
AC11A(3)	429	0.83	,,
AC11A(4)	430	0.83	,,
Control(T309)	508	1.00	Diploid
AC11D(1)	356	0.70	Haploid
AC11D(2)	357	0.70	,,
AC11D(3)	360	0.71	,,
AC11D(4)	356	0.70	,,
AC11D(5)	357	0.70	,,
AC11D(6)	358	0.70	,,
Control(T309)	512	1.00	Diploid
AC12B(1)	504	0.98	,,
AC12B(2)	504	0.98	,,

Table 4B continued.....

Sample code[a]	Relative fluorescence	DNA index	Ploidy
Control(T309)	512	1.00	Diploid
AC12B(3)	505	0.99	,,
AC12B(4)	500	0.98	,,
AC12B(5)	499	0.97	,,
AC12B(6)	503	0.98	,,
AC12B(7)	499	0.97	,,
AC12B(8)	497	0.97	,,
AC12B(9)	504	0.98	,,
AC12B(10)	511	1.00	,,
AC12B(11)	500	0.98	,,
AC12B(12)	509	0.99	,,
AC12B(13)	506	0.99	,,
Control(T309)	501	1.00	Diploid
AC12B(14)	458	0.91	,,
AC12B(15)	458	0.91	,,
AC12B(16)	468	0.93	,,
AC12B(17)	459	0.92	,,
Control(T309)	493	1.00	Diploid
AC12C(1)	509	1.03	,,
AC12C(2)	505	1.02	,,
AC12C(3)	501	1.02	,,
AC12C(4)	512	1.04	,,
AC12C(5)	504	1.02	,,
AC12C(6)	498	1.01	,,
AC12C(7)	507	1.03	,,
AC12C(8)	511	1.04	,,
AC12C(9)	502	1.02	,,
AC12C(10)	495	1.00	,,
AC12C(11)	508	1.03	,,
AC12C(12)	510	1.03	,,
AC12C(13)	511	1.04	,,
AC12C(14)	515	1.04	,,
AC12C(15)	515	1.04	,,
AC12C(16)	507	1.03	,,
AC12C(17)	506	1.03	,,
AC12C(18)	504	1.02	,,
Control(T309)	501	1.00	Diploid
AC12C(19)	461	0.92	,,
AC12C(20)	454	0.91	,,
AC12C(21)	456	0.91	,,
AC12C(22)	458	0.91	,,
AC12C(23)	457	0.91	,,
AC12D(1)	461	0.92	,,
AC12D(2)	462	0.92	,,
AC12D(3)	444	0.89	,,
AC12D(4)	456	0.91	,,
AC12D(5)	460	0.92	,,
AC12D(6)	456	0.91	,,

Table 4B continued.....

Sample code[a]	Relative fluorescence	DNA index	Ploidy
Control(T309)	87	1.00	Diploid
AC12D(7)	453	0.90	"
AC12D(8)	452	0.90	"
AC12E(1)	457	0.91	"
AC12E(2)	458	0.91	"
AC12E(3)	453	0.90	"
AC12E(4)	451	0.90	"
AC4D(P1)	159	1.83	Tetraploid
AC4D(P2)	189	2.17	"
AC4D(P3)	183	2.10	"
AC4D(P5)	191	2.20	"
AC4D(P6)	190	2.18	"
Control(T309)	93	1.00	Diploid
AC4C(P1)	182	1.96	Tetraploid
AC4C(P2)	180	1.94	"
AC4C(P3)	176	1.89	"
AC4C(P4)	181	1.95	"
AC4C(P5)	178	1.91	"
AC4C(P6)	180	1.94	"
AC4C(P7)	179	1.92	"
AC4C(P8)	177	1.90	"
AC4C(P9)	182	1.96	"
AC4C(P10)	178	1.91	"
AC4C(P11)	176	1.89	"

[a]LBN - protoplast-derived plant (LB-origin); MSN - protoplast-derived plant (MSE-origin); JEN - callus-derived plant (ISE-origin); MSCL - callus-derived plant (MSE-origin); AC - callus-derived plant (AN-origin); AC4D(P1) or AC4C(P1) - protoplast-derived plant (AN-origin).

Table 4C Determination of ploidy level by flow cytometry and estimation of percent ploidy from callus and protoplast-derived plants using different explants of *Japonica* rice cultivar Taipei 309

Explant source	*In vitro* regenerated plants from									
	Callus				Protoplast[b]					
	Haploid		Diploid		Diploid		Triploid		Tetraploid	
	No.	%	No.	%	No.	%	No.	%	No.	%
AN[a]	154	74.8	52	25.2	-	-	-	-	17	100.0
MSE[a]	-	-	20	100.0	14	42.4	7	21.2	9	27.3
ISE[a]	1	50.0	1	50.0	-	-	-	-	-	-
LBM[a]	-	-	4	100.0	8	34.8	5	21.7	10	43.5

[a]AN = anther; MSE = mature seed-embryos; ISE = immature seed-embryos; LBM = leaf-base meristems.

[b]One pentaploid (3.0%) and two aneuploid plants (6.1%) were also regenerated from cell suspension-derived protoplast culture (MSE-origin).

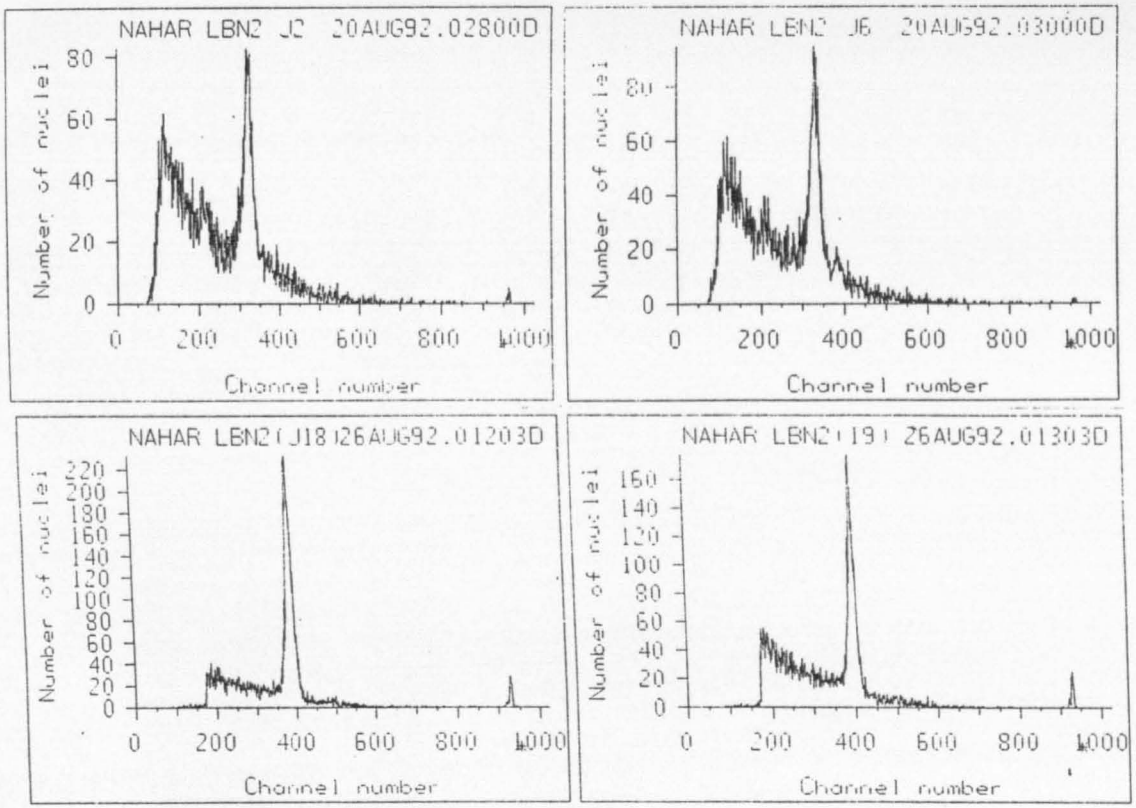


Fig 4.1a. Flow cytometric analysis of nuclear DNA content of triploid plants regenerated from protoplasts, which were isolated from LBM-derived cell suspensions. Nuclei were isolated from freshly chopped leaves and stained with propidium iodide. An aliquot of 20 µl fluorescently labelled beads was mixed with each sample before analysis.

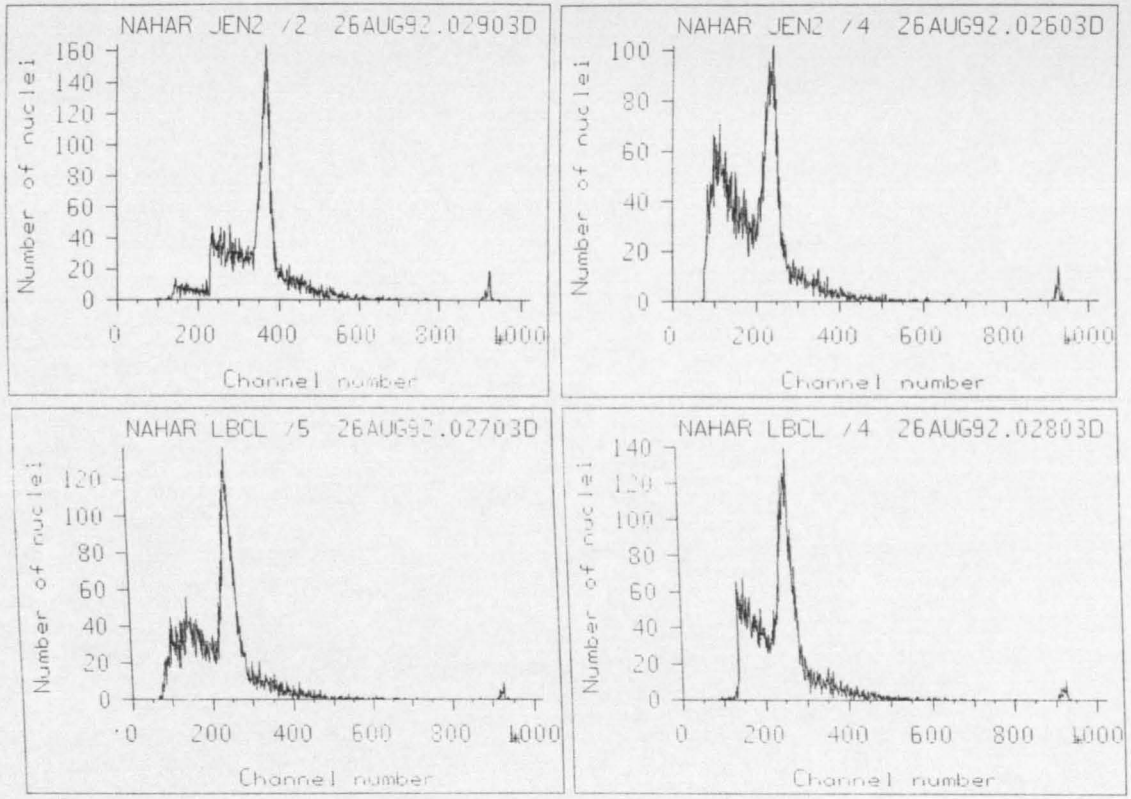


Fig 4.1b. Flow cytometric analysis of nuclear DNA content of diploid and haploid plants regenerated from embryogenic callus culture [ISE-origin (JEN2) and LBM-origin (LBCL)]. The other procedures for isolation, staining and analysis of the nuclei were followed the same.

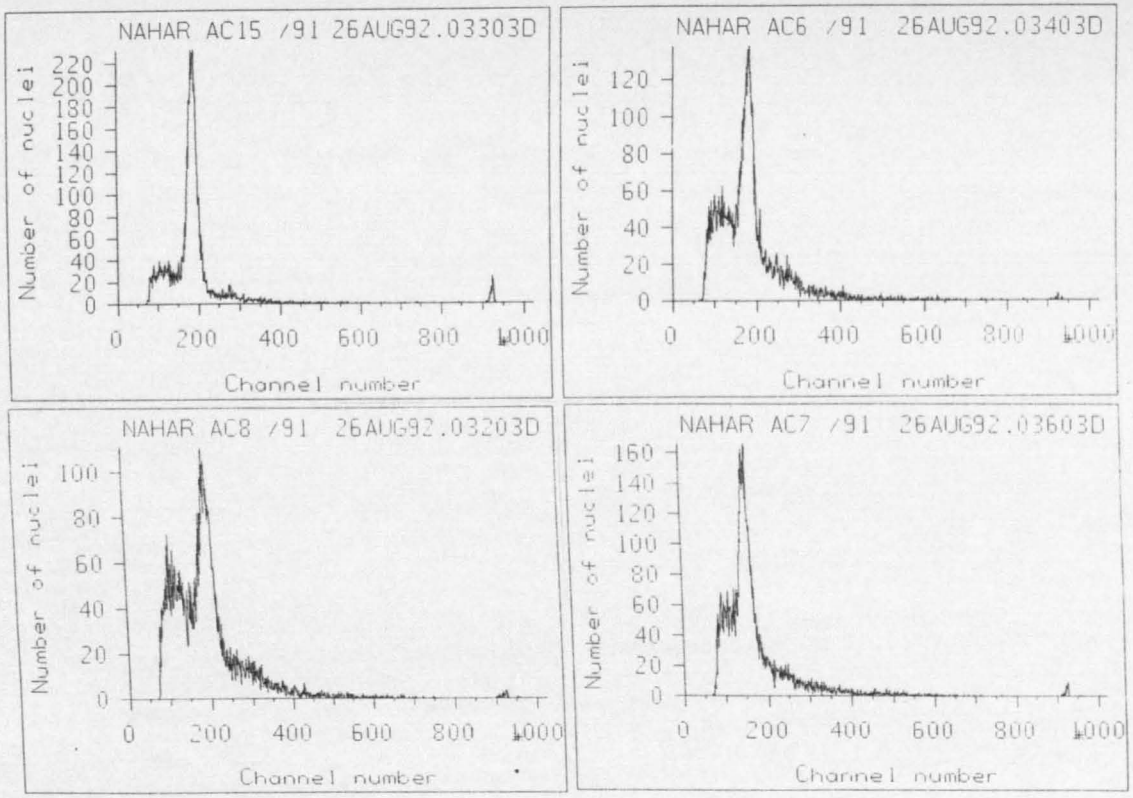


Fig 4.1c. Flow cytometric analysis of nuclear DNA content of haploid plants regenerated from embryogenic callus culture [anther-origin (AC)]. The other procedures for isolation, staining and analysis of the nuclei were followed the same.

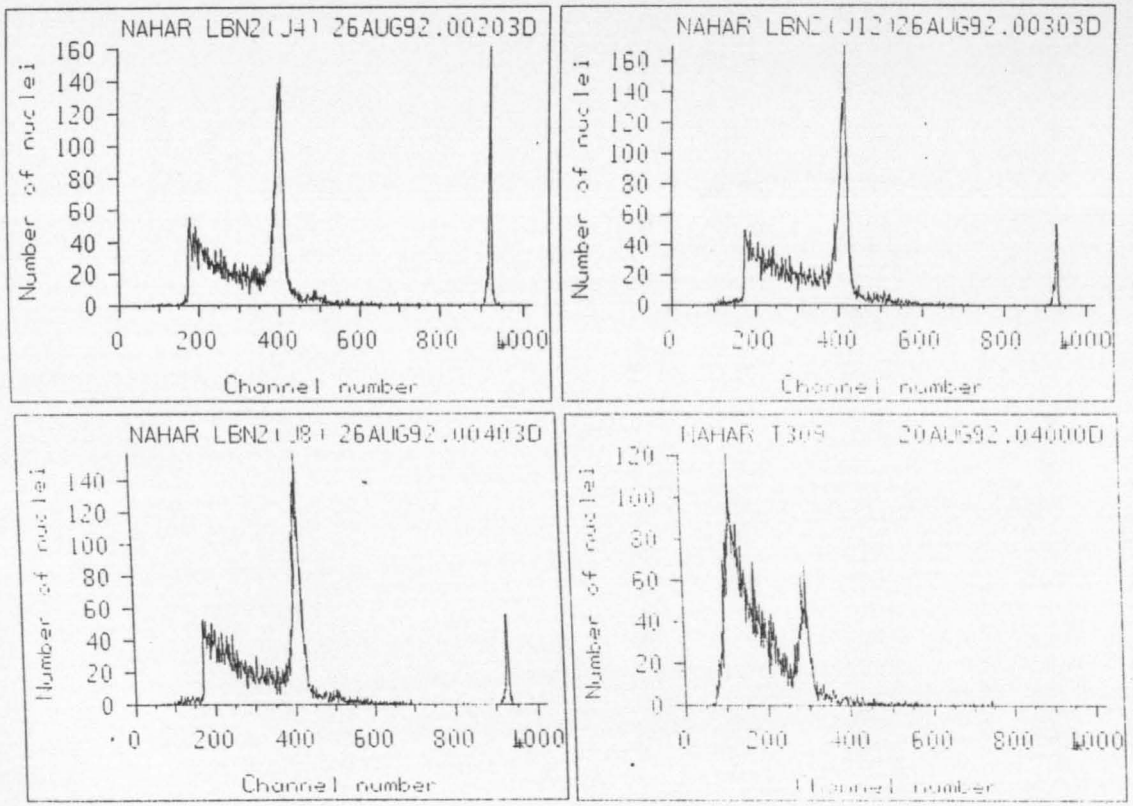


Fig 4.1d. Flow cytometric analysis of nuclear DNA content of tetraploid plants regenerated from protoplasts, which were isolated from LBM-derived cell suspensions compared to diploid Taipei 309 plants grown from seeds. The other procedures for isolation, staining and analysis of the nuclei were followed the same.

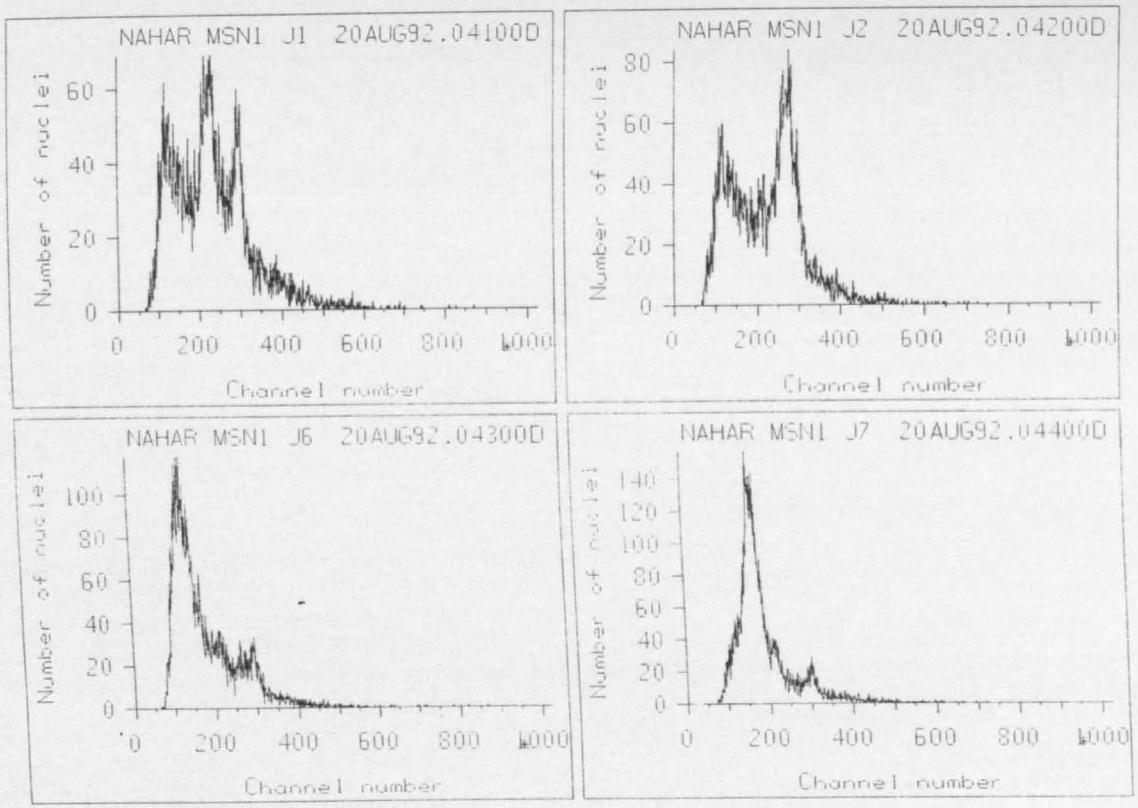


Fig 4.1e. Flow cytometric analysis of nuclear DNA content of regenerated plants from protoplasts having different ploidy levels, which were isolated from MSS-derived cell suspensions. The other procedures for isolation, staining and analysis of the nuclei were followed the same.

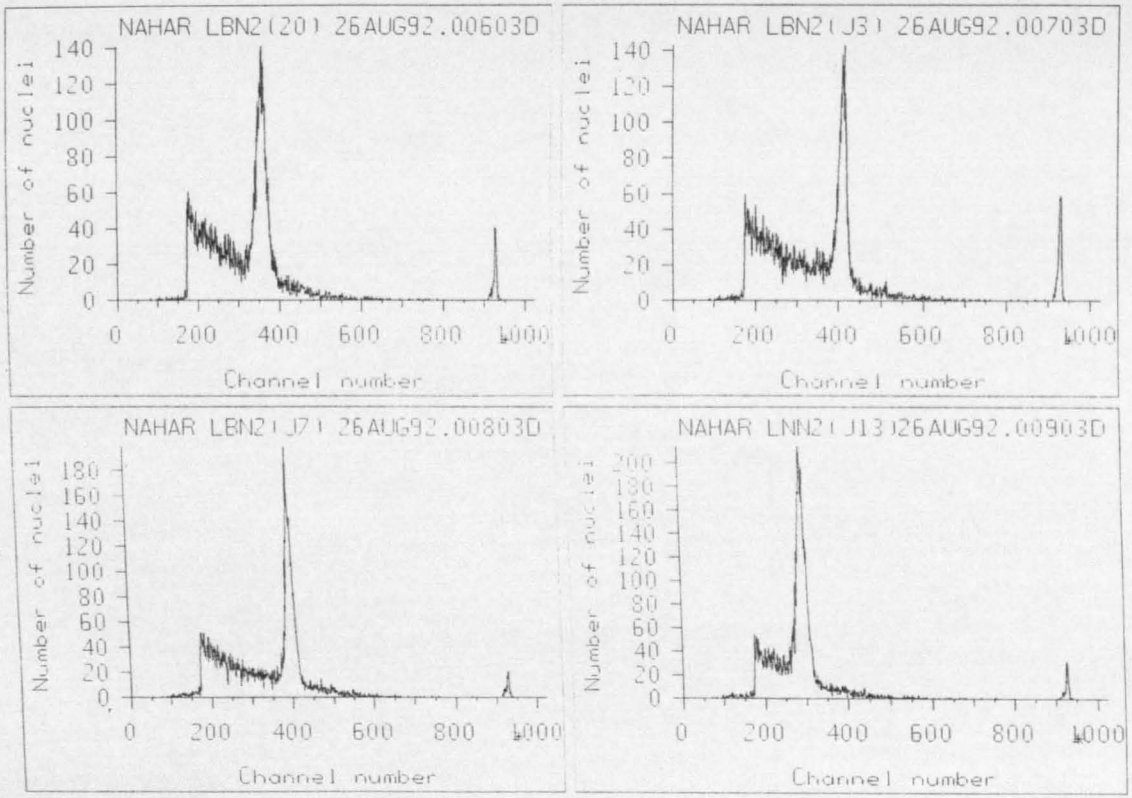


Fig 4.1f. Flow cytometric analysis of nuclear DNA content of regenerated diploid and tetraploid plants from protoplasts, which were isolated from LBM-derived cell suspensions. The other procedures for isolation, staining and analysis of the nuclei were followed the same.

Fig 4.2 Percentage of haploid and diploid plants obtained from callus culture of different explants of Japonica rice cultivar (T309)

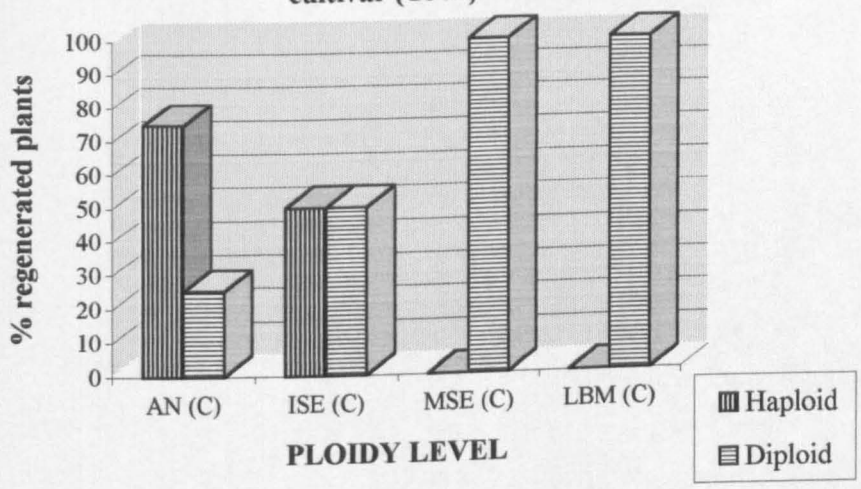
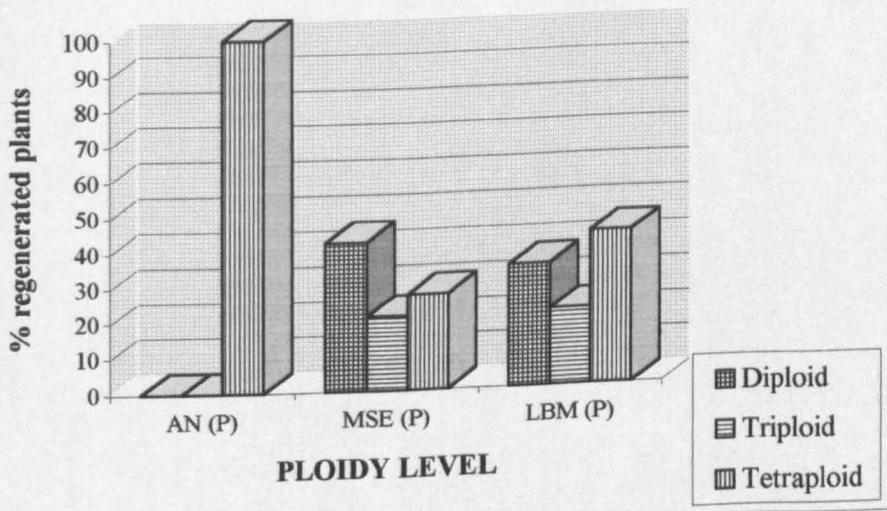


Fig 4.3 Percentage of haploid, diploid and tetraploid plants obtained from protoplast culture using different explants (T309)



Chapter 5

Rice Chromosomes and the counting of chromosomes from root-tip squash preparations

5.1 Introduction

The culture of plant tissues and cells in phytohormone-containing media induces the formation of an unorganized tissue mass, the callus. It is known to be genetically variable. The genetic instability of callus cells has been characterized by the variation of chromosome number. The chromosome number variation has been observed not only in callus cells, but also in callus-derived regenerated plants. The chromosomal constitution of cultured tissues, anther-derived regenerants, protoplast-derived regenerants, somatic hybrids and genetically engineered plants has been studied during the last three decades (Ogura 1990). The cause of chromosome number variation, especially of chromosome number reduction was assumed to be due to nuclear fragmentation at the time of callus initiation (Bennici and D'Amato 1978). However, several factors are considered to play important roles in the chromosomal constitution of cultured tissues and cells. These are :

- nuclear condition of the original explants;
- compositions of the medium, for example, kinds and/or concentrations of the plant growth regulators;
- age of the culture;
- variation due to plant species
- karyotype changes

Auxins and cytokinins, which were used to promote cell division, also caused chromosome number variations even at the concentrations usually used in plant tissue culture media. Cells of many plant species are aneuploids or polyploids during culture. The degrees of aneuploidy or polyploidy due to chromosome number variation seem to be dependent on the particular species cultured. Chromosome structure or karyotype changes of different types, for example, deficiencies, translocations, isochromosomes, dicentric chromosomes are also common in cultured tissues and cells. Although a

considerable number of plant regeneration studies by somatic embryogenesis in rice have been reported until now, but only a few studies on cytological analyses of the regenerants have been performed. In 1981, Wernicke et al. reported eudiploid regenerants derived from rice leaves. It was also described that how the plants regenerated through somatic embryogenesis from cultured immature embryos of 4 wheat cultivars exhibited chromosome number variation(2n = 42) and gave rise to 29% aneuploids (Karp and Maddock 1984). In another study, she and her group described extensive numerical and structural chromosomal variations in dividing protoplasts of bread wheat.

Chromosomal variation observed in the regenerants also constitutes an important part of somaclonal variation. Chen and Lin (1976) observed 23 tetraploids among 165 regenerated plants of 5 *Japonica* varieties of rice. Polyploid and aneuploid regenerants derived from rice embryo callus were also identified (Bajaj and Bidani 1980, 1986). Chu et al. (1985) obtained a mean frequency of 10.2% aneuploids from 1715 plants regenerated by anther culture. Ogura et al. (1987) examined cytologically 126 regenerants derived from protoplast culture of 4 rice cultivars and found 12 chromosome variants which was 9.5% of the total regenerants. Among these variants, 10 were tetraploids, 1 triploid, 1 aneuploid (trisomic, 2n = 25) and the remaining 114 plants were diploids. In another study, the chromosome number was determined in 7 regenerated rice plants, obtained from protoplasts of 7-10 months old callus and found 4 of them tetraploids (4n = 48), 2 aneuploids (4n = 46) and 1 diploid (2n = 24). According to Kanda et al. (1988), the high frequency regeneration of chromosomal variants may presumably be associated with the age of the callus.

Haploid and diploid plants were also regenerated from protoplasts isolated from anther calli-derived cell suspension cultures (Toriyama et al. 1986). Rice chromosomes were observed in protoplast-derived calli of two cultivars (*Japonica*) and in plants regenerated from the calli of five cultivars (*Japonica*). In 43 regenerants observed in the 4 cultivars including 'Nipponbare', 42 were diploids; whereas in the cultivar 'Iwaimochi' tetraploid plants were regenerated at a higher frequency (54.5%), compared to the other four cultivars (2.3%) (Nishibayashi et al. 1989).

The present study was undertaken to establish an efficient method of chromosome squash preparation from meristematic root-tips of *in vitro* grown regenerated rice plants obtained through various culture systems. The chromosome squash preparation from root-tips of *in vitro* seed-grown seedlings was also performed to compare and to use as standards with known ploidy and diploid number of chromosomes (2n = 24). The aims of the work described in this Chapter were :

- to investigate an efficient method of root-tip squash preparation for rice chromosomes;
- to determine the ploidy level of some samples of regenerated plants obtained from various culture systems *in vitro* by counting chromosomes;
- to confirm the results obtained from flow cytometry DNA analysis for the determination of ploidy level.

5.2 Materials and methods

5.2.1 Source of working materials

Seedlings of rice cultivar Taipei 309 were grown *in vitro* at least for 15-20 days from seeds and micropropagated again for 2-3 weeks under cool, white, fluorescent light (1000 lux) in the culture room. Their freshly grown young roots were used for root-tip squash preparation and also as standard of known ploidy level and chromosome number (diploid, 2n = 24). Freshly grown roots from regenerated rice plantlets obtained by callus and protoplast cultures, which have been discussed in Chapter 2, Part I & II, and Chapter 3, Part I & II, were used as major experimental materials.

5.2.2 Preparation of chemicals, collection, fixation and storage of rice root-tip samples

Preparation of pre-treatment solutions before squash preparation

- 8-hydroxyquinoline (8-HQ) : A few drops of 95% ethanol were added to 29 mg of 8-HQ which was then diluted to 100 ml with distilled water (effective concentration 2 mM). The solution was incubated at 60°C until granules had completely dissolved (4-6 hr) and kept for up to one week at 20°C. The root-tip cells were incubated in this solution for 1-4 hr at room temperature.

- α-bromonapthalene (α-BN) stock : One ml of α-BN was added to 100 ml of absolute alcohol, shaken vigorously and kept at room temperature. Before use, the fresh pre-treatment solution was prepared at the ratio of 1 µl stock solution to 1 ml distilled water at room temperature.
- Enzymatic mixture : 4% Cellulase Onozuka RS (Yakult Honsha Co. Ltd. Tokyo) and 1% Pectolyase Y-23 (Seishin Pharmaceutical Co. Ltd. Tokyo) were dissolved in 100 ml distilled water, pH adjusted to 4.2, filtered and stored in the deep-freezer.

Preparation of stain for staining rice chromosomes

The commercial products, for example, Feulgen stain (Schiff's reagent), Aceto-carmine, Acetic orcein and Giemsa stain were purchased from BDH, UK which were used as standard stains for plant chromosomes by many workers. Only Carbol-fuchsin stain was prepared in the laboratory according to Kao (1975) and further modified to obtain better result for rice chromosomes.

- Carbol-fuchsin stain : Stock A-3% basic fuchsin in 70% ethanol
  Stock B-1 to 9 dilution of stock A in 5% aqueous phenol
  Working Stock C-45 ml of stock B plus 6 ml glacial acetic acid plus 6 ml 37% aqueous formaldehyde (formalin)

A modified version of the carbol-fuchsin stain was a 25% solution of the working stock C in 45% acetic acid and 1.8% sorbitol.

Preparation of fixative, solutions for hydrolysis and washing

Fresh fixative was prepared every time before use at the ratio of 3:1, absolute alcohol/95% ethanol : glacial acetic acid. For hydrolysis of roots by incubation at 60°C, 1N HCL was prepared. For washing rice roots, 50% methanol in distilled water was prepared and used.

Collection, pre-treatment, fixation and storage of rice roots

Freshly grown root samples, when they were around 1-2 cm in length growing down to base of the jar, were collected from both seed-grown and regenerated plant samples of known ploidy levels, i.e. haploids, diploids and tetraploids, which were previously determined by DNA analysis on the flow cytometer. All the plant samples were grown *in vitro*, before collection of root samples they were subcultured at least 2-3 weeks

back to get freshly grown roots for each experiment. During subculture for micropropagation, the depth of semi-solid MSN1.5 agarose medium in the jar was ensured (60 ml/jar), so that the roots would not wrap around the base of the jar. For a large plant population which had the same ploidy levels, root samples were collected from 5 plants (maximum) and for a small population, from 1 or 2 plants.

Twenty five to thirty roots (1.5 cm long) were collected from each plant directly into pre-treatment solution from culture. For pre-treatment in 8-HQ, they were treated for 3-4 hr at 20°C and then transferred to fixative. The samples were fixed at least for 24 hr, 2-3 days fixation was better or can be stored for indefinite period before use at 4-6°C in the fridge. For the pre-treatment in α-BN in alcohol, they were treated at least overnight at 4°C in the refrigerator and then transferred to fixative for storage.

For maceration of root-tips in enzyme mixtures, no pre-treatment was applied in order to obtain natural size and shape of rice chromosomes. They were collected in distilled water, fixed immediately in the fixative for 1 hr and then transferred into glacial acetic acid for indefinite storage in the freezer (-20°C) before use.

5.2.3 Use of different root-tip squash preparation techniques

Three different root-tip squash preparation techniques were followed for achieving good spread and staining quality of rice chromosomes. The first method included preparation of root-tip squashes after pre-treatment in either 8-HQ or α-BN and staining with aceto-carmine or acetic orcein or Feulgen (Schiff's reagent). The second method included maceration of root-tips in enzyme mixture before squash preparation and staining with modified carbol-fuchsin stain. The third method was followed exactly like the second one, but staining was performed with Giemsa stain.

The protocols used for the first method :

- The pre-treated and fixed root samples were hydrolysed in 1N HCL at 60°C for 6-10 min.
- After brief washing in distilled water, one root sample was placed on a clean slide for one slide preparation. The root-cap was first removed, then the meristematic region (2-3 mm from the tip) of the root sample was cut and the rest was discarded.

- Following addition of a drop of 45% acetic acid, the root-tip sample was finely chopped with a scalpel blade and thoroughly tapped until dispersed. Any remaining large pieces were removed and a drop of aceto-carmine or acetic orcein was then mixed with the fine tissue pieces.
- A clean coverslip was carefully placed and pressed dry with filter paper. Then the fine root-tip tissues were squashed vertically with the thumb. Make sure that the coverslip should not be moved from side to side during pressing.
- The preparation was examined using a Carl-Zeiss photomicroscope and squashed again if necessary.
- When a good spread was obtained, the coverslip was sealed with transparent nail-varnish. The slide sample was re-examined after the varnish was dry to count the number of chromosomes.
- In case of Feulgen stain, all the root samples were dipped in 1-2 ml of ready-made commercial stain after hydrolysis and before approaching squash preparation.

The protocols used for the second method :

- Fixed roots which were stored in glacial acetic acid were thawed in water bath at 37°C, thoroughly washed in 50% methanol and then in distilled water for 10-20 min by shaking frequently. No pre-treatment was applied before fixation to obtain natural chromosomes.
- As in the first method, the meristematic regions of the root samples were cut on a glass slide and placed into an Eppendorf tube containing 0.5 ml of melted enzyme mixture for maceration at 37°C for 60-70 min.
- After maceration, the enzyme mixture was removed and the root-tips were thoroughly washed with distilled water 4-5 times and subjected to a hypotonic treatment in distilled water for 15-20 min.
- One macerated root-tip was placed on a clean glass slide and tapped with a small stainless steel rod after adding 2-5 drops of fixative successively until the tissue was spread invisibly. All visible tissues and cell clusters were removed from the slide.
- Before placing the coverslip on the slide, one drop of modified carbol-fuchsin stain was added to the spread tissues and thoroughly mixed. Use of excess stain was always avoided to prevent the flow of isolated cells from under the coverslip.

- After sealing the coverslip with nail-varnish, each slide was ready for microscopic observation (to count the number of chromosomes and to take photograph).

The protocols used for the third method was similar except the staining procedure with Giemsa stain :

- In case of Giemsa staining, the slide sample was air-dried, dipped into 100% methanol for 30 sec and then stained with 4% Giemsa solution in phosphate buffer (pH 6.8) for 30 min.
- After staining, the slide sample was washed with tap water, air-dried and ready for microscopic observation. Slides were prepared for all the root-tips of each sample plant using different preparation techniques and observed using a Carl-Zeiss photomicroscope for counting the chromosome number, taking photograph and determining ploidy level.

5.2.4 Ploidy level determination of *in vitro* grown regenerated plants by flow cytometry and confirmation of the results by chromosome counting

Ploidy levels of all the *in vitro* grown regenerated rice plants obtained from various culture systems were determined by measuring the nuclear DNA content of leaf nuclei using a flow cytometer (Coulter EPICS 541). The results and the analysis have been discussed in Chapter 4. For confirmation of the ploidy level obtained from the flow cytometer, attempts were made to analyse and to prepare root-tip squashes from young healthy rice roots grown *in vitro*. To make these attempts successful, different pre-treatment procedures and squash preparation techniques (section 5.2.3) had been used.

5.3 Results and discussions

5.3.1 Problems encountered with rice chromosome spread and staining

In order to obtain good rice chromosome samples suitable for chromosome identification and counting to determine the ploidy level of the test plant, good chromosome spreads at the mitotic pro-metaphase stage must be collected. For cytogenetic or cytological work, some chemical compounds like Colchicine, 8-hydroxyquinoline, α-bromonaphthalene, *p*-dichlorobenzene are commonly employed.

These compounds are used to produce high frequencies of mitotic figures, chromosome contraction and good separation of chromosomes.

In case of rice chromosomes, the specific problem encountered was too much contraction, loss of natural morphology and artificial modification after pre-treating them with either 8-HQ or α-BN. Fukui and Mukai (1988) concluded with three main problems after their detailed study on rice chromosomes; these were :

- Besides the smallness of chromosome size, chromosome shapes were sometimes similar to each other, making karyotype analysis difficult.
- Stainability of small chromosomes was not good, because of very low amount of DNA content, particularly in *Oryza* species (1.2 pg) compared to wheat (34.6) (Bennett and Smith 1976).
- The third problem was specific to plants with cells having thick cell wall, which prevented a good spread of chromosomes by squash method. Moreover, tissues of *Oryza* species are siliconized which make them more rigid.

During pretreatment, the rice chromosomes contract until they are the same size as *E. coli* (1-2 μm) as the mitotic stage proceeds, so it was very difficult to identify each chromosome at mitosis (Fukui 1986a). Therefore, the pre-treatment of rice roots either in 8-HQ or α-BN was avoided in order to obtain the natural morphology in the present study.

Hydrolysis in 1N HCL at 60°C for 6-10 min, which is a routine step to soften root-tip tissues, also did not help to soften the siliconized tissues and thick cell wall of rice root-tips. Therefore, instead of following hydrolysis step, the rice root samples were just stored in glacial acetic acid and kept in the freezer (-20°C), not in the fridge (4°C) to make the tissues soft after thawing. Enzymatic maceration was important to digest cell wall and soften the root-tip tissues, which made the squash preparation easy with a good and clear spread of chromosomes without cytoplasmic debris.

Staining of rice chromosomes was very difficult by using conventional stains, such as aceto-carmine, acetic orcein, Feulgen and even Giemsa stain, which stained not only the chromosomes but the cytoplasm as well. These stains made the chromosomes partially or completely invisible for counting, specially because of very low amount of

DNA content in rice chromosomes (1.2 pg/2C). On the other hand, addition of modified carbol-fuchsin stain on the slide would stain the chromosomes only, leaving the cytoplasm transparent and making the chromosomes more clearly visible for counting under microscope (Fig. 5a & Fig. 5e).

5.3.2 New protocol for rice chromosome preparation

The new procedure, which was established for root-tip squash preparation and staining of rice chromosomes in this study, was different from most other protocols (Gupta and Ghosh 1983; Sree Ramulu et al 1986; Nishibayashi et al. 1989). After fixation of root samples in fixative for 1 hr (section 5.2.2), they were transferred to glacial acetic acid and stored in the freezer (-20°C) until use. The enzymatic maceration of samples was performed in Eppendorf tubes, and incubated in water-bath at 37°C rather than in a moist chamber. Sometimes, 60-70 min maceration was necessary in order to obtain a good spread of chromosomes. Tapping of the macerated root-tip on the slide was performed in fixative with the smooth surface of a stainless steel rod instead of a fine forcep to spread the tissue completely invisible. After removing visible cell clusters, one drop of modified carbol-fuchsin stain (25% of the working stock C) was added to the root-tip suspension and mixed thoroughly. After staining for 8-10 min, a clean coverslip was placed carefully and pressed dry with filter paper. Excess use of stain was always avoided. After sealing the coverslip with nail varnish, the preparation was observed using the photomicroscope to count the chromosome number and to take photograph.

5.3.3 Confirmation of flow cytometry results by chromosome counting

The ploidy level determination of *in vitro* grown regenerated rice plants, which was performed by measuring the nuclear DNA content of isolated nuclei from fresh leaf tissues using the flow cytometer, was confirmed by counting of chromosomes from root-tip squash preparations. A wide range of variability with different ploidy levels, for example, haploid, diploid, triploid, tetraploid, aneuploid and even pentaploid, was observed among the regenerated plants obtained from various culture systems (callus and protoplast).

Fig 5.1 Chromosome squash preparation from root-tip samples of regenerated plants derived from callus and protoplast cultures of *O. sativa*

a) The root -tip squash preparation from an anther callus-derived plant showing diploid number of chromosomes {2n = 24, AC12C (10), Table 5A} (x 70).

b) The chromosome preparation from root-tip of a mature seed-scutellum (MSS) callus-derived plant, demonstrating diploid number of chromosomes {2n = 24, MSCL(14)} (x 140).

c) The preparation from root-tip of *in vitro* grown seedling of Taipei 309, showing diploid number of chromosomes (2n = 24) (x 70).

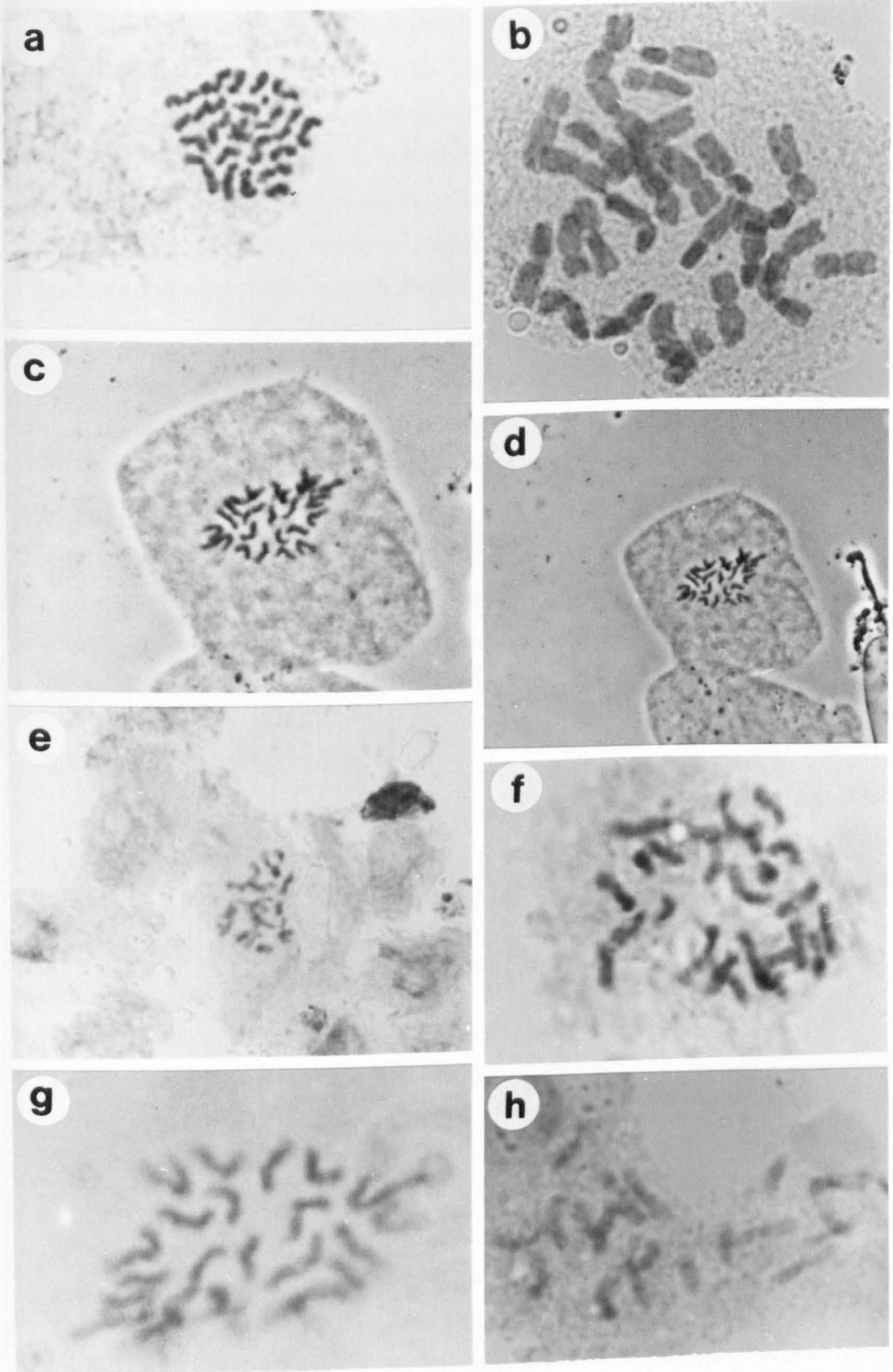
d) The sample in (c) at lower magnification showing 24 chromosomes (x 35).

e) The preparation of root-tip squash from an immature seed-embryo callus-derived plant, showing diploid number of chromosomes {2n = 24, IEN2(2)} (x 35).

f) Diploid number of chromosomes {2n = 24, LBN2(J20)} was obtained from root-tip cells of a protoplast-derived plant, where protoplast source was LBM-derived cell suspension (x 140).

g) The diploid plant MSN1B(45), which was regenerated from cell suspension-derived protoplast (MSS-origin), showing 24 chromosomes in root-tip cells (x 140).

h) One MSS-callus-derived plant, MSCL(9), was aneuploid showing 22 chromosomes in root-tip squash preparation (x 140).


a
b
c
d
e
f
g
h

The plants, regenerated from anther callus, predominantly gave rise to haploids (n = 12, 74.8%) and spontaneous diploids (2n = 24, 29.6%) where the number of chromosomes were duplicated spontaneously during the cultural process, possibly by endomitosis or nuclear fusion. It was interesting to note that the plants, which were derived from callus originated from immature seed-embryos (ISE), were haploids (45%) and diploids (55%), but those originated from mature seed-embryos (MSE) and leaf-base meristems (LBM) were all diploids (100%).

All the plants regenerated from cell suspension-derived protoplast culture, where the callus was initiated from anthers, were tetraploids (100%). The plants regenerated from cell suspension-derived protoplast culture, where the callus was initiated either from MSS/LBM, exhibited different ploidy levels, i.e. diploids (2n = 24, 42.4%/34.8%), triploids (3n = 36, 21.2%/21.7%), tetraploids (4n = 48, 27.3%/43.5%), aneuploids, and pentaploid (see Chapter 4, Graph 4.1 and 4.2). The occurrence of aneuploid (2 plants) and pentaploid (1 plant) were negligible. The results obtained from rice root-tip squash preparations to confirm ploidy levels of sample plants which had been analysed by flow cytometry, are presented in Table 5A.

5.4 Summary and conclusions

The results presented in this Chapter showed that both types of analyses (flow cytometry and chromosome) were equally important for the determination of ploidy level of *in vitro* grown regenerated rice plants obtained from callus, protoplast and anther culture systems. The most important achievement in the present study was that the results obtained from both types of analysis were similar, but flow cytometry analysis is rapid, accurate, convenient and sensitive for the measurement of nuclear DNA content of isolated nuclei. The results also indicated that flow cytometry could be employed to identify precisely those anther-derived plants that developed as haploids. On the other hand, chromosome counting technique required root meristematic regions for the identification of ploidy levels (haploids, diploids, triploids or tetraploids). This in turn required the establishment of root initiation and its healthy growth in *in vitro* culture before the ploidy can be determined. As rice chromosomes are very small (almost the same size as *E. coli*, 1-2 μm), having very low amount of DNA content (1.2 pg/2C), it is very difficult to identify each chromosome at mitosis.

From the experience of the author, it is observed that it is still not an easy task to identify the rice chromosomes objectively, because the identification method also depends much on personal skill and experience. After 4 months of tedious work, the first sample of chromosome squash preparation with a good spread and staining quality was achieved. In the present study, chromosomal variants were not obtained from MSS and anther-callus derived plants, whereas from cell suspension-derived protoplasts culture, these variants were frequently obtained when MSS or LBM-derived callus was used for suspension initiation. Among these variants, diploids, triploids, tetraploids, aneuploids and one pentaploid were obtained. It was assumed that a high frequency regeneration of chromosomal variants was presumably associated with many factors, possibly the age of the callus or the suspension cultures and also the age of the regenerated plants during analysis.

Table 5A Ploidy level determination of control and *in vitro* grown regenerated rice plants by flow cytometry and by chromosome counting

Sample code	Ploidy determination by	
	Flow cytometry	Chromosome counting
Control (T309)	Diploid	2n = 24, Diploid
LBN2 (J1)	,,	,,
LBN2 (J4)	Tetraploid	4n = 48, Tetraploid
LBN2 (J8)	,,	,,
LBN2 (J16)	Triploid	3n = 36, Triploid
LBN2 (J18)	,,	,,
LBN2 (J20)	Diploid	2n = 24, Diploid
LBN4A (2)	,,	,,
LBN4A (6)	,,	,,
Control (T309)	Diploid	2n = 24, Diploid
MSN1 (J1)	Tetraploid	4n = 48, Tetraploid
MSN1 (J2)	Diploid	2n = 24, Diploid
MSN1 (J3)	,,	,,
MSN1 (J7)	,,	,,
MSN1 (J8)	,,	,,
MSN1B (20)	Triploid	3n = 36, Triploid
MSN1B (22)	Tetraploid	4n = 48, Tetraploid
MSN1B (30)	,,	,,
MSN1B (32)	Triploid	3n = 36, Triploid
MSN1B (43)	Diploid	2n = 24, Diploid
MSN1B (45)	,,	,,
MSN3A (5)	,,	,,

Sample code	Ploidy determination by	
	Flow cytometry	Chromosome counting
Control (T309)	Diploid	2n = 24, Diploid
MSN3A (7)	,,	,,
MSN3A (6)	Triploid	3n = 36, Triploid
MSN3A (8)	,,	,,
JEN2 (2)	Diploid	2n = 24, Diploid
JEN2 (4)	Haploid	n = 12, Haploid
LBCL(2)	Diploid	2n = 24, Diploid
LBCL(3)	,,	,,
LBCL(4)	,,	,,
LBCL(5)	,,	,,
MSCL (1)	,,	,,
MSCL (3)	,,	,,
MSCL (6)	,,	2n = 26, Aneuploid
MSCL (9)	,,	2n = 22, Aneuploid
MSCL (12)	,,	2n = 24, Diploid
MSCL (14)	,,	,,
Control (T309)	Diploid	2n = 24, Diploid
AC4A (1)	Haploid	n = 12, Haploid
AC4A (2)	,,	,,
AC4B (3)	,,	,,
AC4B (5)	Haploid	n = 12, Haploid
AC4C (6)	,,	,,
AC4C (10)	,,	,,
AC4D (7)	,,	,,
AC4D (11)	,,	,,
AC4E (3)	,,	,,
AC4E (6)	,,	,,
Control (T309)	Diploid	2n = 24, Diploid
AC5A (2)	Haploid	n = 12, Haploid
AC5A (3)	,,	,,
AC10B (2)	,,	,,
AC10B (3)	,,	,,
AC10B (6)	,,	,,
AC10B (8)	,,	,,
AC10B (10)	,,	,,
AC10B (11)	,,	,,
AC10B (21)	,,	,,
AC10B (25)	,,	,,
AC10B (44)	,,	,,
AC10B (48)	,,	,,
Control (T309)	Diploid	2n = 24, Diploid
AC11A (1)	Haploid	n = 12, Haploid

Table 5A continued.....

Sample code	Ploidy determination by	
	Flow cytometry	Chromosome counting
Control (T309)	Diploid	2n = 24, Diploid
AC11A (4)	Haploid	n = 12, Haploid
AC11D (3)	,,	,,
AC11D (6)	,,	,,
AC12B (3)	Diploid	2n = 24, Diploid
AC12B (4)	,,	,,
AC12B (10)	,,	,,
AC12B (12)	,,	
AC12C (4)	,,	,,
AC12C (6)	,,	,,
AC12C (10)	,,	,,
AC12C (14)	,,	,,
AC12D (3)	,,	,,
Control (T309)	Diploid	2n = 24, Diploid
AC4D(P3)	Tetraploid	Tetraploid
AC4D(P6)	,,	,,
AC4C(P1)	,,	,,
AC4C(P5)	,,	,,
AC4C(P9)	,,	,,

LBN - protoplast-derived plant (LB-origin); MSN - protoplast-derived plant (MSE-origin); JEN - callus-derived plant (ISE-origin); MSCL - callus-derived plant (MSE-origin); LBCL - callus-derived plant (LB-origin); AC - callus-derived plant (AN-origin); AC4D(P3) or AC4C(P1) - protoplast-derived plant (AN-origin).

Chapter 6

The agronomic traits of plants regenerated from protoplasts compared with seed-derived plants

6.1 Introduction

Eukaryote genomes are in a dynamic state of continual change. In higher plants, this is most apparent under *in vitro* conditions where the amount of variability generated may be extensive. Spontaneous genetic variation and mutation breeding have been used to develop new plant varieties through conventional breeding. The success of any crop improvement programme depends on the extent of genetic variability in the base population. However, there is a lack of existing genetic variability in most of the agricultural crops. Extensive studies conducted during the last two decades have shown that cell cultures, especially during periodical subculturing, undergo various morphological and genetic changes (Meins 1983; D'Amato 1985). It was also observed that plants regenerated from somatic cells through tissue and protoplast cultures are not genetically uniform, but exhibit significant variability. This variability has been termed somaclonal variation, which can be utilized for new variety development (Evans and Sharp 1986).

In rice, the presence of variant regenerated plants has been found since plants were first regenerated from cultured cells. For example, the first morphological variants were obtained from seed-derived callus (Nishi et al. 1968), while albinos and polyploids were recovered from pollen callus (Niizeki and Oono 1968; Nishi et al. 1969). These variant regenerated plants were also frequently observed in rice anther culture studies. From a single pollen callus, plants were regenerated showing variation in height and panicle length (Oono 1975, 1984). Among rice plants regenerated from long term cell cultures, a high frequency of variation in the morphological characters and chromosome number was observed even after using an efficient protoplast culture technique (Kanda et al. 1988). Generally, to assess the application of this source of variation for plant breeding purposes, or to improve the genetic stability of a plant regeneration process for the benefit of further genetic manipulation, the origin and the

underlying genetic basis of variation must be determined. This is specifically needed where stable reproduction of a specific genotype is essential.

Phenotypic variation in plants regenerated from cultured cells have been observed to occur since the late 1960s (Skirvin 1978; Larkin and Scowcroft 1981). In the early 1980s, it was reported that the morphological changes observed in plants regenerated from cultured cells were simply physiological changes acquired during the culture of cells through many passages in subculture (Chaleff and Keil 1981). Phenotypic variations may also be induced by genomic, chromosomal, gene and cytoplasmic mutations and by physiological effects. The gene level of mutation in cultured tissues was observed in rice plants regenerated by anther culture. These plants had heritable characters of short culm or chlorophyll deficiencies (Oono 1975). The occurrence of somatic variation in plants regenerated by tissue culture were mostly found in plants normally propagated vegetatively. Specially, in cereal crops, the desirable mutations in plant breeding and in mutation studies are not chromosomal mutations but gene mutations. Analysis of regenerated plants from diploid rice calli confirmed that spontaneous mutations were frequently induced in cultured cells (Oono 1983). After classification of each character, it was shown that reduction of plant height and seed fertility did not influence the gene frequencies of qualitative and quantitative characters.

Mutation by somatic cell culture seems to be very useful for broadening genetic variability. For agricultural applications, efficient methods for mutant induction and selection are also desired (Oono 1981). A few workers have tried to improve rice seed protein and lysine from plants regenerated from anther-derived calli resistant to the lysine analogue S-aminoethylcystein (S-AEC). They also obtained cell lines which showed significant increase in lysine and threonine content compared to the control cells (Zapata 1985; Schaeffer 1986; Chen and Meng 1986). Cell lines resistant to the tryptophan analogue 5-methyltryptophan (5-MT) were selected in seed-derived calli of *Oryza sativa* L (Wasaka and Widholm 1982, 1987). Nitrate reductase-deficient rice cell lines, using anther-derived pollen calli inoculated on a medium containing 300 mM of sodium chlorate and streptomycin-resistant cell lines of rice from seed-derived calli, were also reported (Wasaka et al. 1984; Mikami and Kinoshita 1985). Several research groups have attempted *in vitro* selection for salt tolerance and identified salt-tolerant

varieties such as Pokkali. The tolerance was stable and inheritable (Abrigo et al. 1985; Nabors and Dykes 1985).

Currently, somaclonal variation is widespread and has been observed in most major crop plants. Useful variants can be screened and selected from the somaclonal progeny originated from tissue culture. In most cases, broadening the genetic base through sexual hybridisation risks destroying the plant breeder's genetic improvements. On the other hand, wild relatives have genes for disease, insect resistance and stress tolerance, which could further improve yields if transferred to our modern cultivars. Tissue culture and somaclonal variation may help to exploit the wild relative germplasm for desirable traits. They also enhance genetic variation without risking any possible genetic damage naturally occurring through conventional hybridisation (Scowcroft et al. 1985). There is great potential for *in vitro* generation of genetic variability and also genetic transfer for the improvement of cereals. Rice is one of the systems which has been extensively studied and useful somaclones have been derived from cells, protoplasts, anthers, embryos (Bajaj 1990a). The callus-derived plants of various cereals and grasses have already proved to be a rich source of somaclones. In the late 1980s, a wide range of protoclonal variability, and differences in the crop yield and phenotypic traits in the selfed progeny of protoplast-derived rice plants were also observed (Ogura et al. 1987, 1989).

At the same time, a few reports appeared with regeneration of rice plants from callus and protoplasts having somaclonal, protoclonal and molecular variations in their seed progeny (Abdullah et al. 1989; Zheng et al. 1989; Saleh et al. 1990; Müller et al. 1990; Davey et al. 1991). These reports have been discussed in detail in Chapter 1 (section 1.9.2). Commonly observed variation in tissue or protoplast-derived rice plants includes the number of tillers per plant, plant height, flag leaf length, flag leaf width, days to flowering, panicle length, number of branches per panicle, number of spikelets per panicle, number of grains per plant, grain length, grain width and mean grain weight. The work described in this Chapter deals with the phenotypic variations observed in Taipei 309 protoclones regenerated from cell suspension-derived protoplasts, the source cultures hereby originated from mature seed embryos. The phenotypic characters that are agronomically important, observed in both the seed-

grown plants and the protoplast-derived protoclones were analyzed statistically using the Genstat 5 package.

The aims of the work described in this Chapter were :

- to compare the different important agronomic traits between the protoplast-derived protoclones and the seed grown plants of the same cultivar;
- to analyze statistically the recorded data for various characters to illustrate any significant differences between protoclones and seed plants;
- to develop an improved procedure for the establishment and survival, with minimal loss, of the tissue culture-derived rice plants in the growth room and glasshouse.

6.2 Materials and Methods

6.2.1 Source of working materials

Protoclones were regenerated from cultured protoplasts isolated from mature seed-scutellum-derived (MSS) suspensions of *Oryza sativa* cv. Taipei 309 following the standard method described in Chapter 2 (Part II). At the same time, seed-derived plants grown in the glasshouse were used as controls and treated the same as the protoclones. The details of the protoclones used in this study are listed in Table 6A.

Table 6A. Total number of seed-derived control and protoplast-derived R_0 protoclonal plants transferred to the glasshouse (Taipei 309)

Control and Protoclonal plant source	Total no. of plants transferred	Number of plants survived	Number of plants died	Survival percentage (%)	Mortality percentage (%)
Seed	12	12	0	100.0	0.0
L51	70	15	55	21.4	78.6
L52	114	33	81	28.9	71.1
L55	80	7	73	8.7	91.3
L14R2	4	2	2	50.0	50.0
L2BR1	16	0	16	0.0	100.0

6.2.2 Transfer of the regenerated protoclones (MSS-origin) to the growth room using both standard and improved procedures

About 189 and 95 regenerated green protoclones with profuse root development were transferred to the growth room following the standard and improved procedures respectively for their growth and development to maturity during the 1990-91 season. The improved procedure has already been discussed in Chapter 2 (Part II, section 2.6.9), which was modified and developed by the author after experiencing a high percentage of mortality of the transferred protoclones from culture conditions to the growth room following a standard laboratory procedure.

The standard procedure, which was laid down by Dr. Paul Lynch, is described here as a comparison with the improved protocol. Regenerated green plants from protoplasts (8-10 cm tall) with extensive root development were chosen for transfer. Before potting the plants, a bag of perlite (Silvaperl Ltd., Gainsborough, Lincs., UK) was autoclaved to remove fungal or bacterial contamination. Before filling 3" plastic pots with Perlite, Whatman No 1 filter papers (5.5 cm in diameter) were placed at the bottom of each pot. The filled pots were placed into small propagator trays (46cm x 27cm x 8cm; 8 pots / tray) and the Perlite in the pots were hydrated by filling the tray to a depth of 2 cm with nutrient solution (10 ml of 'Maxi Crop plus sequestered iron' organic feed in 4.5 L of tap water; Maxicrop Ltd., Tonbridge, UK).

Freshly prepared nutrient solution was used every time, and the Perlite-filled pots were left for approx. 1 h until the top of the Perlite in the pots was moist. Regenerated and established green rice plants, grown in MSN1.5 medium were removed from the jars, their roots washed gently with tap water to remove agarose, and one plant was placed in the centre of each pot containing the moist Perlite. After ensuring that the nutrient solution was 2 cm deep in the propagator tray, the plants were covered with a propagator hood (46cm x 27cm x 15cm). After closing the vents of the hood, the trays with plants were kept in a growth room and maintained in a 12 h day : 12 h night cycle with a 70% relative humidity under fluorescent light intensity (1000 lux) at 28°C ± 1°C. Three to 4 days after transfer, the vents of the propagator hood were progressively opened and, as the plants showed more growth, the ventilation was increased. If the plants showed any deterioration during this period, the vents were

closed for 2 days before opening again. The depth of nutrient solution was maintained at 2 cm.

After 1-2 weeks, the vents were fully opened; after a further 1 week, the propagator hood was removed and the plants were fed by spraying with nutrient solution, 3-4 times daily for the next 2 weeks. If the plants showed any deterioration at this stage, the hood was replaced until they recovered. The depth of nutrient solution was maintained as before.

6.2.3 Re-potting of the growth room grown plants showing healthy root growth and their transfer to the glasshouse

As soon as the plants started to grow vigorously, roots began to protrude through the bottom of the pots. When the plants with profuse, healthy root growth were ready to be potted on, they were transferred into 3" plastic pots containing 1 part of Perlite to 2 parts of rice compost (1 part of Fison's M3 soil-less compost to 1 part of John Innes no. 3 compost). They were fed again with fresh nutrient solution and placed in the glasshouse in 100cm x 39cm x 5cm trays. During this period, particularly in summer, the plants were fed 3-4 times a day until they recommenced growth. Once the plants started growing well by producing tillers after about a further 2 weeks, they were transferred and re-potted again into 6" pots containing rice compost, fed by sprinkling once with nutrient solution and subsequently with water 3-4 times a day until the plants began to grow again. The re-potted plants were placed in 100cm x 39cm x 5cm trays; the temperature and humidity were maintained as near as possible to the conditions in the growth room. As the plants started showing healthy, vigorous growth by producing more tillers, the trays were filled with water to a depth of 1 cm, and the water level was raised every week to completely fill the trays after about 4 weeks. Subsequently, *Azolla pinnata*, the floating water fern, was spread on the surface of the water to multiply and to cover the whole surface to control algal growth. For successful transfer of tissue culture-derived plants, it was vital to check the plants regularly 2-3 times a day.

6.2.4 Recording of flowering dates and measurement of the different agronomic traits at maturity

The protoclones regenerated from protoplasts derived from 4 different suspensions

(L51, L52, L55, L14R2), initiated from mature seed-scutella, were transferred from culture from the second week of November 1990 until the second week of January 1991 depending on their growth and extent of their root systems. During the same period, seed-derived plants were transferred to 6" plastic pots containing rice compost (one seedling / pot). The total number of protoclonal plants transferred to the growth room is listed in Table 6A. After about 8 months, during the 17 h day period, the protoclonal plants started to flower from the beginning of July 1991. Most of the plants produced panicles with complete emergence, some of them produced panicles with partial emergence and 8 of them did not produce any panicles.

From the beginning of July 1991, the dates of flowering were recorded on the basis of at least one panicle being produced with complete emergence for most of the plants. For the plants which produced panicles with partial emergence, the dates were recorded following the same criteria but at the time when at least one panicle was partially emerged. It was not possible to record the dates of flowering for 8 plants which did not produce any panicles. Also, the measurement of flag-leaf length and flag-leaf width was not noted for those plants, because it was not possible to recognise their flag-leaves. The same criteria was followed for recording the dates of flowering for the seed-grown control plants.

6.2.5 Collection of rice panicles for recording data on various traits

Panicles were collected from both seed-grown control and protoclonal plants to compare these traits between the two sets of plants. The collection of panicles was initiated from the second week of December 1991 and continued until the first week of January 1992 depending on the different dates of maturity of the protoclonal plants. The date of maturity for the seed-grown control plants was 3 weeks earlier than the protoclonal plants and it was possible to collect all the panicles from each plant and also from a total of 12 plants on the same day. The panicles from the protoclonal plants were collected on 3 different consecutive dates depending on their maturity dates. Generally, the brown colour of the filled mature grains and the white colour of the empty sterile grains were signs of the maturity of the plants.

During collection of panicles, each panicle was collected in a separate paper bag, and a collection of all the panicles for a single plant were kept in a bundle for further

recording of data. At the same time, recording of other plant characters, as for example, plant height, total number of tillers and total number of fertile tillers produced by each plant, random flag-leaf length and flag-leaf width were also completed for each plant. Plant height was measured from the base of the plant at the soil surface up to the tip of the panicle on the first (main) primary tiller. Therefore, before cutting off the panicles, recording of all the plant characters were carried out. Usually, it was advisable to cut off the panicles below the panicle-base to facilitate measurement of the full length of the panicles. Subsequently, the length of each panicle was measured from the base, the total number of primary and secondary branches on both sides of the main stalk, and the total number of spikelets produced on each panicle were counted.

In the present study, the total number of empty and filled grains on each panicle were not counted, while the length and the width of the filled grains on each panicle were also not measured for the seed-grown control plants, because the protoclonal plants did not produce a single filled grain. The reasons why sterile, empty grains were produced by the protoclonal plants are unknown.

6.3 Results and discussions

6.3.1 Comparison of the different agronomic traits of protoplast-derived (protoclones) and seed-derived control plants

A total of 284 protoplast-derived protoclones, 189 from the standard procedure (described in section 6.2.2) and 95 from the improved procedure described in Chapter 2 (Part II, section 2.6.9), were obtained from 5 different suspensions and were transferred to the growth room. Among them, 227 plants (80%) died during their initial growth period in the growth room and the glasshouse. However, a total of 57 plants (20%) survived and were grown to maturity under supplementary fluorescent light conditions (intensity 1500 lux) during the winter (Nov.-April). During the summer of 1991, the temperature ranged from 28-35°C at mid-day and 7-10°C at night and at that period all the plants were at the boot to flowering stage. This could be one of the reasons for 100% sterility of the grains produced by the protoclonal plants. Because the protoplast-derived plants seemed to be more sensitive to these growth conditions in the glasshouse, the sterility problem was encountered with these plants, but it did not occur with the seed-grown control plants. Almost all the plants (184),

transferred by following the standard procedure, died during the course of their growth period in the growth room and in the glasshouse and only 5 plants remained alive. Among 95 plants, transferred by following the improved procedure, 52 survived and 43 plants died.

The reasons for the high plant mortality rate was unknown. The possible reasons were growing of the plants in concentrated standing nutrient solution (10 ml of Maxi Crop plus iron in 4.5 L of tap water) in the trays and the low humidity of the atmosphere in the growth room. The substrate, Perlite, also may have not helped the plants to take up the nutrients directly for healthy root growth. The concentrated nutrient solution may have been toxic to the plants at the initial stage of their growth as shown by the poor growth of the roots (Fig 6.1d), compared to more healthy root growth in M3 soil-less compost (Fig 6.1c). All the control (12) seed-derived seedlings survived and were grown to maturity, with 90-95% seed fertility. The growth and development of protoplast-derived plants is shown in Figs 6.1 (b, e, f, g) and Figs 6.2 (a-i); seed-grown plants in Figs 6.3 (a-f).

Direct correlation could not be made between sterility and plant origin. Sterility was a limitation in further attempts to evaluate the variation observed between the control and R_o parental protoclonal plants, through their R_1 seed progeny. Five phenotypic plant characters, namely, plant height (Pht), total number of tillers and number of fertile tillers (Tottil & Ferttil), together with flag-leaf length and width (Fl & Fw) were recorded for 12 control plants and 57 R_o protoclonal plants. The mean difference was compared between two groups of plants. When the height of protoclonal plants was compared with that of control plant height, the mean plant height was 96.9 cm ranging from 41.7-147.0 cm. However, when it was compared within the protoclonal plant types, the mean plant height was 87.0 cm for protoclones ranging from 62.3-96.5 cm, which was greatly reduced compared with the control plants. Similarly, the mean number of total and fertile tillers for the protoclonal plant type were reduced to slightly greater than half and less than half respectively (7.59 and 3.22) compared to those of control plants 14.67 and 7.58). There was not much variation of mean flag-leaf length and width between these two plant types (ranged from 39.21-31.40 cm for length and 1.20-1.04 cm for width). In contrast, within the 4 protoclonal groups of plant types, derived from 4 suspensions, there were variations for 4 plant characters (plant height

Fig 6.1 Growth and development of protoplast-derived protoclones (MSS-origin) on MSN1.5 medium, in perlite, in M3 soil-less compost and in rice compost; their root development in two different substrates (Taipei 309)

a) Healthy shoot growth and profuse, healthy, white root development from protoplast-derived protoclones on MSN1.5 medium after 3-4 weeks of transfer to the growth room under white, fluorescent light (1000 lux) at 25°C ± 1°C (x 3.6).

b) Unhealthy and weak growth of plants in moist perlite (left 4 pots) compared to healthy growth in M3 soil-less compost (right 4 pots) after 2 months of transfer to the growth room maintained at 28°C ± 1°C, 12 h day and 12 h night cycle, with at least 70% relative humidity (x 2.3).

c) Healthy, loose root development in each plant grown in M3 soil-less compost with the production of 1-2 healthy tillers after 8-10 weeks of transfer to the growth room (x 3.6).

d) Weak, compact root development in each plant grown in moist perlite (keeping the pots in standing nutrient solution), without any production of healthy tiller after 8-10 weeks of transfer to the growth room (x 3.7).

e) Further growth of young, healthy, green protoclonal plants with production of more tillers after 2 months of transfer to rice compost in the glasshouse following 2 months of growth in M3 soil-less compost in the growth room (x 4.0).

f) A healthy, normal protoclonal plant with complete emergence of one or two panicles after 6 months of growth in rice compost under supplementary white, fluorescent light (1500 lux) in the glasshouse at 32°C ± 3°C, maintaining at least 70-80% relative humidity (x 3.8).

g) A healthy looking protoclonal plant with many tillers but with different leaf characteristics (erect, not droopy) and incomplete emergence of many panicles after 6 months of growth in rice compost (initially grown in perlite) under the growing conditions as in (f) (x 3.8).


a
b
c
d
e
f
g

Fig 6.2 Further growth of protoplast-derived protoclones showing different characteristics

a) A healthy protoclonal plant with complete emergence of panicles and with wide, droopy leaves after 8 months of growth in rice compost under supplementary white, fluorescent lights (1500 lux) in the glasshouse at 32°C ± 3°C, maintaining at least 70-80% relative humidity (x 3.2).

b) Complete and incomplete emergence of panicles from the tillers of a protoclonal plant, bearing empty, sterile spikelets with awns and wide, erect, dark green leaves after eight and a half months of growth in rice compost under the growth conditions as in (a) (x 3.3).

c) Production of both healthy green and dead tillers with incomplete emergence of panicles in the same protoclonal plant and mostly with wide, erect, dark green leaves after 6 months of growth in rice compost in the glasshouse (x 1.8).

d) A nine-month-old protoclonal plant with complete and incomplete emergence of panicles bearing sterile spikelets and also with narrow, droopy leaves grown in rice compost under the growth conditions as in (a) (x 4.0).

e) One protoclonal plant with dark green, erect leaves and the other with narrow, erect leaves, each with 2-3 tillers bearing no panicles after 9 months of growth in perlite followed by rice compost in the glasshouse (x 3.8).

f) A short, poorly grown protoplast-derived plant with narrow leaves after 2 months of growth in perlite followed by 6 months of growth in rice compost in the glasshouse under the growth conditions as in (a) (x 3.0).

g) A healthy, seed-derived plant with complete emergence of panicles bearing 85-90% fertile spikelets and narrow, droopy leaves after 7 months of growth in rice compost under the growing conditions as in (a) (x 3.6).

h) A tall and a short protoclonal plants, one with complete and incomplete emergence of panicles and the other without bearing any panicles, both having narrow, droopy leaves after 9 months of growth in the glasshouse (x 3.7).

i) A rice panicle from a protoplast-derived protoclone bearing empty, sterile spikelets with awns on a few primary and secondary branches (x 3.8).

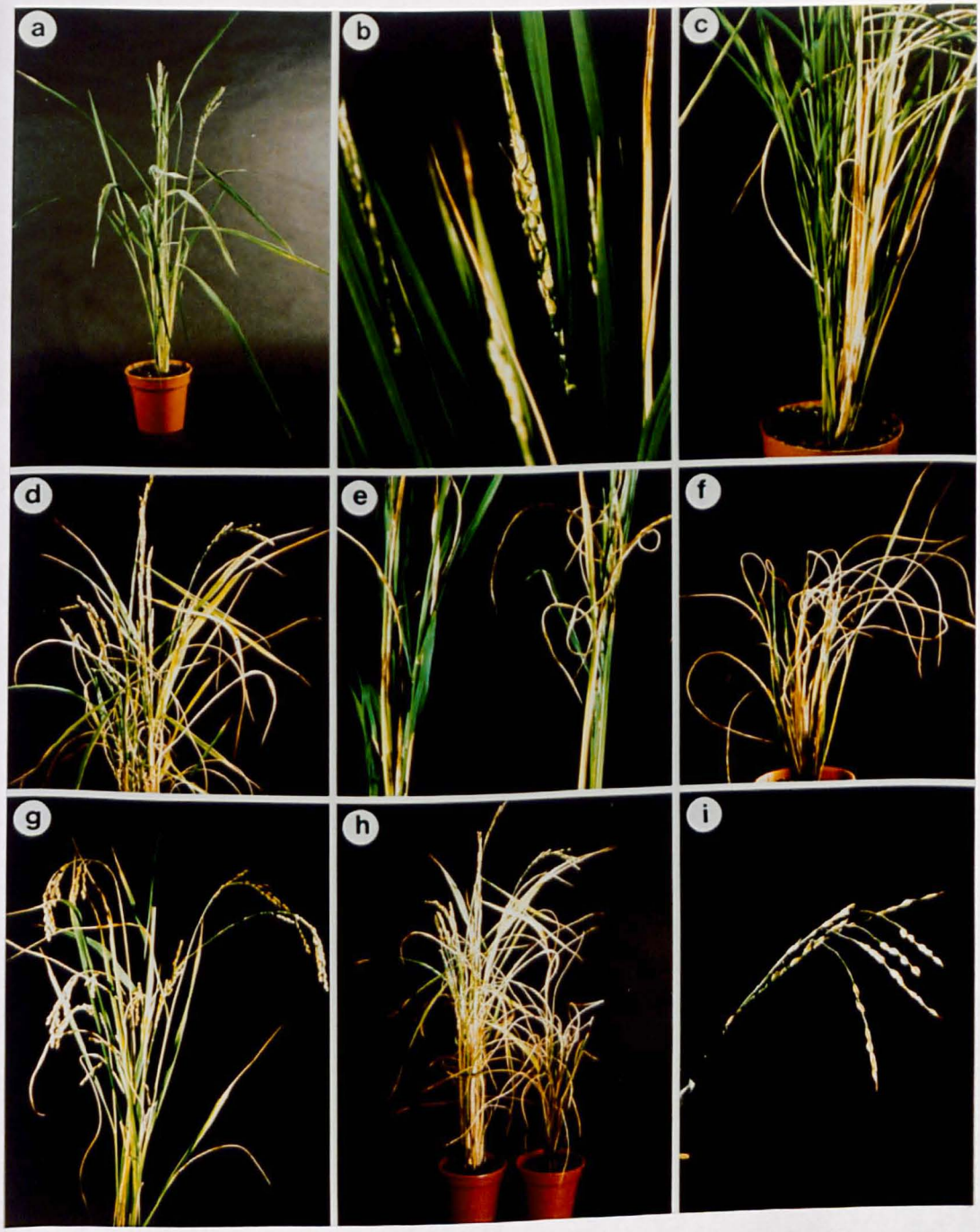

a
b
c
d
e
f
g
h
i

Fig 6.3 Growth and development of seed-grown plants of *O. sativa* (cv. Taipei 309) and production of healthy tillers with complete emergence of panicles bearing fertile spikelets

a) A young seed-grown Taipei 309 plant after 2 months from sowing seeds in rice compost in the glasshouse under the growing conditions as for protoplast-derived protoclones (x 3.7).

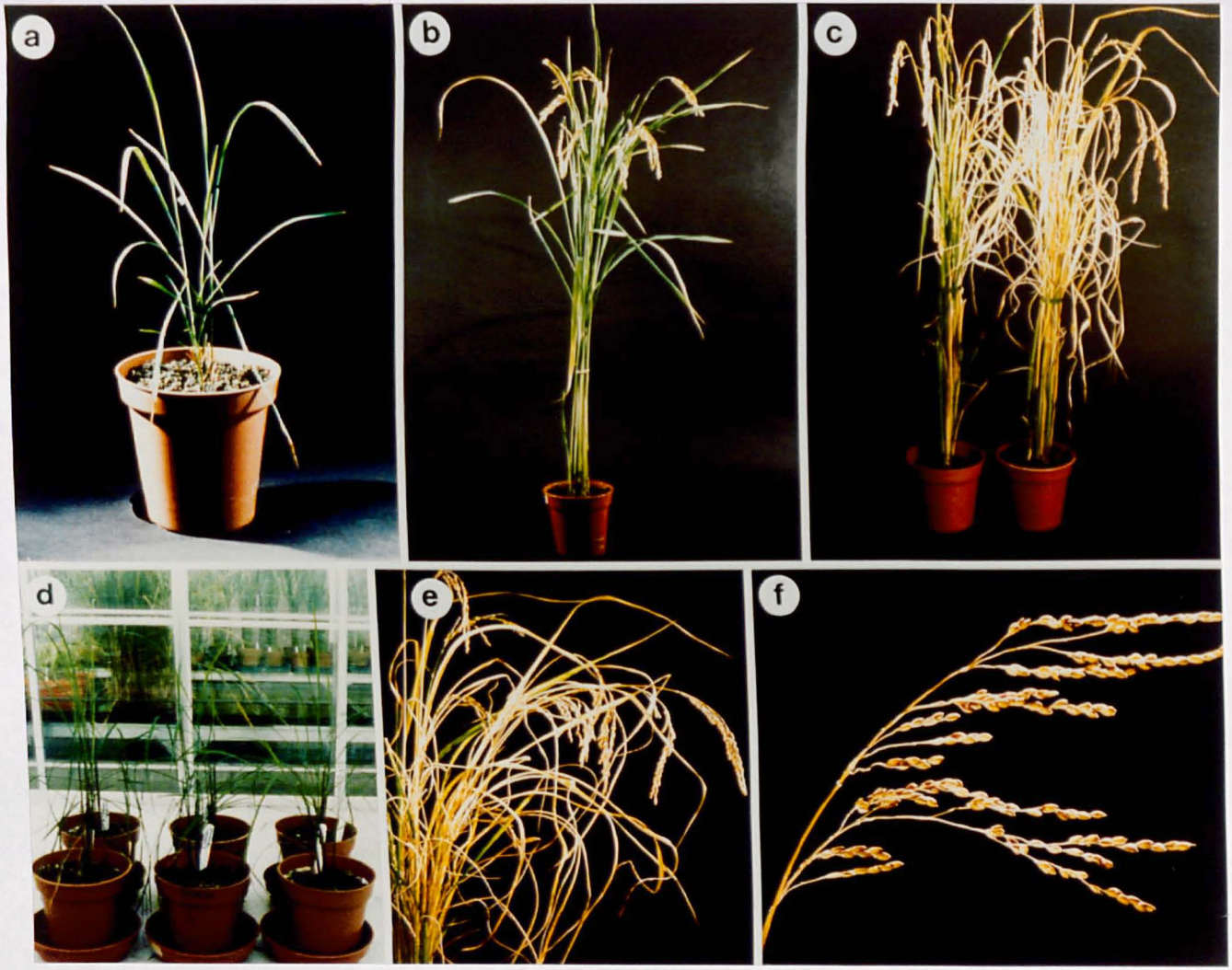
b) A healthy seed-grown plants with fertile tillers, bearing panicles with complete emergence and 85-90% fertile spikelets after 7 months of growth in rice compost in the glasshouse (x 3.9).

c) Two mature seed-grown plants bearing fertile panicles with 85-90% filled grains, taller in height compared to protoclonal plants, erect growth with droopy leaves after 9 months in the glasshouse (x 3.9).

d) A general view of protoclonal and seed-grown plants of Taipei 309 in the glasshouse through the window of the other compartments.

e) Another seed-grown mature plant bearing mature panicles with filled grains, always with narrow, droopy leaves and more number of grains, sometimes having very short awns compared to protoclonal plants with long awns (x 3.7).

f) A mature rice panicle from a seed-grown plant, bearing 95% filled grains with or without short awns and more number of primary and secondary branches per panicle compared to protoclonal panicles (x 3.5).


a
b
c
d
e
f

ranged from 62.3-96.5 cm, number of total and fertile tillers ranged from 3.50-12.76 and 0.73-7.85 respectively, and flag-leaf length ranged from 19.47-41.61 cm). There was no notable variation for flag-leaf width within 4 protoclonal groups, which ranged from 0.95-1.29cm (Appendix A3.1).

On the other hand, 4 phenotypic panicle characters [panicle length (Pl), number of primary and secondary branches (Pbran & Sbran), and number of spikelets (S) on each panicle] were recorded and these characters were compared between control and protoclonal plants and amongst the different protoclonal lines (Appendix A3.2). They were compared for two types of panicles (primary and secondary) between control and protoclonal plants and among the different protoclonal lines (Appendix A3.3). They were also compared for two groups of plants (control and protoclonal) on two types of panicles (Appendix A3.4).

The data showed that the means for these 4 panicle characters varied between two groups of plant types, ranging from 8.86-19.93 cm for Pl, 5.20-7.54 for Pbran, 3.14-10.88 for Sbran and 29.2-74.0 for S. It was clear from the data that there were large differences for Pl, the number of Sbran and S for two groups of plant types (Appendix A3.2). Again, when these characters were compared between two groups of panicle types for control and protoclonal plants, there was variation for Pl and the number of Sbran and S on primary panicles, for Pl and the number of Pbran, Sbran and S on secondary panicles (Appendix A3.3). Finally, when these characters were compared between two groups of plant types (control and protoclonal) and panicle types (primary and secondary), there was variation between Sbran and S only on primary panicles of two plant types (Appendix A3.4). Even within the protoclonal groups of plants, these characters (Sbran and S) were variable on both types of panicles. Surprisingly, 3 of these 4 panicle characters (Pbran, Sbran and S) varied greatly for two types of panicles (primary and secondary) within the seed-derived control plants (Appendix A3.4).

6.3.2 Statistical analysis of the recorded data to illustrate significant difference between protoclones and seed-grown plants

Statistical analysis of the recorded data for various agronomic characters of seed-grown control plants and protoplast-derived protoclones was performed using Genstat

5 on the CCC VME System. In total, 12 control plants were compared with 47 protoclonal plants derived from 4 different suspension lines for 5 plant characters (Appendix A3.1). The analysis was also performed between 12 control and 39 protoclonal plants (with restriction of 8 plants) derived from 2 different suspension lines. The analysis was not performed for those protoclonal plants which did not produce any flag leaves or which did not have flag leaf length and width greater than >.001 or >0.51 cm respectively. For 4 panicle characters (Appendix A3.2), the analysis was performed between two groups of 94 and 276 panicles obtained from control and protoclonal plants respectively. The analysis was also not performed for 28 panicles, because of the few number of observations obtained from each of 3 suspension-derived protoclonal plants.

From the results of the analysis of variance, mean value and standard deviation, it was seen that among 5 plant characters, compared between 2 different plant types (control and protoclonal), plant height and flag leaf width were significantly different, flag leaf length was just significantly different (Appendix A3.5). The number of total and fertile tillers were not significantly different at 5% level, because the analysis was not restricted between control and protoclonal plants obtained from 3 suspension lines only. It was also reavealed from the analysis that when large number of observations (replications) were taken into account, all the plant characters were significantly different at the 5% level between two different (control and protoclonal) plant types (Appendix A3.6). For 4 panicle characters also, comparison was made between two different plant types and lines, two different panicle types (primary and secondary) and lines, two different plant types only, and finally between plant types and panicle types, keeping restriction for the protoclonal plants obtained from one suspension line only.

From the comparison made between plant types and lines, panicle length, the number of secondary branches and spikelets/panicle were significantly different, but the number of primary branches/panicle were not significantly different (Appendix A3.7). The significant difference was also observed for all these panicle characters, when they were compared between two different panicle types (primary and secondary) and lines (Appendix A3.8). The similar result was also noted for panicle length, the number of secondary branches and spikelets/panicle; but significant difference was not observed for the number of primary branches/panicle, when only two different plant types

(control and protoclonal) were compared (Appendix A3.9). Finally, it was noticed that all the 4 panicle characters were different between two different plant types and panicle types and it was highly significant at the 5% level (Appendix A3.4). Data with respect to the mean performance and standard error of the means for the forty seven protoclones and 12 controls for plant characters; 94 control and 276 protoclonal panicles for panicle characters are presented in Figs 6A-J.

Normally, the parent *Japonica* variety, Taipei 309 used in this study is early flowering, has long and narrow flag leaves, panicles with few primary and secondary branches and low number of spikelets per panicle. The protoclonal variability, which was obtained among all the protoplast-derived plants, and also compared to the seed-grown control plants for all the characters evaluated except grain length, grain width and 100-grain weight can be exploited by selection of individual plant from the whole populations. Specifically, the characters which contribute directly for yield improvement can also be exploited for established rice genotypes through somaclonal, protoclonal, gametoclonal and mutation breeding.

6.3.3 Development of an improved procedure for establishing regenerated protoplast-derived rice plants in the glasshouse to minimise loss of the transferred plants

During the course of this present study, 284 protoplast-derived rice plants obtained from 5 different cell suspension lines of the variety T309, were transferred to the growth room for their initial growth and development. The aims of this project were to grow these plants to maturity and to evaluate various important agronomic characters during their growth period (Fig 6.1) and at maturity (Fig 6.2) compared to seed-grown control plants (Fig 6.3) to determine the protoclonal variability generated among these plants. At the initial stage of transfer of these plants to the growth room, the standard procedure used by the previous worker, described in section 6.2.2 of this Chapter, was followed (Fig 6.1). The mortality rate of these transferred protoplast-derived plants was very high and disappointing. [Out of 284 plants, 227 plants died before and after transfer to the glasshouse and 57 plants survived. The possible reasons could be the use of only perlite, the concentration and continuous supply of the nutrient solution in the tray.] After experiencing poor development of roots (Fig 6.1) and high mortality

rate (about 80%), the standard procedure was modified and improved by using Fison's M3 soil-less compost, by keeping the soil just moist and spraying the plants with diluted nutrient solution (5.0 ml in 4.5 L of tap water) 2-3 times a day. The modified, improved procedure has already been described in Chapter 2 (section 2.6.9). By using this method, the mortality rate could be reduced and the healthy growth of the plants was ensured (Fig 6.1 & Fig 6.2).

6.4 Summary and conclusions

The work described in this Chapter shows that the protoclones originated from cell suspension-derived protoplast cultures of 4 different lines, compared to the seed-grown control plants, exhibited variation in almost all the phenotypic characters evaluated. In many cases, the variation was significantly different, especially in those which are directly yield contributing characters, as for example, total and fertile tiller number/plant, number of primary and secondary branches/panicle, individual panicle length, and spikelets number/panicle investigated in this present study and as also described by previous workers (Abdullah et al. 1989; Ogura et al. 1987, 1989). There are other yield contributing characters, 100-grain weight, grain length and grain width, which are also important, but which were not possible to investigate in this study because of sterility. If these characters, which are related directly with yield increase can be improved and exploited through tissue and protoplast culture, further improvement of existing rice varieties cultivated in different rice growing countries can be achieved.

In rice, mainly somaclonal or protoclonal variation could be utililized for theoretical research, obtaining isogenic lines, breeding directly for improved varieties tolerant to saline soil, resistant to diseases, and amino acids or their analogs to increase their seed protein and/or lysine content. Although the mechanism of somaclonal or protoclonal variation is still not clear, the prospect of its application to rice breeding is attractive. The importance of the *in vitro* induction of genetic variability in rice has long been recognized by rice breeders. The success of rice crop improvement programmes mostly depends on the early selection of the desired plants, and is only possible if large variation exists in the base population. Subsequently, the desirable variants can be used as parents in hybridisation programme, or to isolate pure lines for the production of

homozygous varieties, and also as useful mutants. Therefore, the extent of genetic variability is always more important than the total variability. *In vitro* induced genetic variability, through callus tissue or protoplast cultures, may have a special use for plant breeders who are working with crops where there is a problem of sexual incompatibility.

The source of natural variation in plants is always limited and becomes exhausted after a few generations of self pollination and, in these cases, a plant breeder can use various tissue culture methods to create genetic variability in less time and more efficient manner (Reinert and Bajaj 1977). The value of somaclonal variation lies in the ability to recover new genetic variants at high frequency with desirable characteristics. During this approach, two important selection steps occur that serve as sieves and permit the recovery of a population of R_1 plants that are most suitable for a breeding programme. These two important selection steps are :

- The culture medium and the plant regeneration procedure enable a singling out of cells from the original explant which possess genetic competence for plant regeneration;
- Glasshouse selection permits identification of those regenerated plants that are capable of flowering and setting seed. This selection eliminates Ro plants with deleterious genetic information. Therefore, the population that is examined in the field is the most suitable for rapid variety development.

The application of somaclonal or protoclonal variation to crop improvement reflects several unique aspects of this process. The frequency of genetic change is significantly higher for somaclonal or protoclonal variation than observed for spontaneous genetic changes (Evans and sharp 1983). It is also superior to mutation breeding, as regenerated plants are derived from single cells. Therefore, mosaics or variation occur at very low frequency and, in most cases, somaclones can be stabilized in a single generation. It is expected in the next five years that somaclonal variation will be widely used for crop improvement programmes. Because the techniques for producing somaclonal variants are relatively simple compared to those of recombinant DNA technology, they provide a rich source of genetic variability.

There are several specific developments which are also anticipated in the near future :

- To the extent that desirable somaclones can be identified in the Petri dish rather than in field trials, somaclonal variation can be more efficient and cost effective. This will only require a correlation between the cellular and whole plant response to specific chemicals used as selective agents.
- Somaclonal variation also holds promise as an addition to protoplast fusion. In *Nicotiana*, the set of plants obtained by protoplast fusion was much more variable than that produced by sexual hybridisation for interspecific hybrids (Evans et al. 1982). This phenomenon can be used to select somatic hybrids with the appropriate mixture of desirable genetic traits of the two parent lines used for protoplast fusion. As protoplast fusion results in a summation of chromosome number, anther culture can be used to reduce the chromosome number to that which is consistent with sexual hybridization.
- The results of somaclonal variation may also have an impact on gene transfer approaches using recombinant DNA technology, since the knowledge acquired on the use of medium components, which increased or decreased somaclonal variation, may help to optimise regeneration protocols for use in transformation experiments. It is also possible to produce somaclones which could be used to develop more suitable variants for uptake, integration and stable expression of T-DNA.
- In many cases, new somaclonal variants are isogenic stocks of the original parental variety, as this phenomenon results in a large number of simple genetic changes. Sometimes, each somaclone differs from the parent by only a single or small number of genetic changes.

Therefore, if agronomically useful characters can be identified to a greater extent, somaclones or protoclones could be a valuable source of germplasm from which to isolate and identify specific genes of interest. These somaclones and parent lines or varieties could be compared to identify distinct mRNAs associated with a particular phenotype. The mRNAs could be used to prepare cDNA clones and tested for genetic linkage associated with a specific agronomic character. This approach would ultimately result to build cDNA clones for agronomically useful genes and it would be particularly attractive for those crop plants where only a limited gene map exists, like

soybean, sugarcane, coffee. Many of these future prospects for the developments in somaclonal or protoclonal variation are dependent upon the use of these tools by rice breeders. Therefore, a joint approach between tissue culturists, geneticists and breeders is imperative to advance the progress of this technology.

Fig 6A. Plant characteristics (mean and standard deviation) between seed and protoplast-derived plants

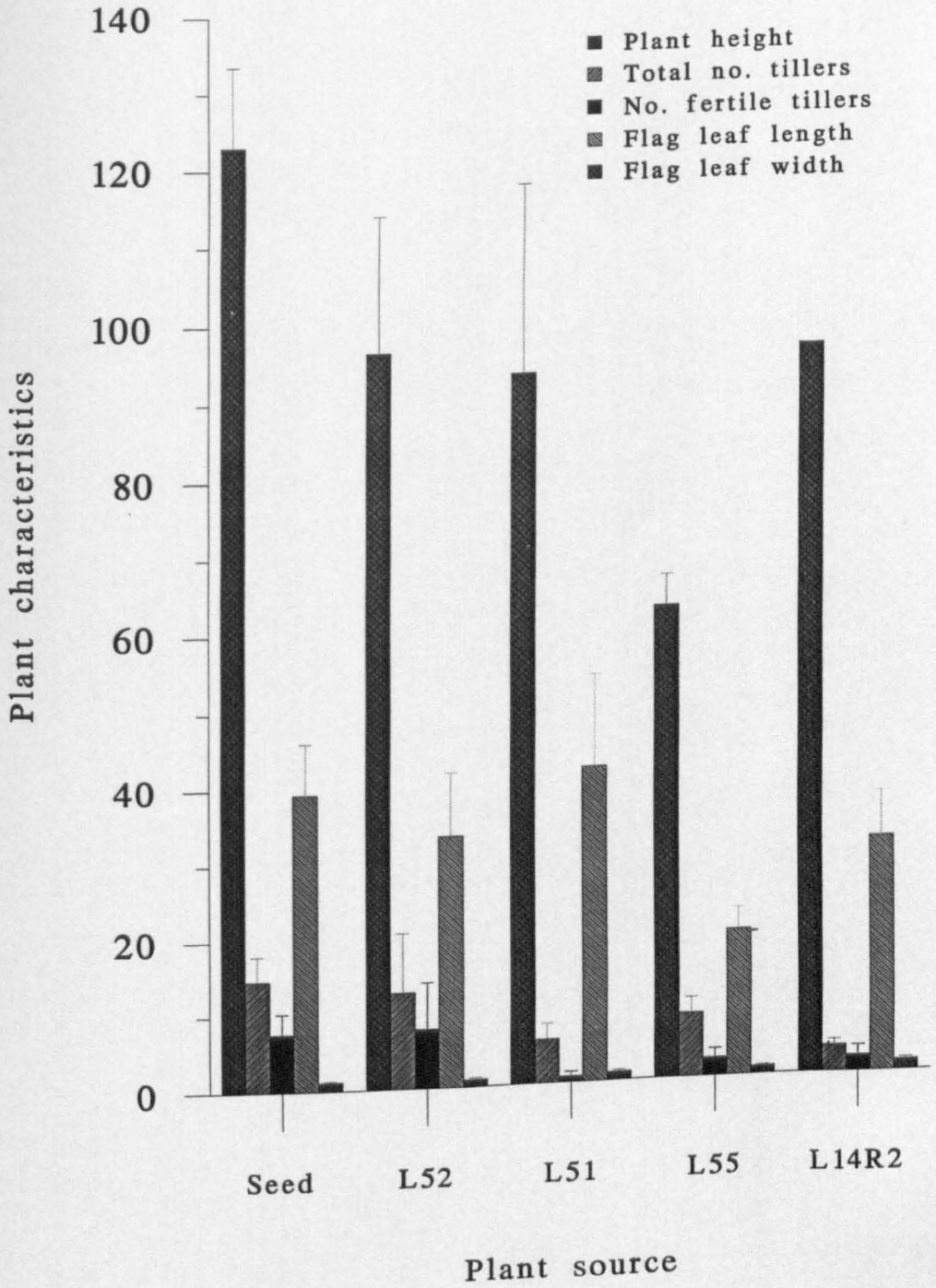


Fig 6B. Panicle characteristics (mean + standard deviation) between seed and protoplast-derived plants

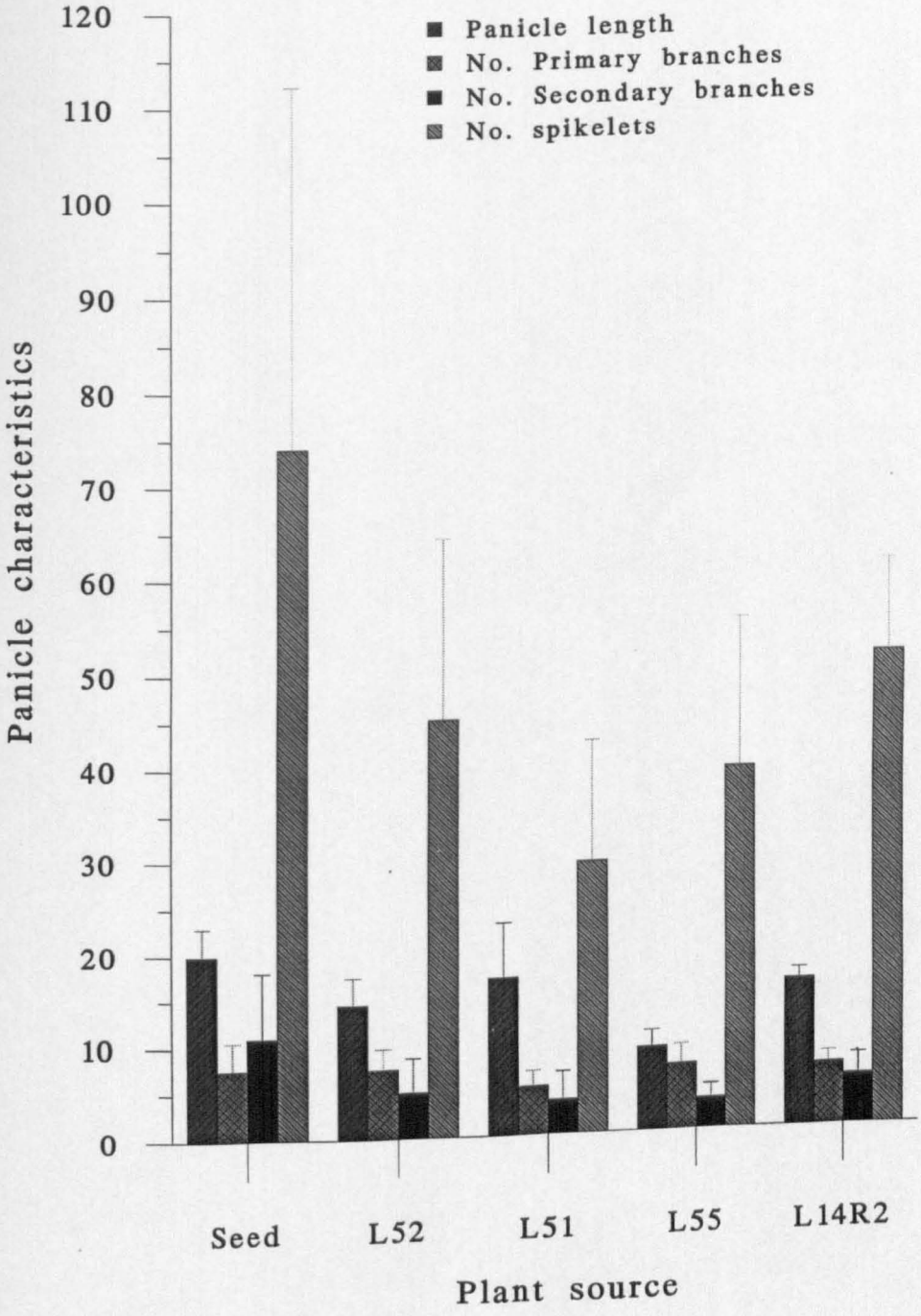


Fig 6C. Panicle length (mean + standard deviation) of primary and secondary panicles of control and protoclonal plants

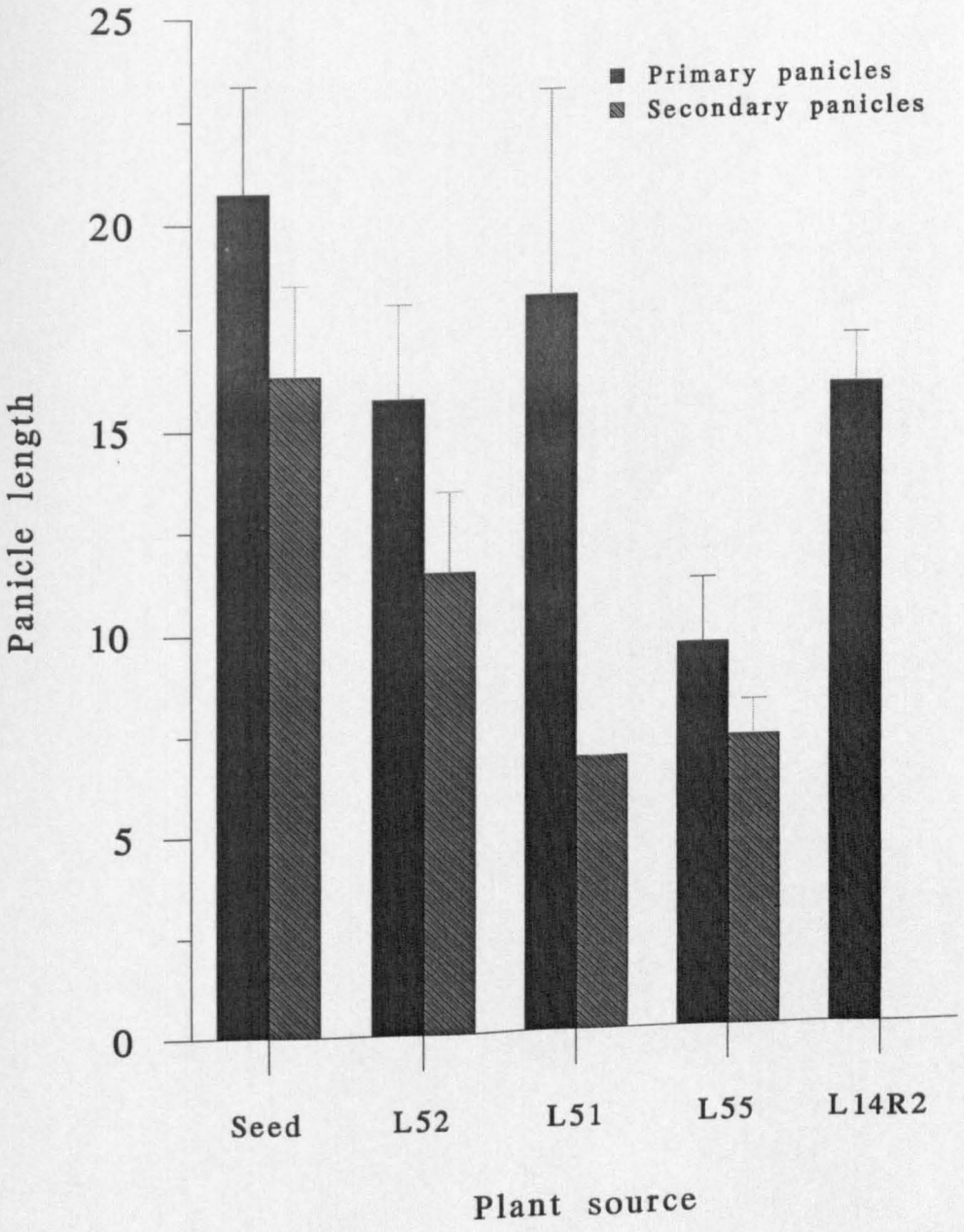


Fig 6D. Number of primary branches (mean + standard deviation) on primary and secondary panicles of control and protoclonal plants

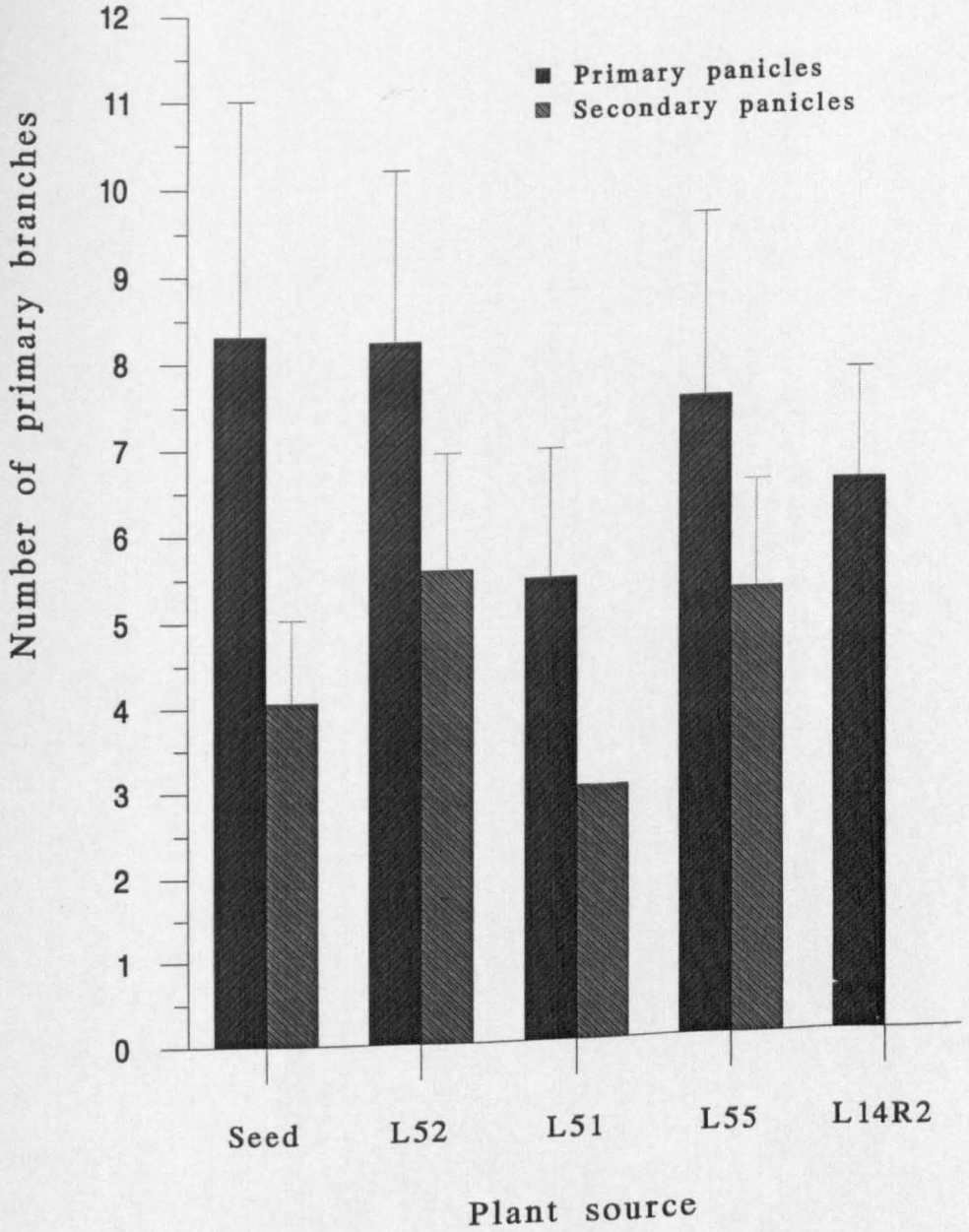


Fig 6E. Number of seccondary branches (mean + standard deviation) on primary and secondary panicles of control and protoclonal plants

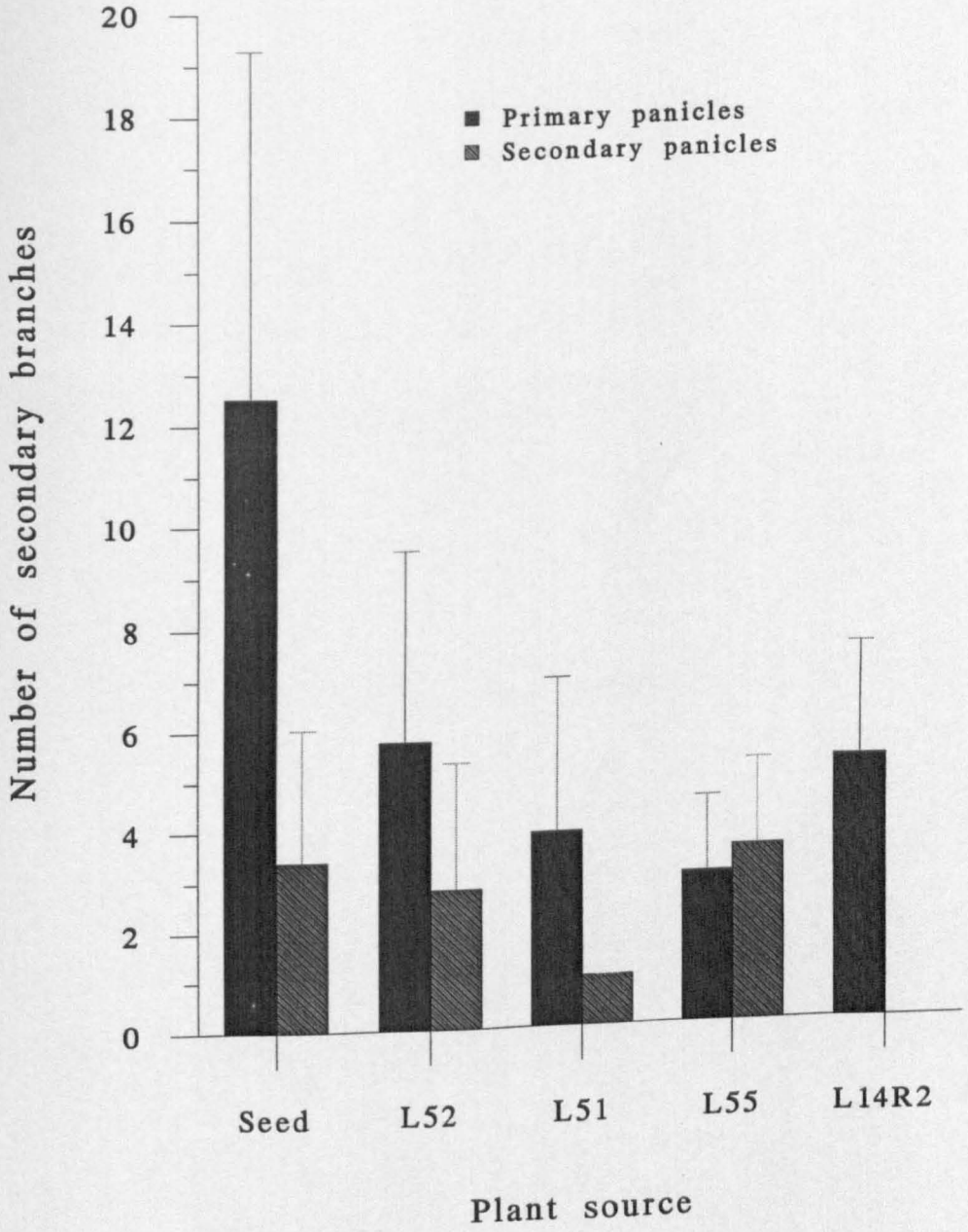


Fig 6F. Number of spikelets (mean + standard deviation) on primary and secondary panicles of control and protoclonal plants

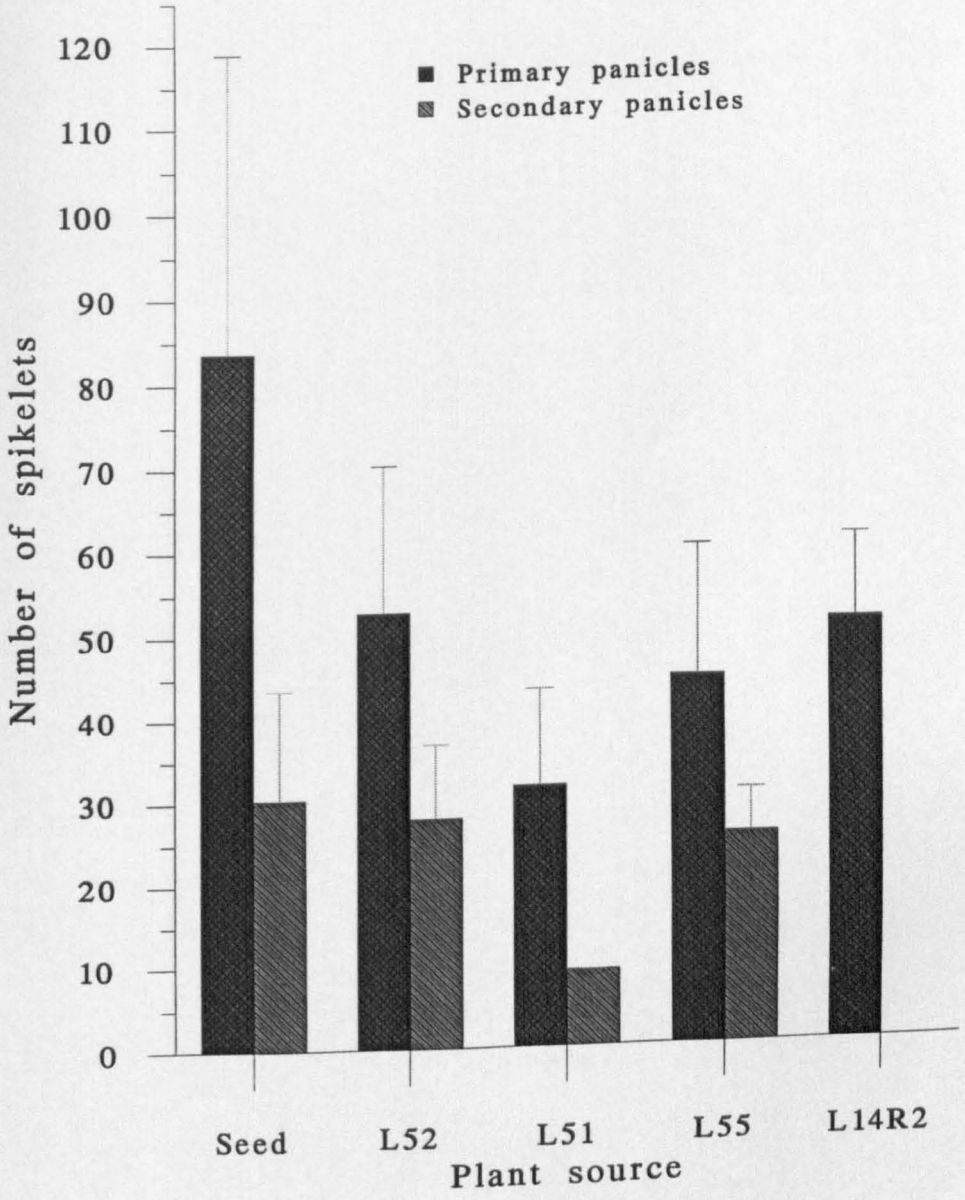


Fig 6G. Panicle length (mean + standard deviation) on primary and secondary panicles of control and protoclonal plants

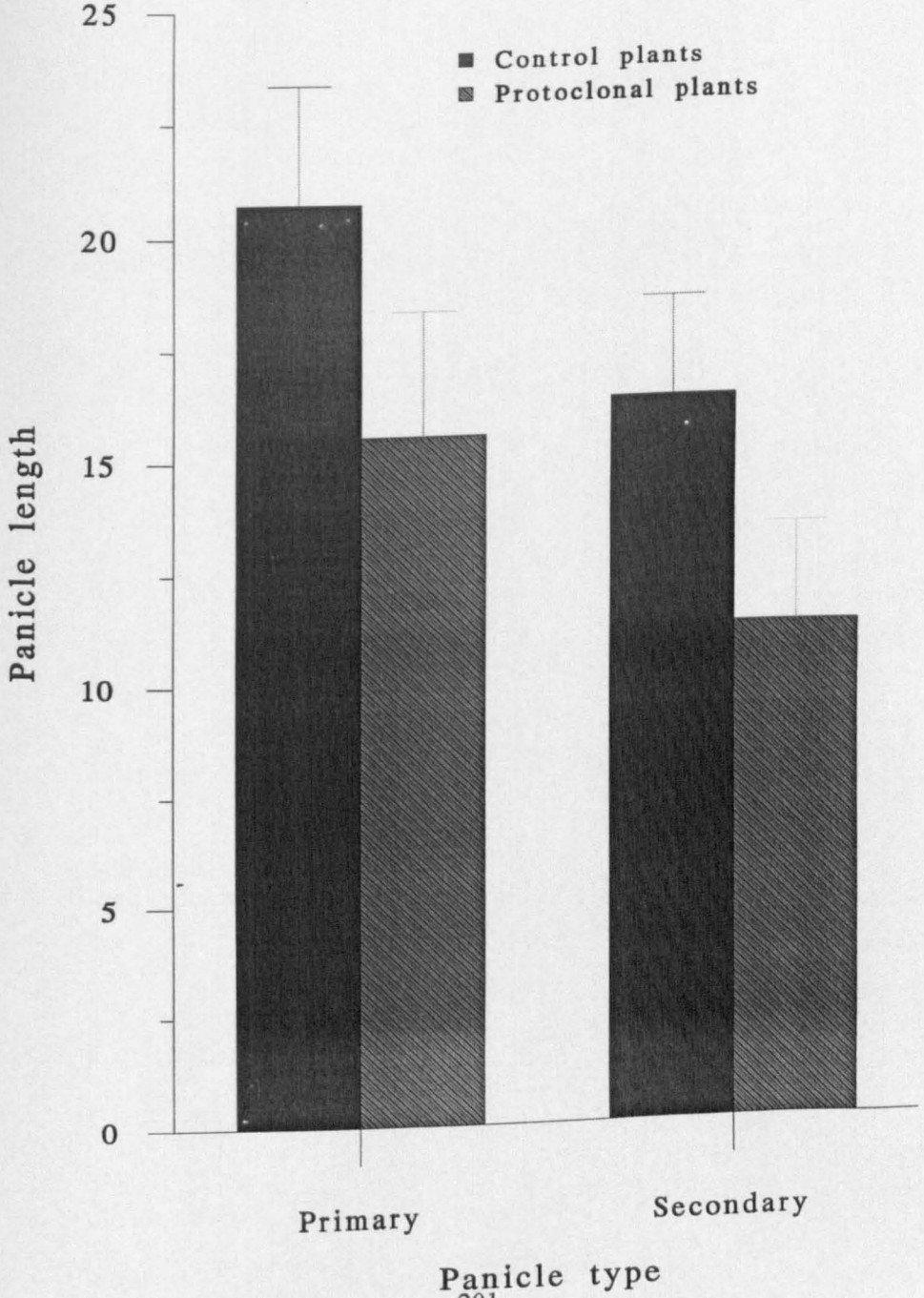


Fig 6H. Number of primary branches (mean + standard deviation) on primary and secondary panicles of control and protoclonal plants

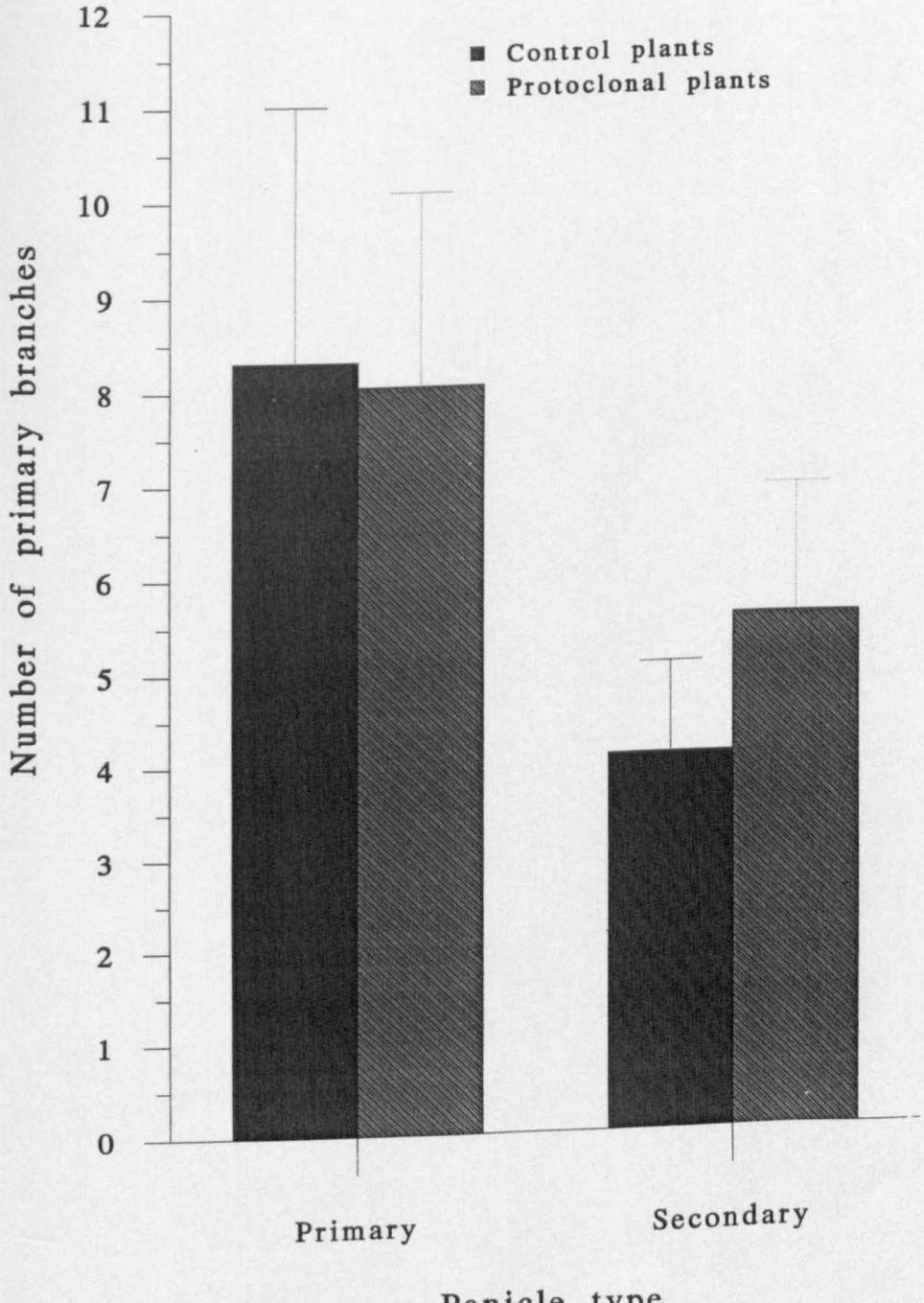


Fig 6I. Number of secondary branches (mean + standard deviation) on primary and secondary panicles of control and protoclonal plants

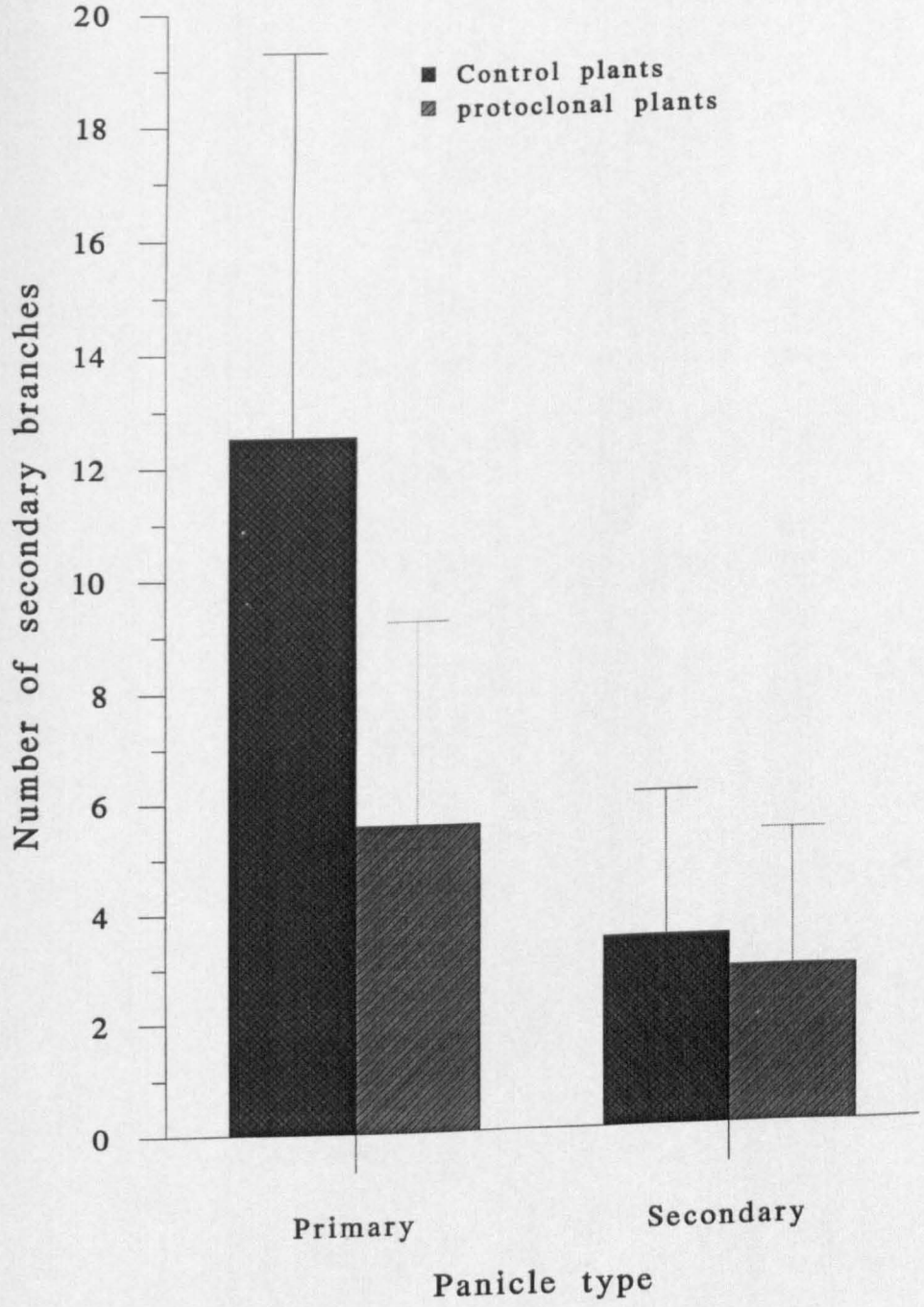
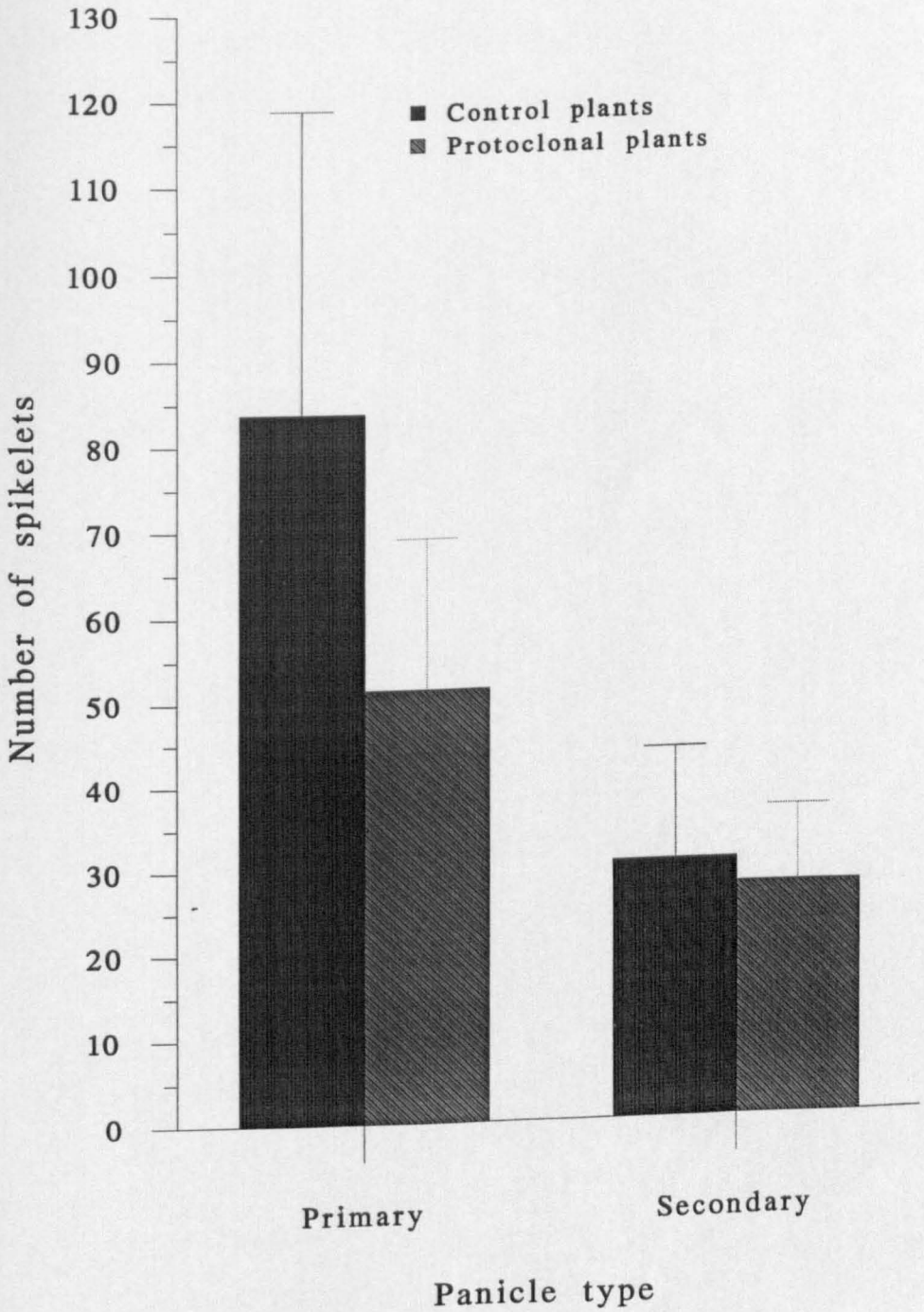


Fig 6J. Number of spikelets (mean + standard deviation) on primary and secondary panicles of control and protoclonal plants



Chapter 7

General Discussion

The major objectives of the work describeded in this thesis were :

- to establish efficient, reproducible, embryogenic callus culture and plant regeneration systems from different explants, for example, excised anthers and isolated microspores, mature seed-scutellum, immature zygotic embryos and leaf-base meristems, using the *Japonica* rice cultivar Taipei 309 (Chapter 2, Part-I and Chapter 3, Part-I),
- to produce and to establish embryogenic cell suspension cultures using embryogenic callus which originated from different explants; to regenerate and to recover green rice plants from cell suspension-derived protoplasts using the same cultivar (Chapter 2, Part-II and Chapter 3, Part-II),
- to estimate and to measure the nuclear DNA content of isolated nuclei by flow cytometry from young green leaf tissues of all regenerated rice plants obtained through different culture systems for the determination of the ploidy level of individual plants (Chapter 4),
- to develop an efficient method of root-tip squash preparation for rice chromosomes, and to determine the ploidy levels of some regenerated plants by chromosome counting in order to confirm the flow cytometry results (Chapter 4 and Chapter 5),
- to characterise and to compare the different important agronomic traits between the protoplast-derived protoclones (MSS-origin) and seed-grown plants of Taipei 309 by statistical analysis of the recorded data (Chapter 6) and
- to develop modified, improved procedures for the survival of the tissue-culture and protoplast-derived plants, and their establishment in the glasshouse.

Major achievements and key points in the present study

During callus culture initiation and maintenance from various explants of rice, it was observed that different factors were important and responsible for successful production of embryogenic callus and subsequent regeneration of green plants

(Chapter 2, section 2.3.1). In this study, immature embryos were found to be more responsive and produced a higher percentage of embryogenic callus compared to other explants (Table 2A). The response of anther callus in liquid medium was very different compared to isolated microspores without any necessity for amino acids such as proline, alanine or glutamine (Tables 3C, 3D and 3G, 3H). Direct proembryo formation, which gave rise mostly to haploid and some diploid plants, was also a frequent phenomenon in the anther callus system. The nutritional requirements for the culture of excised anthers were also much simpler than those of isolated microspores. Presumably, certain factors responsible for the induction of androgenesis of isolated microspores, which might have been provided by the somatic tissues of the anther wall, were absent and needed to be provided through the specific culture medium. Mechanical isolation of cereal or rice microspores has not yet proved to be as efficient as the culture of released microspores in liquid medium (Datta et al. 1990a), which was also true in the present study. Considering all the callus and plant regeneration systems using different explants in the present study, it was found that the anther callus system was the most efficient for plant regeneration.

The most embryogenic cell suspension cultures were established from young, embryogenic callus derived from mature seed-scutellum or immature seed-embryos (2 to 3 months after callus induction) and from callus which had been subcultured and selected over 4-5 transfers. The selection of friable, dry embryogenic callus following its initiation was of major importance for successful establishment of embryogenic cell suspension cultures, in addition to the influence of the explant. Furthermore, the optimum growth of fine, evenly dispersed cell suspensions required selection of cells capable of further growth under the specific conditions of liquid culture. The other essential key factors were the division of cultures into 2 or 3 flasks after 3 weeks of initiation of the suspensions, replacement of 80% of the liquid medium in the culture vessels for the first 5-6 weeks from initiation, together with regular microscopic observations to check the structure and size of the cell clusters and to determine their exponential stage of growth for successful protoplast isolation. The maintenance of regenerable cell suspensions from any particular explant also needed careful management. Regular subculture, using a fixed packed cell volume, was very important and crucial to keep suspensions in their exponential growth phase and to maintain their

normal growth. The production of viable protoplasts, by adequate control of the duration of enzyme treatment, was also essential to ensure high plating efficiencies and plant regeneration frequencies.

The most effective way of achieving high frequency plant regeneration was to adopt a rigid protocol for the initiation of cell suspensions, and the regeneration of green plants (see Chapter 2, sections 2.7.3 and 2.7.4). Certain other factors, for example, the optimal protoplast plating density and the selection of embryogenic, compact protoplast-derived micro-calli, were also essential to obtain high frequency plant regeneration. Furthermore, the culture environment, especially temperature and light intensity, had an important role for establishment and maintenance of anther-derived cell suspensions in this study.

The importance of haploids and doubled haploids in cereal breeding has been emphasised since the early 1980s (Snape and Simpson 1986; Snape 1989). For the study of karyotype instability of cell cultures and for the induction of mutants which are resistant to pests, pathotoxins and herbicides, the use of haploid cell or protoplast systems are more advantageous compared to diploid cell systems. The effective application of co-transformation techniques to produce fertile transgenic *Indica* rice plants carrying agronomically important genes has been reported recently (Peng et al 1991).

Recent advances in rice cell and tissue culture and genetic manipulation studies have proved that novel plants and genetic variants can be obtained in about two years. However, there is still a considerable lack of genetic variability in most agricultural crops. Therefore, efforts should be made to develop innovative biotechnological approaches in addition to conventional methods, for the induction and conservation of genetic variability. In this respect, rice biotechnology employing *in vitro* systems is competent to meet the challenge. One of the major advantages of *in vitro* methods is that some of the useful variants can be selected and stabilised in a single generation.

The frequency of genetic change in somaclones is much higher than the spontaneous genetic changes brought about in the entire plant (Prat 1983). This type of genetic change can be effectively utilised if entire rice plants can be regenerated from somaclonal variant cells. Therefore, somaclonal variation could be used to produce

new rice varieties that retain all the favourable agronomic qualities of existing varieties while improving one additional trait, for example, disease or insect resistance or herbicide tolerance. Immediately after isolating these isogenic lines, they could be used for rice breeding, rice genetic research and as ideal material for genetic transformation. It is now generally accepted that heritable variation exists in somaclones, and some promising lines have already been developed from rice somaclones (Sun and Zheng 1990).

It is also recognised that the increased frequency of genomic structural rearrangement during rice cell and tissue culture provides a new opportunity for alien gene introgression. Furthermore, this can help widen the rice germplasm base, particularly in wide crosses, where alien chromosomes cannot replicate through meiosis. As foreign genes have been successfully transferred to rice protoplasts and plantlets have been regenerated from transformed (Schuh et al. 1993) or fused protoplasts (Toriyama and Hinata 1988b), investigations of both *in vitro* and *in vivo* behaviour of foreign gene(s) present in regenerated plants and their progenies is much needed, because it is going to play an important role in rice improvement. In recent years and in the present study, flow cytometry has proved to be a fast, reliable and highly sensitive tool for DNA analysis to detect variant plants in large populations and also to detect intraspecific variation. The microscopic analysis and counting of rice chromosomes to determine the ploidy levels in large populations of plants is, in comparison, very difficult, tedious and time consuming. This is related to the small size of rice chromosomes and to the very low DNA content of *Oryza* species (1.2 pg) compared to wheat (34.6 pg) (Bennett and Smith 1976).

Recently developed basic and advanced biotechnological approaches for the improvement of rice are now available from different laboratories throughout the world. These include improved procedures for the production of fertile variant rice plants from mesophyll and cell suspension-derived protoplasts of different explant origin, using both *Indica* and *Japonica* varieties (Gupta and Pattanayak 1993; Lee et al. 1989; Datta et al. 1992; Torrizo and Zapata 1992; Jain et al. 1994 and the present work). The production of novel somatic hybrid plants between cultivated rice and its wild relatives to transfer useful agronomic traits (Toriyama and Hinata 1988b; Slamet 1991; Hayashi et al. 1989; Mori et al. 1991) such as environmental stress tolerance or

disease or insect resistance will complement efforts on wide hybridisation using conventional breeding and embryo rescue (Khush 1991). Sexually incompatible interspecific and intergeneric fusions in rice, which are not possible to achieve by conventional breeding techniques, should be a high priority in future somatic hybridisation programmes. The generation of cybrids to transfer cytoplasmic male sterility (Akagi et al. 1989; Finch 1991; Table 2D) is another innovative approach.

The ability to produce fertile transgenic rice plants, which can express agronomically important genes is still limited by the lack of availability of suitable cloned genes. Successful cloning of the coat-protein gene from tungro virus (Hull et al. 1990), which causes the most damaging, widespread viral disease in South and South-East Asia, may enable transgenic rice varieties to be developed with the coat-protein-mediated protection already demonstrated in other crops (Beachy et al 1988). In the near future, successful production of transgenic rice plants with disease and insect resistance genes, and the introduction of genes for grain quality should be realistic possibilities for further improvement of this important crop. When it is feasible to apply biotechnological approaches to the genetic manipulation of rice, they will be a valuable complement to conventional breeding techniques.

Appendices

Appendix 1 Anther and tissue culture media used in this project

Appendix A1.1 Media based on CPW salts (Frearson et al. 1973)

Constituents	Concentration (mgl^{-1})
KH_2PO_4	27.2
KNO_3	101
$CaCl_2 . 2H_2O$	1480
$MgSO_4 . 7H_2O$	246
KI	0.16
$CuSO_4 . 5H_2O$	0.025
Additions for CPW13M :	
Mannitol	13% w/v
Additions for CPW21S :	
Sucrose	21% w/v
All at pH 5.8	Autoclaved

Appendix 1.2 Standard enzyme mixture for protoplast isolation from rice cell suspension cultures

Constituents	Concentration
Cellulase RS	10 gl^{-1}
Pectolyase Y23	1 gl^{-1}
MES buffer	5 mM
CPW salts	for 1 litre
Mannitol	130 gl^{-1}
Made up in CPW 13M medium	pH 5.6

Filter sterilised through a 0.2 μm membrane.

Appendix 1.3 Salt composition of MS based tissue culture media (Murashige and Skoog 1962)

Constituents	Concentration (mgl^{-1})
$CaCl_2.\ 2H_2O$	440
$CoCl_2.\ 6H_2O$	0.025
$CuSO_4.\ 5H_2O$	0.025
$FeSO_4.\ 7H_2O$	27.85
H_3BO_3	6.2
KH_2PO_4	170
KI	0.83
KNO_3	1900
Na_2EDTA	37.25
$Na_2MoO_4.\ 2H_2O$	0.25
NH_4NO_3	1650
$MgSO_4.\ 7H_2O$	370
$MnSO_4.\ 4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
Glycine	2.0
Myo-inositol	100
Nicotinic acid	0.5
Pyridoxine HCL	0.5
Thiamine HCL	0.1

pH 5.8 Autoclaved

LS (Linsmaier and Skoog 1965) = MS salts with 1.0 mgl^{-1} Thiamine HCL and no Nicotinic acid or Pyridoxine HCL. LS is made up as a double strength liquid and added to an equal volume of autoclaved 0.8% aqueous Sigma Type 1 agarose.

Appendix 1.4 Salt composition of K based tissue culture media (Kao et al. 1973)

Constituents	Concentration (mgl^{-1})
$CaCl_2.\ 2H_2O$	600
$CoCl_2.\ 6H_2O$	0.025
$CuSO_4.\ 5H_2O$	0.025
H_3BO_3	3.0
KCl	300
KH_2PO_4	170
KI	0.75
KNO_3	1900
$MgSO_4.\ 7H_2O$	300
$MnSO_4.\ H_2O$	10
$NaMoO_4.\ 2H_2O$	0.25
NH_4NO_3	600
$ZnSO_4.\ 7H_2O$	2.0
Sequestrene-330Fe	28
Ascorbic acid	1.0
Biotin	0.005
Choline chloride	0.5
Folic acid	0.2
Myo-inositol	100
Nicotinamide	1.0
p-ABA	0.01
D-Ca Pantothenate	0.5
Pyridoxine HCL	1.0
Riboflavin	0.1
Thiamine HCL	1.0
Vitamin A	0.005
Vitamin B12	0.01
Vitamin D3	0.005
Citric acid	10
Fumaric acid	10
Malic acid	10
Na-Pyruvate	5.0
Cellobiose	125
Fructose	125
Mannose	125
Rhamnose	125
Ribose	125
Xylose	125
Mannitol	125
Sorbitol	125
Vitamin-free Casamino acids	125
Coconut milk	10 ml

Filter sterilised through a 0.2 μm membrane.

Appendix 1.5 Salt composition of AA based tissue culture medium (Müller and Grafe 1978)

Constituents	Concentration (mg^{-1})
$CaCl_2 . 2H_2O$	440
$CoCl_2 . 6H_2O$	0.025
$CuSO_4 . 5H_2O$	0.025
$FeSO_4 . 7H_2O$	27.85
H_3BO_3	6.2
KCl	2940
KH_2PO_4	170
KI	0.83
Na_2EDTA	37.25
$Na_2MoO_4 . 2H_2O$	0.25
$MgSO_4 . 7H_2O$	370
$MnSO_4 . 4H_2O$	22.3
$ZnSO_4 . 7H_2O$	8.6
Myo-inositol	100
Nicotinic acid	0.5
Pyridoxine HCL	0.1
Thiamine HCL	0.5
L-Arginine	228
L-Aspartic acid	226
L-Glutamine	877
Glycine	75

pH 5.8

Filter sterilised through a 0.2 µm membrane.

Appendix 1.6 Salt composition of Gamborg's B5 (modified) based tissue culture medium (Gamborg et al. 1968)

Constituents	Concentration (mgl^{-1})
KNO_3	2500
$CaCl_2.\ 2H_2O$	150
$MgSO_4.\ 7H_2O$	250
$(NH_4)_2SO_4$	134
$NaH_2PO_4.\ 2H_2O$	150
KI	0.75
H_3BO_3	3.0
$MnSO_4.\ 4H_2O$	10.0
$ZnSO_4.\ 7H_2O$	2.0
$Na_2MoO_4.\ 2H_2O$	0.25
$CuSO_4.\ 5H_2O$	0.025
$CoCl_2.\ 6H_2O$	0.025
$FeSO_4.\ 7H_2O$	27.8
Na_2EDTA	37.3
Myo inositol	160
Pyridoxine HCL	1.0
Thiamine HCL	10.0
Nicotinic acid	1.0

pH 5.8

Filter sterilised through a 0.2 μm membrane.

Appendix 1.7 Composition of tissue culture media used in this project

Code	Salt base	Concentration of supplements (mg l^{-1})											(g l^{-1})		
		ABA	BAP	2,4-D	GA	K	NAA	Z	I	P	A	G	Y	Glu	Suc
AA1		-	-	1.0	0.1	0.2	-	-	-	-	-	-	-	-	20
AA2	AA	-	-	2.0	0.1	0.2	-	-	-	-	-	-	-	-	20
KP8	K	-	-	0.2	-	-	1.0	0.5	-	-	-	-	-	100	0.25
KPR	K	-	-	0.5	-	-	1.0	0.5	-	-	-	-	-	100	0.25
LS2.5	LS	-	-	2.5	-	-	-	-	-	-	-	-	-	-	30
MS0	MS	-	-	-	-	-	-	-	-	-	-	-	-	-	30
MSB2	MS	-	2.0	-	-	-	-	-	-	-	-	-	-	-	50
MSN1.5	MS	-	-	-	-	-	1.5	-	-	-	-	-	-	-	30
MSKN	MS	-	-	-	-	2.0	0.5	-	-	-	-	-	-	-	30
E24	B5	-	0.5	1.0	-	-	0.5	-	-	-	-	-	-	-	20
E10	B5	-	0.5	1.0	-	-	-	-	0.5	-	-	-	-	5	20
E10P	B5	-	0.5	1.0	-	-	-	-	0.5	115	-	-	-	5	20
E10A	B5	-	0.5	1.0	-	-	-	-	0.5	-	89	-	-	5	20
E10G	B5	-	0.5	1.0	-	-	-	-	0.5	-	-	146	-	5	20
N19	MS	-	-	-	-	1.0	1.0	-	-	-	-	-	-	-	30
$N19Y_1$	MS	-	-	-	-	1.0	1.0	-	-	-	-	-	10	-	30
J19	B5	-	-	-	-	1.0	1.0	-	-	-	-	-	-	-	20
$J19Y_1$	B5	-	-	-	-	1.0	1.0	-	-	-	-	-	10	-	20
MSA20	B5	20	-	-	-	1.0	1.0	-	-	-	-	-	-	-	30
MSYo	B5	-	-	-	-	1.0	1.0	-	-	-	-	-	-	-	30

LS2.5, MSB2, MSN1.5 and MSKN were solidified with 0.4% w/v Sigma Type 1 agarose. KP8 and KPR were solidified with 1.2% w/v Sea Plaque agarose (FMC Corp., Rockland MA, USA). MS0 was solidified with 0.8% w/v Sigma agar. K, AA and B5 media were filter sterilized, the rest were autoclaved. Agarose was autoclaved separately from other media components and mixed when sterile during plating. All B5 media were adjusted to pH 5.6 and all other media to pH 5.8.

Appendix 2

A2.1 Sterilization of media and equipment

All equipments and media with non-thermolabile components, which were dispensed into suitable containers, were sterilized by autoclaving at 121°C for 20 min (15 psi). Agarose was autoclaved separately from the rest of the medium components. Media containing thermolabile components, such as amino acids, were filter-sterilized by passage through a 0.2µm cellulose nitrate filter. Enzyme mixtures for cell wall digestion were centrifuged at high speed prior to this process to remove debris.

A2.2 Aseptic techniques

All manipulations for *in vitro* grown tissue and plant materials and operations such as filter sterilization were carried out in a lamina-flow of sterile air provided by a Slee 6HLF horizontal lamina flow cabinet providing an airflow of 0.5m/sec.

Appendix 3

Appendix A3.1 Descriptive statistics of plant characteristics for seed and protoplast-derived plants

Plant source	Days to flowering (mean ± se)	Plant height (mean ± se)	Total no. tillers (mean ± se)	No. fert. tillers (mean ± se)	F-leaf length (mean ± se)	F-leaf width (mean ± se)
T309	115.1 ± 1.54	123.0 ± 10.44	14.667 ± 3.34	7.583 ± 2.74	39.21 ± 6.76	1.203 ± 0.16
L52	230.3 ± 2.25	96.48 ± 17.36	12.758 ± 8.00	7.848 ± 6.21	33.33 ± 8.31	0.953 ± 0.22
L51	219.5 ± 2.30	93.29 ± 24.37	5.667 ± 2.06	0.733 ± 0.59	41.61 ± 12.10	0.977 ± 0.25
L55	198.4 ± 3.84	62.31 ± 4.07	8.429 ± 2.07	2.286 ± 1.25	19.47 ± 2.92	0.947 ± 0.13
L14R2	195.9 ± 8.09	96.00 ± 0.00	3.500 ± 0.71	2.000 ± 1.41	31.20 ± 6.08	1.285 ± 0.26

Appendix A3.2 Descriptive statistics of panicle characteristics for seed and protoplast-derived plants

Plant source	Panicle length (mean ± se)	No. Prim. bran. /pan. (mean ± se)	No. Second. bran. /pan. (mean ± se)	No. spikelets /pan. (mean ± se)
T309	19.93 ± 3.07	7.54 ± 2.97	10.88 ± 7.15	74.0 ± 38.3
L52	14.42 ± 2.97	7.42 ± 2.19	4.87 ± 3.66	45.1 ± 19.2
L51	17.03 ± 5.93	5.20 ± 1.62	3.60 ± 3.03	29.2 ± 13.1
L55	8.86 ± 1.75	6.86 ± 2.14	3.14 ± 1.51	39.0 ± 15.8
L14R2	15.85 ± 1.21	6.50 ± 1.29	5.25 ± 2.22	51.0 ± 9.83

Appendix A 3.3 Descriptive statistics of panicle characteristics for primary and secondary panicles of seed and protoplast-derived plants

Plant source	Panicle length (mean ± se)		No. Prim. bran. /panicle (mean ± se)		No. Second. bran. /panicle (mean ± se)		No. spikelets /panicle (mean ± se)	
	Prim. pan.	Second. pan.	Prim. pan.	Second. pan.	Prim. pan.	Second. pan.	Prim. pan.	Second. pan.
T309	20.73 ± 2.63	16.31 ± 2.22	8.312 ± 2.70	4.059 ± 0.97	12.53 ± 6.77	3.41 ± 2.62	83.65 ± 35.2	30.29 ± 13.27
L52	15.73 ± 2.30	11.43 ± 2.00	8.234 ± 1.97	5.571 ± 1.37	5.77 ± 3.73	2.81 ± 2.51	52.64 ± 17.5	27.70 ± 8.97
L51	18.17 ± 5.01	6.80 ± 0.00	5.444 ± 1.51	3.000 ± 0.00	3.89 ± 3.06	1.00 ± 0.00	31.44 ± 11.7	9.00 ± 0.00
L55	9.52 ± 1.58	7.22 ± 0.87	7.500 ± 2.12	5.250 ± 1.26	3.00 ± 1.49	3.50 ± 1.73	44.40 ± 15.4	25.50 ± 5.20
L14R2	15.85 ± 1.21	0	6.500 ± 1.29	0	5.25 ± 2.22	0	51.00 ± 9.8	0

Appendix A 3.4 Descriptive statistics of panicle characteristics for control and protoplast-derived plants classified according to panicle types

Panicle type	Panicle length (mean ± se)		No. Prim. bran. /panicle (mean ± se)		No. Second. bran. /panicle (mean ± se)		No. spikelets /panicle (mean ± se)	
	Control	Protoclones	Control	Protoclones	Control	Protoclones	Control	Protoclones
Primary	20.73 ± 2.63	15.55 ± 2.79	8.312 ± 2.70	8.051 ± 2.04	12.53 ± 6.77	5.55 ± 3.66	83.65 ± 35.2	51.34 ± 17.6
Secondary	16.31 ± 2.22	11.19 ± 2.19	4.059 ± 0.97	5.528 ± 1.38	3.41 ± 2.62	2.82 ± 2.47	30.29 ± 13.3	27.39 ± 9.00

Appendix A 3.5 Analysis of Variance between control lines and all protoclonal lines (L51, L52, L55, L14R2)

Plant characteristics	Degrees of freedom (d. f.)		Sum of squares (s. s.)		Mean squares (m. s.)		Variance ratio(v. r.)	Probability
	Plant type	Residual	Plant type	Residual	Plant type	Residual		
Plant height (cm)	1	57	7065	19064	7065	335	21.1	< 0.001*
Total tillers/plant	1	57	114	2443	114	43	2.7	0.108
Fertile tillers/plant	1	57	23	1678	23	29	0.8	0.377
Flag leaf length (cm)	1	57	334	5493	334	96	3.5	0.068
Flag leaf width (cm)	1	57	0.4	2	0.4	0.04	11.2	0.001*

*, significant at 5% level.

Appendix A 3.6 Analysis of Variance between control lines and two protoclonal lines (L51, L52)

Plant characteristics	Degrees of freedom (d. f.)		Sum of squares (s. s.)		Mean squares (m. s.)		Variance ratio (v. r.)	Probability
	Lines	Residual	Lines	Residual	Lines	Residual		
Plant height (cm)	2	48	4545	11508	2272	240	9.5	< 0.001*
Total tillers/plant	2	48	549	1753	275	37	7.5	0.001*
Fertile tillers/plant	2	48	450	1109	225	23	9.7	< 0.001*
Flag leaf length (cm)	2	48	620	3565	310	74	4.2	0.021*
Flag leaf width (cm)	2	48	0.4	2	0.2	0.04	5.8	0.006*

*, significant at 5% level.

Appendix A 3.7 Analysis of Variance between control line (T309) and protoclonal line (L52)

Panicle Characters	Degrees of freedom (d. f.)		Sum of squares (s. s.)		Mean squares (m. s.)		Variance ratio (v. r.)	Probability
	Plant types	Residual	Plant types	Residual	Plant types	Residual		
Panicle length (cm)	1	368	2130	3303	2130	9	237.3	< 0.001*
Prim. branches/panicle	1	368	1	2135	1	6	0.2	0.680
Second. bran./panicle	1	368	2536	8439	2536	23	110.6	< 0.001*
Total spikelets/panicle	1	368	58764	238135	58764	647	90.8	< 0.001*

*, significant at 5% level

Appendix A 3.8 Analysis of Variance between control line (T309) and protoclonal line (L52)

Panicle Characters	Degrees of freedom (d. f.)		Sum of squares (s. s.)		Mean squares (m. s.)		Variance ratio (v. r.)	Probability
	Panicle types	Residual	Panicle types	Residual	Panicle types	Residual		
Panicle length (cm)	1	368	1776	3658	1776	10	178.7	< 0.001*
Prim. branches/panicle	1	368	635	1501	635	4	155.6	< 0.001*
Second. bran./panicle	1	368	1689	9286	1689	25	66.9	< 0.001*
Total spikelets/panicle	1	368	81808	215091	81808	585	140.0	< 0.001*

*, significant at 5% level

Appendix A 3.9 Analysis of Variance between control line (T309) and protoclonal line (L52)

Panicle Characters	Degrees of freedom (d. f.)			Sum of squares (s.s.)			Mean squares (m. s.)			Variance ratio	Probability	Variance ratio	Probability
	Plant type	Plant type & Panicle type	Residual	Plant type	Plant type & Panicle type	Residual	Plant type	Plant type & Panicle type	Residual				
Panicle length (cm)	1	2	366	2130	1357	1946	2130	679	5	400.6	< 0.001*	127.6	< 0.001*
Prim. branches/panicle	1	2	366	1	666	1468	1	333	4	0.3	0.620	83.0	< 0.001*
Second. bran./panicle	1	2	366	2536	1671	6768	2536	835	18	137.1	< 0.001*	45.2	< 0.001*
Total spikelets/panicle	1	2	366	58764	75984	162151	58764	37992	443	132.6	< 0.001*	85.8	< 0.001*

*, significant at 5% level.

References

Abdullah R (1987). The development of a reproducible system for efficient plant regeneration from rice (*Oryza sativa* L.) protoplasts. Ph.D. Thesis, University of Nottingham, UK.

Abdullah R, Cocking EC, Thompson JA (1986). Efficient plant regeneration from rice protoplasts through somatic embryogenesis. Bio/Technol. 4 : 1087-1090.

Abdullah R, Thompson JA, Khush GS, Kaushik RP, Cocking EC (1989). Protoclonal variation in the seed progeny of plants regenerated from rice protoplasts. Plant Sci. 65: 97-101.

Abe T, Futsuhara Y (1984). Varietal difference of plant regeneration from root callus tissues in rice. Jpn. J. Breed. 34 : 147-155.

Abe T, Futsuhara Y (1985). Efficient plant regeneration by somatic embryogenesis from root callus tissues of rice (*Oryza sativa* L.). J. Plant Physiol. 121 : 111-118.

Abe T, Futsuhara Y (1986). Genotypic variability for callus formation and plant regeneration in rice (*Oryza sativa* L.). Theor. Appl. Genet. 72 : 3-10.

Abrigo WM, Novero AU, Coronel VP, Cabuslay GS, Blanco LC, Parao FT, Yoshida S (1985). Somatic cell culture at IRRI. In: Biotechnology in International Agricultural Research. Int. Rice Res. Inst., Manila, Philippines, p 149-158.

Afonso CL, Harkins KR, Thomas-Compton MA, Krejci AE, Galbraith DW (1985). Selection of somatic hybrid plants in *Nicotiana* through fluorescence-activated sorting of protoplasts. Bio/Technol. 3 : 811-816.

Akagi H, Sakamoto M, Negishi T, Fujimura T (1989). Construction of rice cybrid plants. Mol. Gen. Genet. 215 : 501-506.

Akhmed I (1989). Advanced agricultural biotechnologies. Some empirical findings on their social impact. Internat. Labour Rev. 128: 553-572.

Alexander RG, Cocking EC, Jackson PJ, Jett JH (1985). The characterization and isolation of plant heterokaryons by flow cytometry. Protoplasma 128 : 52-58.

Allchin FR (1969). Early cultivated plants in India and Pakistan. In:The domestication and exploitation of plants and animals. Ucko PJ and Dimbleby GW eds., Aldine, Gerald Duckworth, London, Chicago, p 323-329.

Ameniya A. Akemine H, Toriyama K (1956). Culture conditions and growth of immature embryo in rice plant (Studies on the embryo culture in rice plant I). Bull. Natl. Inst. Agric. Sci. (Jap.) Ser. D(6) : 1-40.

Andrus JR, Mohammed AF (1958). The economy of Pakistan. Oxford University Press, Oxford.

Asselin de Beauville M (1980). Obtention d'haploïdes in vitro à partir d'ovaires non fécondés de riz, *Oryza sativa* L. CR Acad. Sci. Paris D 296 : 489-492.

Arumuganathan K, Earle ED (1991a). Nuclear DNA content of some important plant species. Plant Mol. Biol. Rep. 9 : 208-218.

Arumuganathan K, Earle ED (1991b). Estimation of nuclear DNA content of plants by flow cytometry. Plant Mol. Biol. Rep. 9 : 229-233.

Baba A, Hasezawa S and Syono K (1986). Cultivation of rice protoplasts and their transformation mediated by *Agrobacterium* spheroplasts. Plant Cell Physiol. 27 : 463-471.

Baenziger PS, Schaeffer GW (1983). Dihaploids via anthers cultured *in vitro*. In: Genetic Engineering: Applications to Agriculture. Beltsville Symposia in Agricultural Research 7. Owens LD ed., Rowman & Allanheld, Totowa, New Jersey, p 269-284.

Bajaj YPS (1983a). *In vitro* production of haploids. In: Handbook of plant cell culture, Vol. 1. Evans DA, Sharp WR, Ammirato PV, Yamada Y eds., MacMillan, New York, p 228-287.

Bajaj YPS (1983b). Haploid protoplasts. *In*: Plant protoplasts. Giles KL ed., Academic Press, London, New York, p 113-141.

Bajaj YPS (1989b). Recent advances in the isolation and culture of protoplasts and their implications in crop improvement. In: Biotechnology in Agriculture and Forestry, Vol. 8. Plant Protoplasts and Genetic Engineering I. Bajaj YPS ed., Springer-Verlag, Berlin, Heidelberg, New York, Tokyo, p 3-22.

Bajaj YPS (1989c). Genetic engineering and *in vitro* manipulation of plant cells–technical advances. In: Biotechnology in Agriculture and Forestry, Vol. 9. Plant Protoplasts and Genetic Engineering II. Bajaj YPS ed., Springer-Verlag, Berlin, Heidelberg, New York, Tokyo, p 1-25.

Bajaj YPS (1990a). Somaclonal Variation–Origin, induction, cryopreservation, and implications in plant breeding. In: Biotechnology in Agriculture and Forestry, Vol. 11, Somaclonal Variation in Crop Improvement I. Bajaj YPS ed., Springer-Verlag, Berlin, Heidelberg, New York, Tokyo, p 3-48.

Bajaj YPS (1990b). *In vitro* production of haploids and their use in cell genetics and plant breeding. In: Biotechnology in Agriculture and Forestry, Vol. 12, Haploids in Plant Improvement I. Bajaj YPS ed., Springer-Verlag, Berlin, Heidelberg, New York, Tokyo, p 3-44.

Bajaj YPS (1991). Biotechnology in rice improvement. In: Biotechnology in Agriculture and Forestry, Vol. 14. Rice. Bajaj YPS ed., Springer-Verlag, Berlin, Heidelberg, New York, Tokyo, p 3-18.

Bajaj YPS, Bidani M (1980). Differentiation of genetically variable plants from embryo-derived callus of rice. Phytomorphol. 30 : 290-294.

Bajaj YPS, Bidani M (1986). *In vitro* induction of genetic variability in rice (*Oryza sativa* L.). In: New Genetical Approaches to Crop Improvement. Siddiqui KA, Faruqui AM eds., PIDC, Karachi, Pakistan, p 63-74.

Bajaj YPS, Davey MR (1974). The isolation and ultrastructure of pollen protoplasts. In: Fertilization in higher plants. Linskens HF ed., North-Holland, Amsterdam, p 73-80.

Bajaj YPS, Davey MR, Grout BW (1975). Pollen tetrad protoplasts: A model system for the study of ontogeny of pollen. In: Gamete competition in plants and animals. Mulcahy DL ed., North-Holland, Amsterdam, p 7-18.

Barker R, Herdt RW, Rose B (1985). The rice economy of Asia. Resources for future, Inc., Washington D.C. 324p.

Baset A (1992). Protoplast to plant regeneration systems for cultivated and wild rices. Ph.D. Thesis, University of Nottingham, UK.

Baset A, Finch RP, Cocking EC (1991). Plant regeneration from protoplasts of wild rice (*Oryza rufipogon* Griff.). Plant Cell Rep. 10 : 200-203.

Bayard DT (1970). Excavations of Non Nok Tha, Northeastern Thailand. Asian Perspectives 13 : 109-143.

Beachy RN, Nelson RS, Regester III J, Fraley RT, Tumer N (1988). Transformation to produce virus-resistant plants. In: Genetic Improvements of Agriculturally Important Crops. Fraley RT, Frey NM, Schell JS eds., Cold Spring Harbour Curr. Comm., p 47-53.

Bennett MD and Smith JB (1976). Nuclear DNA amounts in Angiosperms. Phil. Trans. R. Soc. Lond. B274 : 227-274.

Bennici A, D'Amato F (1978). *In vitro* regeneration of durun wheat plants. I. Chromosome numbers of regenerated plants. Z. pflanzenzücht 81 : 305-311.

Bergounioux C, Perennes C, Miege C & Gadal P (1986). The effect of male sterility on protoplast division in *Petunia hybrida*. Cell cycle comparison by flow cytometry. Protoplasma 130 : 138-144.

Bergounioux C, Brown SC, Petit PX (1992). Flow cytometry and plant protoplast cell biology. Physiol. Plant. 85 : 374-386.

Bezancon G, Second G (1979). Prospection of African Rice, Mission to Zambia, 1978 Campaign. In: Mimeo. Report, IRAT-ORSTOM (1974-1984), Paris.

Binding H, Binding K, Straub J (1970). Selektion in Gewebekulturen mit haploiden Zellen. Naturwissenschaften 57 : 138-139.

Binh DQ, Heszky LE, Gyulai G, Csillag A (1992). Plant regeneration of NaCl-pretreated cells from long-term suspension culture of rice (*Oryza sativa* L.) in high saline conditions. Plant Cell, Tiss. Org. Cult. 29 : 75-82.

Biswas GCG, Zapata FJ (1990). Callus formation and plant regeneration from protoplasts derived from suspension culture of rice (*Oryza sativa* L. cv. Taipei 177). Plant Breed. 105: 95-100.

Biswas GCG, Zapata FJ (1991a). Embryogenic cell suspension and plant regeneration from protoplasts of Indica rice. Int. Rice Res. Newslett. 16 : 9-10.

Biswas GCG, Zapata FJ (1991b). Morphogenesis in anther derived cell suspension protoplasts of Indica rice (*Oryza sativa* L. cv. Tetep). J. Genet. & Breed. 45 : 301-306.

Biswas GCG, Zapata FJ (1992). Plant regeneration from long-term cell suspension cultures of Indica rice (*Oryza sativa* L. cv. IR43). J. Plant Physiol. 139 :523-527.

Biswas GCG, Zapata FJ (1993). High-frequency plant regeneration from protoplasts of Indica rice (*Oryza sativa* L.) using maltose. J. Plant Physiol. 141 :470-475.

Biswas GCG, Burkhardt PK, Wünn J, Klöti A, Potrykus I (1994). Fertile Indica rice plants regenerated from protoplasts isolated from scutellar tissue of immature embryos. Plant Cell Rep. 13 : 528-532.

Botti C, Vasil IK (1983). Plant regeneration by somatic embryogenesis from parts of cultured mature embryos of *Pennisetum americanum* (L.) K. Schum. Z. für Pflanzenphysiol. 111 : 319-325.

Bray F (1986). The Rice Economics. Blackwell, NY, USA.

Bright SWJ (1985). Selection *in vitro*. In: Cereal tissue and cell culture. Bright SWJ, Jones MGK eds., Martinus Nijhoff / Dr. W Junk publ., Dordrecht, Boston, Lancaster, p 231-260.

Bright SWJ, Jones MGK (1985). Cereal tissue and cell culture. Martinus Nijhoff / Dr. W Junk publ., Dordrecht, Boston, Lancaster.

Brown PTH, Kyozuka J, Sukekiyo Y, Kimura Y, Shimamoto K, Lörz H (1990). Molecular changes in protoplast-derived rice plants. Mol. Gen. Genet. 223 : 324-328.

Brown SC, Jullien M, Coutos-Thevenot P, Muller P, Renaudin J-P (1986). Present developments of flow cytometry in plant biology. Biology of the Cell 58 : 173-178.

Candolle A. De (1886). Origin of cultivated plants (English translation), 2nd ed. Hafner Publishing, New York.

Cao J, Zhang W, McElroy D, Wu R (1991). Assessment of rice genetic transformation techniques. In: Rice Biotechnology. Toenniessen G, Khush GS eds., IRRI and Rockefeller Foundation, p 175-198.

Carlson PS (1970). Induction and isolation of auxotrophic mutants in somatic cultures of *Nicotiana tabacum*. Science 168 : 487-489.

Carlson PS (1973a). The use of protoplasts for genetic research. Proc. Natl. Acad. Sci. USA 70 : 598-602.

Carlson PS (1973b). Methionine sulfoximine-resistant mutants of tobacco. Science 180 : 1366.

Chaleff RS, Stolarz A (1981). Factors influencing the frequency of callus formation among cultured rice (*Oryza sativa*) anthers. Physiol. Plant. 51 : 201-206.

Chaleff RS, Keil RL (1981). Genetic and physiological variability among cultured cells and regenerated plants of *Nicotiana tabacum*. Mol. Gen. Genet. 181 : 254-258.

Chang TT (1976a). The rice cultures. Phil. Trans. R. Soc. (London), B275 : 143-157.

Chang TT (1976b). The origin, evolution, cultivation, dissemination, and diversification of Asian and African rices. Euphytica 25 : 425-441.

Chang TT (1983). The origins and early cultures of the cereal grains and food legumes. In: The origins of Chinese cultivation. Keightley DN ed., Univ. of California Press, p 65-94.

Chang TT (1984). Conservation of rice genetic resources : luxury or necessity? Science 224 : 251-256.

Chang TT (1985). Crop history and genetic conservation : Rice-a case study. Iowa State J. Res. 59(4) : 425-455.

Chang TT (1987). The impact of rice on human civilization and population expansion. Inter. Sci. Rev., Vol. 12, No. 1, p 63-69.

Chang TT (1988). The ethnobotany of rice in island southeast Asia. Asian Perspectives 26 : 69-76.

Chang H, Liu T, Wang Y (1978). A preliminary observation on histogenesis and organogenesis of the in vitro developments from rice microspores into plantlets. In: Proc. Symp. Plant Tissue Culture, Science Press, Peking, p 125-132.

Chen CC (1977). *In vitro* development of plants from microspores of rice. In Vitro 13 : 484-489.

Chen CC, Chen CM (1979). A method for anther culture of rice. Tissue Cult. Assoc. Manual 5 : 1051-1053.

Chen CC, Chen CM (1980). Changes in chromosome number in microspore callus of rice during successive subcultures. Can. J. Genet. Cytol. 22 : 607-614.

Chen CC, Lin MH (1976). Induction of rice plantlets from anther culture. Bot. Bull. Acad. Sin. 17 : 18-24.

Chen CC, Wu YH (1983). Segmentations in microspores of rice during anther culture. Proc. Natl. Sci. Counc. B 7 : 151-157.

Chen CC, Chiu WL, Yu LJ, Ren SS, Yu WJ, and Lin MH (1983). Genetic analysis of anther -derived plants of rice: tests for independent assortment of unlinked genes. Can. J. Genet. Cytol. 25 : 324-328.

Chen CC, Tsay HS, Huang CR (1986). Rice (*Oryza sativa* L.) : Factors affecting androgenesis. In: Biotechnology in Agriculture and Forestry, Vol. 2:Crops I. Bajaj YPS ed., Springer-Verlag, Berlin, Heidelberg, p 123-138.

Chen CC, Tsay HS, Huang CR (1991). Factors affecting androgenesis in rice (*Oryza sativa* L.). In: Biotechnology in Agriculture and Forestry, Vol. 14. Rice. Bajaj YPS ed., Springer-Verlag, Berlin, Heidelberg, New York, Tokyo, p 193-215.

Chen JJ, Tsay HS (1984). The callus-forming ability of rice anther originated from tillers of different order and from spikelets of different positions and branches on the panicles. J Agric. Res. China 33(4) : 354-362.

Chen JJ, Chen LJ, Tsay HS (1984). Relationship between the time for callus initiation, callus age and the ability of organ regeneration from rice anther culture. J. Agric. Assoc. China 126 : 2-9.

Chen JJ, Hsu JY, Tsay HS (1986). Effect of iron on rice anther culture. J. Agric. Res. China 35(3) : 244-252.

Chen Y, Wang RF, Tian WZ, Zuo QX, Zheng SW, Lu DY, Zhang GH (1980). Studies on pollen culture *in vitro* and induction of plantlets in *Oryza sativa* subsp. Keng. Acta Genet. Sin. 7 : 46-54.

Chen Y, Zuo QX, Li SY, Lu DY, Zheng SW (1981a). Green plants regenerated from isolated rice pollen grains *in vitro* and the induction factors. Acta Genet. Sin. 8 : 158-163.

Chen Y, Zuo QZ, Li SY, Qu RD (1982). Plant regeneration from isolated rice pollen culture and some factors affecting induction frequency. In: Plant Tissue Culture 1982. Proc. 5th Intl. Cong. Plant Tissue & Cell Culture. Fujiwara A ed., Maruzen, Tokyo, p 559-560.

Chen Y (1983). Anther and pollen culture of rice in China. In: Proc. of Workshop on Cell and Tissue Culture Techniques for Cereal Crop Improvement. Science Press, Beijing, China and IRRI, Manila, Philippines, p 11-26.

Chen Y (1986a). Anther and pollen culture of rice. In: Haploids of higher plants *in vitro*. Hu H, Yang H eds., China Acad. Publ., Beijing, and Springer-verlag, Berlin, Heidelberg, p 3-15.

Chen Y, Meng Z (1986). Isolation of a rice mutant resistant to S-(2-aminoethyl)-cysteine (AEC). Abstr. Int. Congr. Plant Tissue Cell Cult., 6th, Univ. Minn. p 439.

Cheng TK (1974). The prehistory of China. T'oung Pao 60 : 1-11.

Cho MS, Zapata FJ (1988). Callus formation and plant regeneration in isolated pollen culture of rice (*Oryza sativa* L. cv. Taipei 309). Plant Sci. 58 : 239-244.

Chu CC (1982a). Haploids in plant improvement. In: Plant Improvement and Somatic Cell Genetics. Vasil IK, Scowcroft WR, Frey KJ eds., Academic Press, London, New York, p 129-158.

Chu CC, Wang CS, Sun CS, Hsu C, Yin KC, Chu CY, Bi FY (1975). Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci. Sin. 18 : 659-668.

Chu Q, Zhang Z, Gao Y (1985). Cytogenetical analysis on aneuploids from protoclones of rice (*Oryza sativa* L.). Theor. Appl. Genet. 71 : 506-512.

Chung GS (1987). Application of anther culture technique for rice (*Oryza sativa* L.) improvement. In: Proc. Korea-China Plant Tiss. Cult. Symp., Nov. 17-23, Suweon, Korea, Korean Soc. of plant tissue culture, Agricultural Sci. Inst. and Inst. of Bot., Academia Sinica publishers, RDA and China, p 36-56.

Chuong PV, Beversdorf WD, Powell AD, Pauls KP (1988). Somatic transfer of cytoplasmic traits in *Brassica napus* L. by haploid protoplast fusion. Mol. Gen. Genet. 211 : 197-201.

Clayton WD (1968). Studies in *Gramineae* : XVII. West African wild rice. Kew Bulletin 21 : 485-488.

Cocking EC (1987). Plant cell biology in the 21st Century : the needs of plant cell and tissue culture. In: Plant Tissue and Cell Culture. Proc. VIth Intern. Cong. on Plant Tiss. and Cell Cult., August 3-8, 1986, Minnesota, USA. Green CE, Somers DA, Hackett WP, Biesboer DD eds., Alan R. Liss, Inc., New York, p 3-15.

Cocking EC (1988). Gene transfer in rice, using protoplast fusion and recombinant DNA technology. The Second Shouichi Yoshida Memorial Lecture, Intern. Rice Res. Conf., Hangzhou, China, 24 Sept., 1987, Intern. Rice Res. Inst., Manila, Philippines, p 1-20.

Cornejo-Martin MJ, Primmo-Millo E (1981). Anther and pollen grain culture of rice (*Oryza sativa* L.). Euphytica 30 : 541-546.

Costich DE, Ortiz R, Meagher TR, Bruederle LP, Vorsa N (1993). Determination of ploidy level and nuclear DNA content in blueberry by flow cytometry. Theor. Appl. Genet. 86 : 1001-1006.

Coulibaly MY, Demarly Y (1986). Regeneration of plantlets from protoplasts of rice, *Oryza sativa* L. Z. Pflanzenzücht. 96 : 79-81.

Croughan TP, Chu QR (1991). Rice (*Oryza sativa* L.): Establishment of callus cultures and the regeneration of plants. In: Biotechnology in Agriculture and Forestry, Vol. 14. Rice. Bajaj YPS ed., Springer-Verlag, Berlin, Heidelberg, New York, Tokyo, p 20-37.

D'Amato F (1965). Endopolyploidy as a factor in plant tissue development. In: Plant Tissue Culture. White P, Grove RA eds., McCutchan, Berkeley, USA, p 449-462.

D'Amato F (1985). Cytogenetics of plant cell and tissue cultures and their regenerates. CRC Critical Rev. Plant Sci. 3 : 73-112.

Datta SK, Datta K, Potrykus I (1990a). Embryogenesis and plant regeneration from microspores of both 'Indica' and 'Japonica' rice (*Oryza sativa* L.). Plant Sci. 67 : 83-88.

Datta SK, Datta K, Potrykus I (1990b). Fertile Indica rice plants regenerated from protoplasts isolated from microspore derived cell suspensions. Plant Cell Rep. 9: 253-256.

Datta SK, Peterhans A, Datta K, Potrykus I (1990c). Genetically engineered fertile indica-rice recovered from protoplasts. Bio/Technol. 8 : 736-740.

Datta K, Potrykus I, Datta SK (1992). Efficient fertile plant regeneration from protoplasts of the Indica rice breeding line IR72 (*Oryza sativa* L.). Plant Cell Rep. 11 : 229-233.

Davey MR, Rech EL, Mulligan BJ (1989). Direct DNA transfer to plant cells. Plant Mol. Biol. 13 : 273-285.

Davey MR, Kothari SL, Zhang H, Rech EL, Cocking EC, Lynch PT (1991). Transgenic rice : characterization of protoplast-derived plants and their seed progeny. J. Exp. Bot. 42: 1159-1169.

De Datta SK (1981). Principles and practices of rice production. John Wiley and Sons, London.

De Laat AMM, & Blaas J (1984). Flow cytometric characterization and sorting of plant chromosomes. Theor. Appl. Genet. 67 : 463-467.

De Laat AMM, Schel JHN (1986). The integrity of metaphase chromosomes of *Haplopappus gracilis*. (Nutt.). Gray isolated by flow cytometry. Plant sci. 47 : 145-151.

De Laat AMM, Verhoeven HA & Sree Ramulu K (1989). Chromosome transplantation and application of flow cytometry in plants. In: Biotechnology in Agriculture and Forestry, Vol. 9. Plant Protoplasts and Genetic Engineering II. Bajaj YPS ed., Springer-Verlag, Berlin, Heidelberg, New York, Tokyo, p 343-359.

De Laat AMM, Göhde W & Vogelzang MJDC (1987). Determination of ploidy of single plants and plant populations by flow cytometry. Plant Breed. 99 : 303-307.

Deka PC and Sen SK (1976). Differentiation in calli originated from isolated protoplasts of rice (*Oryza sativa* L.) through plating technique. Mol. Gen. Genet. 145 : 239-243.

Dekeyser R, Claes B, Marichal M, Van Montagu M, Caplan A (1989). Evaluation of selectable markers for rice transformation. Plant Physiol. 90 : 217-223.

Delanny X, LaVallee BJ, Proksch RK, Fuchs RL, Sims SR, Greenplate JT, Marrone PG, Dodson RB, Augustine JJ, Layton JG, Fischhof DA (1989). Field performance of transgenic tomato plants expressing the *Bacillus thuringiensis* var. *kurstaki* insect control protein. Bio/Technol. 7 : 1265-1269.

Dunwell JM (1985). Anther and ovary culture. In: Cereal tissue and cell culture. Bright SWJ and Jones MGK eds., Martinus Nijhoff / Dr. W Junk publ., Dordrecht, Boston, Lancaster, p 1-44.

Dyer AF (1963). The use of lacto-propionic orcein in rapid squash methods for chromosome preparations. Stain Technol. 38 : 85-90.

Dykes TA, Nabors MW (1986). Tissue culture in rice and its application in selecting for stress tolerance. In: Rice Genetics : Proc. 1st Int. Rice Genet. Symp. 27-31 May 1985, IRRI, Los Baños, Philippines, p 799-810.

Evans DA, Flick CE, Kut SA and Reed SM (1982). Comparison of *Nicotiana tabacum* and *Nicotiana nisophila* hybrids produced by ovule culture and protoplast fusion. Theor. Appl. Genet. 62 : 193-198.

Evans DA, Sharp WR (1983). Single gene mutations in tomato plants regenerated from tissue culture. Science 221 : 949-951.

Evans DA, Sharp WR, Madina-Filho HP (1984). Somaclonal and gametoclonal variation. Am. J. Bot. 71 : 759-774.

Evans DA, Sharp WR (1986). Applications of somaclonal variation. Bio/Technol. 4 : 528-532.

FAO (1988). Production Year Book. Vol. 41, FAO, Rome.

Finch RP (1991). Development of protoplast systems for the genetic manipulation of rice. Ph.D. Thesis, University of Nottingham, UK.

Finch RP, Lynch PT, Jotham JP, Cocking EC (1991). In: Biotechnology in Agriculture and Forestry, Vol. 14. Rice. Bajaj YPS ed., Springer-Verlag, Berlin, Heidelberg, New York, Tokyo, p 251-268.

Flick CE, Evans DA, Sharp WR (1983). Organogenesis. In: Handbook of plant cell culture, vol 1. Evans DA, Sharp WR, Ammirato PV, Yamada Y eds., Macmillan, New York, p 13-81.

Frearson EM, Power JB, Cocking EC (1973). The isolation, culture and regeneration of *Petunia* leaf protoplasts. Dev. Biol. 33 : 130-137.

Fujimura T, Sakurai M, Akagi H, Negishi T, Hirose A (1985). Regeneration of rice plants from protoplasts. Plant Tissue Cult. Lett. 2 : 74-75.

Furuhashi K, Yatazawa M (1964). Indefinite culture of rice stem node callus. Kagaku (Japan) 34 : 623.

Galbraith DW & Shields BA (1982). The effect of inhibitors of cell wall synthesis on tobacco protoplast development. Physiol. Plant. 55 : 25-30.

Galbraith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP & Firoozabady E (1983). Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Science 220 : 1049-1051.

Galbraith DW, Afonso CL, Harkins KR (1984). Flow sorting and culture of protoplasts: conditions for high-frequency recovery, growth and morphogenesis from sorted protoplasts of suspension cultures of *Nicotiana*. Plant Cell Rep. 3 : 151-155.

Gamborg OL, Miller RA, Ojima K (1968). Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell. Res. 50 : 151-158.

Genovesi AD and Magill CW (1979). Improved rate of callus and green plant production from rice anther culture following cold shock. Crop Sci. 19 : 662-664.

Glaszmann JC (1986). A varietal classification of Asian cultivated rice (*Oryza sativa* L.) based on isozyme polymorphism. In: Rice Genetics. Proc. Intern. Rice. Gen. Symp., 27-31 May, 1985, IRRI, Manila, Philippines, p 83-90.

Glaszmann JC (1987). Isozymes and classification of Asian rice varieties. Theor. Appl. Genet. 74 : 21-30.

Glaszmann JC, Benoit H, Arnaud M (1984). Classification des riz cultivés (*Oryza sativa* L.) Utilisation de la variabilité isoenzymatique. Agron. Trop. (Paris) 39 : 51-66.

Gorman C (1977). A *Priori* Models and Thai Prehistory : A reconsideration of the beginnings of Agriculture in Southeast Asia. In: Origins of Agriculture. Reed CA ed., Mouton, The Hague, p 321-355.

Gresshoff PM, Doy CH (1972).Development and differentiation of haploid *Lycopersicon esculentum* (tomato). Planta 107 : 161-170.

Grist DH (1983). Rice (6th ed.). Longman, London.

Guha S, Maheshwari SC (1964). *In vitro* production of embryos from anthers of *Datura*. Nature 204 : 497.

Guha S, Maheshwari SC (1966). Cell division and differentiation of embryos in the pollen grains of *Datura in vitro*. Nature 212 : 97-98.

Guha S and Mukherjee (1973). Genotypic differences in the *in vitro* formation of embryoids from rice pollen. J. Exp. Bot. 24 (78) : 139-144.

Guiderdoni E, Courtois B, Glaszmann JC (1990). Use of isozyme markers to monitor recombination and assess gametic selection among anther culture derivatives of remote crosses of rice (*Oryza sativa* L.). In: Review of Advances in Plant Biotechnology, 1985-1988. Mujeeb-Kazi A, Sitch LA eds., Internat. Maize and Wheat Improvement Center, Mexico, and Internat. Rice Res. Inst., Manila, Philippines.

Guiderdoni E, Courtois B, Boissot N, Valdez M (1991b). Rice somatic tissue and anther cultures : current status in France. In: Biotechnology in Agriculture and Forestry, Vol. 14. Rice. Bajaj YPS ed., Springer-Verlag, Berlin, Heidelberg, New York, Tokyo, p 591-618.

Guiderdoni E, Chaïr H (1992). Plant regeneration from haploid cell suspension-derived protoplasts of Mediterranean rice (*Oryza sativa* L. cv. Miara). Plant Cell Rep. 11 : 618-622.

Gunn RE and Day PR (1986). *In vitro* culture in plant breeding. In: Plant Tissue Culture and Its Agricultural Applications. Withers LA, Anderson PG eds., Butterworths, London, p 313-336.

Gupta HS, Borthakur DN (1987). Improved rate of callus induction from rice anther culture following microscopic staging of microspores in iron alum-haematoxylin. Theor. Appl. Genet. 74 : 95-99.

Gupta HS, Pattanayak A (1993). Plant regeneration from mesophyll protoplasts of rice (*Oryza sativa* L.). Bio/Technol. 11 : 90-94.

Hammatt N, Lister A, Blackhall NW, Gartland J, Ghose TK, Gilmour DM, Power JB, Davey MR, Cocking EC (1990). Selection of plant heterokaryons from diverse origins by flow cytometry. Protoplasma 154 : 34-44.

Hammatt N, Blackhall NW, Davey MR (1991). Variation in the DNA content of *Glycine* species. J. Exp. Bot. 42 : 659-665.

Harkins KR, Galbraith DW (1984). Flow sorting and culture of plant protoplasts. Physiol. Plant. 60 : 43-52.

Harlan JR (1973). Genetic resources of some major field crops in Africa. In: Survey of crop genetic resources in their centres of diversity. Frankel OH ed., FAO/IBP, Rome, Italy, p 45-64.

Hargrove TR, Coffman WR, Cabanilla VL (1980). Ancestry of improved cultivars of Asian rice. Crop Sci. 20 : 721-727.

Harn C (1973). Fusion of protoplasts isolated from rice callus. SABRAO Newsletter 5 : 107-110.

Hayashi Y, Kyozuka J, Shimamoto K (1988). Hybrids of rice (*Oryza sativa* L.) and wild *Oryza* species obtained by cell fusion. Mol. Gen. Genet. 214 : 6-10.

Hayashimoto A, Li Z, Murai N (1990). A polyethylene glycol-mediated protoplast transformation system for production of fertile transgenic rice plants. Plant Physiol. 93 : 857-863.

Heyser JW, Dykes TA, DeMott KJ, Nabors MW (1983). High frequency, long term regeneration of rice from callus cultures. Plant Sci. Lett. 29 : 175-182.

Higham CFW (1984). Prehistoric rice cultivation in Southeast Asia. Sci. Am. 250(4) : 100-107.

Ho PT (1975). The cradle of the east. The Chinese University of Hong Kong Pub. Office, Hong Kong.

Hodges TK, Peng J, Lee L, Koetje DS (1990). *In vitro* culture of rice : transformation and regeneration of protoplasts. In: Gene Manipulation in Plant Improvement II. Gustafson JP ed., Proc. 19th Stadler Genet Symp. 1989. Plenum Press, NY, USA, p 163-183.

Hodges TK, Peng J, Lyznik LA, Koetje DS (1991). Transformation and regeneration of rice protoplasts. In: Rice Biotechnology. Toenniessen G, Khush GS eds., IRRI and Rockefeller Foundation, p 157-174.

Hu H (1985) Use of haploids in crop improvement. In: Biotechnology in International Agricultural Research. Proc. The Inter-Center Seminar on International Agricultural

Research Centers (IARCs) and Biotechnology, 23-27 April 1984, IRRI, Manila, Philippines, p 75-84.

Huang CR, Wu YH, Chen CC (1986). Effects of plant growth substances on callus formation and plant regeneration in anther culture of rice. In: Rice Genetics : Proc. 1st Int. Rice Genet. Symp. 27-31 May 1985, IRRI, Los Baños, Philippines, p 763-771.

Hull R, Jones MC, Dasgupta I, Cliffe JM, MinginsC, Lee G, Davies JW (1991). Molecular biology of rice tungro viruses : evidence for a new retroid virus. In: Rice Genetics II. Proc. 2nd Intern. Rice Gen. Symp., 14-18 May 1990, IRRI, Manila, Philippines.

Inoue M, Maeda E (1980). Effects of auxins and cytokinins on the occurrence of green regions in rice callus cultures. Jpn. J. Crop Sci. 49 : 167-174.

IRRI (1987). World Rice Statistics. IRRI, The Philippines.

IRRI (1989). The challenge of the future. In: The IRRI Reporter 2 : 3.

IRRI Rice Facts (1988). International Rice Research Institute, Los Baños, Manila, The Philippines.

Jain RK, Gosal SS, Khehra GS, Marchant R, Blackhall NW, Davey MR, Power JB, Cocking EC (1994). An efficient protoplast-to-plant system for the Indian Indica rice varieties Pusa Basmati 1 and Jaya (abstract). In: Proc. of the Annual Meeting of the Rockefeller Foundation's Intern. Program on Rice Biotechnology, Bali, Indonesia.

Jähne A, Lazzeri PA, Jäger-Gussen M, Lörz H (1991a). Plant regeneration from embryogenic cell suspensions derived from anther cultures of barley (*Hordeum vulgare* L.). Theor. Appl. Genet. 82 : 74-80.

Jähne A, Lazzeri PA, Lörz H (1991b). Regeneration of fertile plants from protoplasts derived from embryogenic cell suspensions of barley (*Hordeum vulgare* L.). Plant Cell Rep. 10 : 1-6.

Jena KK, Khush GS (1986). Production of monosomic alien addition lines of *Oryza sativa* having a single chromosome of *O. officinalis*. In: Rice Genetics : Proc. 1st Int. Rice Genet. Symp. 27-31 May 1985, IRRI, Los Baños, Philippines, p 199-208.

Jena KK, Khush GS (1989). Wide hybridization for rice Improvement. In: Progress in Irrigated Rice Research. Proc. International Rice Research Conference. IRRI, Manila, Philippines, Sept. 21-25, 1987, p274.

Jena KK, Khush GS (1990). Introgression of genes from *Oryza officinalis* Well. ex Watt to cultivated rice, *Oryza sativa* L. Theor. Appl. Genet. 80 : 737-745.

Jia YJ, Abe T, Futsuhara Y (1987). Int. Rice Genet. Newslett. 4 : 109-110.

Jones TJ, Rost TL (1989). The developmental anatomy and ultrastructure of somatic embryos from rice (*Oryza sativa* L.) scutellum epithelium cells. Bot. Gaz. 150 : 31-42.

Juliano BO (1985). Rice. J. Plant Foods 6 : 129-145.

Kameya T, Hinata K (1970). Induction of haploid plants from pollen grains of *Brassica*. Jpn. J. Breed. 20 : 82-87.

Kamo KK, Chang KL, Lynn ME, Hodges TK (1987). Embryogenic callus formation from maize protoplasts. Planta 172 : 245-251.

Kanda M, Kikuchi S, Takaiwa F and Oono K (1988).Regeneration of variant plants from rice (*Oryza sativa* L.) protoplasts derived from long term cultures. Jpn. J. Genet. 63 : 127-136.

Kao KN, Gamborg MR, Michayluk WA, Keller WA, Miller RA (1973). The effect of sugars and inorganic salts on cell regeneration and sustained division in plant protoplasts.

Kao KN, Michayluk MR (1975). Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at a very low population density in liquid media. Planta 126 : 105-110.

Karp A, Maddock SE (1984). Chromosome variation in wheat plants regenerated from cultured immature embryos. Theor. Appl. Genet. 67 : 249-255.

Karp A, Wu QS, Steele SH, Jones MGK (1987). Chromosome variation in dividing protoplasts and cell suspensions of wheat. Theor. Appl. Genet. 74 : 140-146.

Kasha KJ, Kao KN (1970). High frequency haploid production in barley (*Hordeum vulgare* L.). Nature 225 : 874-876.

Kasha KJ (1974). Haploids from somatic cells. In: Haploids in higher plants : advances and potential. Kasha KJ ed., Univ. Press, Guelph, Canada, p 67-87.

Kawata S, Ishihara A (1968). The regeneration of rice plant in the callus derived from the seminal root. Proc. Jpn. Acad. 44 : 549-553.

Kawata M, Harada S, Antonio B, Oono K (1992). Protoclonal variation of plant regeneration in rice. Plant Cell, Tiss. Org. Cult. 28 : 1-10.

Keller WA, Arnison PG, Brian JC (1987). Haploids from gametophytic cells - Recent developments and future prospects. In: Plant Tissue and Cell Culture. Proc. VIth Intern. Cong. on Plant Tiss. and Cell Cult., August 3-8, 1986, Minnesota, USA. Green CE, Somers DA, Hackett WP, Biesboer DD eds., Alan R. Liss, Inc., New York, p 223-241.

Kermicle JL (1973). Androgenesis and the indeterminate gametophyte mutation : Source of the cytoplasm. Maize Genet. Coop. Newslett. 47 : 208-209.

Kimber G and Riley R (1963). Haploid angiosperms. Bot. Rev. 29 : 480-631.

Khush GS (1977a). Disease and insect resistance in rice. Adv. Agron. 29 : 265-341.

Khush GS, Coffman WR (1977b). Genetic evaluation and utilization (GEU) program. The rice improvement program of the International Rice Research Institute Theor. Appl. Genet. 51 : 97-110.

Khush, Ling KC, Aquino RC, Aguiero VM (1977c). Breeding for resistance to grassy stunt in rice. In: Proc. 3rd Int. Congr. SABRAO. Canberra, Australia. Plant Breed. Papers. 4:3-9.

Khush GS (1984a). IRRI breeding program and its worldwide impact on increasing rice production. In: Gene Manipulation in Plant Improvement. Gustafson JP ed., USDA-ARS, Columbia, Missouri, USA, p 61-94.

Khush GS (1984b). Terminology for rice growing environments. In: Terminology for Rice Growing Environments. International Rice Research Institute, Los Baños, Manila, Philippines, p 5-10.

Khush GS, Virmani SS (1985). Some plant breeding problems needing biotechnology. In: Biotechnology in International Agricultural Research. IRRI, Manila, Philippines, p 51-63.

Khush GS, Jena KK (1986). Capabilities and limitations of conventional plant breeding and the role of distant hybridization. In: Proc. Workshop on Biotechnology for Crop Improvement : Potentials and Limitations. IRRI, Philippines, Oct.13-17, 1986.

Khush GS (1991). Wide hybridization for rice improvement. In: Internal Review Rep. of Biotechnology. IRRI, Philippines, Sept. 9-13, 1991.

King J (1986). Plant cells and the isolation of conditional lethal mutants. Enzyme Microb. Technol. 9 : 514-522.

Koetje DS, Grimes HD, Wang Y-C, Hodges TK (1989). Regeneration of Indica rice (*Oryza sativa* L.) from primary callus derived from immature embryos. J. Plant Physiol. 135 : 184-190.

Kohlenbach HW, Wernicke W, Lang H (1978). Androgenetic development of isolated microspores of *Hyocyamus* and *Nicotiana*. In: Production of natural compounds by cell culture methods. Alfermenn AW, Reinhard E eds., Ges Strahlen Umweltforsch, Munich, p 284-294.

Kumar A, Cocking EC (1987) Protoplast fusion : A novel approach to organelle genetics in higher plants. Amer. J. Bot. 74 : 1289-1303.

Kyozuka J, Hayashi Y, Shimamoto K (1987). High frequency plant regeneration from rice protoplasts by novel nurse culture methods. Mol. Gen. Genet. 206 : 408-413.

Kyozuka J, Otoo E, Shimamoto K (1988). Plant regeneration of protoplasts of Indica rice : genotypic differences in culture response. Theor. Appl. Genet. 76 : 887-890.

Kyozuka J, Kaneda T, Shimamoto K (1989). Production of cytoplasmic male sterile rice (*Oryza sativa* L.) by cell fusion. Bio/Technol. 7 : 1171-1174.

Lai KL, Liu LF (1982). Induction and plant regeneration of callus from immature embryos of rice plants (*Oryza sativa* L.). Jpn. J. Crop Sci. 51 : 70-74.

Lai KL, Liu LF (1986). Further studies on the variability of plant regeneration from young embryo callus cultures in rice plants (*Oryza sativa* L.). Jpn. J. Crop Sci. 55 : 41-46.

Lai KL, Liu LF (1988). Increased plant regeneration frequency in water-stressed rice tissue cultures. Jpn. J. Crop Sci. 57 : 553-557.

Lai LZ (1986). A promising line derived from ovary culture of rice. In: Abstr. 3rd Natl. Meet. Chinese Soc. Cell Biology, Chengdu, China, p 141-142.

Larkin PJ, Scowcroft WR (1981). Somaclonal variation – a novel source of variability from cell culture for plant improvement. Theor. Appl. Genet. 60 : 197-214.

Lazzeri PA, Brettschneider R, Lührs R, Lörz R (1991). Stable transformation of barley via PEG-induced direct DNA uptake into protoplasts. Theor. Appl. Genet. 81 : 437-444

Lee SY, Lee YT, Lee MS (1988). Studies on the anther culture of *Oryza sativa* L. 3. Growing environment of donor plant in anther culture, effects of photoperiod and light intensity. Res. Rep. Rural Dev. Admin., Suweon, Korea 30 : 7-12.

Lee CH, Power JB (1988). Intraspecific gametosomatic hybridisation in *Petunia hybrida*. Plant Cell Rep. 7 : 17-18.

Lee L, Schroll RE, Grimes HD, Hodges TK (1989). Plant regeneration from indica rice (*Oryza sativa* L.) protoplasts. Planta 178 : 325-333.

Lee L, Schroll RE, Grimes HD, Hodges TK (1990). Regeneration from Indica rice protoplasts. Rice Biotechnol. Quart. 1 : 11.

Li C-C, Gan D, Xu J (1986). New progress on screening resistant mutant of rice *in vitro*. Sci. Agric. Sin. 2 : 93-94.

Li MF, Ni PC, Shen JH (1988). Application of anther culture to 'convergent improvement' in rice breeding. In: Genetic Manipulation in Crops. IRRI and Academia Sinica eds., Cassell Tycooly, Philadelphia, p 39-41.

Liang HM (1978). The advance of studies on medium for anther culture of rice in China. In: Proc. of the Symposium on Plant Tissue Culture. Science Press, Beijing, p 57-64.

Lin SC, Yuan LP (1980). Hybrid rice breeding in China. In: Innovative Approaches to Rice Breeding. International Rice Research Institute, Manila, Philippines, p 35-51.

Ling SC, Min SK, Huang YX, Zu DM (1986). Cross-breeding of rice. In: Rice cultivation science in China. CAAS Agronomic Press, Beijing, p 271-330.

Ling D, Vidhyaseharan P, Borromeo ES, Zapata FJ, Donghua M (1986). *In vitro* screening of rice germ-plasm for resistance to brown spot disease using phytotoxin [in Chinese]. Acta Genet. Sin. 13 : 194-200.

Ling D, Ma Z, Chen W, Chen M (1988). Variation of somaclonal male sterile lines of indica rice by cell culture. Acta Genet. Sin. 15 : 9-14.

Linsmaier EM, Skoog F (1965). Organic growth factor requirements of tobacco tissue culture. Physiol. Plant. 18 : 100-127.

Liu LF, Lai KL (1991). Enhancement of regeneration in rice tissue cultures by water and salt stress. In: Biotechnology in Agriculture and Forestry, Vol. 14. Rice. Bajaj YPS ed., Springer-Verlag, Berlin, Heidelberg, New York, Tokyo, p 47-57.

Liu ZL, Zhou C (1984). Investigation of ploidy and other characters of the gynogenic plants in *Oryza sativa* L. Acta Genet. Sin. 11 : 113-119.

Loo SW, Xu ZH (1991). Anther culture for rice improvement in China. In: Biotechnology in Agriculture and Forestry, Vol. 2 : Crops I. Bajaj YPS ed., Springer-Verlag, Berlin, Heidelberg, p 139-156.

Loo SW, Xu ZH (1991). Anther culture for rice improvement in China. In: Biotechnology in Agriculture and Forestry, Vol. 14. Rice. Bajaj YPS ed., Springer-Verlag, Berlin, Heidelberg, New York, Tokyo, p 151-179.

Lörz H (1987). *In vitro* culture and genetic transformation of cereal cells. In: Molecular Mechanisms in the Regulation of Cell Behavior. Waymouth C ed., Alan R. Liss, Inc., New York, p 285-290.

Lörz H, Baker B, Schell J (1985). Gene transfer to cereal cells mediated by protoplast transformation. Mol. Gen. Genet. 199 : 178-182.

Lörz H, Brown PTH (1986). Variability in tissue culture derived plants – possible origin, advantages and drawbacks. In: Genetic Manipulation in Plant Breeding. Horn W, Jensen CJ, Odenbach W, Schieder O eds., Walter de Gruyter, Berlin, New York, p 513-534.

Lu JJ, Chang TT (1980). Rice in its temporal and spatial perspectives. In: Rice : Production and Utilization. Luh BS ed., ? Westport, Conn. USA, p 1-74.

Lupotto E (1982). Improvement in plantlet differentiation from anther culture of rice. Genet. Agrar. 36 : 129-142.

Lührs R, Lörz H (1987). Plant regeneration *in vitro* from embryogenic cultures of spring- and winter-type barley (*Hordeum vulgare* L.). Theor. Appl. Genet. 75 : 16-25.

Lührs R, Lörz H (1988). Initiation of morphogenic cell-suspension and protoplast cultures of barley (*Hordeum vulgare* L.). Planta 175 : 71-81.

Lynch PT, Finch RP, Davey MR, Cocking EC (1991). Rice tissue culture and its application. In: Rice Biotechnology. Toenniessen G, Khush G eds., Rockefeller Foundation and IRRI, Los Baños, Philippines, p 135-155.

Lyznik LA, Ryan RD, Ritchie SW, Hodges TK (1989). Stable co-transformation of maize protoplasts with *gusA* and *neo* genes. Plant Mol. biol. 13 : 151-161.

Maeda E, Hagiwara T (1974). Enzymatic isolation of protoplasts from the rice leaves and callus cultures. Proc. Crop Sci. Japan 43 : 68-76.

Magoon ML, Khanna KR (1963). Haploids. Caryologia 16 : 191-235.

Maheshwari SC, Tyagi AK, Malhotra K, Sopory SK (1980). Induction of haploidy from pollen grains in angiosperms–the current status. Theor. Appl. Genet. 58 : 193-206.

Maheshwari SC, Rashid A, Tyagi AK (1982). Haploids from pollen grains–retrospect and prospect. Amer. J. Bot. 69(5) : 865-879.

Marton L, Dung TM, Mendel RR, Maliga P (1982). Nitrate reductase deficient cell lines from haploid protoplast cultures of *Nicotiana plumbaginifolia*. Mol. Gen. Genet. 186 : 301-304.

Mascarenhas JP (1989). The male gametophyte of flowering plants. The Plant Cell 1 : 657-664.

McCoy TJ, Phillips RL, Rines HW (1982). Cytogenetic analysis of plants regenerated from oat (*Avena sativa*) tissue cultures : high frequency of partial chromosome loss. Can. J. Genet. Cytol. 24 : 37-50.

McElroy D, Zhang W, Cao J, Wu R (1990a). Isolation of an efficient actin promoter for use in rice transformation. The Plant Cell 2 : 163-171.

McElroy D, Rothenberg M, Wu R (1990b). Structural characterization of a rice actin gene. Plant Mol. Biol. 14 : 163-171.

Meins F (1983). Heritable variation in plant cell culture. Annu. Rev. Plant Physiol. 34 : 327-346.

Mercy ST, Zapata FJ (1987). Position of anthers at plating and its influence on anther callusing in rice. Plant Cell Rep. 6 : 318-319.

Miezahn K, Second G (1979). Prospection of the traditional varieties and wild species of rice in Tanzania. In: Mimeo. Mission report. IRAT-ORSTOM (1974-1984), p 28-91.

Mikami T, and Kinoshita T (1988). Genotypic effects on the callus formation from different explants of rice (*Oryza sativa* L.). Plant Cell Tiss. Org. Cult. 12 : 311-314.

Mori KI (1991). Genetic constitution of somatic hybrids between *O. sativa* and wild relative *Oryza* species. Abstr., VIII Intern. Protoplast Symp. Uppsala, Sweden, 16-20 June, 1991.

Morinaga T and Kuriyama H (1958). Intermediate type of rice in the subcontinent of India and Java. Jpn. J. Breed. 7 : 253-259.

Morishima H, Sono Y, Oka HI (1984a). Differentiation of perennial and annual types due to habitat conditions in the wild rice *Oryza perennis*. Plant Syst. Evol. 144 : 119-135.

Müller AJ, Grafe R (1978). Isolation and characterization of cell lines of *Nicotiana tabacum* lacking nitrate reductase. Mol. Gen. Genet. 161 : 67-76.

Müller E, Brown PTH, Hartke S, Lörz H (1990). DNA variation in tissue culture-derived rice plants. Theor. Appl. Genet. 80 : 673-679.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15 : 473-497.

Murata M, Orton TJ (1983). Chromosome structural change in cultured celery cells. In Vitro 19 : 83-89.

Nabors MW, Dykes TA (1985). Tissue culture of cereal cultivars with increased salt, drought, and acid tolerance. In: Biotechnology in International Agricultural Research. International Rice Research Institute, Manila, Philippines, p 121-138.

Nakano H and Maeda E (1979). Shoot differentiation in callus of *Oryza sativa* L. Z. Pflanzenphysiol. Bd. 93 : 449-458.

Negrutiu I, De Brouwer D, Dirks R, Jacobs M (1985). Amino acid auxotrophs from protoplast cultures of *Nicotiana plumbaginifolia, Viviani*. I. BUdR enrichment selection, plant regeneration, and general characterisation. Mol. Gen. Genet. 199 : 330-337.

Neuhaus G, Spangenberg G, Mittelsten Scheid O, Schweiger HG (1987) Transgenic rapeseed plants obtained by the microinjection of DNA into microspore-derived embryoids. Theor. Appl. Genet. 75 : 30-36.

Niizeki H, Oono K (1968). Induction of haploid rice plant from anther culture. Proc. Jpn. Acad. 44 : 554-557.

Niizeki H, Oono K (1971). Rice plants obtained by anther culture. In: Les cultures de tissus de plantes. Hirth ML, Morel MG eds., Colloq. Int. CNRS, Paris, 193 : 251-257.

Niizeki M, Kita F (1981). Cell division of rice and soybean and their fused protoplasts. Japan J. Breed. 31 : 161-167.

Nishi T and Mitsuoka S (1969). Occurrence of various ploidy plants from anther and ovary culture of rice plants. Jpn. J. Genet. 44 : 341-346.

Nishi T, Yamada Y, Takahashi E (1968). Organ redifferentiation and plant restoration in rice callus. Nature 219 : 508-509.

Nishibayashi S, Hayashi Y, Kyozuka J, Shimamoto K (1989). Chromosome variations in protoplast-derived calli and in plants regenerated from the calli of cultivated rice (*Oryza sativa* L.). Jpn. J. Genet. 64 : 355-361.

Nitsch C (1974b). Pollen culture–a new technique for mass production of haploid and homozygous plants. In: Haploids in Higher Plants–Advances and Potentials. Kasha KJ ed., University of Guelph, Guelph, Canada, p 123-135.

Nitsch C (1981). Production of isogenic lines : basic technical aspects of androgenesis. In: Plant Tissue Culture, Methods and Applications in Agriculture. Thorpe TA ed., Academic Press, New York, p 241-252.

Nitsch JP, Nitsch C (1969). Haploid plants from pollen grains. Science 163 : 85-87.

Novak FJ, Vyskot B (1975). Karyology of callus cultures derived from *Nicotiana tabacum* haploids and ploidy of regenerants. Z. Pflanzenzücht 75 : 62-70.

Ogura H (1990). Chromosome variation in plant tissue culture. In: Biotechnology in Agriculture and Forestry, Vol. 11. Somaclonal Variation in Crop Improvement I. Bajaj YPS ed., Springer, Berlin, Heidelberg, New York, Tokyo, P 49-83.

Ogura H, Kyozuka J, Hayashi Y, Koba T, Shimamoto K (1987). Field performance and cytology of protoplast-derived rice (*Oryza sativa*) : high yield and low degree of variation of four japonica cultivars. Theor. Appl. Genet. 74 : 670-676.

Ogura H, Kyozuka J, Hayashi Y, Shimamoto K (1989). Yielding ability and phenotypic traits in the selfed progeny of protoplast-derived rice plants. Jpn. J. Breed. 39 : 47-56.

Oka HI (1953a). Variations in various characters and character combinations among rice varieties. Jpn. J. Breed. 3 : 33-43.

Oka HI (1953b). Phylogenetic differentiation of cultivated rice. 6. The mechanism of sterility in the intervarietal hybrid. Jpn. J. Breed. 4 : 101-110.

Oka HI (1958). Intervarietal variation and classification of cultivated rice. Ind. J. Genet. Plant Breed. 18 : 79-89.

Oka HI (1974). Experimental studies on the origin of cultivated rice. Genetics 78 : 475-486.

Oka HI (1978). An observation of wild rice species in tropical Australia : Report of trip, 1978. Rep. Natl. Inst. Genet., Jap., 24pp.

Oka HI (1988). Origin of cultivated rice. Developments in Crop Science, Vol. 14, Jpn. Sci. Soc. Press, Tokyo & Elsevier Press, Amsterdam, Oxford, New York, Tokyo.

Oono K (1975). Production of haploid plants of rice (*Oryza sativa*) by anther culture and their use for breeding. Bull. Natl. Inst. Agric. Sci. Ser. D 26 : 139-222.

Oono K (1981). *In vitro* methods applied to rice. In: Plant Tissue Culture, Methods and Applications in Agriculture. Thorpe TA ed., Academic Press, New York, London, p 273-298.

Oono K (1983a). Genetic variability in rice plants regenerated from cell culture. In: Proc. of the Workshop on Cell and Tissue Culture Techniques for Cereal Crop Improvement. Science Press. Beijing, China and IRRI, Manila, Philippines, p 95-104.

Oono K (1983b). Tissue culture and genetic engineering in rice. In: Biology of rice. Tsunoda S and Takahashi N eds., Jpn. Sci. Soc. Press, Japan, p 339-358.

Oono K (1988). Rice breeding through tissue culture. In: Plant Breeding and Genetic Engineering. Zakri AH ed., SABRAO, Malaysia, p 339-358.

Oram R (1982). Plant breeding and genetic engineering. In: Genetic engineering for agriculture-the substance behind the promise. Sutton BG, Williams P eds., Aust. Inst. Agric. Occ. Publ. 2, p 73-79.

Orton TJ (1980). Chromosomal variability in tissue cultures and regenerated plants of *Hordeum*. Theor Appl. Genet. 56 : 101-112.

Ozawa K, Komamine A (1989). Establishment of a system of high-frequency embryogenesis from long-term cell suspension cultures of rice (*Oryza sativa* L.). Theor. Appl. Genet. 77 : 205-211.

Parisi L, Picard E (1986). Disease response of doubled haploid lines and their original cultivars in wheat (*Triticum aestivum* L.). Z. Pflanzenzücht. 96 : 63-78.

Paul WRC (1945). Paddy Cultivation. Ceylon Govt. Press, Colombo.

Peng J, Lyznik LA, Hodges TK (1990). Co-transformation of indica rice protoplasts with *gusA* and *neo* genes. Plant Cell Rep. 9 : 168-172.

Peng J, Lyznik LA, Hodges TK (1991). Co-transformation of indica rice *via* PEG-mediated DNA uptake. In: Proceedings of the Second International Rice Genetics Symposium 14-18 May 1990, IRRI, Los Banõs, Philippines, p 563-574.

Peng J, Kononowicz H, Hodges TK (1992). Transgenic indica rice plants. Theor. Appl. Genet. 83 : 855-863.

Petit P, Diolez P, Müller P, Brown S (1986b). Binding of Concanavalin A to the outer membrane of potato tuber mitochondria detected by flow cytometry. FEBS Lett. 196 : 65-70.

Pirrie A, Power JB (1986). The production of fertile, triploid somatic hybrid plants [*Nicotiana glutinosa* (n) + *N. tabacum* (2n)] via gametic : somatic protoplast fusion. Theor. Appl. Genet 72 : 48-52.

Ponnamperuma FN (1978). Electrochemical changes in submerged soils and the growth of rice. In: Soils and Rice. IRRI, Philippines, p 421-424.

Portères R (1956). Taxonomie agrobotanique des riz cultivés *O. sativa* Linné. et *O. glaberrima* Steudel. J. Agric.Trop. Bot. Appl. 3 : 341-384, 541-580, 627-700, 821-856.

Potrykus I, Saul MW, Petruska J, Paszkowski J, Shillito RD (1985a). Direct gene transfer to cells of a graminaceous monocot. Mol. Gen. Genet. 199 : 183-188.

Potrykus I (1990a). Gene transfer to cereals : an assessment. Bio/Technol. 8 : 535-542.

Potrykus I (1990b). Gene transfer to plants : assessment and perspectives. Physiol. Plant. 79 : 125-134.

Powell W (1988b). The influence of genotype and temperature pre-treatment on anther culture response in barley (*Hordeum vulgare* L.). Plant Cell Tiss. Org. Cult. 12 : 291-297.

Powell W, Borrino EM, Goodall V (1988). The effect of anther orientation on microspore-derived plant production in barley (*Hordeum vulgare* L.). Euphytica 38 : 159-163.

Prat D (1983). Genetic variability induced in *Nicotiana sylvestris* by protoplast culture. Theor. Appl. Genet. 67 : 17-24.

Puite KJ (1992). Progress in plant protoplast research. Physiol. Plant. 85 : 403-410.

Pulver EL, Jennings PR (1986). Application of anther culture to high volume rice breeding. In: Rice Genetics: Proc. of the First International Rice Genetics Symposium. International Rice Research Institute, Manila, Philippines, p 811-820.

Raghava-Ram NV and Nabors MW (1984). Cytokinin mediated long-term, high-frequency plant regeneration in rice tissue culture. Z. für Pflanzenphysiol. 113 : 315-323.

Raghava-Ram NV and Nabors MW (1985). Plant regeneration from tissue cultures of Pokkali rice is promoted by optimizing callus to medium volume ratio and by a medium-conditioning factor produced by embryogenic callus. Plant Cell, Tiss. Org. Cult. 4 : 241-248.

Raina SK, Satish P, Sarma SK (1987). Plant regeneration from *in vitro* cultures of anthers and mature seeds of rice (*Oryza sativa* L.) cv. Basmati-370. Plant Cell Rep. 6 : 43-45.

Raina SK (1989). Tissue culture in rice improvement : status and potential. Adv. Agro. 42 : 339-397.

Raineri DM, Bottino P, Gordon MP, Nester EW (1990). *Agrobacterium*-mediated transformation of rice (*Oryza sativa* L.). Bio/Technol. 8 : 33-38.

Reinert J, Bajaj YPS, Heberle E (1975). Induction of haploid tobacco plants from isolated pollen. Protoplasma 84 : 191-196.

Reinert J, Bajaj YPS (1977). Applied and Fundamental Aspects of Plant Cell, Tissue, and Organ Culture. Springer-Verlag, Berlin, Heidelberg, New York.

Rhodes CA, Lowe KS, Ruby KL (1988). Plant regeneration from protoplasts isolated from embryogenic maize cell cultures. Bio/Technol. 6 : 56-60.

Roger PA, Watanabe I (1986). Technologies for utilizing biological nitrogen fixation in wetland rice: potentialities, current usage, and limiting factors. In: Nitrogen Economy of Flooded Rice Soils. De Datta SK, Patrick WH eds., Kluwer Academic Pub., Dordrecht, Boston, Lancaster, p 39-77.

Sacristan MD (1971). Karyotypic changes in callus cultures from haploid and diploid plants of *Crepis capillaris*. Chromosoma 33 : 273-283.

Saka H, Maeda E (1969). Effect of kinetin on organ formation in callus tissue derived from rice embryos. Proc. Crop Sci. Soc. Jpn 38 : 668-674.

Saleh NM, Gupta HS, Finch RP, Cocking EC, Mulligan BJ (1990). Stability of mitochondrial DNA in tissue-cultured cells of rice. Theor. Appl. Genet. 79 : 342-346.

Schaeffer GW, Sharpe FT, Cregan PB (1984). Variation for improved protein and yield from rice anther culture. Theor. Appl. Genet. 67 : 383-389.

Schaeffer GW (1986). Selection of rice, *Oryza sativa*, cells from inhibitory levels of lysine plus threonine and the characterization of the progeny. In: Abstr. Int. Congr. Plant Tissue Cell Cult., 6th, Univ. Minnesota, USA, p 439.

Schell JS (1987). Transgenic plants as tools to study the molecular organization of plant genes. Science 237 : 1176-1183.

Schieder O, Köhn H (1986). Protoplast fusion and generation of somatic hybrids. In: Cell Culture and Somatic Cell Genetics of Plants, Vol. 3 : Plant regeneration and genetic variability. Vasil IK ed., Academic Press, New York, London, p 569-584.

Schocher RJ, Shillito RD, Saul MW, Paszkowski J, Potrykus I (1986). Co-transformation of unlinked foreign genes into plants by direct gene transfer. Bio/Technol. 4 : 1093-1096.

Schuh W, Nelson MR, Bigelow DM, Orum TV, Orth CE, Lynch PT, Eyles PS, Blackhall NW, Jones J, Cocking EC and Davey MR (1993). The phenotypic characterisation of R_2 generation transgenic rice plants under field conditions. Plant Sci. 89 : 69-79.

Scowcroft WR, Ryan SA, Brettel RIS and Larkin PJ (1985). Somaclonal variation in crop improvement. In: Biotechnology in International Agricultural Research. IRRI, Manila, Philippines, p 99-109.

Second G (1982). Origin of the genic diversity of cultivated rice (*Oryza* spp.) : study of the polymorphism scored at 40 isozyme loci. Jpn. J. Genet. 57 : 25-57.

Second G (1985). Evolutionary relationships in the *sativa* group of *Oryza* based on isozyme data. Génet. Sél. Evol. 17 : 89-114.

Sharma DP, Firoozabady E, Ayres NM & Galbraith DW (1983). Improvement of anther culture in *Nicotiana*: Media, cultural conditions and flow cytometric determination of ploidy levels. Z. Pflanzenphysiol. 111 : 441-451.

Shen JH, Li MF, Chen YQ, Zhang ZH (1983a). Improving rice by anther culture. In: Cell and Tissue Culture Techniques for Cereal Crop Improvement. Science Press, Beijing, China and IRRI, Manila, Philippines, p 183-205.

Shillito RD, Carswell GK, Johnson CM, DiMaio JJ, Harms CT (1989). Regeneration of fertile plants from protoplasts of elite inbred maize. Bio/ Technol. 7 : 581-587.

Shimamoto K, King PJ (1983). Isolation of a histidine auxotroph of *Hyoscyamus muticus* during attempts to apply BUdR enrichment. Mol. Gen. Genet. 189 : 69-72.

Shimamoto K, Terada R, Izawa T, Fujimoto H (1989) Fertile transgenic rice plants regenerated from transformed protoplasts. Nature 338 : 274-276.

Siriwardana S, Nabors MW (1983). Tryptophan enhancement of somatic embryogenesis in rice. Plant Physiol. 73 : 143-146.

Skirvin RM (1978). Natural and induced variation in tissue culture. Euphytica 27 : 241-266.

Slamet IH (1991). Protoplast culture and somatic hybridisation of Indica and Japonica rice cultivars. Ph.D. Thesis, University of Nottingham, UK.

Snape JW (1989). Doubled haploid breeding : theoretical basis and practical applications. In: Review of Advances in Plant Biotechnology, 1985-1988. Proc. 2nd Intern. Symp. Genetic Manipulation in Crops. Mujeeb-Kazi A, Sitch LA eds., Internat. Maize and Wheat Improvement Center, Mexico and IRRI, Manila, Philippines, p 19-30.

Snape JW, Simpson E (1986). The utilization of double haploid lines in quantitative genetics. Bull. Soc. Bot. Fr. 133, Actualités Bot. 4 : 59-66.

Solheim WG II (1972). An earlier agricultural revolution. Sci. Amer. 226 : 34-41.

Song KM, Osborn TC, William PH (1988). *Brassica* taxonomy based on nuclear restriction fragment length polymorphisms (RFLP). Theor. Appl. Genet. 75 : 784-794.

Spencer JE (1963). The migration of rice from mainland Southeast Asia into Indonesia. In: Plants and the migration of Pacific peoples. Barrau J ed., Bishop Museum Press, Honolulu, USA, p 83-89.

Steffen A, Schieder O (1984). Biochemical and genetical characterization of nitrate reductase deficient mutants of *Petunia*. Plant Cell Rep. 3 : 134-137.

Sun L-H, She J-M, Lu X-F (1986). *In vitro* selection of *Xanthomonas oryzae*-resistant mutants in rice. I. Induction of resistant callus and screening regenerated plants. Acta Genet. Sin. 13 : 188-193.

Sun ZX, Zheng KL (1990). Somaclonal variation in rice. In: Biotechnology in Agriculture and Forestry, Vol. 11. Somaclonal Variation in Crop Improvement I. Bajaj YPS ed., Springer, Berlin, Heidelberg, New York, Tokyo, p 288-325.

Sundberg E, Glimelius K (1986). A method for production of interspecific hybrids within *Brassiceae* via somatic hybridization, using resynthesis of *Brassica napus* as a model. Plant Sci. 43 : 155-162.

Sundberg E, Landgren M, Glimelius K (1987). Fertility and chromosome stability in *Brassica napus* resynthesized by protoplast fusion. Theor. Appl. Genet. 75 : 96-104.

Sunderland N (1983). The concept of morphogenetic competence with reference to anther and pollen culture. In: Plant Cell Culture in Crop Improvement. Sen SK, Giles KL eds., Plenum Press, New York, p 125-139.

Swaminathan MS (1984a). Rice. Sci. Amer. 250 : 63-72.

Swaminathan MS (1986). Plant research and world agriculture. Plant Mol. Bio. Rep. 4 : 1-17.

Tada Y, Sakamoto M, Fujimura T (1990). Efficient gene introduction into rice by electroporation and analysis of transgenic plants : use of electroporation buffer lacking chloride ions. Theor. Appl. Genet. 80 : 475-480.

Tamaki A, Hatade I (1974). Climate : The Earth and Human History. Heibonsha, Tokyo.

Tanksley SD, Young ND, Paterson AH, Bonierbale MW (1989). RFLP mapping in plant breeding-new tools for an old science. Bio/Technol. 7 : 257-264.

Tateoka T (1964). Notes on some grasses XVI. Embryo structure of the genus *Oryza* in relation to the systematics. Am. J. Bot. 51 : 539-543.

Tateoka T (1965). *Porteresia*, a new genus of *Gramineae*. Bull. Natl. Sci. Mus. Tokyo 8 : 405-406.

Terada R, Kyozuka J, Nishibayashi S, Shimamoto K (1987). Plantlet regeneration from somatic hybrids of rice (*Oryza sativa* L.) and barnyard grass (*Echinochloa oryzicola* Vasing). Mol. Gen. Genet. 210 : 39-43.

Terada R, Shimamoto K (1990). Expression of CaMV35S-GUS gene in transgenic rice plants. Mol. Gen. Genet. 220 : 389-392.

Thompson JA, Abdullah R, Cocking EC (1986). Protoplast culture of rice (*Oryza sativa* L.) using media solidified with agarose. Plant Sci. 47 : 123-133.

Thompson JA, Abdullah R, Chen WH, Gartland KMA (1987). Enhanced protoplast division in rice (*Oryza sativa* L.) following heat shock treatment. J. Plant Physiol. 127 : 367-370.

Ting Y (1949). Chronological studies of the cultivation and the distribution of rice varieties, keng and sen. Sun Yatsen Univ. Agron. Bull. 6 : 1-32.

Toriyama K, Hinata K (1985a). Cell suspension and protoplast culture in rice. Plant Sci. 41 : 179-183.

Toriyama K, Hinata K (1985b). Panicle culture in liquid media for obtaining anther calli and protoplasts in rice. Japan J. Breed. 35 : 449-452.

Toriyama K, Hinata K, Sasaki T (1986) Haploid and diploid plant regeneration from protoplasts of anther callus in rice. Theor. Appl. Genet. 73 : 16-19.

Toriyama K, Arimoto Y, Uchimiya H, Hinata K (1988a). Transgenic rice plants after direct gene transfer into protoplasts. Bio/Technol. 6 : 1072-1074.

Toriyama K, Hinata K (1988b). Diploid somatic-hybrid plants regenerated from rice cultivars. Theor. Appl. Genet. 76 : 665-668.

Torrizo LB and Zapata FJ (1992). High efficiency plant regeneration from protoplasts of the Indica cultivar IR58. J. Exp.Bot. 43 : 1633-1637.

Tsay SS, Tsay HS, Chao CY (1986). Cytochemical studies of callus development from microspore in cultured anther of rice. Plant Cell Rep. 5 : 119-123.

Tsukahara M, Hirosawa T (1992). Simple dehydration treatment promotes plantlet regeneration of rice (*Oryza sativa* L.) callus. Plant Cell Rep. 11 : 550-553.

Uchimiya H, Fushimi T, Hashimoto H, Harada H, Syono K, Sugawara Y (1986a). Expression of a foreign gene in callus derived from DNA-treated protoplasts of rice (*Oryza sativa* L.). Mol. Gen. Genet. 204 : 208.

Uchimiya H, Handa T, Brar DS (1989). Transgenic plants. Journal of Biotechnol. 12:1-20.

Vasil IK (1982). Plant cell culture and somatic cell genetics of cereals and grasses. In: Plant Improvement and Somatic Cell Genetics. Vasil IK, Scowcroft WR, Frey KJ eds., Academic Press, New York, USA, p 179-203.

Vasil IK (1983c). Isolation and culture of protoplasts of grasses. In: Plant Protoplasts. Intern. Rev. Cytol. 14, Giles KL ed., Academic Press, New York, p 79-88.

Vasil IK (1987). Developing cell and tissue culture systems for the improvement of cereal and grass crops. J. Plant Physiol. 128 : 193-218.

Vasil IK (1988). Progress in the regeneration and genetic manipulation of cereal crops. Bio/Technol. 6 : 397-402.

Vasil IK, Vasil V (1986). Regeneration in cereal and other grass species. In: Cell Culture and Somatic Cell Genetics of Plants, Volume 3, Plant regeneration and Genetic Variability. Vasil IK ed., Academic Press, Orlando, p 121-150.

Vasil IK, Vasil V (1992). Advances in cereal protoplast research. Physiol. Plant. 85 : 279-283.

Vasil V, Vasil IK (1982). Characterization of an embryogenic cell suspension culture derived from inflorescences of *Pennisetum americanum* (pearl millet; Gramineae). Amer. J. Bot. 69 : 1441-1449.

Vasil V, Vasil IK (1984). Induction and maintenance of embryogenic callus cultures of *Gramineae*. In: Cell Culture and Somatic Cell Genetics of Plants. Vol. 1. Vasil IK ed., Academic Press, Florida, USA, p 36-42.

Vasil V, Vasil IK (1987). Formation of callus and somatic embryos from protoplasts of a commercial hybrid of maize (*Zea mays* L.). Theor. Appl. Genet. 73 : 793-798.

Vasil V, Redway F, Vasil IK (1990). Regeneration of plants from embryogenic suspension culture protoplasts of wheat (*Triticum aestivum* L.). Bio/Technol. 8 : 429-434.

Vaughan DA, Sitch LA (1991). Gene flow from the jungle to farmers. Biosci. 41 : 22-28.

Virmani SS, Edwards IB (1984). Current status and future prospect of breeding hybrid rice and wheat. Adv. Agron. 36 : 145-214.

Vishnu-Mittre (1974). Paleobotanical evidence in India. In: Evolutionary studies on world crops. Hutchinson JB ed., Cambridge University Press, Cambridge, p 3-30.

Wang CC, Sun CS, Chu Z (1974). On the conditions for the induction of rice pollen plantlets and certain factors affecting the frequency of induction. Acta Bot. Sin. 16: 43-53.

Wang D, Miller PD, Söndahl MR (1989). Plant regeneration from protoplasts of Indica type rice and CMS rice. Plant Cell Rep. 8 : 329-332.

Wang X, Hu H (1985). The chromosome constitution of plants derived from pollen of hexaploid triticale x common wheat F_1 hybrids. Theor. Appl. Genet. 70 : 92-96.

Wang ZX and Iwata N (1991). Production of n+1 plants and tetrasomics by means of anther culture of trisomic plants in rice (*Oryza sativa* L.). Theor. Appl. Genet. 83 : 12-16.

Wang ZY, Tanksley SD (1989). Restriction fragment length polymorphism in *Oryza sativa* L. Genome 32 : 1113-1118.

Wasaka K, Kobayashi M, Kamada H (1984). Colony formation from protoplasts of nitrate reductase-deficient rice cell lines. J. Plant Physiol. 117 : 223-231.

Wasaka K, Widholm JM (1982). Regeneration of resistant cells of tobacco and rice to amino acids and amino acid analogs. In: Plant tissue culture 1982. Proc. Int. Congr. Plant Tissue Cell Cult., 5th, Fujiwara A ed., Maruzen, Tokyo, p 455-456.

Wasaka K, Widholm JM (1987). A 5-methyltryptophan resistant rice mutant MT9YC selected in tissue culture. Theor. Appl. Genet. 74 : 49-54.

Wernicke W, Brettell R, Wakizuka T, Potrykus I (1981). Adventitious embryoid and root formation from rice leaves. Z. Pflanzenphysiol. 103 : 361-365.

Widholm JM (1972). The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Technol. 47 : 189-194.

Willmitzer L (1988). The use of transgenic plants to study plant gene expression. Trends Genet. 4 : 13-18.

Whyte RO (1972). The *Gramineae*, wild and cultivated of monsoonal and equatorial Asia. I. Southeast Asia. Asian Perspectives 15 : 127-151.

Woo SC, Huang CY (1978). Anther culture of *Oryza sativa* L. and *Oryza perennis* Moench hybrids. Bot. Bull. Acad. Sin. 19 : 171-178.

Woo SC, Chen CC (1982). Rice anther culture in Taiwan. In: Rice Tissue Culture Planning Conference. International Rice Research Institute, Manila, Philippines, p 83-90.

Xie QJ, Rush MC (1990). Heritability of somaclonal variation and plant regeneration from protoplasts in rice. In: Abstr. Proc. 4th Annual Meeting of the Rockefeller Foundation's International Program on Rice Biotechnology. IRRI, Los Baños, Philippines, p ?.

Xu ZH, Huang B (1984). Anther factor(s) in barley anther culture. Acta Bot. Sin. 26:1-10.

Yamada Y, Loh WH (1984). Rice. In: Handbook of Plant Cell Culture. Vol 3. Ammirato PV, Evans DA, Sharp WR, Yamada Y eds., Macmillan, New York, London, p 151-170.

Yamada Y, Yang ZQ, Tang DT (1986). Plant regeneration from protoplast-derived callus of rice (*Oryza sativa* L.). Plant Cell Rep. 5 : 85-88.

Yang HY, Zhou C, Tian H, Liu Z, Cai D, Yan H (1984). *In vitro* gynogenesis in unfertilized ovaries and ovules. In: Abstr. of the Intern. Symp. Genet. Manipul. Crops, Beijing, p 10.

Yang HY, Zhou C, Cai D, Yan H, Wu Y, Chen (1986). *In vitro* culture of unfertilized ovules in *Helianthus annus*. In: Haploids of higher plants in vitro. Hu H, Yang HY eds., China Acad. Publ., Beijing; Springer, Berlin, Heidelberg, New York, Tokyo, p 182-191.

Yang H, Zhang HM, Davey MR, Mulligan BJ, Cocking EC (1988). Production of kanamycin resistant rice tissues following DNA uptake into protoplasts. Plant Cell Rep. 7 : 421-425.

Yang S (1978). On the remains of cultivated rice discovered at Shixia, Guangdon. Wenwu 7 : 23-28.

Yang Z-Q, Shikanai T, Yamada Y (1988). Asymmetric hybridization between cytoplasmic male-sterile (CMS) and fertile rice (*Oryza sativa* L.) protoplasts. Theor. Appl. Genet. 76 : 801-808.

Yang Z-Q, Shikanai T, Mori K, Yamada Y (1989). Plant regeneration from cytoplasmic hybrids of rice (*Oryza sativa* L.). Theor. Appl. Genet. 77 : 305-310.

Yatazawa M, Furuhashi K, Suzuki T (1967). Growth of callus tissue from rice roots *in vitro*. Plant Cell Physiol. 8 : 363-373.

Yeh CC, Tsay HS (1988). The effect of temperature treatment and medium composition on rice anther culture. J. Agric. Res. China 36 : 247-253.

Yin KC, Hsu C, Chu CY, Pi FY, Wang ST, Liu TY, Chu CC, Wang CC, Sun CS (1976). A study of the new cultivar of rice raised by haploid breeding method. Sci. Sinica 19 : 227-242.

Yuan LP, Virmani SS (1986). Status of hybrid rice research and development. In: Hybrid Rice. IRRI, Manila, Philippines, p 7-24.

Zakri AH (1986). Somaclonal variation in rice. In: Rice Genetics : Proc.1st Int. Rice Genet. Symp. 27-31 May 1985, IRRI, Los Baños, Manila, Philippines, p 753-765.

Zapata FJ, Torrizo LB, Romero RO, Alezar MS (1982b). Androgenesis in *Oryza sativa*. In: Plant Tissue Culture 1982. Proc. 5th Intl. Cong. Plant Tissue & Cell Culture. Fujiwara A ed., Maruzen, Tokyo, p 531-532.

Zapata FJ, Khush GS, Crill JP, Neu MH, Romero RO, Torrizo LB, Alezar MS (1983). Rice anther culture at IRRI. In: Cell and Tissue Culture Techniques for Cereal Crop Improvement. Science Press, Beijing, China and IRRI, Manila, Philippines, p 27-46.

Zapata FJ (1985). Rice anther culture at IRRI. In: Biotechnology in International Agricultural Research. Proc. Inter-Center Seminar on International Agricultural Research Centers (IARCS) and Biotechnology. Burn K ed., IRRI, Manila, Philippines, p 85-95.

Zapata FJ and Torrizo LB (1986). Heat treatment to increase callus induction efficiency in anther cultutr of IR42. Int. Rice Res. Newsl. 11 : 25-26.

Zapata FJ, Aldemita RR, Novero AU, Torrizo LB, Magaling LB, Mazaredo AM, Visperas RM, Lim MS, Moon HP (1986a). IRRI-Korea collaborative project for the development of cold-tolerant lines through anther culture. Res. Paper Ser. No. 118. International Rice Research Institute, Manila, Philippines.

Zapata FJ, Aldemita RR, Torrizo LB, Novero AU, Raina SK, Rola RR (1986b). Anther culture of Basmati 370 at IRRI. A. Gamma ray-induced green plant regeneration. Int. Rice Res. Newsl. 11 : 22.

Zapata FJ, Aldemita RR, Ella ES, Cho MS (1991). Isolated microspore culture of rice at the International Rice Research Institute. In: Proceedings of the Second International Rice Genetics Symposium, 14-18 May, 1990, IRRI, Los Baños, Philippines, p 311-319.

Zhang HM (1990). Production of transgenic rice plants. PhD Thesis, University of Nottingham, UK.

Zhang HM, Yang H, Rech EL, Golds TJ, Davis AS, Mulligan BJ, Cocking EC, Davey MR (1988). Transgenic rice plants produced by electroporation-mediated plasmid uptake into protoplasts. Plant Cell Rep. 7 : 379-384.

Zhang ZH, Chu QR (1986). Advances in rice anther culture for varietal improvement in China. J. Agric. Sci. (China) Suppl. 2 : 10-16.

Zhang L-N, Chu Q-R (1984). Characteristic and chromosome variation of somaclones and its progeny in rice (*Oryza sativa* L.). Sci. Agric. Sin. 4 : 14-20.

Zhang W, Wu R (1988). Efficient regeneration of transgenic plants from rice protoplasts and correctly regulated expression of the foreign gene in the plants. Theor. Appl. Genet. 76 : 835-840.

Zheng K-L, Zhan Z-M, Wang G-L, Luo Y-K, Xiong Z-M (1989). Somatic cell culture of rice cultivars with different grain types : somaclonal variation in some grain and quality characters. Plant Cell Tiss. Org. Cult. 18 : 201-208.

Zheng Z-L, Chu Q-R, Zhang C-M (1985). Pathogenicity of culture filtrate from *Pyricularia oryzae* on rice. Acta Agric. Shanghai 1 : 85-90.

Zhonglai L, Chang Z (1984). Investigation of ploidy and other characters of the gynogenic plants in *Oryza sativa* L. Acta Genet. Sin. 11 : 113-119.

Zhou C, Yang HY (1980). *In vitro* induction of haploid plantlets from unpollinated young ovaries of *Oryza sativa* L. Acta Genet. Sin. 7 : 287-288.

Zhou C, Yang HY (1981). Induction of haploid rice plantlets by ovary culture. Plant Sci. Lett. 20 : 231-237.

Zhou C, Yang HY, Tian HQ, Zhonglai L, Yan H (1986). *In vitro* culture of unpollinated ovaries in *Oryza sativa* L. In: Haploids of higher plants in vitro. Hu H, Yang HY eds., China Acad. Publ., Beijing; Springer, Berlin, Heidelberg, New York, Tokyo, p 165-181.

Zhou C, Yang HY (1991). *In vitro* production of haploids in rice through ovary culture. In: Biotechnology in Agriculture and Forestry, Vol. 14. Rice. Bajaj YPS ed., Springer, Berlin, Heidelberg, New York, Tokyo, p 180-192.

Ziauddin A, Kasha KJ (1990a). Genetic stability in haploid cell cultures. In: Biotechnology in Agriculture and Forestry, Vol. 12, Haploids in Plant Improvement I. Bajaj YPS ed., Springer-Verlag, Berlin, Heidelberg, New York, Tokyo, p 83-98.

Zou G-Z, Yei M-M, Jia Y-F (1986). Establishment of rice cell line and plantlet regeneration from single cell culture. Acta Agric. Shanghai 2 : 1-8..