TISSUE CULTURE IN CEREALS

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BY

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CONTENTS

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DECLARATION

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(Dr. A. F. Mascarenhas)

KEY TO ABBREVIATIONS

[I] MEDIA

MS	-	Murashige & Skoog's medium (1962)
SH	-	Schenk & Hildebrandt's medium (1972)
B_5	-	Gamborg's medium (1968)
V-47	-	Binding's medium (1974)

[II] HORMONES

ABA	-	Abscisic acid
BAP	-	6 Benzylamino purine
Dicamba	-	3,6 dichloroanisic acid
IAA	-	Indole-3-acetic acid
Ki	-	Kinetin (6-furfuryl amino purine)
NAA	-	α Naphthalene acetic acid
Picloram	-	4 amino-3,5,6-trichloro picolinic acid
Z	-	Zeatin (6-[4-hydroxy-3-methyl-but-2-enyl] amino purine
2,4-D	-	2,4-dichlorophenoxy acetic acid
2,4,5-T	-	2,4,5-trichlorophenoxy acetic acid
2ip	-	6-τ-τ-dimethyl allylamino purine

[III] ORGANIC ADDITIVES

CAA	-	Casamino acids (Casein hydrolysate)
CM	-	Coconut milk

[IV] CALLUS TYPES

E calli	-	Embryogenic calli
NE calli	-	Non-embryogenic calli

ABSTRACT

Cereals constitute an important group of plants in view of animal nutrition. Earlier *in vitro* studies have revealed this group as being highly recalcitrant. However, recently, the development of such systems in all the major cereals has been made a possibility. In part, this success has been due to the screening of several genotypes for their regeneration potential.

The present study was initiated with the primary aim of establishing tissue culture systems in a major cereal - wheat. Screening of various indigeneous genotypes (which have been developed and adapted for Indian environments), as well as various explants for their embryogenic potential was carried out. Further, attempts have been made to establish suspension cultures and to resolve the various cellular and biochemical parameters associated with the process of somatic embryogenesis.

The thesis consists of seven chapters, followed by a resume and a list of references. The chapters included are :

Chapter I	:	General Introduction
Chapter II	:	Materials And Methods
Chapter III	:	Establishment Of An Embryogenic System From Immature Embryos
Chapter IV	:	Establishment Of Morphogenic Systems From Leaf Bases
Chapter V	:	Establishment Of *In Vitro* Systems From Other Explants
Chapter VI	:	Establishment Of Cell Suspension Cultures
Chapter VII	:	Isoenzyme Profiles During The Course Of Somatic Embryogenesis

CHAPTER I : GENERAL INTRODUCTION

This chapter describes the current state of research in cereals with respect to the following aspects :

1. Morphogenesis in cereal cultures.
2. Selection of mutants / variants and resistant cell lines.
3. Somatic hybridisation.
4. Gene transfer and transformation.
5. Molecular biology.

CHAPTER II : MATERIALS AND METHODS

This chapter describes the plasticware, glassware apparatus, the various cultural methods and media used in the course of studies. A table listing the various genotypes used, with their characteristics has been included. The histological, cytological and biochemical methods used for evaluating results have also been described.

CHAPTER III : ESTABLISHMENT OF AN EMBRYOGENIC SYSTEM FROM IMMATURE EMBRYOS

Experiments were carried out to initiate, maintain and regenerate plantlets from embryogenic cultures derived from immature embryos of wheat.

[I] Screening of genotypes : Of the thirty three genotypes screened for their embryogenic response, five were identified as being highly embryogenic. These include the genotypes NI - 5439, NI - 9272, VL-614, CPAN-2005 and Raj-1777. The studies further suggested a possible relation

between precocious germination, somatic embryogenesis and plantlet regeneration.

[II] **Nature of somatic embryogenesis** : The embryogenic callus was characterised by its organised and compact nature, nodular appearence and yellowish color. Somatic embryogenesis was seen to be of the atypical and indirect type, involving an intermediate callus phase.

[III] **Optimising the embryogenic response and regeneration**: The five highly embryogenic genotypes were used to standardise several parameters known to be involved in somatic embryogenesis. These included use of various basal media and auxins, organic additives and antibiotics, screening embryos at different ages and a comparison of scutellar callus Vs. epiblast derived callus. Further, for conversion of somatic embryos to plantlets, several cytokinins were tested.

[IV] **Maintenence of embryogenic cultures** : Cultures not exhibiting precocious germination could be maintained by visual selection of the embryogenic callus and consequent subculture. In precociously germinating cultures, maintenence involved an inhibition of germination. Several factors were tested to achieve this. These included - change of photoperoiod, addition of varying concentrations of abscisic acid and silver nitrate to the medium, varying osmoticum and auxin levels in the medium. Thus, embryogenic lines could be maintained for upto 8 months without significant decrease in their embryogenic potential.

CHAPTER IV : ESTABLISHMENT OF MORPHOGENIC SYSTEMS FROM LEAF BASES

The developmental gradients along cereal leaves, which reflect on the *in vitro* response (described frequently in literature) was evident in leaves from seedlings older than 5 days. Hence to avoid such developmental gradients, younger tissues from 3 and 4 day old seedlings were used which displayed a greater plasticity. Several combimations of auxins [2,4-D and dicamba] and cytokinins [kinetin, benzylaminopurine (BAP) and zeatin] were used for culture initiation. Two types of responses were observed *in vitro* :

1. **Atypical, indirect somatic embryogenesis** : Callus developed from the cultured leaf bases on all the media tested, except those containing combinations of dicamba and BAP. This callus, further gave rise to somatic embryos.
2. **Direct morphogenesis** : On media containing combinations of dicamba and BAP, shoot buds and somatic embryos were formed directly from the cells of the cultured leaf explants. On maintaining these cultures on the same medium for an extended period of time, secondary morphogenesis also occurred. This response was observed exclusively in three genotypes *viz.* Raj-1777, VL-614 and CPAN-2005.

Histological studies to confirm the nature of the above processes was also carried out.

CHAPTER V : ESTABLISHMENT OF *IN VITRO* SYSTEMS FROM OTHER EXPLANTS

This chapter describes the use of various explants like immature inflorescences, root tips and mature embryos for initiating *in vitro* systems in the five selected genotypes.

[I] Immature Inflorescences : The embryogenic response from these explants was studied as an interplay between genotype, age and the type, concentration and combinations of plant growth regulators in the medium. Histological studies were also carried out to confirm the process of somatic embryogenesis. The frequencies of somatic embryogenesis and plantlet regeneration from this explant was found to be comparable to those in the case with immature embryos.

[II] Mature Embryos : Atypical, indirect embryogenesis was observed to occur from the calli initiated from the embryo axis of the mature embryo. Microtillering was also induced from the basal shoot node of the embryo axis. The number of tillers increased with the frequency of subculturing upto the 20^{th} subculture, after which the number of shoots were seen to decrease and the rate of albino shoot production increased.

[III] Root Tips : On auxin containing media (2,4-D or dicamba at 2 mg/l each), callussing occurred. This callus after 2 - 3 subcultures, gave rise to nodular structures on its surface. However, plantlet regeneration from these nodular structures could not be achieved.

CHAPTER VI : ESTABLISHMENT OF CELL SUSPENSION CULTURES

Experiments were carried out to initiate, maintain and regenerate calli from cell suspension cultures. The following aspects are covered in this chapter :

1. Source of inoculum used for initiating cultures. The best source proved to be root tips.

2. Monitoring of subculture schedule and fresh weight changes.
3. Callus regeneration.

CHAPTER VII : ISOENZYME PROFILES DURING THE COURSE OF SOMATIC EMBRYOGENESIS

The developmental patterns of isozymes of some key enzymes were investigated. The transition of the immature embryos to the formation of embryogenic or non-embryogenic callus and finally to the germination of the somatic embryos was associated with the selective expression or repression of peroxidase, esterase, glutamate dehydrogenase, hexokinase, phosphogluco-isomerase, 6-phosphogluconate dehydrogenase and malate dehydrogenase. Embryogenesis and callus specific isoforms were detected. Each developmental stage is associated with a definite isoenzyme profile.

CHAPTER I : GENERAL INTRODUCTION

Today, in the third world countries, the prognosis that agricultural productivity requires a sustained rate of increase for securing food is justified as a counter - measure of population growth. The testimony of the past, when conventional breeding has directly accounted for upto 50% in the increased yield of crops such as wheat, maize and barley (Bingham 1981) is witness to this compulsion. The efficiency of breeding practices depends on increasing the genetic variability, selection, fixation and rapid propagation of plants at the individual levels.

However, the genetic limitations of conventional methods of crop improvement have been manifested in several ways. These include - the genetic linkage of desirable genes with unwanted ones, difficulties in the assessment of complex traits from the phenotype, identification of spontaneous mutations - which could be unfavourable, and lastly, but not the least - the very long time scales of many breeding programs. All these factors slow down the production and release of varieties which are specific improvements over pre - existing cultivars. The delayed time - scales involved between the development and release of an improved cultivar is especially important in view of disease - resistant varieties. This is because, over the years, changes in the genome of the pathogen may be significant enough to neutralise the mechanism of resistance in the improved plant and thereby render it sensitive to the pathogen.

Rapid developments have occurred in the field of crop improvement within the last few years. These include techniques for the selection and characterisation of mutants, haploidy to obtain homozygous individuals and to

provide more efficient screening among recombinants, somatic hybridisation for recombining genomes of sexually incompatible species and embryo culture for recovering inviable hybrids. The use of cell culture for rapid multiplication, to generate genetic variability as well as providing convenient systems for the transfer, integration, expression and selection of genes (Larkin and Scowcraft 1981) are also envisaged to play a significant role in the improvement of plants faster than was possible in the past, and in turning them into wholly acceptable crops.

Cereals form one of the most important group of plants in view of human and animal nutrition. This fact, by itself, justifies a major share of plant research being diverted towards these species. Thus, over the past few decades, cereals have been the subject of detailed genetic and physiological studies. It would hence be rightfully expected that the recently developed techniques of cellular and molecular biology be applied to this economically important group of crops in view of producing plants with enhanced consumer or agronomic qualities.

However, in practice, a considerable amount of research has been carried out only with the so - called "model" systems like carrot, tobacco, petunia, tomato etc. which are considered to be more responsive to *in vitro* culture. All along it had been inferred that the accomplishments made in these species could be extended to the economically important crops, with slight modifications if necessary. Unfortunately, this could not be brought about as easily as it had been predicted.

The major obstacle in cereal research was their relatively unresponsive behaviour in tissue culture, by

which, these crop species came to be branded as being highly recalcitrant to *in vitro* culture. Significant progress has been made in the last decade in cellular manipulations in cereals - the major breakthrough has been the successful establishment of tissue culture systems. The first cereal tissue to be successfully cultured *in vitro* was the endosperm of immature maize kernels of an unidentified genotype (La Rue 1949). Following this, several attempts have been made to culture the major cereals like rice, wheat, maize, sorghum, rye,etc in view of obtaining organogenesis and somatic embryogenesis.

Such *in vitro* organogenic and embryogenic systems provide standard, consistent and reproducible methods for growing plant material under controlled conditions for selection and modification at the single cell, protoplast or cultured tissue levels. Further, regeneration from these cultures is an absolute necessity if the expression and the inheritence of the alterations made in the plant are to be analysed.

The matter presented in this chapter reviews the advances made in cereal research with reference to the following topics -

1. Morphogenesis in cereal cultures.
2. Selection of mutant/ variant and resistant cell lines.
3. Somatic hybridisation.
4. Gene transfer and transformation.
5. Genetics and molecular biology.

1.1 MORPHOGENESIS IN CEREAL TISSUES

The demonstration of totipotency of any growing tissue requires the sustained division of cells. For this to happen, the cells themselves should be competent to the stimuli that might stimulate cell proliferation and morphogenesis. Totipotency may not be exhibited by every cell type, so the ability to establish regenerating callus depends on the tissues selected to be cultured. The distinction between competent and non - competent cells is particularly marked in the Gramineae. In these crops, cells lose their capacity for growth *in vitro* at a very early stage in differentiation.

Till the 1980's, regeneration in cereals was sporadic and transient in nature, the number of plants recovered was rather small, and the regeneration potential could not be maintained for long periods of time. Plant regeneration in tissue cultures of the Gramineae, is recognised to take place by three principal pathways -

1. By the derepression of previously existing shoot meristems resulting in " microtillering" (King *et al.* 1978, Dunstan *et al.* 1979, Green 1982, Tanzarella and Greco 1985). Probably the best example of this type of micropropagation is the system of shoot tip culture in several cereals and forage grasses like *Lolium multiflorum* (Dalton and Dale 1981), sugarcane (Hendre *et al.* 1975) etc. The isolated shoot tip is placed on a cytokinin containing medium which interferes with the normal apical dominance and results in very rapid tiller production . Such types of cultures have proved to be useful for pathogen elimination, storage and micropropagation.

2. By the *do novo* organisation of meristems (Rangan 1974, Nakano and Maeda 1979, Springer *et al.* 1979) - Organogenesis in the form of rhizogenesis rarely leads to viable plantlets. Shoot forming cultures can, however, produce plantlets readily by subsequent rooting. Histologically, the vascular tissue of the regenerated shoot buds typically extends into the parental tissue. Further, the shoots and roots may not show vascular connection to each other especially if regenerated independently from different sectors.

3. By the production of somatic embryos - Several accounts of plantlet regeneration in cereals *via* soamatic embryogenesis have been described in the last ten years. Somatic embryogenesis is the process by which haploid or diploid somatic cells develop into differentiated plants through characteristic embryological stages without the fusion of gametes. The master unit involved in this process is the 'somatic embryo' or 'embryoid ' which has been defined as an asexually produced bipolar structure that lacks a vascular connection with the mother tissue and resembles a zygotic embryo and functions like one, in giving rise to a new plant (Haccius, 1978).

The detailed characterisation of embryogenic cereal cultures highlighted the need for using -

(i) immature explants which are in a relatively undifferentiated state,

(ii) high concentrations of plant growth regulators (usually 2,4-D) and

(iii) screening genotypes capable of eliciting an optimum *in vitro* response.

TABLE 1 : A REVIEW OF MORPHOGENESIS IN CEREALS

No.	CROP	IMMATURE EMBRYOS	MATURE EMBRYOS	IMMATURE INFLORESCENCES	ANTHERS /POLLEN	LEAVES / SHOOT TIPS
1.	MAIZE	Organogenesis				
		Green & Philip (1975) Springer *et al.* (1979)	Harms *et al.* (1976) Mott & Cure (1978) Mascarenhas *et al.* (1975)		Ting *et al.* (1981) Genovesi & Collins (1982) Nitch *et al.* (1982)	
		Somatic Embryogenesis				
		Lu *et al.* (1982) Vasil *et al.* (1983) Torne *et al.* (1984) Duncan *et al.* (1985) Tomes & Smith (1985) Armstrong & Green (1985) Kamo & Hodges (1986) Close & Ludeman (1987) Lee & Philips (1987) McCain *et al.* (1988) Vain *et al.* (1989) Close & Gallagher -Ludeman (1989) Franz & Schel (1991a) Franz & Schel (1991b)	Abou-Mandour & Hartung (1986)	Rhodes *et al.* (1986)	Ku *et al.*(1978) Brettell *et al.* (1981) Brettell *et al.* (1981) Petolino & Jones (1986) Tsay *et al.* (1986) Barnabas *et al.* (1987) Sun *et al.* (1989) Gaillard *et al.* (1991) Reiffer & Freire (1990)	Chang (1983) Santos *et al.* (1984) Conger *et al.* (1987) Sinha Ray & Ghosh (1990)
		Organogenesis And Somatic Embryogenesis				
		Lowe *et al.* (1985) Rapela *et al.*(1985)				

Contd....

No.	CROP	IMMATURE EMBRYOS	MATURE EMBRYOS	IMMATURE INFLORESCENCES	ANTHERS /POLLEN	LEAVES / SHOOT TIPS
2.	WHEAT	Organogenesis				
		Gosch-Wackerle *et al.* (1978) Shimada (1978) Shimada & Yamada (1978) Bapen & Rao (1982a) Sears & Deckard (1982) Lazar *et al.*(1983) Maddock *et al.* (1983) Mathias *et al.* (1986) Bartok & Sagi(1990)	Gosch-Wackerle *et al.* (1978) Bapen & Rao (1982a) Ozias-Akins & Vasil (1983c) Lazar *et al.* (1983) Mascarenhas *et al.* (1985)	Gosch-Wackerle *et al.* (1978) Maddock *et al.* (1983)	Schaeffer *et al.* (1979) Huang (1987)	Ahuja (1982) Zamora & Scott (1983) Greco *et al.* (1984)
		Somatic Embryogenesis				
		Ozias-Akins & Vasil (1982, 1983a, 1983b) Magnusson & Bornman (1985) Heyser *et al.* (1985) He *et al.* (1986) Mathias & Boyd (1986) Breiman *et al.* (1987) Carman *et al.* (1987a, 1987b) Papenfuss & Carman (1987) Galiba & Yamada (1988) Qureshi *et al.* (1989) Duncan & Widholm (1989) He *et al.* (1991)	Heyser *et al.* (1985) MacKinnon *et al.* (1987)	Ozias-Akins & Vasil (1982) Wang (1990)	Picard & DeBuyser (1977) Henry *et al.* (1984) Lazar *et al.* (1985) Datta & Wenzel (1987) Jones & Petolino (1987) Chu & Hill (1988) Kudirka *et al.* (1989) Feng & Ouyang (1989) Huaping & Konzak (1989) Simmonds (1989) Sagi & Barnabas (1989) Chu *et al.* (1990) Orshinsky *et al.* (1990) Foroughi-Wehr & Zehr (1990)Lu *et al.* (1990) Last & Brettell (1990) Ziegler *et al.* (1990) Rybzynski *et al.* (1991) Zhou *et al.* (1991)	

Contd....

No.	CROP	IMMATURE EMBRYOS	MATURE EMBRYOS	IMMATURE INFLORESCENCES	ANTHERS /POLLEN	LEAVES / SHOOT TIPS
	WHEAT(Contd.)	Organogenesis And Somatic Embryogenesis				
		Ahloowalia (1983) Brown *et al.* (1989)			Armstrong *et al.* (1987) Simmonds (1989)	
3.	**RICE**	Organogenesis				
		Maeda (1968)	Nishi *et al.* (1968) Nakano & Maeda (1974) Wu & Li (1971) Inou & Maeda (1981) Kavi Kishor (1987)	Ling *et al.* (1983) Kavi Kishor *et al.* (1989)	Iyer & Raina (1972) Chen *et al.* (1978) Cornejo-Martin & Prima-Millo (1981) Torrizo & Zapata (1981) Tsay *et al.* (1986) Kavi Kishor *et al.* (1989) Cho & Zapata (1988) Gupta & Borthakur (1987) Raina *et al.* (1987) Wang *et al* (1989)	Yan & Zhao (1982)
3.	**RICE**	Somatic Embryogenesis				
		Heyser *et al.* (1983) Ling *et al.* (1983) Siriwardana & Nabors (1983) Abe & Futsuhara (1986) Maggioni *et al.* (1989) Jones & Rost (1989)	Heyser *et al.* (1983) Raghava Ram & Nabors (1984) Abe & Futsuhara (1986) Raina *et al.* (1987) Maggioni *et al.* (1989) Wang *et al.* (1987)	Ling *et al.* (1983) Chen *et al.* (1985) Wang *et al.* (1987)	Genovesi & Magill (1982) Reddy *et al.* (1985) Torrizo & Zapata (1986) Schaeffer *et al.* (1986) Mercy & Zapata (1987) Raina *et al.* (1987) Datta *et al.* (1990)	Wernicke *et al.* (1981)

Contd....

No.	CROP	IMMATURE EMBRYOS	MATURE EMBRYOS	IMMATURE INFLORESCENCES	ANTHERS /POLLEN	LEAVES / SHOOT TIPS
4.	**BARLEY**	Organogenesis				
		Cheng & Smith (1975)	Bayliss & Dunn (1979) Lupotto (1984)		Foroughi-Wehr *et al.* (1976) Zenktler & Stefanik (1982) Huang & Sunderland (1982) Dunwell *et al.* (1987)	Chen & Smith (1975) Koblitz & Saalbach (1976)
		Somatic Embryogenesis				
		Norstog (1970) Dale & Deambrogio (1979) Kott & Kasha (1984) Hanzel *et al.*(1985) Breiman (1985) Thomas & Scott (1985) Goldstein & Kronstadt (1986) Rengel (1986) Caligari *et al.* (1987) Luhrs & Lorz (1987)		Chu *et al.* (1984) Thomas & Scott (1985)	Hesemann & Schroder (1982) Shannon *et al.* (1985) Datta (1987) Ziauddin *et al.* (1990) Roberts-Oehlschanger & Dunwell (1990) Zhu *et al.* (1990) Kao *et al.* (1991) Thompson *et al.* (1991)	Weigel & Huges (1985) Rengel & Jelaska (1986) Mohanty & Ghosh (1988) Barcelo *et al.* (1991)
5.	**SORGHUM**	Organogenesis				
		Mastellar & Holdren (1970) Gamborg *et al.* (1977) Dunstan *et al.* (1978) Brar *et al.* (1979)	Strgonov (1968) Mastelar & Holden (1970) Mascarenhas *et al.* (1975) Rao & Kavi Kishor (1989)			Bhaskaran *et al.* (1983) Cai *et al.* (1987)

Contd....

No.	CROP	IMMATURE EMBRYOS	MATURE EMBRYOS	IMMATURE INFLORESCENCES	ANTHERS /POLLEN	LEAVES / SHOOT TIPS
	SORGHUM(Contd.)	Somatic Embryogenesis				
		Thomas *et al.*(1977) Ma *et al.* (1987)	Bhaskaran *et al.* (1985) Mac Kinnon *et al.* (1987)	Brettell *et al.* (1980) Boyes & Vasil(1984) George & and Eapen (1988) Cai & Butler (1991)	Rose *et al.* (1986)	Wernicke & Brettell (1980) Wernick *et al.* (1982) Cai *et al.* (1987) Bhaskaran & Smith (1988)
6.	OATS	Organogenesis				
		Cummings *et al.* (1976) Rines & McCoy (1981) Nabors *et al.* (1982)	Carter *et al.*(1967) Lorz *et al.* (1976) Cure & Mott (1978)		Rines (1983)	
		Somatic Embryogenesis				
		Heyser & Nabors (1982) Bregitzer *et al.* (1989)	Heyser & Nabors (1982) Bregitzer *et al.* (1991)			
7.	RYE	Organogenesis				
		Rybcynski (1979)	Eapen & Rao (1982b)	Rybcynski *et al.* (1980)		
		Somatic Embryogenesis				
		Lu *et al.* (1984) Zimny & Lorz (1989)	Linacero & Vazquez (1986) Vazequez *et al.* (1991)	Vazequez *et al.* (1991)		Vazequez *et al.* (1991)
		Organogenesis And Somatic Embryogenesis				
		Rybczynski & Zdunczyk (1986)				

Contd....

No.	CROP	IMMATURE EMBRYOS	MATURE EMBRYOS	IMMATURE INFLORESCENCES	ANTHERS /POLLEN	LEAVES / SHOOT TIPS
8.	MILLETS	Organogenesis				
				Bajaj & Dhanju (1981)	Bui Dang & Pernes (1982) Nitsch *et al.*(1982)	
		Somatic Embryogenesis				
		Vasil & Vasil(1981) Vasil & Vasil (1982a) Lu & Vasil (1982a) Hanna & Vasil(1984)	Heyser (1982) Botti & Vasil(1983)	Botti & Vasil(1984) Vasil & Vasil (1982b) Wang & Vasil(1981)	Haydu & Vasil(1981) Lu & Vasil(1981) Rangan & Vasil (1981) Chandler & Vasil (1984a) Taylor & Vasil (1987) Rajasekharan *et al.* (1987)	Haydu & Vasil(1981)

Notable milestones in the *in vitro* morphogenesis in cultures of the major cereal species with respect to organogenesis, somatic embryogenesis and regeneration from several explants, are listed in Table 1.

Thus, plantlet regeneration *via* organogenesis and somatic embryogenesis is now feasible in all the major cereals. Further, attempts have also been made to achieve regeneration from suspension and protoplast cultures. The initiation and maintainence of stable embryogenic suspension cultures depends on several factors which include the source tissue, frequency of subculture, dilution ratio with fresh medium, composition of the medium, etc. Similarly, the regeneration from cereal protoplasts has been realised as being extremely difficult to achieve - possibly due to the wide array of variables involved in the process. These include :

1. Donor plant / cell variables - Genotype, explant, cell types and their degree of differentiation, extent of senescence, environmental history, cultural conditions, etc.
2. Isolation variables - Isolation procedures, concentrations of enzymes used, buffers used, etc.
3. Culture variables - Protoplast density, chemical cultural conditions, physical cultural conditions, etc.

In spite of all these hurdles, regenerable suspension and protoplast cultures have been established in all the major cereals. Some of these reports are summarised in Table 2.

TABLE 2 : REVIEW OF SUSPENSION AND PROTOPLAST CULTURES OF CEREALS

NO.	CROP	SUSPENSION CULTURES		PROTOPLAST CULTURES	
		Regeneration	Reference	Regeneration	Reference
1.	MAIZE	Callus	Potrykus *et al.* (1979)	Callus	Potrykus *et al.* (1977)
		Plants	Vasil & Vasil (1986)	Callus	Potrykus *et al.* (1979)
				Callus	Chourey & Zurawski (1981)
				Callus	Ludwig *et al.* (1985)
				Callus,somatic embryos	Vasil & Vasil (1987)
				Callus,somatic embryos,leaf-like structures, roots	Kamo *et al.* (1987)
				Microcolonies	Birnberg *et al.* (1988)
				Plants	Rhodes *et al.* (1988)
				Callus	Carswell *et al.* (1989)
				Plants	Sun *et al.* (1989)
				Plants	Prioli & Sondahl (1989)
				Plants	Morocz *et al.* (1990)
2.	WHEAT	Roots	Gamborg & Eveleigh (1968)	Callus	Maddock (1985)
		Non-morphogenic	Dudits *et al.* (1977)	Callus, Embryoids & Albino plants	Hayashi & Shimamoto (1988)
		Plants	Ahuja *et al.* (1982)	Plants	Harris *et al.* (1988)
		Plants	Wang *et al.* (1983)	Callus, shoot meristems & leaves	Lee *et al.* (1988)
		Non-morphogenic	Chen *et al.* (1985)		
		Single Plant	Maddock *et al.*(1985)		
		Plants	Fedak *et al.* (1987)	Plants	Vasil *et al.* (1990)
		Plants	Harris *et al.* (1988)		
		Embryoids & Plants	Inagaki *et al.*(1988)		
		Plants	Redway *et al.* (1990)		
		Plants	Wang & Nguyen (1990)		

Contd....

3.	**RICE**	Plants Plants	Ye (1984) Zimny & Lorz(1986)	Roots Microcolonies Callus & Embryoids Plants Plants Plants Plants Plants Plants Plants Plants Plants Plants	Deka & Sen (1976) Wakasa *et al.* (1984) Abdullah & Cocking (1986) Abdullah *et al.* (1986) Toriyama *et al.* (1986) Yamada *et al.* (1986) Kyozuka *et al.* (1987) Ogura *et al.* (1987) Kyozuka *et al.* (1988) Lee *et al.* (1989) Masuda *et al.* (1989) Shimamoto *et al.* (1989) Wang *et al.* (1989) Brown *et al.* (1990) Li & Murai (1990)
4.	**BARLEY**	Plants Plants	Kott & Kasha (1984) Jahne *et al.* (1991)	Plants	Jahne *et al.* (1991)
5.	**SORGHUM**	Callus Callus Plants	Brar *et al.* (1980) Chourey & Sharpe (1985) Wei & Xu	Callus Callus Plants	Brar *et al.* (1980) Chourey & Sharpe (1985) Wei & Xu
6.	**OATS**			First few divisions Microcalli	Kaur-Sawhney *et al.* (1980) Hahne *et al.* (1990)
7.	**RYE**			First few divisions	Koblitz (1976)
8.	**MILLETS**	Plants	Lu & Vasil (1981) Vasil & Vasil (1981) Vasil & Vasil (1982) Karlsson & Vasil (1986)	Plants Plants Plants Callus Callus	Vasil & Vasil(1980) Lu *et al.* (1981) Vasil *et al.*(1983) Heyser (1983b) Heyser (1984)

1.2 SELECTION OF MUTANT / VARIANT / RESISTANT CELL LINES

Cultured cells are known to display extensive genetic variability (Bayliss 1980, D 'Amato 1985, Muller *et al.* 1990). Part of this variation is due to the mixoploid nature of differentiated somatic tissues (Swedlund and Vasil 1985), but some genetic changes occur in culture, particularly in friable, non - morphogenic cultures (Vasil 1988). Such genetic changes have been designated as somaclonal variation (Larkin and Scowcroft 1981). This random variability present or induced in cultured cells may be useful in plant breeding and improvement.

Tissue culture variability, even though genotype dependent, is pervasive, and has been adequately documented in many crops such as sugarcane (Nickell and Heinz 1973; Liu *et al.* 1977), oat (Cummings *et al.* 1976), maize (Gegenbach *et al.* 1977; Green 1977; Brettell and Ingram 1979; Zehr *et al.* 1987; Armstrong and Philips 1988; Miao *et al.* 1988; Williams *et al.*1991), barley (Deambrogio and Dale 1980), sorghum (Gamborg *et al.* 1977; Smith and Bhaskaran 1988), rice (Nishi *et al.* 1968; Henke *et al.* 1978; Oono 1975; 1978), wheat (Mascarenhas *et al.* 1977; Karp and Maddock 1984; Maddock and Semple 1986; Breimann *et al.* 1987; Ryan and Scowcroft 1987; Mahmant and Nabors 1990, 1991; Hashim *et al.* 1991), pearl millet (Morrish *et al.* 1990), and many more.

However, most of the somaclonal variants described so far in literature are either of limited value or have no direct impact on crop improvement (Lorz 1987). A possible application of somaclonal variation for cereal species is seen primarily in the combination of *in vitro* culture with *in vitro* selection. Further, *in vitro* selection in cultured

suspensions and isolated protoplasts would make possible the handling of very large numbers of individuals which otherwise would demand large growing areas and intensive labour if equivalent numbers are treated as plants in the soil.

Thus, cereal tissue cultures have been utilised to isolate cell lines that are resistant to specific drugs, antimetabolites, metabolite analogs or toxins produced by pathogenic organisms (Maliga 1980). Selection of cells resistant to S - 2 aminoethyl cysteine, 5 - methyl DL trypto phan or high levels of L-lysine + L-threonine has been carried out in rice (Chaleff and Carlson 1975; Shaeffer and Sharp 1981; Chen and Chen 1979; Lee and Kameya 1989), barley (Bright *et al.* 1979; Cattoir - Reynaerts *et al.* 1981; Bright *et al.* 1982), wheat (Tantau and Dorffling 1991), sorghum (Singh and Axtell 1973), maize (Green and Donovan 1980; Widholm 1988; Miao *et al.* 1988; Zehr & Widholm 1988) and napier grass (Redway and Vasil 1990).

Soils in many arid regions of the world are unsuitable for agriculture owing to their high salinity. One of the possible remedies to this problem is the selection of salt tolerant cell lines and regenerate plants from such cell lines. Such salt tolerant plants have been recovered in rice (Yano *et al.* 1982; Kavi Kishor 1985; Vajrabhaya *et al.* 1989; Prakash and Padayatty 1989), sorghum (Bhaskaran and Smith 1983), sugarcane (Yasuda *et al.* 1982), napier grass (Chandler and Vasil 1984; Bajaj and Gupta 1984), barley (Ye *et al.* 1987) and pearl millet (Bajaj and Gupta 1984).

Tissue culture selection has also been used to obtain disease - resistant clones of sugarcane that are resistant to *Helminthosporium sacchari*, Fuji disease and *Celerospora sacchari* (Heinz *et al.* 1977). Similarly, cell lines and

plants of maize resistant to *Helminthosporium maydis* toxin (Gegenbach *et al.* 1977; Bretell and Ingram 1979), barley yellow mosaic virus resistant anther culture derived cell lines (Foroughi - Wehr and Friedt 1984), barley and wheat for resistance to *Helminthosporium sativum* (Chawla and Wenzel 1987a; Wenzel and Foroughi - Wehr 1990) and fusaric acid resistant barley plants (Chawla and Wenzel 1987b) have been isolated.

1.3 SOMATIC HYBRIDISATION

Interspecific as well as intergeneric hybrids, which cannot be obtained by conventional breeding methods, can be produced by protoplast fusion (Gleba and Hoffmann 1980). Recent successes with plant regeneration from cereal protoplasts, albeit limited, have encouraged work on somatic hybridisation. This led to the recovery of somatic hybrid cell lines of pearl millet + Guinea grass, pearl millet + Einkorn wheat (Vasil *et al.* 1988), somatic hybrid embryos of pearl millet + sugarcane (Tabaeizadeh *et al.* 1986), somatic hybrid plantlets of rice + barnyard grass and mature hybrid plants of cultivated + wild rice (Terada *et al.* 1987; Finch *et al.*1990; Toriyama and Hinata 1988).

Rearrangements, recombination and selective amplification of the mitochondrial genome has been observed on several of the somatic hybrid cell lines. As cytoplasmic male sterility is regulated by the mitochondrial genome, somatic hybrids / cybrids may be useful for the transfer of this important trait (Zelcher *et al.* 1978). Maize is known to be susceptible to the pathogen *Helminthosporium maydis*; the site of action of the HmT toxin is proposed to be primarily the mitochondria (York *et al.* 1980). Resistance to the HmT toxin could be conferred to somatic hybrids by fusing corn mesophyll protoplasts with soybean protoplasts which are resistant to the toxin (Earle and Gracen 1980). However, plantlet regeneration remains a limiting factor, as yet. Development of useful somatic hybrids with unique combination of nuclear or organelle genomes conferring tolerence to herbicides or pathogens must await the development of efficient protocols for the recovery of plants from fused / unfused protoplasts.

1.4 GENE TRANSFER AND TRANSFORMATION

The last decade has brought to prominence in the minds of cereal plant breeders the issues of genetic vulnerability and narrow genetic bases (Larkin 1985). It is argued that such a narrow genetic base means that our crops are vulnerable to the ever changing spectrum of challenges from diseases and pests. It may further mean that the important genetic components of crop improvement may soon be exhausted unless breeding strategies are radically altered. Given that the narrow genetic base is a reality, two approaches may be made to alter the situation : firstly, the existing germplasm in adapted cell lines should be exploited to its fullest extent, and secondly, the introduction of a wide range of exotic and unadapted germplasm into such crops may be established.

The work reported so far on gene transfer in cereals with the ultimate aim of crop improvement may be categorised as follows :

1. Protoplast manipulations.
2. Direct DNA transfer.
3. Biolistic transformation.
4. Pollen transformations, macroinjections and DNA imbibition by seeds.

1.4.1 Protoplast manipulations :

Isolated protoplasts are being considered ideal tools for generating genetic variability because of the fusion of two genomes to produce a transformed plant with improved characteristics. The following manipulations in cereal protoplasts have been reported :

1.4.1.1 Somatic Hybridisation -

This has been discussed in detail in section 1.3.

1.4.1.2 Direct DNA Transfer -

Isolated genes have been transferred directly into protoplasts either by imbibition or by electroporation.

(a) Imbibition :

Lorz *et al.* (1985) have reported the transformation of *Triticum monococcum* protoplasts with plasmid DNA containing the Neomycin Phosphotransferase (NPT II) gene under the influence of the Nopaline Synthase (NOS) promotor. Consecutively, Potrykus *et al.* reported a similar transformation in *Lolium multiflorum* using the NPT II gene under the control of Cauliflower Mosaic Virus (CaMV) promotor. Soon after, several similar reports followed in rice (Uchimiya *et al.* 1986; Datta *et al.* 1988; Yang *et al.* 1988,1990; De Keyser *et al.* 1989; Hayashimoto *et al.* 1990) and barley (Lazzeri *et al.* 1991).

(b) Electroporation :

Fromm *et al.*(1986) were the first to report the successful transformation of maize with the bacterial Chloramphenicol Acetyl Transferase (CAT) gene. This was soon followed by the stable expression of the NPT II gene in the maize system (Fromm *et al.* 1986). Since then, several more reports of transient as well as stable expression of genes in other major cereals followed e.g. rice (Ou-Lee *et al.* 1986; Zhang *et al.* 1988; Toriyama *et al.* 1988; Yang *et al.* 1988; Okada *et al.* 1988; Shimamoto *et al.* 1989; Mulligan *et al.* 1989; Peng *et al.* 1990; Davey *et al.* 1991) and maize (Rhodes *et al.* 1988; Mulligan *et al.* 1989; Planckaert and Walbot 1989; Antonelli and Staedler 1990).

1.4.1.3 Use Of Vectors -

The plant pathogen *Agrobacterium tumefaciens* is known to cause crown gall disease in dicots by transferring a

defined DNA segment (the T - DNA) from its Ti (tumor - inducing plasmid) to the host genome. This T - DNA is normally integrated into the nuclear DNA of the host and is transmitted to the progeny in a normal Mendelian fashion.

Two types of agrobacterium based vectors have been developed : integrative and binary. In the integrative type, advantage is taken of the fact that it is possible to replace all the tumor inducing genes with the desirable gene(s), and the newly developed construct can be used for infection. In the binary vectors, the concept applied is that the virulence (vir) and the border regions of the T - DNA may be located on two separate plasmids without affecting infectivity. Agrobacteria with these two plasmids are also capable of stably integrating the DNA lying between the T - DNA borders into the plant genome.

The first report of successful agroinfection was reported by Graves and Goldman (1986) - in maize. This was followed by other reports in maize (Grimsley *et al.* 1987 & 1988; Gould *et al.* 1991), wheat (Hayes *et al.* 1988; Woolston *et al.* 1988; Mooney and Goldwin 1991; Mooney *et al.* 1991), rice (Raineri *et al.* 1990) and barley (Creisen *et al.*1990). In most cases, the agrobacterium - mediated transformation has been reported to be roughly as efficient as in dicot plants - hence this system of gene transfer is believed to hold potential for future work.

1.4.1.4 Biolistic Transformations -

Variably called the biolistic, bioblaster, particle acceleration, particle gun, microprojectile or gene gun technique, it is the most recent and widely used procedure developed to transfer genes into plants. Klein *et al.* (1988a & 1988b) first reported the transformation of maize

suspension cultures with highly accelerated tungsten particles coated with a plasmid harbouring the CAT gene. After this, several reports in cereals followed. These included - maize (Rhodes *et al.* 1988; Klein *et al.* 1989a; Lyznik *et al.*1989; Cao *et al.* 1990; Fromm *et al.* 1990; Oard *et al.* 1990; Gordon - Kaum *et al.* 1990; Izawa *et al.* 1991; Reggiardo *et al.* 1991), wheat (Lonsdale *et al.* 1990; Oard *et al.* 1990; Chibbar *et al.* 1991; Daniell *et al.* 1991; Vasil *et al.* 1991), barley (Ahokas 1989; Kartha *et al.* 1989; Creissen *et al.* 1990; Gopalkrishnan *et al.* 1991), pearl millet (Taylor and Vasil 1991), rice (Dekeyser *et al.* 1990) and sorghum (Hagio and Browse 1991).

At present, there seems to be no inherent limitation in the species of tissue that can be treated by this procedure. Thus the technique is believed to hold immense potential towards the goal of achieving transgenic plants.

1.4.1.5 Pollen Transformations, Macroinjections and DNA Imbibition by Seeds :

In view of the difficulties associated with the regeneration of plants from cereal protoplasts and suspension cultures, it may be advisable to develop transformation systems that are independent of *in vitro* culture techniques. The approach of pollen transformations goes back to the early seventies and is based on the hope that DNA could be taken up into germinating pollen and either integrate into the sperm nucleus or reach the zygote with the pollen tube. Ohta (1986) applied a paste of donor DNA (obtained from a donor plant with a different phenotype) along with pollen to receptive stigmas of maize plants and claimed a high frequency of transformation. However, the

experiment could not be reproduced in other plants (Sanford *et al.* 1985; Booy *et al.* 1989).

De la Pena *et al.* (1987) have reported successful transformation by injecting a DNA solution containing the NPT II gene into the stem below the floral meristem of rye. Although the authors reported successful transformation, it is difficult to understand how DNA could reach the sporogenic cells over several layers of neighbouring cells in this experimental design. Such an experiment needs to be reproduced on a large - scale to establish proof. Further work along these lines has not been reported.

More recently, the uptake and expression of chimeric genes by imbibition of embryos in DNA solutions has been described in wheat (Topfer *et al.* 1989, 1990) and also in other cereals - which includes maize, rice, oats, triticale and barley (Topfer *et al.* 1989). This seems a very simple method to achieve transformation.

In all the above described techniques, the markers used to detect transformants include resistance to antibiotics, or the presence of bacterial metabolites - opines (in agroinfection), or development of viral symptoms. Such transformations cannot be the ultimate aim of gene transfer, but provide excellent avenues to assess the success and feasibility of each system. Since the last few years, genes of agronomic importance have begun to be isolated and characterised. A majority of these genes belong to multigene families. Thus, any transformation system that is developed further, will have to be flexible enough to be applicable for genes from multigene families.

Until recently, it was a matter of speculation whether the insertion of modified genes would affect the stability

of the host's genetic machinery and lead to imbalances in the functioning of the inserted and other genes. Neil *et al.* (1987) have reported the expression of a wheat α-gliadin gene in yeast cells; consecutively, the synthesis and secretion of wheat α-amylase in yeast cells was also reported (Rothstein *et al.* 1987). Wallace *et al.* (1988) have also reported the modification of the zein gene (to increase its lysine content) and secretion of the protein on its insertion into a completely alien environment (frog oocyte). Such experiments may eventually make possible the ultimate aim of plant biotechnology *viz.* that to produce improved crops for the future *via* directed gene transfer.

1.5 GENETICS AND MOLECULAR BIOLOGY

"Molecular biology implies not so much a technique as an approach from the view-point of the so-called basic sciences, with the leading idea of searching below, large-scale manifestations of classical biology for the corresponding molecular plan" - Astbury 1950. Molecular biology is now begining to provide new insights into the essence of the living state, within the framework of plant cell and tissue culture. Possibly, within the next decade, the needs of plant cell and tissue culture will increasingly be inextricably linked to the molecular aspects of plant cell biology (Cocking 1987).

Thus, studies involving cell culture, somatic cell genetics and molecular biology are begining to provide an interesting and deeper understanding of certain nucleo-cytoplasmic interactions in the control of gene expression and plant development. Some of the reported variability observed in cytoplasmic traits may be caused by the recombination or loss of mtDNA, mutations or intracellular heterogeneity of mtDNA (Gegenbach *et al.* 1977; Brettell *et al.* 1979; Smith and Choudhury 1991).

Work is also being conducted on several aspects of gene structure, expression and regulation (Kaneko *et al.* 1990; Reina *et al.* 1990; Montoliu *et al.* 1990; Rundle and Zielinski 1991; Tagu *et al.* 1991; Guidet *et al.* 1991; Khayat *et al.* 1991; Harcourt and Dale 1991 etc.), fertility elements/ nuclear restoration genes (Kemble and Bedbrook 1980; Smith and Choudhury 1991), photosynthesis (Rundle and Zielinski 1991; Galili *et al.* 1991; Okkels *et al.* 1991), seed storage proteins (Heubner *et al.* 1991; Puckett and Kriz 1991; Wilson 1991; Cruz-Alvarez *et al.* 1991; Quale *et al.*

1991), pathogen specificity and resistance (Andersen and Smedegaard-Petersen 1988; Rebmann *et al.* 1991; Casacuberta *et al.* 1991; Kogel *et al.*1991), heat and drought tolerance (Clark and Critchley 1990; Terzi *et al.* 1991), hormone regulation (Morris *et al.* 1991; Bartels *et al.* 1991), etc. These studies represent just a fraction of the work carried out in the field of cereal molecular biology within the past two years.

Considerable interest has also spurged in the restriction fragment length polymorphism (RFLP) probing techniques, which have been described as one of the first and most powerful tools of biotechnology that are useful in the field (Ratner 1990). Thus, RFLP's have been used in corn to identify the genes responsible for oligogenic and monogenic traits such as yield (Garst Seed, ICI, London). In addition to its field application, RFLP'S are also being studied as part of research programs to resolve the enigma of gene functioning (Hartcourt and Gale 1991, Liao and Niks 1991).

The preceeding pages thus provide strong and convincing evidences of the rapid and encouraging progress in cell culture, genetics and molecular biology of cereal crops. It is thus certain that plant biotechnology in future will certainly supplement and complement conventional breeding in plant improvement. This will require a long-term commitment and a greater understanding of plant growth, development, physiology and gene structure and functioning. It is also essential that better dialogue and interactions take place between cellular and molecular biologists, plant breeders and geneticists.

The driving force behind economic, social and cultural development since pre-civilisation times has been the ability of man to "domesticate" cereal and other crops and improvise upon them. This is evident even today as is seen from the dramatic effects of the Green Revolution which was a result of the introduction of high - yielding and superior varieties of wheat (developed at the Centre for Maize and Wheat Improvement, Mexico) and rice (International Rice Research Institute, Philippines).

In India, wheat production has increased three - fold in less than twenty years (1965 - 1983). However, as a counter - measure of the increase in the population, the present day food production is not sufficient. This then, is the challange for plant biotechnology. If the latter has to initiate another green revolution in the 21st century, its success or failure must be viewed against the backdrop of the acievements of the agricultural advances of the past and the benefits of traditional agricultural practices.

Thus, the initiation of any sort of an economic revolution in India would require the improvement of indigeneous varieties of crops (that have been well - proved to be suitable for Indian environments). This essentially requires the development of reliable and reproducible *in vitro* systems which may then be used in further manipulations for improvement of agronomic characters.

However, despite the vast amount of literature, the difficulties encountered on obtaining the desired response from a cereal explant are numerous. Growth regulator requirements for callus induction and plant regeneration are as varied as the explants. For most systems, the identity of cells that are triggered into embryogenesis and/or

organogenesis remain elusive. Even lesser is the information concerning the biochemical and genetic events initiating and regulating these processes. Hormones are intricately associated with plant growth and development, yet their precise role in embryogenesis and organogenesis from cultured tissue is poorly understood. Thus, there are no general strategies that can be applied to any cereal with a certainty of establishing morphogenetically competent cell cultures.

The studies presented in this thesis are an approach towards such developments in the field of plant biotechnology in India.

CHAPTER II : MATERIALS AND METHODS

2.1 PLASTICWARE

Laxbro (India) brand of plasticware were used. Sterile, disposable petri dishes (55mm and 85mm diameter) for culture experiments and autoclavable Eppendorf tubes and microtips were used during the protein extraction procedures.

2.2 GLASSWARE

Test tubes (25 X 150 mm), petridishes (55 mm and 85 mm diameter), conical flasks (100ml and 250 ml capacity) and pipettes (1,2,5 and 10 ml capacity) of Corning/Borosil (India) brand were used in most of the experiments.

Glassware was cleaned by an initial boiling in a saturated solution of sodium bicarbonate for 1h and subsequent washing in tap water. It was then immersed in 30% nitric acid solution for 30 minutes followed by a thorough wash with tap water. This washed glassware was then rinsed with distilled water and allowed to dry.

Test tubes and flasks were plugged with absorbant cotton and petri plates and pipettes wrapped in brown paper for sterilisation by autoclaving. All dissection instruments and filter paper pads required for preparation of explants were autoclaved in plastic bags. Autoclaving was carried out at 121^{o}C for 1h.

2.3 CHEMICALS

Inorganic salts used were of Analar grade (BDH, India). Vitamins, hormones and other organic additives were obtained from Sigma (U.S.A), Difco Laboratories (India) or Loba Chemicals (India). Sucrose and agar used were from Glindia (India).

Coconut milk was collected from tender coconuts and stored at -20^{o}C after autoclaving and filtering through Whatman filter paper No. 1.

2.4 PREPARATION OF MEDIA

Glass distilled water was used to prepare culture media. The pH of the medium was adjusted after dissolving all the constituents except the solidifying agent, using 1N NaOH or 1N HCl. 0.4% agar (Glindia, Bombay) was then added, and the medium heated to melt and then dispersed into test tubes or flasks. All culture media were autoclaved at 121^{o}C for 20 mins. When required, autoclaved media were poured into sterile petri dishes before gelling. Heat labile additives were filter sterilised through Millpore membranes (0.22μm) and added to the autoclaved medium before gelling. The concentrations of the macroelement and microelement salts and organic constituents of the various basal media used during the course of experimentation are listed in Tables nos. 3a, 3b and 3c respectively.

2.5 PREPARATION OF EXPLANTS AND CULTURE CONDITIONS

All explants were first washed with tap water, then with a commercial detergent solution, followed by 70% alcohol for 30 seconds and 0.1% mercuric chloride for 10 - 15 minutes. This was followed by final washes (3 - 4 times) with sterile distilled water.

Inoculations were caried out in a laminar air flow cabinet. All dissections required for preparation of the explant were done on sterile filter paper or petri dishes. Instruments used for dissection were flamed intermittantly after dipping in rectified spirit. Petri dishes were sealed with plastic film (Klin wrap, Flexo Film Wraps, Bombay) before incubation. The photoperiods used during culturing have been specified in the description of each culture.

TABLE 3a : COMPOSITION OF MACROELEMENT SALTS IN VARIOUS BASAL MEDIA (mg/l)

SALT	MS (1962)	WHITE (1954)	B_5 (1968)	V-47 (1974)	SH (1972)
KNO3	1900	80	3000	1480	2500
NH4NO3	1650	-	-	1444	-
Ca(NO3)2.4H2O	-	300	-	-	-
CaCl2.2H2O	440	-	150	735	200
MgSO4.7H2O	370	720	500	984	400
KH2PO4	170	-	-	68	-
KCl	-	10	-	-	-
NaH2PO4.H2O	-	16.5	150	-	-
Na2SO4	-	200	-	-	-
(NH4)2SO4	-	-	134	-	-
NH4H2PO4	-	-	-	-	300

TABLE 3b : COMPOSITION OF MICROELEMENT SALTS IN VARIOUS BASAL MEDIA (mg/l)

SALT	MS (1962)	WHITE (1954)	B_5 (1968)	V-47 (1974)	SH (1972)
MnSO4.4H2O	22.3	7.0	1000	500	10.0
ZnSO4.7H2O	8.6	3.0	200	150	1.0
H3BO3	6.2	1.5	300	200	5.0
KCl	0.83	0.75	75	25	1.0
CuSO4.5H2O	0.025	-	2.5	1.5	0.2
Na2MoO4.2H2O	0.25	0.25	25	10	0.1
CuCl2	-	0.27	-	-	-
CoCl2.6H2O	0.025	-	2.5	1.5	0.1
FeSO4.7H2O	27.8	-	27.8	27.8	15.0
Na2EDTA.2H2O	37.3	-	37.3	37.3	20.0
Fe Citrate	-	5.0	-	-	-

TABLE 3c : COMPOSITION OF ORGANIC SUPPLEMENTS IN VARIOUS BASAL MEDIA (mg/l)

SUPPLEMENT	MS (1962)	WHITE (1954)	B_5 (1968)	V-47 (1974)	SH (1972)
VITAMINS					
Thiamine HCl	0.4	-	10.0	4.0	5.0
Nicotinic acid	0.5	-	1.0	4.0	5.0
Pyridoxine HCl	0.5	-	1.0	0.7	0.5
Inositol	100.0	-	100.0	100.0	100.0
Glycine	2.0	3.0	-	1.4	-
Biotin	-	-	0.1	-	-
Folic acid	-	-	0.1	-	-
CARBOHYDRATES					
Sucrose	30,000	30,000	30,000	17,000	30,000
Glucose	-	-	-	9,900	

2.6 SCREENING OF GENOTYPES

Studies were initiated using thiry three genotypes of wheat obtained from the Agricultural Research Centre, Niphad, Maharashtra. These were selected on the basis of their special characteristics (Table 4). Three genotypes viz. N -59, MACS - 1967 and HD - 2278 were Triticum durum (2n = 4x = 28), while the rest of them were Triticum aestivum (2n = 4x = 42).

2.7 HISTOLOGICAL PROCEDURES

Histological studies were carried out according to standard procedures (Johansen 1940). The genotype used in these histological studies was Raj - 1777. Tissues were fixed in FAA solution (Formaldehyde : Acetic acid : Ethanol - 90 : 5 : 5) for 24h. These were then passed through the alcohol - xylene dehydration series and embedded in parrafin wax (m.p.60^{o}C). Serial sections 8 - 10 μ thick were cut with a rotary microtome, floated in water and applied to slides. Staining of the sections was done with haematoxylin and safranin after passing through the xylene - alcohol series.

2.8 CYTOLOGICAL STUDIES

Root tips from at least five regenerated plantlets of each of the five selected genotypes were analysed cytologically. Cytology was done according to the procedures described by Sharma and Sharma (1980). Root tips were initially subjected to a cold pretreatment (4^{o}C) for 24h. These were then fixed in an acetic - alcohol solution (Acetic acid : Ethanol - 1 : 3) for 24h, followed by 70% alcohol. For cytological observations, the root tips were hydrolysed with 1N HCl for 8 minutes and the acetocarmine

TABLE 4 : WHEAT GENOTYPES SCREENED FOR THEIR EMBRYOGENIC POTENTIAL

No.	CHARACTERISTICS	GENOTYPES
1.	Rust - resistance	VL-614, CPAN-1994, CPAN-2005, Kite
2.	Cultivation in rain-fed areas	Hy-65, MACS-1967
3.	Cultivation in irrigated areas	HD-2278, HD-2189, HD-4502
4.	Early maturity	NI-9272, NI-8289, Sonalika
5.	Late maturity	NI-8611, NI-8629, NI-8729
6.	Tall varieties	NI-345, NI-747-19, NI-917
7.	Dwarf varieties	NI-977
8.	High tillering	NI-5439, NI-5643
9.	Thermoinsensitive	Raj-1777
10.	Released earlier	CC-464, N-59, Kalyan Sona
11.	Good chapati (bread quality)	NI-4
12.	Promising for Indian soils	NI-9065, NI-9075, NI-8796, MP-845, MP-847, MACS-2067,NI-8188

(1%) squash technik was followed for determining chromosome numbers. A minimum of 20 metaphase plates were counted for determining the cytological status of each plant.

2.9 STATISTICAL PROCEDURES

Statistical methods were used for comparison of treatment means during optimising of parameters for standardising the embryogenic response and regeneration from immature embryos. Initially, an analysis of variance (ANOVA) was carried out, followed by a ranking of means using the student's "t" test. The formulae used in these procedures were as described by Snedecor and Cochran (1967).

2.10 CALCULATION OF Rm VALUES

During the studies on isozyme profiles during the course of somatic embryogenesis in wheat, the different isoforms were identified on the basis of their differential migration in polyacrylamide gels. This differential migration was identified as Rm values (Rate of migration) of each band, which is defined by the following ratio:

$$Rm = \frac{\text{Distance traversed by the band}}{\text{Distance traversed by the dye front}}$$

CHAPTER III : ESTABLISHMENT OF AN EMBRYOGENIC SYSTEM FROM IMMATURE EMBRYOS

3.1 INTRODUCTION :

While considering the conditions under which differentiated cells are able to express their potential in the production of somatic embryos, it must be realised that this ability is not confined specially to the artificial conditions of *in vitro* culture. Adventive or accessory embryogenesis is known to occur in fully or partially differentiated plants *in vivo*, following interruption of the whole plant integrity. In fact, these manifestations of totipotency are of such importance in nature, that they constitute major mechanisms of reproduction in some plants (Raghavan 1976).

3.1.1 Adventive embryogenesis - as it occurs in nature

Adventive embryony is known to occur in the Buxaceae, Euphorbiaceae, Cactaceae, Myrtaceae, Orchidaceae and Crassulaceae. In the bog orchid, *Malaxis paludosa*, the mature leaf produces numerous flask - shaped embryoids partially enclosed within a jacket layer of cells. In *Bryophyllum calcycinum*, meristematic groups of cells in the leaf margins form a pair of leaves, a shoot apex and two roots. Again, in *Kalanchoe daigremontiana*, the leaves produce tiny plantlets while still attached to the parent plant. In *Brynesia weinbergii*, the meristems in the leaf remain dormant until provoked. In all these instances, severing of the leaf from the plant results in the production of daughter plantlets.

In angiosperms, the most common source for spontaneous origin of embryo - like structures are the synergids, which become egg - like and develop into embryos with or without fertilisation. Occasionally, embryoids may also originate from the anti - podals or endosperm nuclei through

irregularities in the fertilisation process. The cells of the integument and nucellus of plants like *Citrus*, *Mangifera* and *Eugenia* form accessory embryos routinely as a normal part of their developmental cycle. The conversion of the diploid cells of the ovular tissue into a diploid embryo involves active divisions of cells which gradually push their way into the embryo sac cavity where they compete with each other and complete their development in a chaotic environment. In this case, a sharp demarcation of adventive embryos from zygotic embryos cannot be drawn except by their lateral position and lack of well - defined suspensor. This phenomenon has also been demonstrated in two Gramineae species viz. *Pennisetum mezianum* and *Cenchrus ciliaris*, where the nucellar embryos develop with the total absence of embryo sacs (Shantamma and Narayan 1977). In the nucellus, the stimulus to divide and form embryoids is believed to be furnished by the surrounding degenerative cells, much in the way a wound hormone acts (Haberlandt 1922) or due to discharge of substances from the pollen tubes (Raghavan 1976).

3.1.2 *In Vitro* Somatic Embryogenesis

The extension of nature to the laboratory, as is manifested by successful regeneration of plants from a wide variety of organs, tissues and cells from a wide spectrum of plant species *via* somatic embryogenesis, has caused a considerable upsurge of interest and activity. It is speculated that the genes involved in somatic embryogenesis are, possibly, a large subset of the genes functioning during the process of zygotic embryogenesis (Christianson 1985). This is possibly the only reason that selection over the past few million years has retained the information for

producing embryoids *in vitro* even in plants that do not produce accessory embryos in nature. Moreover, light and electron microscopic examinations of somatic embryogenesis have revealed morphological, histological and developmental similarities between zygotic and somatic embryony. Thus, somatic embryogenesis possibly shares the same developmental programme as zygotic embryony.

3.1.3 Nature of embryogenic cells :

Currently, somatic embryogenesis is best known as a pathway for induced regeneration from haploid and diploid somatic cells through characteristic embryological stages without the fusion of gametes (Williams and Maheshwaran 1986). Further, somatic embryos may be formed either directly from cells of organised structures like stem segments, leaves, zygotic embryos, hypocotyl regions, cotyledons etc. or indirectly from callus derived from organised structures, cell suspensions or protoplasts.

In all the systems, embryogenic cells *viz.* cells which ultimately form the embryoid show a number of common features. These are generally small, round or isodiametric, microvacuolated with dense cytoplasmic contents, having large nuclei with prominent nucleoli and are filled with starch grains (Carman 1990). Histochemical studies have suggested intense RNA synthesis and metabolic activity in these cells. Ultrastructurally, embryogenic cells have numerous free ribosomes, mitochondria and plastids with starch grains. The cytoplasm also contains small vacuoles and abundant endoplasmic reticulum (Thorpe 1988). In some species, lipid and multivesicular bodies are also present (Street and Withers 1974).

3.1.4 What influences somatic embryogenesis *in vitro* !

Somatic embryogenesis *in vitro* has been shown to be influenced by four major factors *viz.* the explant, the genotype of the donor plant, the culture medium and the culture environment (Thorpe 1988). The developmental stage and the physiological condition of the explant has been shown to be critical in establishing embryogenic cultures. It has been suggested that, most explants show a "window" of competence, i.e. a short period during development when embryogenic competence is maximally expressed (Roberts *et al.*1989). In general, although somatic embryogenesis has been reported from a wide variety of tissues, young and meristematic tissues have proved to be most amenable.

3.1.4.1 Genotype dependency

It is also clear from the number of documented reports that the genotype of the cultured tissue significantly affects the rate of cell proliferation as well as the embryogenic response (Hodges *et al.* 1986, Komatsuda and Ohyama 1988, Trolinder and Xhixian 1989, Lu *et al.* 1991). Genetic control of regenerative capacity, which is thought to be polygenic, has been reported in all the major cereals. Genes that control the synthesis of plant growth regulators seem to be under developmental/physiological control (Vasil 1988).

3.1.4.2 Nutritional dependency

The nutritional requirements of plant tissues *in vitro* are essentially similar to those of intact plants growing in nature. However, the most critical chemical factors involved in the induction of somatic embryogenesis include the source of nitrogen and the hormone components in the medium (Vajrabhaya 1988). Embryoid formation is promoted when

reduced nitrogen in the form of NH_4Cl or KNO_3 is present in the medium (Wetherell and Dougall 1976), Besides NH_4^+ and NO_3^-, casein hydrolysate, alanine and glutamine have also proved useful (Vajrabhaya 1988). Cations like K^+, $Fe^{+3}$and Na^+ play a cardinal role in somatic embryogenesis (Rangaswamy 1986). The source of carbohydrate is usually sucrose, however, galactose, lactose, raffinose, melibiose and maltose may also promote embryoid formation (Kochba *et al.* 1978, Sorvari and Scheider 1987, Orshinsky *et al.* 1990).

In addition to carbohydrates, inorganic salts and vitamins, tissue cultures require an exogenous source of hormones - the requirement differs with the species and the explant. In general, it is realised that primary culture *viz.* committment of the system towards embryogenesis requires an auxin. NAA and 2,4-D respectively have been used in 27% and 57% of successful cases (Evans *et al.* 1981). During secondary culture, a lower level of auxin induces the development of embryoids - at this stage, other phytohormones like cytokinins and abscisic acid may also be used.

3.1.4.3 Physical requirements

Cultural conditions such as temperature, photoperiod, light intensity, culture vessel, position of the tissue on the medium, the physical state of the medium, shaking speed, osmotic stress etc. have all been reported to affect somatic embryogenesis in certain tissues. Heat or cold treatments at specific stages have also improved embryogenesis and germination of somatic embryos.

Thus, as is seen in all cases of organised development *in vitro*, there is an interplay between the explant, the culture medium and the culture conditions. To obtain

optimum responses in a particular system, these factors must be resolved. In the following chapter, the establishment of an embryogenic system in wheat, using immature embryo explants and the effect of various factors on this system are described.

3.2 RESULTS AND DISCUSSION

3.2.1 Screening of genotypes

Using immature embryos as the explants, thirty three genotypes were screened for their embryogenic potential. These genotypes have been listed in Chapter II (Table 4). Dehusked, immature caryopses (12 - 14 days after anthesis) were surface sterilised as described in Chapter II. Immature embryos (1.5 - 2.5 mm in length) were excised aseptically under a stereomicroscope and cultured with the embryo axes in contact with the medium, in petri dishes (85 mm diameter) containing 10 ml solid MS medium supplemented with 2 mg/l 2,4-D (this medium was selected after a literature survey on cereals). Between 20 - 25 embryos were placed in each petri dish; a total of 100 embryos per genotype were inoculated. Cultures were maintained at 27^{o}C with a 16h photoperiod. Illumination was by cool, white fluorescent light of intensity 11.7 $\mu E\ m^{-2}s^{-1}$.

After 12 - 15 days, cultures were scored for somatic embryogenesis. Embryogenic (E) calli, when observed under the microscope, were identified by the presence of distinct bipolar structures on their surface. For regeneration, calli were transferred to basal MS medium supplemented with 1 mg/l kinetin and 3% sucrose. The percentage of regenerating calli was determined after 10 - 12 days on this medium. For shoot elongation, the regenerating embryoids were transferred onto filter paper supports in a liquid medium comprising of half - strength MS salts, full - strength MS vitamins and 2% sucrose.

3.2.1.1 Nature Of Somatic Embryogenesis

This was studied at two levels *viz.* under the light microscope and in histological sections.

(a) Light microscope studies :

Callus developed from the scutellum as well as the embryo axis, 5 - 7 days after incubation on the callus induction medium. However, when the explants were inoculated with the embryo axis facing downwards on the medium, only the scutellar proliferations were capable of forming embryogenic callus. The embryogenic callus was characterised by its organised and compact nature, nodular appearance and white to yellowish color (Fig.1). On the other hand, non - embryogenic callus was translucent, watery or crystalline in nature, friable and apparently unorganised (Fig.2). This type of callus usually remained non - morphogenic, but occasionally, gave rise to roots.

The nodular appearance of the embryogenic callus was due to the presence of distinct bipolar structures on its surface which resemble zygotic embryos. These structures are referred to as somatic embryos. Within 7 - 10 days after transfer to the regeneration medium, each somatic embryo gave rise to a green, leafy structure (considered as being equivalent to the scutellum), from the base of which arise multiple shoot primordia (Fig.3). Thus, the pathway by which the somatic embryos are formed can be described as indirect, since the explant initially undergoes callusing and embryoids develop from the callus cells.

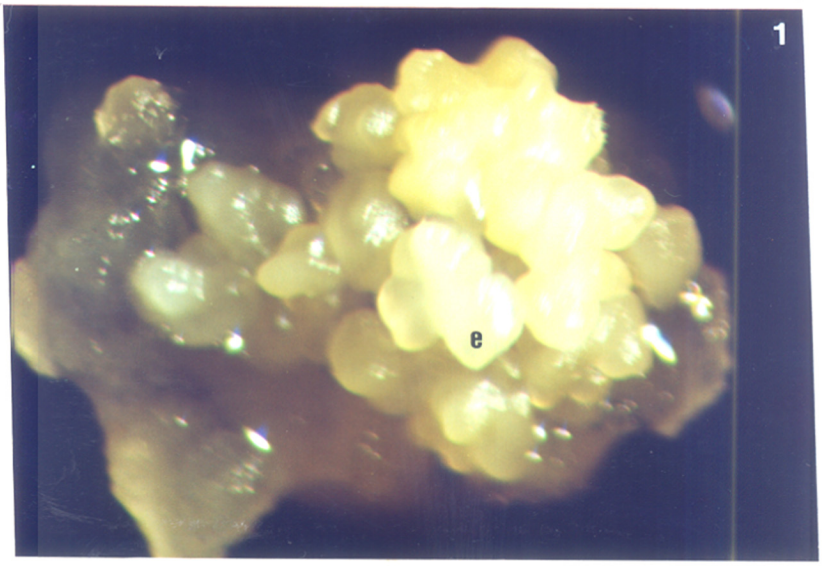
(b) Histological studies :

To trace the pattern of somatic embryogenesis histologically, tissues were harvested and fixed at specific stages during the process. These stages were :

(i) Immature embryos - collected 12 - 14 days after anthesis.

Fig. 1 : Immature embryo derived embryogenic callus of genotype Raj-1777 (e-embryoid).

Fig. 2 : Friable, non-embryogenic callus of genotype Raj-1777.


1
e

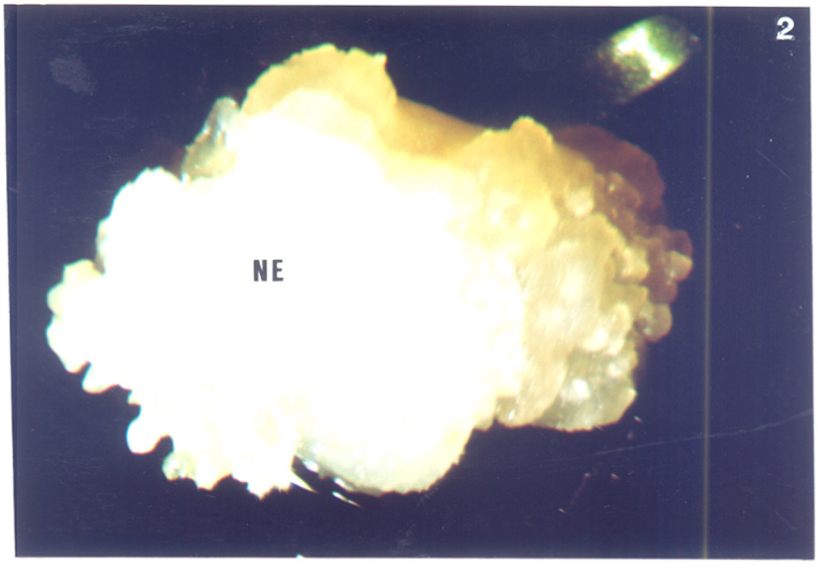
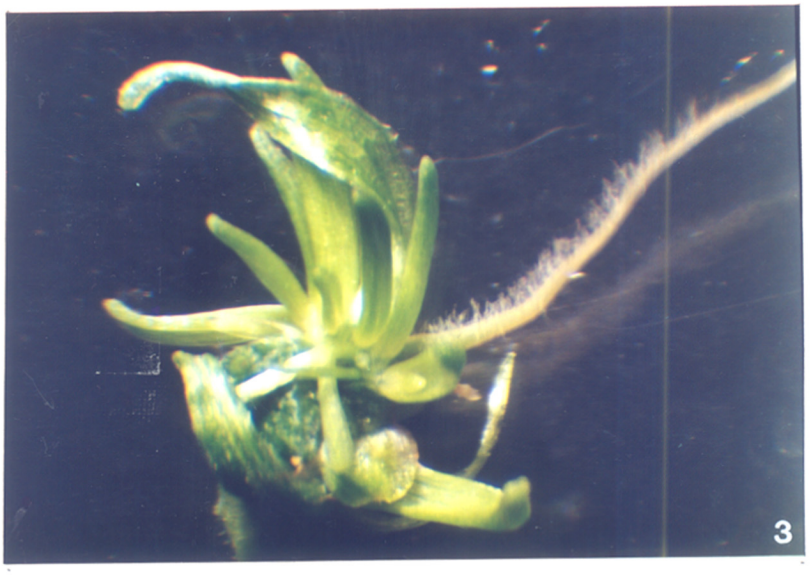

2
NE

Fig. 3 : Regenerating somatic embryo of genotype Raj-1777.


3

(ii) Induction phase - collected 5 - 7 days after culture initiation.

(iii) Embryogenic (E) calli - collected two weeks after culture initiation.

(iv) Non - embryogenic (NE) calli - collected at the same period as the E calli.

These tissues were processed for histological studies as described in Chapter II.

The initial immature embryo explant had a scutellum, coleoptile, shoot and root primordia at a very early stage of development (Fig. 4). After 5 - 7 days of incubation on 2,4-D containing medium, active cell divisions could be seen in the scutellar and shoot - root regions. In the scutellum, maximum meristematic activity occurred in the epidermal and sub - epidermal cells and resulted in small, compactly arranged cells with a dense cytoplasm and enlarged nucleus. Subsequent divisions gave rise to undulations and callussing on the scutellar surface which led to the formation of protuberances (Fig. 5).

Within another week of incubation, the callus assumed a nodular appearance and the scutellar protuberances developed into distinct bipolar structures (Fig. 6). Further development of the bipolar structures ensued after transfer to the regeneration medium (Fig. 7). The activity in these bipolar structures finally led to the formation of well - organised embryonic axes with a closed vascular system (Fig. 8). On the other hand, the non - embryogenic (NE) callus exhibited large, elongated and highly vacuolated cells. This NE callus did not develop any bipolar structures either after prolonged culture on the 2,4-D

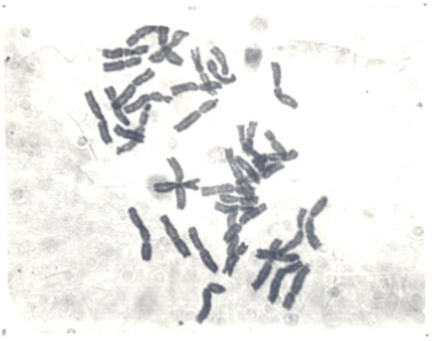



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Fig. 10: Chromosome counts in root tips of regenerated plants of genotype Raj-1777 (2n = 6x = 42). Magnification = 100X

Fig. 11: Wheat plants (genotype : Raj-1777) regenerated via somatic embryogenesis.

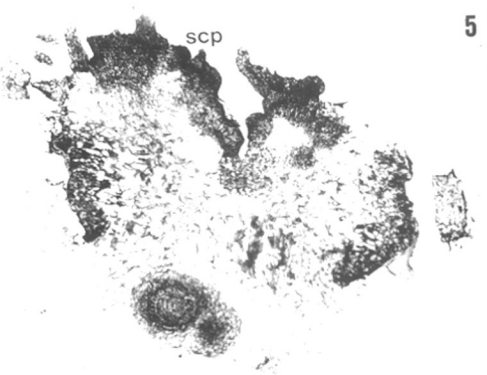
Fig. 4 : Section through immature embryo at time of culture (sc-scutellum, cp-coleoptile, s-shoot pole, r-root pole, cr-coleorhiza). Magnification = 50X

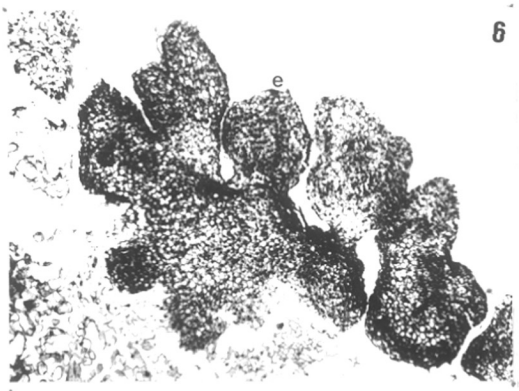
Fig. 5 : Formation of protuberances (scp) from the scutellum. Magnification = 25X

Fig. 6 : Formation of bipolar structures (e-embryoid). Magnification = 60X

Fig. 7 : Somatic embryos 4 days after transfer to regeneration medium. Magnification = 75X


4
cp
s
sc
r
cr


scp
5


6
e


7

Fig. 8 : Bipolar somatic embryo with a well organised embryonic axis. (cp-coleoptile, s-shoot pole, r-root pole). Magnification = 50X.

8

containing medium or on subsequent transfer to the regeneration medium.

Some of the somatic embryos formed differed from the zygotic ones in that they lacked an epiblast and a coleorhiza, which form parts of a typical zygotic embryo. In these somatic embryos, the suspensor may or may not be formed during development. A split coleoptile was also often observed in such somatic embryos, which is not seen in zygotic embryos. Due to these differences, such somatic embryos are described in literature as atypical (Ozias - Akins and Vasil 1982). On the other hand, typical somatic embryos showed the presence of a suspensor at the globular stage and during further differentiation, form an epiblast and coleorhiza. In the present study, both - typical as well as atypical somatic embryos were formed, with the latter being more frequently formed than the former.

Considerable controversy exists regarding the origin of somatic embryos. The single - cell origin has been demonstrated in pearl millet (Vasil 1983), orchard grass (Trigiano *et al.* 1989) and wheat (Magnusson and Bornmann 1985). Contrarily, the formation of somatic embryos has been traced to groups of meristematic cells, termed as pro - embryogenic cells eg. in maize (Springer *et al.*1979, Vasil *et al.* 1985), sorghum (Wernicke *et al.* 1982) and wheat (He *et al.* 1990). In the present study, groups of actively dividing cells in the epidermal and sub - epidermal layers of the scutellum seem to contribute towards the formation of somatic embryos.

3.2.1.2 Genotype dependency of somatic embryogenesis and precocious germination

The present study demonstrates that in wheat, the frequencies of somatic embryo formation and plantlet regeneration is under genotypic influence (Table 5). Immature embryos of all the genotypes responded to *in vitro* culture by forming callus in frequencies ranging from 2% - 100%. However, only 22 genotypes elicited an embryogenic response (ranging from 9.3% - 83.0%), while the rest formed friable, non - embryogenic callus (Table 5). Of the 22 genotypes, five (Raj - 1777, CPAN - 2005, VL - 614, NI - 9272 and NI - 5439) exhibited a high frequency of somatic embryogenesis (70 - 83%), six others (HD - 2278, NI - 8188, NI - 8629, NI -917, MACS - 1967 and NI - 345) exhibited a medium frequency of embryogenesis (50 - 69.9%), while the remaining genotypes were poorly embryogenic (9.3 - 49.9%). Plantlet regeneration was obtained from somatic embryos of all the embryogenic genotypes in frequencies ranging from 16% (MP - 845) to 100% (NI - 345, NI - 5439, NI - 8629, CPAN - 2005, Raj - 1777 and HD - 2278).

Thus, the present results seem to be in agreement with earlier observations on the genotype - dependent variations in embryogenic callus formation and plantlet regeneration in cereals. The superiority of japonica varieties of rice for somatic emryogenesis and plantlet regeneration over several indica varieties and japonica - indica hybrids has been documented (Abe and Futsuhara 1984, 1986). The effect of genotype on the tissue culture response has also been emphasised in recent years in wheat (Sears and Deckard 1982, Lazar *et al.* 1983, Galiba *et al.* 1986, Mathias and Simpson 1986, Carman *et al.* 1987a, Higgins and Mathias 1987, He *et*

TABLE 5 : GENOTYPIC EFFECT ON THE FREQUENCIES OF SOMATIC EMBRYOGENESIS* , PRECOCIOUS GERMINATION AND PLANTLET REGENERATION FROM CULTURED IMMATURE EMBRYOS

No	GENOTYPE	% PRECOCIOUS GERMINATION OF ZYGOTIC EMBRYOS	% OF RESPONDING EMBRYOS	% SOMATIC EMBRYOGENESIS	% PRECOCIOUS GERMINATION OF SOMATIC EMBRYOS	% REGENERATION FROM SOMATIC EMBRYOS
1	NI-4	-	100	38.8	48.4	44.8
2	NI-345	87.4	100	51.0	42.3	100
3	NI-747-19	-	50	-	-	-
4	NI-917	-	95	57.0	-	17
5	NI-977	1.5	85	15.7	9.5	43.8
6	NI-5439	-	100	70	69.6	100
7	NI-5643	-	100	29	51.7	85.7
8	NI-8188	61.6	98	63.2	52.7	88.3
9	NI-8289	-	59	-	-	-
10	NI-8611	-	38	-	-	-
11	NI-8629	77.8	100	63	56.9	100
12	NI-8729	-	2	-	-	-
13	NI-8796	10.3	100	20.5	-	40
14	NI-9065	-	56.6	-	-	-
15	NI-9075	-	100	17	-	43.8
16	NI-9272	1.0	100	71	7	73.8
17	CPAN-1994	-	2	-	-	-
18	CPAN-2005	14.3	100	71.4	84.3	100
19	MACS-1967	0.6	100	52.1	14.8	30.2
20	MACS-2067	-	45	9.3	-	45
21	MP-845	43.0	100	36	52.9	16
22	MP-847	31.0	100	-	-	-
23	VL-614	40.3	40.3	70.3	51.3	97.7
24	HY-65	-	38	-	-	-
25	CC-464	-	76	-	-	-
26	KITE	2.0	100	29	-	18.2
27	KALYAN SONA	1.0	100	41	4.9	17.1
28	SONALIKA	20.0	98	25	17	18.2
29	Raj-1777	43.2	100	83	43.2	100
30	N-59	-	63	25	-	20
31	HD-2189	-	71	-	-	-
32	HD-2278	87.8	98	55	67.7	100
33	HD-4502	-	30	-	-	-

* - Cultures were scored after 15-20 days of culture

Explant - Immature Embryos (Scutellar Tissue)

Medium - MS + 2,4-D (2mgl/l)

Temperature - 27 $\pm$ 2 $^{\circ}$C

Photperiod - 16h

Light Intensity - 11.7 uE m^{-2} s^{-1}

al. 1988, Rajyalakshmi *et al.* 1988), corn (Green and Philips 1975, Duncan *et al.* 1985), barley (Hanzel *et al.* 1985), sorghum (Ma *et al.* 1987), oats (Cummins *et al.* 1976, Rines and Mc Coy 1981) and triticale (Nakamura and Keller 1982). It has been suggested (Bayliss and Dunn 1977), that in different genotypes, differential regulation of the endogenous levels of growth substances may occur, which is manifested by a varied response of the genotypes to *in vitro* culture.

Genetic studies have revealed that the major genes controlling tissue culture responses are either dominant or partially dominant. Hodges *et al.*(1986) have suggested the involvement of one or two nuclear genes in somatic embryogenesis and plantlet regeneration, while the study of Tomes and Smith (1985) indicates significant negative maternal effects. In barley anther cultures, the capacity for callus induction and plantlet regeneration is under the control of nuclear genes and is inherited (Foroughi - Wehr *et al.* 1982). In wheat, the genetic control of callus growth and plantlet regeneration from immature embryos has been shown to involve the group 2 chromosomes in a highly responsive genotype (Kaleikau *et al.* 1989a, 1989b). Peng and Hodges (1989) have presented evidence in rice cultures that plant regeneration is under the control of nuclear genes. Wilman *et al.* (1989) suggest that at least one or a block of genes controls the expression of somatic embryogenesis from maize tissue cultures. On the other hand, Close and Gallagher - Ludeman (1989) presented evidence that the induction of regenerable callus in maize is purely a physiological phenomenon dependent on external

growth regulators and independent of the genetic background of the explant.

One of the known targets of auxin action is the inhibition of germination of embryos. Auxin depletion or its lowered levels have also been reported to be necessary for the maturation of somatic embryos (Ozias - Akins and Vasil 1983, Thorpe 1988). In the present study, precocious germination (i.e. premature shoot and root emergence in the presence of auxin) was noted in case of both - zygotic as well as somatic embryos. Based on the occurrence of precocious germination, the twenty two genotypes which exhibited somatic embryogenesis could be categorised into four classes -

[I] genotypes showing precocious germination exclusively of zygotic embryos - this class includes the genotypes NI - 8796 and Kite.

[II] genotypes showing precocious germination exclusively of somatic embryos - this class includes the genotypes NI - 4, NI - 5439 and NI - 5643.

[III]genotypes not showing precocious germination of either somatic or zygotic embryos - this class includes the genotypes NI - 917, NI - 9075, MACS - 2067 and N - 59.

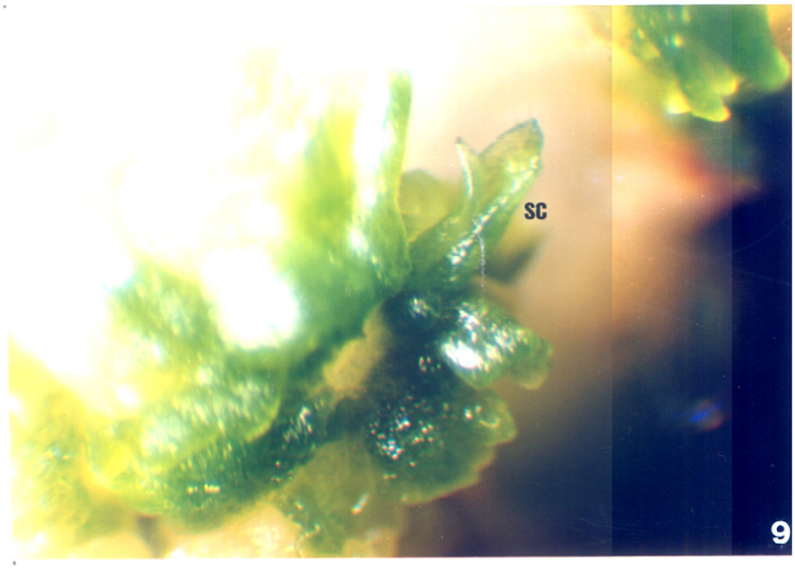
[IV] genotypes showing precocious germination of both zygotic as well as somatic embryos - this class encompasses the remaining thirteen genotypes.

The present study thus, suggests a genotypic influence on precocious germination of zygotic and somatic embryos. Further, a possible relation between precocious germination, somatic embryogenesis and plantlet regeneration is also suggested. Except for the genotypes NI-5439 (Class II, somatic embryogenesis = 70%) and NI-917 (Class III, somatic

embryogenesis = 57%), all the genotypes that fall under the first three classes are poorly embryogenic (somatic embryogenesis < 49.9%). Class IV however, includes a greater number of embryogenically superior genotypes. This class is the one that exhibits precocious germination of zygotic as well as of somatic embryos. Moreover, embryogenic calli of genotypes from Class IV produce shoots within 4 - 5 days after transfer to the regeneration medium, whereas embryos not undergoing precocious germination require longer (7 - 10 days) to develop shoots. The percentage of regenerating calli was also higher in genotypes showing precocious germination (Classes I, II and IV) than those of Class III genotypes. Thus, precocious germination seems to be favorable for the regeneration of plantlets from somatic embryos. However, once precocious germination is initiated, the somatic embryos cannot undergo secondary embryogenesis. This limits the long - term maintenance of embryogenic cultures.

A peculiar phenomenon was observed with the genotypes NI - 5439 and NI - 5643. Both these genotypes showed precocious germination of somatic embryos on the callus inducing medium which resulted in the formation of scutelli. At this stage, however, the shoot primordia did not develop further. On transfer to the regeneration medium, the scutelli multiplied extensively (Fig. 9) and on further depletion of hormones from the medium multiple shoot primordia were formed at the base of each scutellum, which developed into shoots. Thus, effectively, a larger number of shoots were regenerated per embryogenic callus. These two genotypes are characteristically high - tillering, and

Fig. 9 : Scutellar (sc) multiplication in genotype NI-5439


SC
9

it is possible that the phenomenon of scutellar multiplication may be related to this character.

Regenerated plants were kept at room temperatures for 3-5 days before transfer into a soil : sand (1:3) mixture. Potted plants were kept under highly humid conditions for the first 15-20 days to improve survival. The frequencies of plantlet survival were as follows :

NI - 5439 : 50%
NI - 9272 : 60%
VL - 614 : 62%
CPAN - 2005 : 72%
Raj - 1777 : 68%

The surviving plantlets showed the expected chromosome number of 2n = 6x = 42 ((Fig. 10) in all five genotypes which are normally hexaploid. Further, these plantlets were found to be normal in growth and development (Fig 11).

3.2.2 Screening of parameters

From the thirty three genotypes screened, five exhibiting high percentage of somatic embryogenesis were identified. These genotypes were used in all further experiments. Several physical and chemical parameters were tested with a view of improving on the embryogenic system with respect to two main criteria :.

3.2.2.1 Optimising the embryogenic response and regeneration.

3.2.2.2 Maintaining the embryogenic response.

3.2.2.1 Optimising the embryogenic response and regeneration :

The embryogenic response studied during genotype screening was done in the cultural conditions identified from previous literature. To further enhance the embryogenic response and regeneration in the five Indian genotypes identified to be highly embryogenic , several parameters were studied. In each of these parameters, 50 embryos of each genotype were inoculated on the medium specific for each parameter. Further, the number of somatic embryos that develop in the embryogenic callus two weeks later were scored and these embryo counts were employed in statistical analyses, in order to improve the understanding of various treatments on the embryogenic system by discussing differences among treatments.

(a) **Effect of different basal media** The basal salts and vitamins utilised were MS, V - 47, SH, B_5 and White. All the basal media were supplemented with 2 mg/l 2,4-D.

The variations in the embryogenic response was not seen to be significant between the different genotypes (Table 6a). However, significant differences were observed in the

embryogenic response within each individual genotype on different media. On application of the student's "t" test (Table 6b), the following ranking has been reached :

NI - 5439 : MS > V - 47 > SH > B_5 > WHITE

NI - 9272 : V - 47 > MS > SH > B_5 > WHITE

VL - 614 : MS > V - 47 > SH > B_5 > WHITE

CPAN - 2005 : MS > V - 47 > SH > B_5 > WHITE

Raj - 1777 : MS > SH > V - 47 > B_5 > WHITE

Thus, MS medium was seen to evoke the best embryogenic response in four genotypes, while in the genotype NI - 9272, the basal salts and vitamins of V - 47 medium gave the best results. On MS, V - 47 and LS media, the E calli formed were fast growing, compact and hard in nature. However, the use of SH medium is lowered because of an increased percentage of precocious germination of zygotic embryos. All the genotypes, on B_5 and White's medium gave rise to a slow growing callus which underwent subsequent necrosis.

The MS medium has been the most frequently reported medium for the induction of somatic embryogenesis from immature embryos of wheat (Ozias - Akins and Vasil 1982, He *et al.* 1986, Carman *et al.* 1987b, Rajyalakshmi *et al.* 1988). In the present study, cultures incubated on MS medium appeared healthier and morphologically more active throughout the incubation period , which was manifested by an increase in the number of embryoids during incubation. Thus, MS medium was ideal for eliciting somatic embryogenesis in a majority of genotypes tested.

(b) Effect of various auxins : The auxins under study included 2,4-D, 2,4,5-T, picloram, dicamba, IAA and NAA. These auxins were added at a concentration of 2 mg/l to the basal MS medium.

TABLE 6a : OVERALL ANOVA FOR MEDIA TREATMENTS

Media used - MS, SH, WHite, B5 & V-47
Auxin used - 2,4-D (2mg/l)
Genotypes - NI-5439, NI-9272, VL-614, CPAN-2005 & Raj-1777

SOURCE	d.f.	S.S.	M.S.S.	F VALUE
BETWEEN GENOTYPES	4	1.00	0.25	0.32^{N}
BETWEEN DIFFERENT MEDIA WITHIN GENOTYPES	16	5327.64	332.98	425.10^{+}
ERROR	11846	9278.98	0.78	
TOTAL	11866	14607.63	1.23	

N - Not Significant
+ - $p < 0.01$

TABLE 6b : SIGNIFICANCE OF MEDIA TREATMENTS

No	TREATMENT COMPARISON	NI-5439	NI-9272	VL-614	CPAN-2005	Raj-1777
1	MS Vs SH	4.84^{+}	0.14^{N}	1.65^{N}	10.94^{+}	9.22^{+}
2	MS Vs White	7.60^{+}	6.24^{+}	9.04^{+}	14.51^{+}	25.70^{+}
3	MS Vs B5	7.43^{+}	2.16^{N}	6.41^{+}	12.40^{+}	17.75^{+}
4	MS Vs V-47	0.04^{N}	2.44^{++}	0.51^{N}	1.78^{N}	9.26^{+}
5	SH Vs White	4.06^{+}	6.32^{+}	7.94^{+}	7.32^{+}	19.28^{+}
6	SH Vs B5	2.54^{+}	2.28^{++}	4.64^{+}	1.86^{+}	8.65^{+}
7	SH Vs V-47	4.90^{+}	2.29^{++}	1.10^{N}	9.05^{+}	0.02^{N}
8	35 Vs V-47	7.46^{+}	4.51^{+}	5.72^{+}	10.58^{+}	8.73^{+}
9	White Vs V-47	7.62^{+}	7.91^{+}	8.16^{+}	13.28^{+}	19.36^{+}

N - Not Significant
+ - $p < 0.01$
++ - $p < 0.05$

The embryogenic response was seen to be significantly different within the genotypes as well as between the different auxins used (Table 7a). The student's "t" test (Table 7b) has revealed the following ranking for the individual genotypes :

NI - 5439 : 2,4-D > Dicamba > Picloram > 2,4,5-T

NI - 9272 : Dicamba > Picloram > 2,4-D > 2,4,5-T

VL - 614 : Dicamba > Picloram > 2,4-D > 2,4,5-T

CPAN - 2005 : Dicamba > Picloram > 2,4-D > 2,4,5-T

Raj - 1777 : Picloram > Dicamba > 2,4-D > 2,4,5-T

2,4-D has been reported to be the most effective auxin for the *in vitro* culture of cereals (Bright and Jones 1985, Bhaskaran and Smith 1988, Vasil 1988 etc.). Dicamba, too, has been reported to be effective in eliciting embryogenesis (Duncan *et al.* 1985, Songstadt and Conger 1986), possibly because of its being more rapidly metabolised than 2,4-D in certain tissues (Papenfuss and Carman1987). Picloram and 2,4,5-T have also been used for callus induction and plantlet regeneration in cereals (Conger *et al.* 1982. Rengel and Jelaska 1986).

The present results suggest that the embryogenic response involves an interplay between auxin and genotypic effects under a given set of cultural conditions. The auxin 2,4-D has proved to be the best for the genotype NI - 5439, dicamba for the genotypes NI - 9272, VL - 614 and CPAN - 2005 and picloram for the genotype Raj - 1777. The auxins IAA and NAA failed to elicit somatic embryogenesis from immature embryos of wheat - the same has been reported in barley (Luhrs and Lorz 1987), while the auxin 2,4,5-T induced embryogenesis at a lower frequency than either 2,4-D, dicamba or picloram.

TABLE 7a : OVERALL ANOVA FOR AUXIN TREATMENTS

Medium - MS
Auxins - 2,4-D, Picloram, Dicamba & 2,4,5-T
Concentration of auxin - 2 mg/l
Genotypes - NI-5439, NI-9272, VL-614, CPAN-2005 & Raj-1777

SOURCE	d.f.	S.S.	M.S.S.	F VALUE
BETWEEN GENOTYPES	4	1830.76	457.69	49.94+
BETWEEN DIFFERENT MEDIA WITHIN GENOTYPES	12	2614.34	217.86	23.77+
ERROR	698	6397.42	9.17	
TOTAL	714	10842.53	15.19	

+ - $p < 0.01$

TABLE 7b : SIGNIFICANCE OF MEDIA TREATMENTS

No	TREATMENT COMPARISON	NI-5439	NI-9272	VL-614	CPAN-2005	Raj-1777
1	2,4-D Vs Picloram	2.57+	0.61N	1.58N	0.37+	3.69+
2	2,4-D Vs Dicamba	0.82N	1.16N	2.20N	1.50N	2.41++
3	2,4-D Vs 2,4,5-T	6.41+	1.42N	0.63N	0.38N	1.20N
4	Picloram Vs Dicamba	1.62N	0.53N	0.77N	1.11N	1.24N
5	Dicamba Vs 2,4,5-T	5.62+	2.51++	2.64++	1.79N	3.40+
6	Picloram Vs 2,4,5-T	2.74++	1.97N	1.99N	0.72N	4.58+

N - Not Significant
+ - $p < 0.01$
++ - $p < 0.05$

(c) Effect of organic additives : Effects of several additives to basal MS medium containing 2 mg/l 2,4-D were studied. These additives were :

(i) Casamino acids - 500 mg/l and 1000 mg/l,

(ii) Cefotaxime - 50 µg/l and 100 µg/l - Cefotaxime is commercially available as clafaron.

(iii) Coconut milk - 5 % and 10% .

(i) Casamino acids - Casein hydrolysate or casamino acids have been reported to promote embryo germination at low concentrations (Carman *et al.* 1987a), while at higher concentrations, may help prevent germination (Ozias - Akins and Vasil 1982). Luhrs and Lorz (1987) have reported that casein hydrolysate increased the induction of E callus in barley.

The variations in the embryogenic response was not significant between the different genotypes (Table 8). Differences were noted in the frequencies of embryogenesis at different casamino acid concentrations. However, on application of the student's "t" test, these differences were not seen to be of a significant magnitude. Moreover, there was no marked effect on embryo germination. Hence, casamino acids could be eliminated from the medium without any adverse effect on the frequency of somatic embryogenesis.

(ii) Effect of cefotaxime - As in the case of Casamino acid treatments, variations in the embryogenic response were not significant between the different genotypes for different concentrations of cefotaxime (Table 9), nor were the variations within each individual genotype of statistical significance. Mathias *et al.*(1987) have reported an enhanced effect of cefotaxime on callus growth

TABLE 8 : OVERALL ANOVA FOR CASAMINO ACID CONCENTRATION TREATMENTS

Medium - MS
Auxin - 2,4-D (2 mg/l)
Concentrations (CAA) - 500 mg/l & 1000 mg/l
Genotypes - NI-5439, NI-9272, VL-614, CPAN-2005 & Raj-1777

SOURCE	d.f.	S.S.	M.S.S.	F VALUE
BETWEEN GENOTYPES	4	1.00	0.25	0.01^{N}
BETWEEN DIFFERENT MEDIA WITHIN GENOTYPES	8	869.29	108.66	3.30^{+}
ERROR	522	17186.53	32.92	
TOTAL	534	18056.83	33.81	

N - Not Significant
+ - $p < 0.01$

On application of Student's "t" test, no significant results were obtained for Casamino acid treatments.

TABLE 9 : OVERALL ANOVA FOR CEFOTAXIME TREATMENTS

Medium - MS
Auxin - 2,4-D (2 mg/l)
Concentrations (Cefotaxime) - 50 mg/l & 100 mg/l
Genotypes - NI-5439, NI-9272, VL-614, CPAN-2005 & Raj-1777

SOURCE	d.f.	S.S.	M.S.S.	F VALUE
BETWEEN GENOTYPES	4	1.00	0.25	0.02^{N}
BETWEEN DIFFERENT MEDIA WITHIN GENOTYPES	8	762.57	95.32	6.84^{+}
ERROR	508	7075.48	13.93	
TOTAL	520	7839.06	15.08	

N - Not Significant
+ - $p < 0.01$

On application of Student's "t" test, no significant results were obtained for Cefotaxime treatments.

and regeneration in wheat, tobacco and barley. They attribute these beneficial effects of cefotaxime to some "direct physiological action" of the antibiotic on plant cells rather than an indirect effect resulting from the activity of the compound against bacterial infections. However, in the present study, the results obtained indicated that cefotaxime could be eliminated from the medium without affecting the embryogenic response.

(iii) Effect of coconut milk : CM is known to promote embryo germination which has been attributed to its cytokinin - like constituents (Moore 1979). Hence, cultures initiated on media containing CM rapidly produce plantlets (Maddock *et al.* 1983). However, in such cultures, very little embryogenic activity is evident on long - term storage (Carman *et al.* 1987a). In the present study, a similar effect was observed. However, in a system that is already capable of high conversion, this result does hold sufficient significance.

On the whole, there was very little variation in the responses between the genotypes (Table 10a). However, at different CM concentrations there was considerable variance in the genotype NI - 9272 (Table 10b), which showed enhanced embryogenesis at 5% and 10% concentrations. Since this effect is to a comparable level at both concentrations, 5% CM was further included in the induction medium to increase the frequency of somatic embryogenesis in this genotype.

(d) Effect of age of the embryo : This was studied as a function of the size of the embryo. Thus, the size ranges within which embryos were selected and cultured on basal MS medium with 2 mg/l 2,4-D were -

TABLE 10a : OVERALL ANOVA FOR COCONUT MILK TREATMENTS

Medium - MS
Auxin - 2,4-D (2 mg/l)
Concentrations (CM) - 500 mg/l & 1000 mg/l
Genotypes - NI-5439, NI-9272, VL-614, CPAN-2005 & Raj-1777

SOURCE	d.f.	S.S.	M.S.S.	F VALUE
BETWEEN GENOTYPES	4	1.00	0.25	0.01^{N}
BETWEEN DIFFERENT MEDIA WITHIN GENOTYPES	8	802.89	100.36	5.14^{+}
ERROR	575	11231.77	19.53	
TOTAL	587	12035.66	20.50	

+ - $p < 0.01$
N - Not Significant

TABLE 10b : SIGNIFICANCE OF COCONNUT MILK TREATMENTS

No	TREATMENT COMPARISON	NI-5439	NI-9272	VL-614	CPAN-2005	Raj-1777
1	0% Vs 5%	1.07^{N}	2.18^{++}	1.16^{N}	1.14^{N}	0.09^{N}
2	0% Vs 10%	0.93^{N}	2.44^{++}	0.51^{N}	0.54^{N}	0.52^{N}
3	5% Vs 10%	0.17^{N}	0.25^{N}	0.64^{N}	0.61^{N}	0.43^{N}

N - Not Significant
+ - $p < 0.01$
++ - $p < 0.05$

[A] 0.5 - 1.0 mm,

[B] 1.1 - 2.0 mm,

[C] 2.1 - 3.0 mm and

[D] 3.1 - 4.0 mm.

As the rate of embryo development is affected by the growth conditions and the genotype, morphological characters like size of the embryo can be considered a more reliable index of embryo development rather than the time after anthesis. Thus, immature embryos of wheat, harvested 12 - 14 days after anthesis could be classified into the four size ranges as described above. The embryogenic response did not vary significantly between the different genotypes (Table 11a). However, there were drastic differences in the responses from embryos of different sizes. The embryogenic response was poor from the first size range (0.5 - 1.0 mm) as most of the embryos underwent rapid necrosis and turned brown or remained white without callusing. The embryogenic response of embryos from the next two size ranges (1.1 - 2.0 mm) and (2.1 - 3.0 mm) did not differ much in the genotypes NI - 5439 and CPAN - 2005 (Table 11b); while in the remaining three genotypes the size range (1.1 - 2.0 mm) evoked the best response. Embryos belonging to the size range (3.1 - 4.0 mm) showed decreased embryogenic potential - this was possibly because of precocious germination of the zygotic embryos and the formation of friable, NE callus from the radicles.

(e) Effect of embryo orientation : The orientation of the embryo on the medium is known to determine the origin of the embryogenic callus. Thus, using MS basal medium supplemented with 2 mg/l 2,4-D, two types of calli were developed for comparison -

TABLE 11a : OVERALL ANOVA FOR AGE TREATMENTS

Medium - MS
Auxin - 2,4-D (2 mg/l)
Size Ranges -
A : 0.5mm - 1.0mm
B : 1.1mm - 2.0mm
C : 2.1mm - 3.0mm
D : 3.1mm - 4.0mm
Genotypes - NI-5439, NI-9272, VL-614, CPAN-2005 & Raj-1777

SOURCE	d.f.	S.S.	M.S.S.	F VALUE
BETWEEN GENOTYPES	4	1.01	0.25	0.02^{N}
BETWEEN DIFFERENT AGES WITHIN GENOTYPES	12	7955.96	663.00	48.75^{+}
ERROR	542	7371.46	13.60	
TOTAL	558	155328.43	27.47	

+ - $p < 0.01$
N - Not significant

TABLE 11b : SIGNIFICANCE OF AGE TREATMENTS

No	TREATMENT COMPARISON	NI-5439	NI-9272	VL-614	CPAN-2005	Raj-1777
1	A Vs B	5.56	5.56^{N}	10.68^{N}	29.77^{+}	42.69^{+}
2	A Vs C	4.82	20.72^{N}	22.52^{N}	21.50^{N}	45.12^{+}
3	A Vs D	13.1^{+}	24.54^{N}	10.66^{N}	20.38^{N}	33.05^{+}
4	B Vs C	0.62^{N}	4.65^{N}	3.27^{N}	0.11^{N}	17.61^{N}
5	B Vs D	5.78^{+}	6.55^{++}	0.64^{+}	2.59^{N}	5.13^{+}
6	C Vs D	4.35^{+}	8.69^{N}	6.09^{N}	4.52^{N}	4.58^{+}

N - Not Significant
+ - $p < 0.01$
++ - $p < 0.05$

(i) Scutellum - derived embryogenic callus : this develops when the embryos were inoculated with the scutellum facing upwards on the medium and the embryo axis facing downwards and in contact with the medium.

(ii) Epiblast - derived embryogenic callus : this develops when the embryos were inoculated with the scutellum facing downwards and the embryo axis (and thereby the epiblast) facing upwards and away from the medium. In general, in hexaploid wheat, scutellar callus is believed to be formed at a higher frequency than epiblast callus, while in tetraploid wheat, only scutellar callus is formed (He *et al.* 1988). Magnusson and Bornmann (1985) have suggested that more oxygen availability or low water potential may be the reason for the higher embryogenecity of the scutellum over the epiblast.

In the present study, variations in the embryogenic response were not significant within the genotypes (Table 12a). In the genotypes NI - 5439 and Raj - 1777, there was considerable variation in the response towards orientation treatments (Table 12b), whereas in the remaining three genotypes, the differences between the scutellar and epiblast embryogenesis were not statistically significant.

It is possible that the scutellum and epiblast are not in the same physiological state at the time of excision, because the *in vivo* development of the scutellum is known to occur much earlier than the epiblast (Percival 1971). Such development dependent factors may give rise to varied responses from meristematic cells at different locations on the same explant. Thus, the scutellum in the present, selected developmental stage of the immature embryo, gave rise to a more compact and larger callus than that derived

TABLE 12a : OVERALL ANOVA FOR ORIENTATION TREATMENTS

Medium - MS
Auxin - 2,4-D (2 mg/l)
Genotypes - NI-5439, NI-9272, VL-614, CPAN-2005 & Raj-1777
Orientation - Scutellar Vs. Epiblast

SOURCE	d.f.	S.S.	M.S.S.	F VALUE
BETWEEN GENOTYPES	4	1.00	0.25	0.02^{N}
BETWEEN DIFFERENT ORIENTATIONS WITHIN GENOTYPES	8	889.92	111.24	7.68^{+}
ERROR	343	4967.65	14.48	
TOTAL	355	5858.57	16.50	

+ - $p < 0.01$
N - Not Significant

TABLE 12b : SIGNIFICANCE OF ORIENTATION TREATMENTS

GENOTYPE	"t"
NI-5439	2.35^{++}
NI-9272	1.63^{N}
VL-614	1.69^{N}
CPAN-2005	0.64^{N}
Raj-1777	5.36^{+}

N - Not Significant
+ - $p < 0.01$
++ - $p < 0.05$

from the epiblast, and also produced a higher frequency of normal embryoids.

(f) Effect of cytokinins on regeneration : Twenty five embryogenic calli of each genotype, which did not exhibit precocious germination of somatic embryos were transferred to -

(i) basal MS medium without any hormones,

(ii) basal MS medium with 1 mg/l Ki,

(iii)basal MS medium with 1 mg/l BAP,

(iv) basal MS medium with 10 µg/l Z and

(v) basal MS medium with 100 µg/l 2ip.

The number of somatic embryos per E callus forming plantlets 10 - 12 days after transfer to the regeneration medium was determined and used for comparative analyses. Developing plantlets were allowed to elongate on liquid MS medium (as described earlier). These were then transferred to sterile soil mixtures in polythene bags for acclimatisation. Surviving plantlets were later potted.

The regenerative response was not seen to vary significantly within the genotypes (Table 13). Differences were noted in the frequencies of regeneration on the various media used. However, on application of the student's "t" test, these differences were not seen to be statistically significant. The plantlets regenerated on either kinetin or zeatin containing medium were more readily acclimatised than those regenerated on the other media. In the genotype NI - 5439, the scutelli of germinating somatic embryos multiplied extensively on the kinetin containing medium. Further, in this genotype, shoot elongation required a complete removal of hormones, hence regeneration here was a two step process. Use of the cytokinin BAP is not advisable because it led to a slight increase in the friable, non - embryogenic sectors

TABLE 13 : OVERALL ANOVA FOR CYTOKININ TREATMENTS

Medium - MS
Cytokinins - Kinetin, BAP. Zeatin & 2ip
Genotypes - NI-5439, NI-9272, VL-614, CPAN-2005 & Raj-1777

SOURCE	d.f.	S.S.	M.S.S.	F VALUE
BETWEEN GENOTYPES	4	1.01	0.25	0.03^{N}
BETWEEN DIFFERENT MEDIA WITHIN GENOTYPES	12	397.66	33.14	4.14^{+}
ERROR	183	1464.29	8.00	
TOTAL	199	1862.96	9.36	

+ - $p < 0.01$
N - Not Significant

On application of Student's "t" test, no significant results were obtained for cytokinin treatments.

within the E calli before shoot formation ensued. This could possibly result in a greater degree of cytological variation in the different sectors of the callus and thereby, in the resulting plantlets.

3.2.2.2 Maintenance of embryogenic cultures :

The maintenance of embryogenic cereal cultures has proved to be difficult because repeated subculture is known to be associated with decline in the embryogenecity as well as regenerative potential of the callus. Moreover, the occurrence of precocious germination, observed during genotype screening, although advantageous in view of ready regeneration of plants from cultures, has inherent drawbacks. The major problem posed by the phenomenon is in the maintenance of embryogenic lines. Such cultures cannot be maintained either on the induction medium or on one containing lowered levels of auxins, because of the tendency of the somatic embryos to germinate and form plantlets. Further, if these precociously germinating calli are not transferred to the regeneration medium, callusing sets in the plantlet, giving rise to friable callus and abnormal plantlets. Hence in view of maintaining embryogenic cultures, it is necessary to inhibit or reduce the frequency of precocious germination of the somatic embryos. For this, the following parameters were tested in two genotypes Raj - 1777 and CPAN - 2005:

(a) Effect of light : Incubation of cultures in total darkness - in contrast to the 16h photoperiod.

As seen in Fig. 12, incubation of cultures in the dark as compared to the 16h photoperiod did not influence the patterns of somatic embryogenesis and/or precocious germination considerably. Significant difference was

Fig. 12: SEsis - Somatic Embryogenesis
PG - Precocious Germination

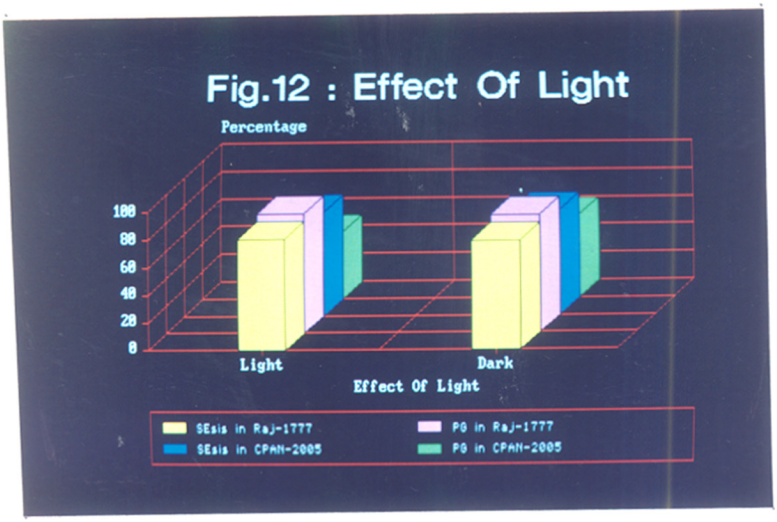


Fig.12 : Effect Of Light
Percentage
100
80
60
40
20
0
Light
Dark
Effect Of Light
SEsis in Raj-1777
SEsis in CPAN-2005
PB in Raj-1777
PB in CPAN-2005

observed only in the frequency of precocious germination of somatic embryos in the genotype CPAN - 2005, which increased by 12% on incubation in the dark. The other three observations viz. the frequencies of somatic embryogenesis in Raj-1777 and CPAN-2005 and precocious germination in Raj-1777 did not vary to a great extent under the different light conditions.

It is well documented that light is one of the most important factors that triggers off conversion of an embryo into a plantlet (Raghavan 1976). In the present study, it is indicated that precocious germination is not only an effect of physiological factors like the photoperiod, but probably involves several factors as well.

(b) Additives to the medium : The effect of certain additives on precocious germination was studied. For this purpose, induction of somatic embryogenesis was initiated on MS basal medium containing 2 mg/l 2,4-D and one week after induction, the cultures were transferred to fresh medium containing the additive and the same level of 2,4-D. The additives tested were two growth regulators - one a phytohormone (ABA) and the other an inhibitor ($AgNO_3$) of a phytohormone (Ethylene).

(i) Abscisic acid (ABA) - at concentrations of 50 and 100 µg/l.

(ii) Silver nitrate ($AgNO_3$) - at concentrations of 5, 10 and 20 mg/l. Cultures were further incubated in the dark because of the known photosensitivity of $AgNO_3$.

(i) Effect of ABA -

The inclusion of ABA in the medium had a marked effect on somatic embryogenesis as well as precocious germination of somatic embryos (Fig. 13a). In the genotype Raj-1777,

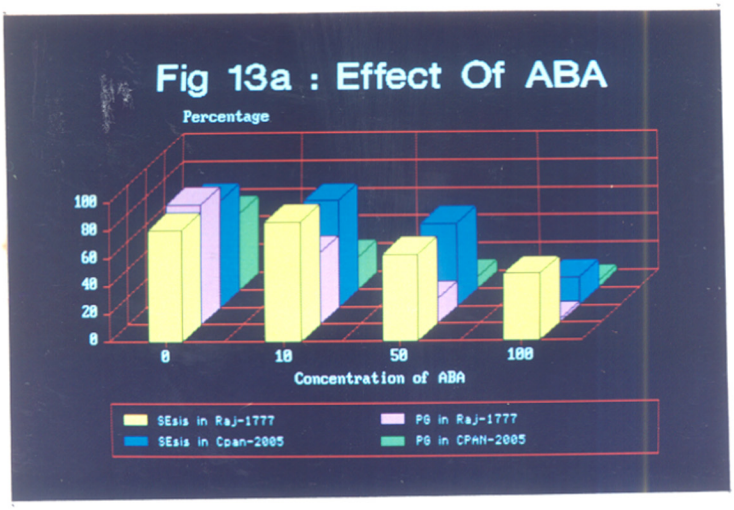


Fig 13a : Effect Of ABA
Percentage
100
80
60
40
20
0
0
10
50
100
Concentration of ABA
SEsis in Raj-1777
SEsis in Cpan-2005
PG in Raj-1777
PG in CPAN-2005

somatic embryogenesis was enhanced by the incorporation of ABA in the medium at low concentrations (10 µg/l). Further, at this concentration there was not much variation in the embryogenic response in CPAN-2005 as compared to the control. However, even at this low concentration, there was a distinct decrease in the precocious germination of somatic embryos of both the genotypes. On further increasing the concentrations of ABA, the incidence of precocious germination was reduced to as low as 2% and 4% (in CPAN - 2005 and Raj - 1777 respectively, at 100 µg/l ABA concentration). However, there was a corresponding decline in the embryogenic response , which is naturally, not desired.

ABA has been implicated in many stress effects on cereal plant metabolism (Walton 1987). It has also proved useful in normalising somatic embryony in caraway (Ammirato 1977), carrot (Ammirato 1983), pearl millet (Vasil and Vasil 1982) and soybean (Ranch *et al.*1985). Further, ABA has also been reported to increase embryogenesis and shoot regeneration in barley (Rengel 1986), maize (Close and Ludeman 1987) and wheat (Brown *et al.*1989, Qureshi *et al.* 1989). Yet another role of ABA suggested is in the prevention of precocious germination (Quatrano 1986).

The present results generally agree with these reports by retaining the embryogenic potential and decreasing the frequency of precocious germination. The optimum level of ABA to be used seems to be 10 µg/l since at this concentration, somatic embryogenesis is at a maximum possible level, with a corresponding decline in precocious germination.

(ii) **Effect of $AgNO_3$ -**

$AgNO_3$ is generally used in tissue culture as an inhibitor of ethylene action. $AgNO_3$ has been suggested to play a role in shoot regeneration (Purnhauser *et al.* 1987, Songstadt *et al.* 1988) as well as in embryogenic callus initiation (Vain *et al.*1989, Songstadt *et al.* 1989). In the present study, another role *viz.* maturation of somatic embryos by inhibition of precocious germination is suggested.

As seen in Fig.13b, the frequency of somatic embryogenesis was not adversely affected with increasing concentrations of $AgNO_3$. Only in the genotype CPAN-2005, there was a slight decline in somatic embryogenesis of upto 10% (at a concentration of 20 ug/l). Correspondingly, there was a steady increase in the frequencies of precocious germination in both the genotypes - about 70% and 40% in Raj-1777 and Cpan-2005 respectively. Hence, the use of $AgNO_3$ in these two genotypes proved to be beneficial in view of maintaining the embryogenic potential of cultures by promoting somatic embryo maturation and inhibiting precocious germination.

(c) Effect of osmoticum : The osmoticum was changed by using 1%, 2%, 3% and 4% sucrose in the medium.

High osmotic potential of the culture medium is known to prevent precocious germination of the zygotic embryos (Rijven 1952, Norstog and Klein 1972). Fig. 14 shows the response of one week old calli to varying concentrations of sucrose in the medium. In the absence of sucrose (not shown in the figure), the E calli became necrotic and turned brown. Use of 3% sucrose retained maximum embryogenic potential but did not check precocious germination.

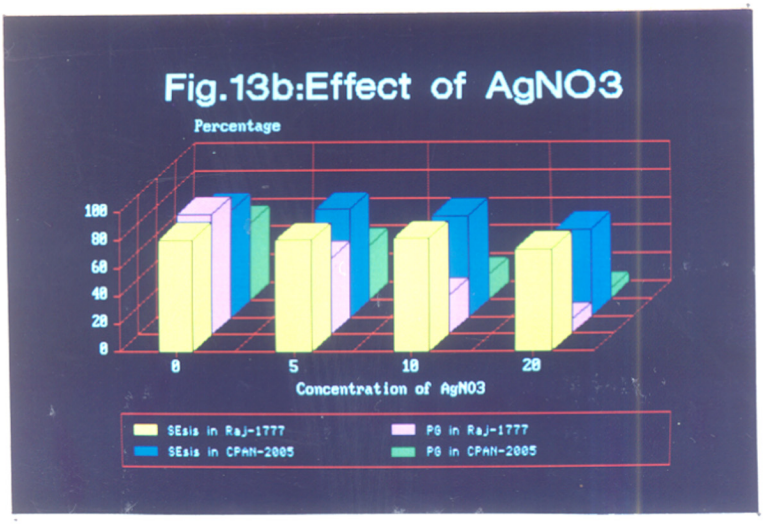


Fig.13b:Effect of AgNO3
Percentage
100
80
60
40
20
0
0
5
10
20
Concentration of AgNO3
SEsis in Raj-1777
SEsis in CPAN-2005
PG in Raj-1777
PG in CPAN-2005

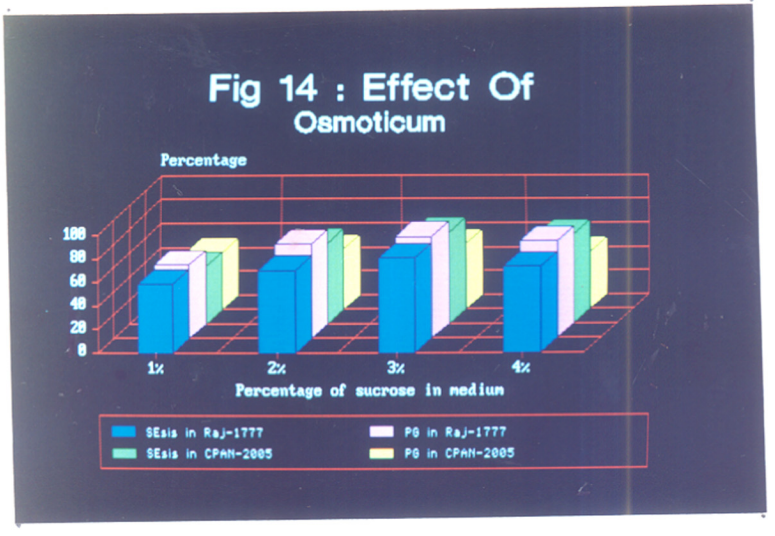


Fig 14 : Effect Of
Osmoticum
Percentage
100
80
60
40
20
0
1%
2%
3%
4%
Percentage of sucrose in medium
SEsis in Raj-1777
SEsis in CPAN-2005
PG in Raj-1777
PG in CPAN-2005

Increase or decrease in the sucrose concentration reduced the frequency of somatic embryogenesis without affecting precocious germination significantly. Thus, osmoregulation by varying the sucrose content of the maintenance medium is not an ideal solution to resolve the problem of precocious germination.

(d) Effect of increased concentrations of auxins : The effect of four auxins *viz.* 2,4-D, 2,4,5-T, picloram and dicamba on precocious germination was studied. The concentrations used were 1, 2, 4 and 8 mg/l.

The effect of the four individual auxins on induction of somatic embryogenesis in the five genotypes has been described earlier. Increasing or decreasing the concentrations of the four auxins in the maintenance medium did not seem to have any profound effect on somatic embryogenesis and precocious germination, although the individual variations of each auxin were evident (Figs. 15a, 15b, 15c and 15d).

Auxin 2,4-D gave optimum embryogenic response at concentration of 2 mg/l; at other concentrations, precocious germination was not inhibited to any significant extent, but the frequency of somatic embryogenesis decreased. On the other hand, use of 4 mg/l 2,4,5-T proved to be better because of an increase in embryogenesis and a corresponding decrease in precocious germination in both the genotypes. Lazar *et al.*(1983) have indicated that 2,4,5-T may be a better auxin than either 2,4-D or IAA for cereal cultures. In the present study, the use of 2,4,5-T was limited to maintaining embryogenic cultures since its frequency for eliciting an embryogenic response is lesser than those of

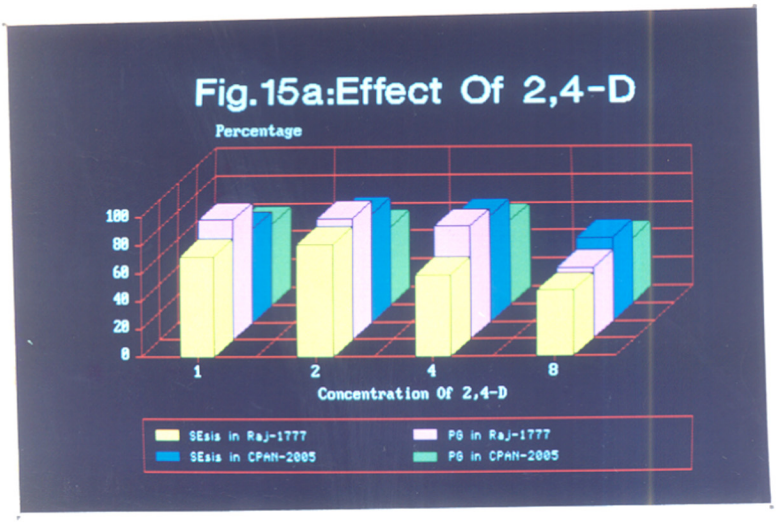


Fig.15a:Effect Of 2,4-D
Percentage
100
80
60
40
20
0
1
2
4
8
Concentration Of 2,4-D
SEsis in Raj-1777
SEsis in CPAN-2005
PG in Raj-1777
PG in CPAN-2005

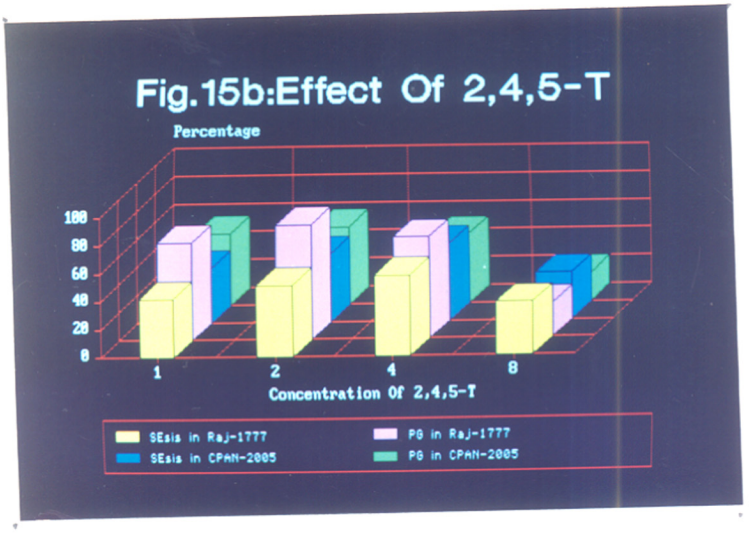


Fig.15b:Effect Of 2,4,5-T
Percentage
100
80
60
40
20
0
1
2
4
8
Concentration Of 2,4,5-T
SEsis in Raj-1777
SEsis in CPAN-2005
PG in Raj-1777
PG in CPAN-2005


Fig.15c:Effect Of Picloram
Percentage
100
80
60
40
20
0
1
2
4
8
Concentration Of Picloran
SEsis in Raj-1777
SEsis in CPAN-2005
PG in Raj-1777
PG in CPAN-2005

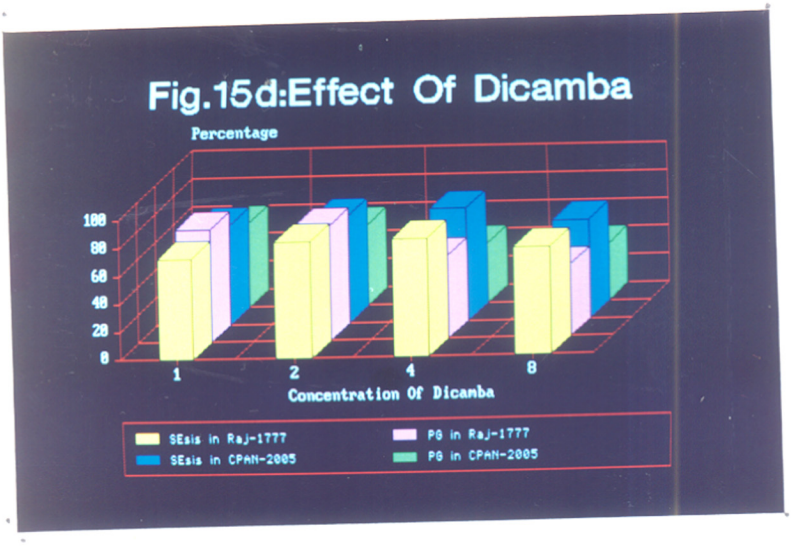


Fig.15d:Effect Of Dicamba
Percentage
100
80
60
40
20
0
1
2
4
8
Concentration Of Dicamba
SEsis in Raj-1777
SEsis in CPAN-2005
PG in Raj-1777
PG in CPAN-2005

the three auxins tested (as was concluded from the statistical analyses).

Like 2,4-D, picloram too, gave an optimum embryogenic response at a concentration of 2 mg/l; at other concentrations, it did not significantly reduce the frequency of precocious germination while retaining a comparable level of embryogenesis. The use of increased concentrations of dicamba proved to be beneficial because of a slight increase in embryogenic potential (at 4 mg/l concentration) corresponding to a considerable decrease in precocious germination of the somatic embryos of both the genotypes.

3.2.2.3 Maintenance of embryogenic calli which did not exhibit precocious germination

Calli which did not exhibit precocious germination were maintained by subculturing on the induction medium (MS + 2 mg/l 2,4-D). At each subculture, embryogenic sectors of the calli (identified on the basis of their morphology) were separated out and further cultured on fresh medium (MS medium supplemented with 1 mg/l 2,4-D) every 25-30 days, while non - embryogenic sectors were discarded. Thus, embryogenic lines could be maintained for upto 8 months without any decrease in the regeneration potential. Thereafter, the capacity of cultures to regenerate plants gradually decreased and finally by the 13th or 14th subculture, these cultures became non - morphogenic.

3.3 CONCLUSION

Plant regeneration from immature, embryo callus cultures thus, appears to be a stepwise process, consisting of embryogenic callus formation, somatic embryo germination and shoot and root elongation. Each stage appeared to have a specific requirement of growth regulators. Whereas 2,4-D was essential in the first stage, kinetin was required for somatic embryo germination (when precocious germination did not precede initiation of germination), while plantlet elongation occurred on medium devoid of hormones.

During the course of somatic embryogenesis, both - typical and atypical embryos were formed; however, the latter were more frequent. These somatic embryos seem to arise from groups of dividing cells in the epidermal and sub-epidermal cells of the scutellum. Very rarely was a single cell origin from the epidermis suggested.

The present study also demonstrates that in wheat, the frequency of somatic embryo formation and plant regeneration is under genotypic control. Thus, before embarking upon a programme on wheat improvement using *in vitro* techniks, it is of utmost importance to screen the cultivars available in that particular region and further, determine the Genotype X Environment variations that are significant. The data and results in the present study suggest that each genotype has an optimal physiological stage of the explant and an optimal source of embryogenic callus (even from different parts of the same explant), which depends on the growth environment of the donor plant and the *in vitro* environment applied.

Thus, the establishment of an efficient and reproducible *in vitro* system in wheat has been carried out for plant regeneration via somatic embryogenesis. However,

an obstacle to the establishment of long-term embryogenic cultures remains in the form of precocious germination (the frequency of which can be reduced but not eliminated completely) and the decline in the totipotency of callus cultures. In the future, more research directed towards the phenomenon of precocious germination in our selected cultivars will go a long way in resolving these problems that are common in most cereal systems.

CHAPTER IV : ESTABLISHMENT OF MORPHOGENIC SYSTEMS FROM LEAF BASES

4.1 INTRODUCTION

It is often argued that meristematic or embryonal cells have a basic pattern of gene expression and that they are in a state where if they receive the appropriate signals, they can embark upon a diversity of pathways of differentiation, each involving a shift to a specific new pattern of gene expression (Street 1977). Differentiation *in vitro* is generally taken to embrace cytodifferentiation, organogenesis and embryogenesis, and these are the various options that a cell, earlier engaged upon unorganised proliferation can commit itself to.

The concept of totipotency implies that while the determination of an induced cell towards differentiation is definite, it is capable of reversal to a state of no committment (Kohlenbach 1977). This statement by itself, poses several questions - What are the differentiation specific signals ? What is unique about the step of determination towards differentiation? To what extent do the metabolic alterations affect the processes of expression of totipotency and differentiation ?

In the Gramineae, it is realised that the capacity for cell division is manifested in the basal meristematic regions to give rise to new leaves and tillers, but not in the more distal differentiated regions (Joarder *et al.* 1986). These leaves therby provide ideal systems for studies on cytodifferentiation, since they posses a natural gradient of cells along the leaf lamina, at different levels of differentiation - from the basal meristematic regions which are amenable to *in vitro* culture, to the highly differentiated cells present towards the leaf tips. Unfortunately, leaf tissue has been used for such purposes

to a very limited extent in cereals. This has been mainly because of the relative ease with which embryonal tissues like the immature embryo can be manipulated *in vitro*. However, it has been realised that leaves may be more ideal sources of experimental material because they can be kept in an uniform, abundant supply by *in vitro* shoot tip culture, or can be harvested from greenhouse grown plants.

Further, in the last decade, the potential of mesophyll tissue in gene transfer studies has also been considered. Leaf disc transformations have been widely applied in a large number of dicots like tobacco, tomato and potato (Cleene and Deley 1976) and a few monocots like asparagus (Hernalsteens *et al.* 1984), *Cholorophytum capense* (Hoykaas Van Slogteren *et al.* 1984). Recently, in maize, Grimsley *et al.* (1988) have shown that meristematic regions of young stems and leaves are most susceptible to agroinfection, and thus provide reliable and sensitive assay systems for gene transfer. Mesophyll cells also have certain advantages over other cells in view of protoplast isolation (Ozias - Akins and Lorz 1984), *viz.* :

1. They are relatively easier to isolate as compared to isolation from other sources.
2. Protoplasts coming directly from the plant are less likely to harbour genetic variation.
3. Mutagenesis of haploid protoplasts (from haploid plants), and the application of selection pressure allows the rapid recovery of recessive mutants.

In view of the wide applicability of mesophyll tissues, the establishment of morphogenic systems from such explants could prove beneficial in view of basic studies on gene

expression as well as in crop improvement programs in the future. In the following chapter, the establishment of morphogenic systems from the most meristematic regions of cereal leaves *viz.* the leaf bases are described.

4.2 RESULTS AND DISCUSSION

Mature seeds of the five selected genotypes were surface sterilized as outlined in Chapter II. These seeds were allowed to germinate on moistened filter paper supports in the dark, and the basal portion (4-5 mm) of the leaves were excised (as described by Bhaskaran and Smith; 1988). The coleoptile was removed and the leaf split longitudinally into 3-4 pieces which were cultured in petri dishes (55 mm diameter). Incubation was done in the dark at 27^{o}C for 15 - 20 days. Three replicates were made for each parameter. Two main sources of variation (besides genotype) were investigated : age of the leaf tissue and the type, concentration and combinations of hormones used in addition to the basal medium which was the MS medium (Table 14).

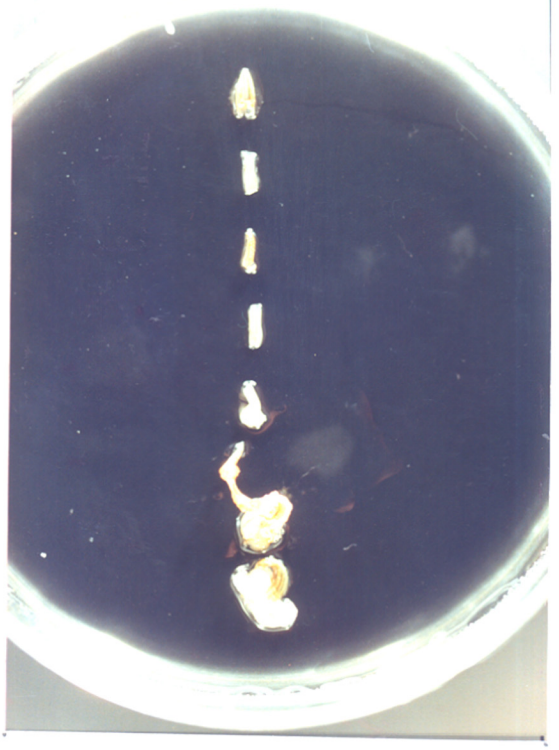
4.2.1 Age of the leaf tissues

To determine the optimum age for culture, leaf bases were collected and cultured from 3,4,5,6 and 7 day old wheat seedlings germinated as described earlier. The medium used was MS medium supplemented with 2 mg/l 2,4-D.

Initially, leaves from 3,4,5,6 and 7 day old seedlings were cultured on MS medium supplemented with 2 mg/l 2,4-D to assess their *in vitro* response. The 3,4 and some of the 5 day old seedlings usually had a single foliage leaf enclosed within the coleoptile, while the 6,7 and occasionally, 5 day old plants contained 2 leaves. The coleoptilar tissue was excluded from culture since, earlier it had been observed to give rise to non - embryogenic, friable and watery callus.

The developmental gradients along cereal leaves which reflect on the *in vitro* response as described by several authors (Haydu and Vasil 1981, Wernicke *et al.* 1982, Rajyalakshmi *et al.* 1991), was evident in the first leaves

FIG. 16 : Gradient in the *in vitro* response in wheat leaves (Genotype : VL-614).



from 5,6 and 7 day old seedlings (Fig.16). In these leaves, it was observed that the cells that segregated from the apical meristem to form a part of the differentiated leaf lamina, rapidly lost competence to dedifferentiate and form callus. It has been suggested that this loss of totipotency may be due to qualitative and quantitative changes in the nuclear DNA (Hesemann and Schroder 1982), the cell cycle status of cells or alterations in the endogenous levels of hormones and nutrients due to leaf senescence or aging (Morrish *et al.* 1987). However, younger leaves from 3,4 and some of the 5 day old plants displayed greater plasticity since virtually the entire leaf lamina would form callus within 7 - 10 days on the 2,4-D containing medium. Hence, in further experiments to standardize the nature of the response with respect to the type, concentration and combination of hormones to be used for induction of the specific response, leaf bases at these ages were used for culture.

This range of ages of the explant selected in the present study (3-5 days) is much lower than that usually reported by other authors (usually from plants at least a few weeks old). In cereals, since it is believed that competence *in vitro* may be correlated with continuing meristematic activity *in vivo* (Wernicke and Bretell 1980), the use of younger explants could be beneficial for initiating embryogenic systems. Further, the study of such undifferentiated leaf culture systems may enable us to define more precisely the step in differentiation leading to the loss of competence.

TABLE 14 : INDUCTION MEDIA USED AND CORRESPONDING RESPONSES OF CULTURED LEAF BASES (Basal Medium = MS)

MEDIUM NO.	HORMONES						RESPONSE
	DICAMBA	2,4-D	NAA	Ki	BAP	ZEATIN	
1.1	-	1mg/l	-	-	-	-	Sh.E
1.2	-	2mg/l	-	-	-	-	IE
1.3	-	5mg/l	-	-	-	-	IE
1.4	-	10mg/l	-	-	-	-	IE + B
1.5	-	20mg/l	-	-	-	-	B
2.1	-	2mg/l	-	1mg/l	-	-	IE
2.2	-	2mg/l	-	2mg/l	-	-	IE
2.3	-	2mg/l	-	3ng/l	-	-	IE
2.4	-	2mg/l	-	4mg/l	-	-	IE
2.5	-	2mg/l	-	5mg/l	-	-	IE + Sh.E
3.1	-	2mg/l	-	-	1mg/l	-	IE
3.2	-	2mg/l	-	-	2mg/l	-	IE
3.3	-	2mg/l	-	-	3mg/l	-	F
3.4	-	2mg/l	-	-	4mg/l	-	F
3.5	-	2mg/l	-	-	5mg/l	-	F
4.1	-	2mg/l	-	-	-	50ug/l	IE
4.2	-	2mg/l	-	-	-	100ug/l	IE
5.1	1mg/l	-	-	-	-	-	Sh.E
5.2	2mg/l	-	-	-	-	-	IE
5.3	5mg/l	-	-	-	-	-	IE
5.4	10mg/l	-	-	-	-	-	IE + B
5.5	20mg/l	-	-	-	-	-	B
6.1	2mg/l	-	-	1mg/l	-	-	IE
6.2	2mg/l	-	-	2mg/l	-	-	IE
6.3	2mg/l	-	-	3mg/l	-	-	IE
6.4	2mg/l	-	-	4mg/l	-	-	IE
6.5	2mg/l	-	-	5mg/l	-	-	IE + Sh.E
7.1	2mg/l	-	-	-	1mg/l	-	DM
7.2	2mg/l	-	-	-	2mg/l	-	DM
7.3	2mg/l	-	-	-	3mg/l	-	DM
7.4	2mg/l	-	-	-	4mg/l	-	DM + B
7.5	2mg/l	-	-	-	5mg/l	-	B
8.1	2mg/l	-	-	-	-	50ug/l	IE
8.2	2mg/l	-	-	-	-	100ug/l	IE
9.1	-	-	1mg/l	-	-	-	Sh.E
9.2	-	-	2mg/l	-	-	-	F
9.3	-	-	5mg/l	-	-	-	F
9.4	-	-	10mg/l	-	-	-	F + B
9.5	-	-	20mg/l	-	-	-	F + B

10.1	-	-	2mg/l	1mg/l	-	-	Sh.E
10.2	-	-	2mg/l	2mg/l	-	-	Sh.E
10.3	-	-	2mg/l	3mg/l	-	-	Sh.E
10.4	-	-	2mg/l	4mg/l	-	-	Sh.E + B
10.5	-	-	2mg/l	5mg/l	-	-	Sh.E + B
11.1	-	-	2mg/l	-	1mg/l	-	Sh.E
11.2	-	-	2mg/l	-	2mg/l	-	Sh.E + F
11.3	-	-	2mg/l	-	3mg/l	-	F
11.4	-	-	2mg/l	-	4mg/l	-	F + B
11.5	-	-	2mg/l	-	5mg/l	-	F + B
12.1	-	-	2mg/l	-	-	50ug/l	F
12.2	-	-	2mg/l	-	-	100ug/l	F

Sh.E	-	Shoot Elongation
IE	-	Indirect Somatic Embryogenesis
DM	-	Direct Morphogenesis
B	-	Browning of explant
F	-	Friable, non -embryogenic callus

TABLE 15 : MATURATION MEDIA USED AND CORRESPONDING RESPONSES OF INDUCED SOMATIC EMBRYOS (Basal Medium = MS)

MEDIUM NO.	ADDITIVES						RESPONSE
	DICAMBA	2,4-D	Ki	BAP	ABA	AgNO3	
A1	-	-1mg/l	-	-	-	-	MEC
A2	-	1mg/l	1mg/l	-	-	-	MEC
A3	-	1mg/l	-	1mg/l	-	-	E-F
A4	-	1mg/l	1mg/l	-	10ug/	-	MEC
A5	-	1mg/l	1mg/l	-	150ug	-	MEC
A6	-	1mg/l	1mg/l	-	/l-	10mg/l	MEC
A7	-	1mg/l-	1mg/l	-	-	20mg/l	MEC
B1	1mg/l	-	-	-	-	-	F
B2	1mg/l	-	1mg/l	-	-	-	F
B3	1mg/l	-	-	1mg/l	-	-	F
B4	1mg/l	-	-	1mg/l	10ug/	-	GSE
B5	1mg/l	-	-	1mg/l	150ug	-	GSE
B6	1mg/l	-	-	1mg/l	/l-	10mg/l	GSE
B7	1mg/l	-	-	1mg/l	-	20mg/l	GSE

MEC - Maintenence of embryogenic callus
E-F - Conversion of embyrogenic callus to friable callus
GSE - Globular somatic embryo
F - Friable callus

4.2.2 Effect of type, concentration and combination of hormones in the medium on the nature of the reponse

It was seen that the response of the cultured leaf bases was significantly affected by the type, concentration and combination of hormones in the medium. Leaf bases in which the entire leaf lamina could callus (3, 4 and 5 day old leaves) were used in these experiments. Several media (listed in Table 14) were tested for the induction of morphogenesis. The auxins used were 2,4-D, dicamba and NAA while the cytokinins used were kinetin, BAP and zeatin.

Thus, depending on the composition of the medium , two types of responses were observed in the cultured leaf bases *viz* indirect, atypical somatic embryogenesis (IE) and direct morphogenesis *viz.* organogenesis and embryogenesis (DM).

4.2.2.1 Indirect, somatic embryogenesis

The media used to elicit this type of response include all those listed in Table 14, exception being medium no. 7. Depending on the medium tested, one of the following changes occurred in the cultured explants :

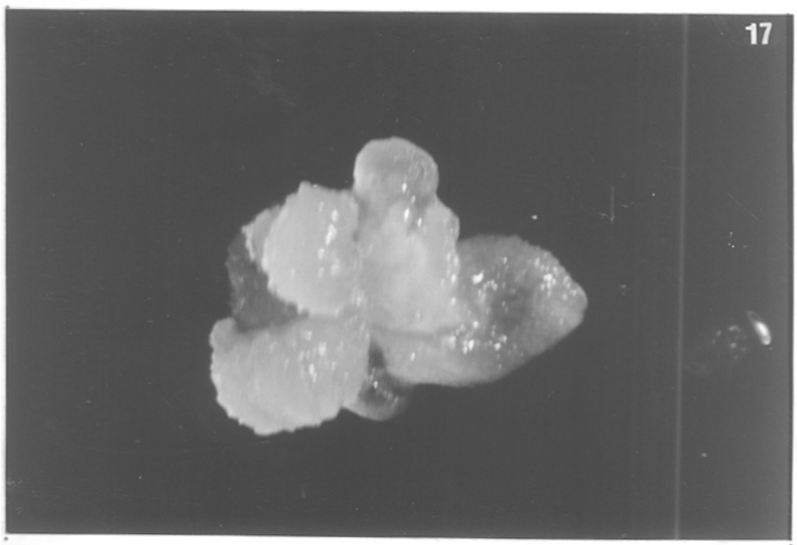
1. browning of the explant followed by necrosis,
2. formation of a crystalline and friable callus which is non-embryogenic (Fig. 17), and
3. formation of a smooth and compact - SE callus (Fig. 18)

The SE callus thus formed would not differentiate embryoids, but required lower concentration of auxin (either 2,4-D or dicamba)in the medium for the formation of embryogenic callus (Fig.18). Maturation of the cultures was tested on several media viz. A_1, A_2, A_3, A_4, A_5, A_6 & A_7 (Table 15). Of these, best was the A_6 medium containing 1 mg/l each of 2,4-D and kinetin and 10 mg/l $AgNO_3$ since it gave rise to an increased number of embryoids per explant.

FIG. 17 : Leaf base derived crystalline, friable, NE callus (Genotype : VL-614).

FIG. 18 : Leaf base derived E callus (Genotype : VL-614).


18
e


17

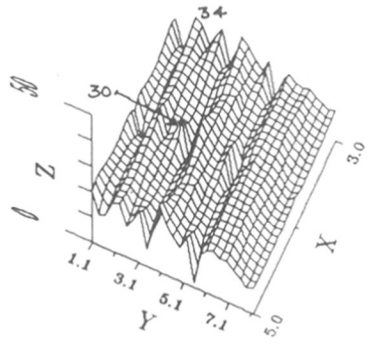
Thus, for a comparative study, the cultures, 15 - 20 days after initiation on the media listed in Table 14 (except media 7), were transferred to the A_6 medium and scored for somatic embryogenesis. The results of this comparative study are depicted in Fig. 19 as an interplay between the genotypes, age and medium composition.

As is seen from this figure, a distinct genotypic variation is evident in the frequency of somatic embryogenesis, with the genotype Raj - 1777 exhibiting maximum embryogenecity (42%) on medium 5.2 (containing 2 mg/l dicamba), while the genotype NI - 9272 gave the poorest embryogenic response (18%) on the same medium at the same age of the explant. Although the leaf bases permitted embryogenic callus formation at all the ages of the explant tested, maximal frequency of embryogenesis was observed in the leaf bases derived from 4 day old seedlings. In explants from 3 day old seedlings, the frequency of browning followed by necrosis of the lamina was more frequent than at other stages. In a few explants from 5 day old seedlings, the second foliage leaf was also formed. In such cases, the first leaf contained differentiated sectors which did not respond *in vitro*.

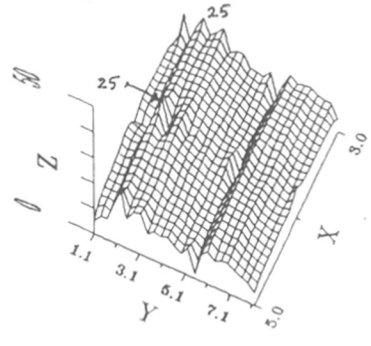
In all the genotypes, the best embryogenic response was observed either on media nos. 5.2 or 5.3 (which contained 2 and 5 mg/l dicamba respectively) , while a comparable response was exhibited on media nos. 1.2 and 1.3 (which contained 2 and 5 mg/l respectively of 2,4-D). At lower auxin concentrations, some of the explants tended to elongate and form shoots without callusing. On the other hand, at higher auxin concentrations (media nos. 1.5 and 5.5 which contained the respective auxin at a concentration of

FIG. 19 : INDIRECT SOMATIC EMBRYOGENESIS FROM LEAF BASES AS AN INTERPLAY BETWEEN GENOTYPE, AGE AND MEDIUM COMPOSITION

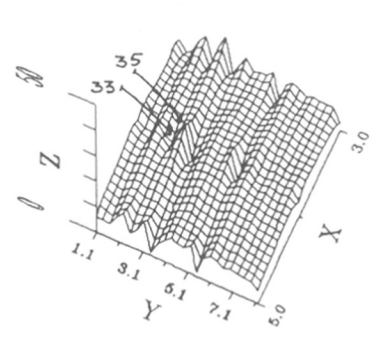
NI - 5439



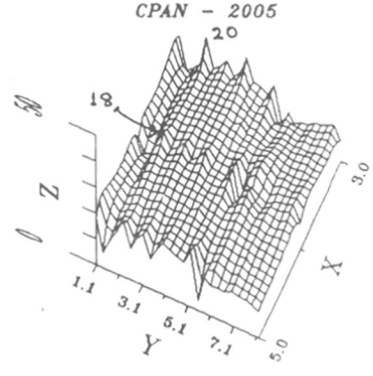
NI - 9272



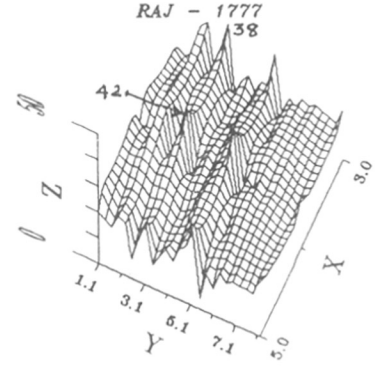
VL - 614



CPAN - 2005



RAJ - 1777



LEGENDS TO FIGURES

X-axis : Age of leaf

Y-axis : Medium Used

Z-axis : Percentage of Somatic Embryogenesis

20 mg/l), somatic embryogenesis was almost totally inhibited and the explants turned brown.

In media containing a combination of an auxin (2,4-D or dicamba) with a cytokinin (media nos. 2, 3, 4, 6 and 8), the embryogenic response varied depending on the type of cytokinin used (either BAP or kinetin). Thus, both the auxins in combination with 3 mg/l kinetin (media nos. 2.3 and 6.3) elicited a better embryogenic response than the combinations with BAP or Zeatin. In media containing higher concentrations of BAP (media nos. 3.4 & 3.5 - containing 4 mg/l & 5 mg/l BAP respectively ,in addition to 2 mg/l 2,4-D), the explants gave rise to a friable callus with a lowered frequency of somatic embryogenesis.

In media containing NAA as the auxin (*viz.* medium nos. 9.1, 9.2, 9.3, 9.4 & 9.5), a friable callus was formed and somatic embryogenesis did not ensue. On the other hand, on media containing a combination of NAA with kinetin (medium nos. 10.1, 10.2, 10.3, 10.4 & 10.5), elongation of the explants occurred, with no detectable callussing / somatic embryogenesis. In medium nos. 11.1 & 11.2 (containing a combination of NAA at 2 mg/l concentration with 1 mg/l and 2 mg/l BAP respectively), a similar elongation of the explants occurred. At higher BAP concentrations (medium nos. 11.3, 11.4 & 11.5 - containing 3, 4 & 5 mg/l BAP respectiely), friable callus was initiated at a high frequency. Similarly, in media nos. 12.1 & 12.2, containing NAA at a concentration of 2 mg/l in combination with 50 ug/l & 100 ug/l zeatin respectively), the explants gave rise to a friable callus. Thus, effectively, the use of NAA in the initiation medium was eliminated.

As described earlier, the embryogenic callus could be maintained for long periods of time by repeated subculture on A_1, A_2, A_3, A_4, A_5 & A_6media (Table 15). On the A_3 medium (containing 1 mg/l each of 2,4-D & BAP), the embryogenic callus was rapidly converted to a friable, non-embryogenic type. On the other hand, on the A_6 medium, each subculture gave rise to an increased number of embryoids per explant. Hence, this medium was consistantly used for maintainence.

For conversion into plantlets, cultures were initially transferred onto a low salt medium (1/2 MS) containing 0.25% activated charcoal. After seven days of incubation on this medium (in the dark at 27^{o}C), cultures were transferred to culture tubes containing 20 ml regeneration medium (Table 16) and placed under a 16h photoperiod regime. On transfer of the E callus to the regeneration media, germination of embryoids ensued. No statistically significant differences were observed with respect to regeneration on either media. However, the plants regenerated on media R_1 & R_3 occasionally underwent callussing at the base of their shoots. Hence medium R_2 (containing 1 mg/l kinetin was used for regeneration. For further shoot elongation, plantlets were transferred onto filter paper supports in liquid 1/2 MS medium (without any hormones) before they were planted in the soil. Chromosome counts were carried out in root tip preparations for determining the cytological status of the regenerated plants. None of the regenerated plantlets exhibited abnormal ploidy levels. Plantlets were potted after elongation and hardening.

4.2.2.2 Direct Morphogenesis

Besides indirect, atypical somatic embryogenesis from cultured leaf bases, yet another type of response was

observed, *viz.* that of direct morphogenesis (organogenesis and somatic embryogenesis). This response was observed only in genotypes Raj-1777, V.-614 and CPAN-2005. Further in the genotype Raj 1777, the frequency of the response was much higher than in the other two genotypes.

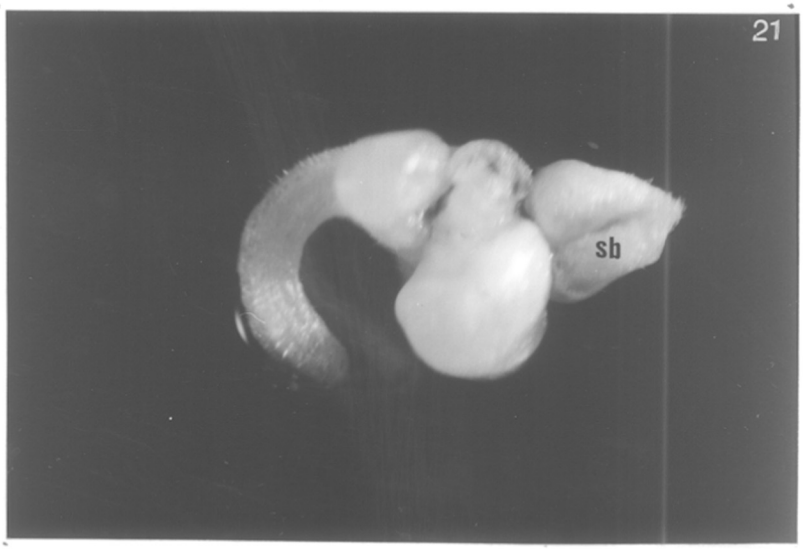
The media responsible for direct morphogenesis were those containing a combination of dicamba and BAP (media nos. 7.1, 7.2, 7.3, and to a lesser frequency, 7.4). About 50% of the leaves cultured on medium no. 7.4 and all of those cultured on medium no.7.5 did not develop any adventitious structures. These remained white upto two weeks before senescening and turning brown. On the other media, leaf explants which did not senesce (50 - 70%), had swollen stubs. Besides these basal stubs, sections of the lamina wounded during dissection also showed swelling, and occasional protuberences appeared on the abaxial surface of leaf (Fig. 20).

Contrary to the systems of immature embryos and immature inflorescences (Chapters III & V respectively), maturation of the morphogenic unit (either the somatic embryo or shoot bud) in both the types of responses - direct as well as indirect, did not occur on the induction medium, but required low auxin-cytokinin ratios. Thus, 15 - 20 days after induction, several media were tested for maturation (Table 15). Maturation was allowed to proceed in the dark at 27^{o}C for 10 - 15 days.

However, on transfer of these explants to the maturation media (B_1, B_2, B_3, B_4, B_5 & B_6 - Table 15), differences in responses were observed. On the B_1, B_2 & B_3 media, callusing set in and non - morphogenic callus was produced. However, on media B_4, B_5, B_6 & B_7 the swollen


20

FIG. 20 : Induction of morphogenesis (Genotype : Raj-1777).


21
sb

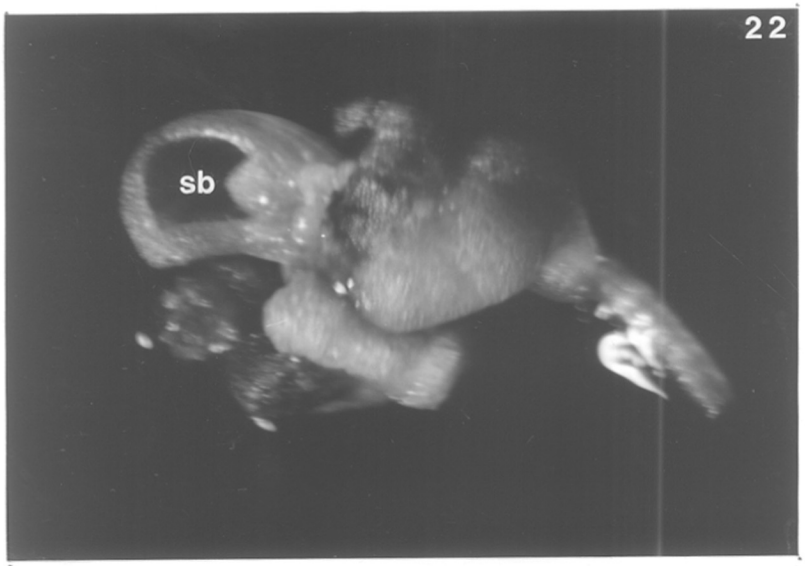
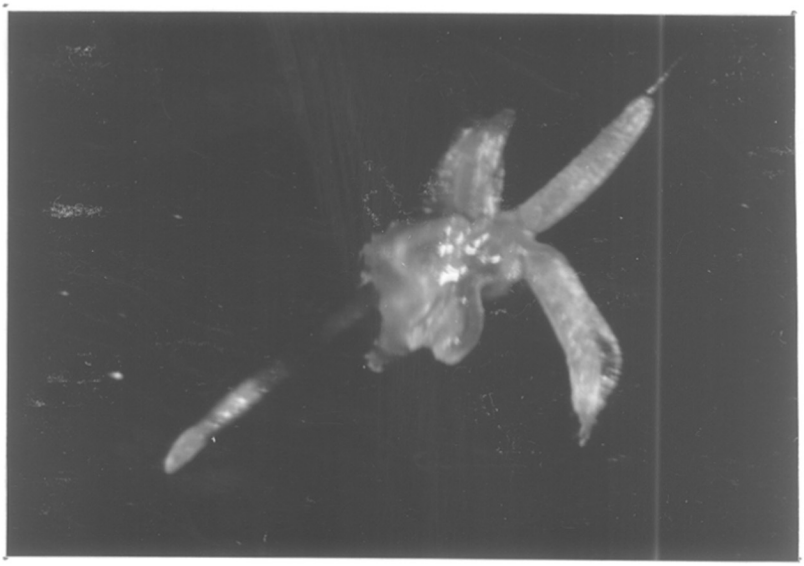
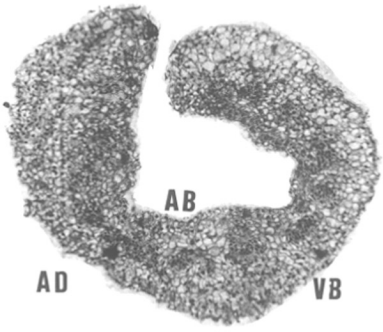

22
sb

FIG. 23 : Elongating somatic embryo (Genotype : Raj-1777).

FIG. 24 : Section through leaf base at time of cuture
(VB - Vascular Bunde, AB - Abaxial leaf surface,
AD - Adaxial leaf surface)
Magnification = 50X.




AB
AD
VB

structures on the explant were transformed into distinct globular ones within 5 - 7 days (Fig. 21). By the 10^{th} day after transfer, majority of the explants initiated shoot bud or embryoid formation. However, the number of differentiating structures (shoot buds or somatic embryos) produced per leaf explant cultured did not exeed 4-5.

Explants exhibiting such structures were transferred to a charcoal containing medium, where further differentiation occurred (Fig. 22). However, on this medium, buds could not elongate to form plantlets. For this purpose, the explants were transferred to the regeneration media (Table 16), where elongation of the shoot and occasionally of the root (from somatic embryos) occurred (Fig. 23). There were no significant differences in the statistics of regeneration on either media. Transfer to filter paper supports in liquid MS medium devoid of hormones was required in some cases where rooting did not occur earlier on the regeneration medium.

The plantlets formed were potted after subsequent hardening treatment. Thus, regeneration from the basal stubs and wounded lamina did not involve an intermediate callus phase.

4.2.3 Histological Studies :

These were carried out to confirm the nature of the two processes of morphogenesis described above. Techniques used in these studies have been described earlier in Chapter II.

4.2.3.1 Indirect, atypical somatic embryogenesis

Histological studies of the cultured leaf segments revealed that distinct vascular tissue differentiation had occurred at the time of culture (Fig. 24). Younger leaves, in which such structural differentiation had not been

TABLE 16 : REGENERATION MEDIA USED AND CORRESPONDING RESPONSES OF INDUCED SOMATIC EMBRYOS (Basal Medium = MS)

MEDIUM NO.	HORMONES (mg/l)		RESPONSE
	BAP	Ki	
R1	1	1	Healthy plantlets; occasional callussing at base of shoots
R2	1	-	Healthy plantlets; occasional callussing at base of shoots
R3	-	1	Healthy plantlets

completed did not respond in culture, but necrosed and were observed to undergo browning.

The centres of cell division were primarily in the xylem and phloem parenchyma, mesophyll and epidermis of the abaxial (lower) surface of the leaf. A similar origin of embryoids has been described earlier in sugarcane (Ho and Vasil 1983). These divisions gave rise to protuberances from the callus derived from the abaxial surface of the leaf (Fig. 25) , which later differentiated into embryoids. The surface of such calli became irregular as a result of extensive localised cell divisions. On transferring the E callus to the regeneration media (Table 16), leafy, bipolar structures - described earlier in the case of immature embryos were formed (Fig. 26) which gave rise to plantlets.

4.2.3.2 Direct Morphogenesis

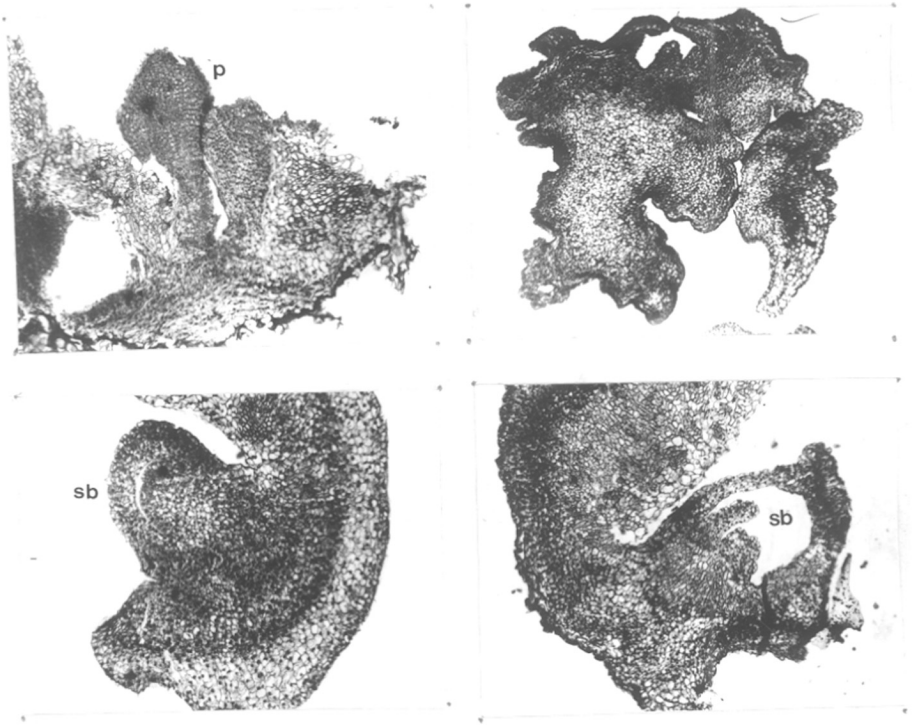
No cell divisions could be seen in the epidermis of the adaxial (upper) surface of the leaf. Dividing cells were located primarily in the mesophyll and the epidermis of the abaxial (lower) surface of the leaf. Two different types of morphogenesis were observed to originate from different tissues.

(a) Direct caulogenesis (shoot bud formation) - In this case, dividing cells were restricted to the first layer of the exposed (by wounding) mesophyll. Thus, these cells led to the formation of distinct shoot buds directly from the mesophyll cells (Figs. 27 & 28). Each shoot bud exhibited a coleoptile which encased the shoot apex. During further differentiation, more than one leaf could be seen in association with the coleoptile (Fig. 28). In no case were shoot buds seen to be produced from the epidermal layer.

FIG. 25 : Protuberances (p) arising in the E calli.
Magnification = 50X.

FIG. 26 : Formation of leafy, bipolar structures.
Magnification = 50X.

FIGS. 27 & 28 : Direct shoot bud (sb) formation.
Magnification (for both figures) = 63X.


p
sb
sb

(b) Direct somatic embryogenesis - In instances where the centres of cell division were localised in the epidermis of the abaxial surface of the leaf in addition to the mesophyll cells, somatic embryos were produced directly, without an intervening callus phase. These globular stage somatic embryos were seen to be linked to the mother explant either *via* a multiseriate suspensor (Fig. 29 & 30) or by a broad base (in the absence of a suspensor). Further differentiation was detected by the formation of a lateral scutellar notch (Fig. 31). This notch, further extended into the proembryo and formed a bipolar structure with a scutellum, shoot apex and a root apex (Fig. 32). On transfer to the medium containing activated charcoal, distinct bipolar embryos were formed.

Yet another unique feature was observed with the above system of somatic embryogenesis. On prolonged culture of the explant on the maturation medium, differentiation of the somatic embryo was initiated. Further, the coleoptile formed gave rise to an occasional shoot bud from the epidermis of its outer (exposed) surface. Fig. 33 depicts this phenomenon; - the somatic embryo has been dettatched from the explant during the histological procedures; however, the presence of a suspensor confirms its nature.

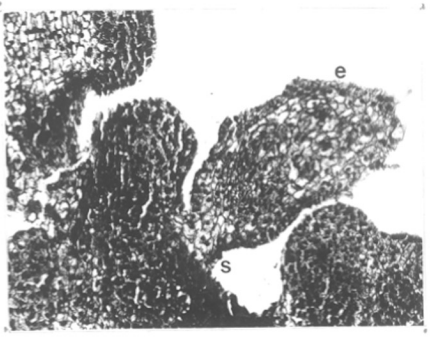
FIG. 29 : Somatic embryo (e) attached by suspensor (s) to the explant. Magnification = 75X.

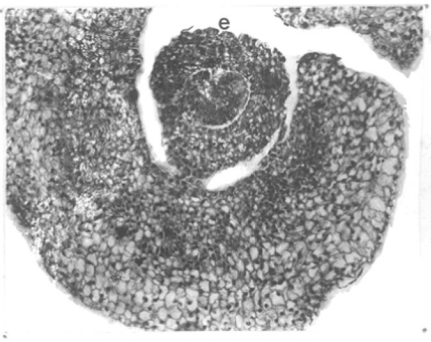
FIG. 30 : Gobular stage somatic embryo (e). Magnification = 75X.

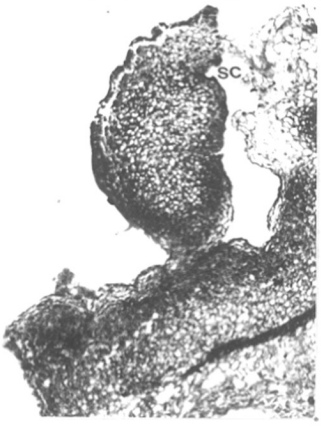
FIG. 31 : Initiation of scutellar notch (sc). Magnification = 50X.

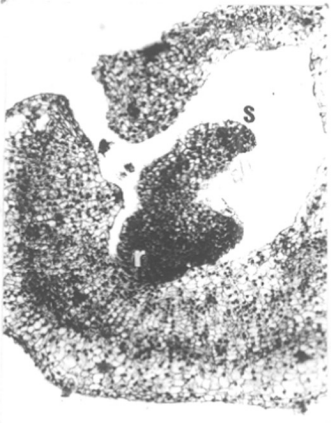
FIG. 32 : Somatic embryo formed directly from the explant. (s-shoot, r-root). Magnification = 50X.

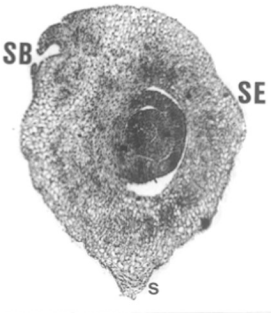
FIG. 33 : Secondary morphogenesis (e-somatic embryo, sb-shoot bud, s-suspensor). Magnification = 30X.


e
s


e


sc


s
r


SB
SE
s

4.3 Conclusion

The globular stage of the somatic embryos originating directly from the leaf tissues closely resembled the proembryo stage of the zygotic embryo. After this stage, usually the shoot - root axis is formed in a majority of cases (Van Lamneren 1987). However, in literature, several reports omit the demonstration of a distinct shoot-root axis at this stage - its absence has been explained by the presence of auxin which has a growth suppressive effect at or in the vicinity of the apical meristems (Pereira and Dale 1982, Wernicke *et al.*1986). Thus, it may be speculated that the formation of shoot buds rather than bipolar structures could be the manifestations of aberrant forms of somatic embryogenesis. This is indicated from the fact that somatic embryos and shoot buds were formed from the same tissue *viz.* wounded mesophyll (Fig. 34) on exposure to identical concentrations of hormones and nutrients.

Earlier, a similar interpretation of organogenesis as an aberrent form of somatic embryogenesis has been made (Walker *et al.*1979). The roots of certain alfalfa cultures become somatic embryos with improvements in the culture medium (Stuart *et al.* 1985). Contrarily, Christianson and Warnick (1985) have shown that in cultures of *Convolvulus*, shoots and roots arise from different parts of the same callus, and not as if the organs represent simulation of either the root or shoot pole of an aberrant embryo. Hence, the controversy of organogenesis being an aberrant manifestation of somatic embryogenesis remains, unresolved.

More recently, it has been suggested that the degree of methylation of the tissue may affect its *in vitro* response. It has been shown that when a gene regulatory sequence is

FIG . 34 : Simultaneous shoot bud (sb) and somatic embryo (e) formation. Magnification = 50X.


sb
e

methylated, the associated gene is inactivated. Further, somatic embryogenesis cannot occur when DNA methylation is specifically inhibited (LoSchiavo *et al.* 1989). In a study of changes in DNA methylation in zones of the first leaf of wheat seedlings, it was observed that in the absence of replication of the cells of the growing point, the degree of methylation decreased considerably in the actively dividing cells (Kirnyus *et al.* 1989). These modulations appeared to be associated with the repair synthesis of DNA.

Okkels *et al.*(1987) suggest that treatment of *in vitro* explants with auxins results in the production of ethylene which depletes the pool of S - adenosylmethionine. Since this compound serves as the methyl group donor for DNA methylation, the process of methylation is thereby inhibited in the presence of the auxin. This produces callus in the same state of dedifferentiation as zygotic embryonic cells - both types contain demethylated DNA. When the auxin is removed, the same genes become active as in zygotic cells and undergo differentiation.

Another study by LoSchiavo *et al.*(1989) appears to modify Okkels and his coworker's proposition. These workers report that carrot cells grown in the presence of ethoxy - carbonyl pyrimidine (a compound that inhibits DNA methylation), show restoration of embryogenic potential on treatment with an auxin, which is associated with an increase in the degree of methylation. In the absence of the auxin, carrot cell embryogenesis is associated with a decrease in the methylation of cytosine, followed by an increase during late embryogenesis in the presence of an auxin. They further suggest that each tissue has its own

basal state - corresponding to a specific level of methylation, which changes during embryogenesis.

In contrast with the above studies, Morrish and Vasil (1989) have shown that in leaves of napiergrass, the loss of embryogenic potential is not linked to a gross alteration in DNA methylation. However, the study of LoSchiavo *et al.* (1989) seems more convincing because even in the present study, it was indicated that each and every explant needed a definite hormonal balance at a particular developmental stage (possibly corresponding with a specific degree of cellular differentiation and DNA methylation?) for an ideal *in vitro* response. Leaf bases provide an ideal *in vitro* system to assess the influence of differentiation and/or DNA methylation on embryogenic competence.

The above studies reveal that several different types of *in vitro* responses can be induced from the same explant merely by modifying the external mileau of the tissue. The role of genotype in eliciting such responses is also evident. However, from the ongoing discussion, it must be realised that the basic questions concerning differentiation are nowhere towards being resolved. In fact, additional questions emerge from the present study - if the two types of differentiation responses (organogenesis and embryogenesis) arise from the same tissue under the same set of environmental signals, then, do the mesophyll cells in the plant itself harbour genetic variation? Or is one form of differentiation (organogenesis) an aberrent form of the other (embryogenesis)? Hope however can be expressed that more work in this area may be carried out to further resolve the queries associated with differentiation.

CHAPTER V : ESTABLISHMENT OF *IN VITRO* SYSTEMS FROM OTHER EXPLANTS

5.1 INTRODUCTION

The growth of plant cells involves integration of two separate processes - cell division and cell expansion. These two processes are readily visible in the meristematic tissues, which are considered to be relatively undifferentiated tissues. Among Graminaceous plants, meristematic tissues have gained importance due to their potential to consistently elicit a favorable *in vitro* response and further, due to the recognition that cells of these species lose their capacity to differentiate as they mature. Besides the state of differentiation, several other cellular, molecular and genetic factors like change in cell shape, external and internal gradients, cell-cell interactions and cell divisions cooperatively lead to the final event of morphogenesis (Van *et al.* 1985).

Plant growth regulators too, elicit multiple responses in the differentiation and developmental programmes of whole plants, organs or cells *in vivo* and *in vitro*. There are five classes of plant growth regulators : auxins, cytokinins, abscisic acid, gibberelic acid and ethylene. Auxins bring about cell enlargement, cytokinins are associated with plastid development, cell division and organogenesis, abscisic acid promotes embryogenesis and seed development besides playing a major role in abscision and senescence - while ethylene is associated with fruit ripening (Parther 1989). Although there is voluminous indirect evidence that plant hormones operate by stimulating expression of genes, direct evidence, however is lacking. However, Marcotte *et al.* (1988) have reported the transfer of a wheat promotor gene into rice protoplasts and its expression and normal regulation when induced by ABA. This

provides the first direct evidence that phytohormones regulate gene transcription.

It has been known for some time that cultured plant cells evolve ethylene (La Rue and Gamborg 1971). Ethylene was found to enhance embryogenesis from anther cultures of *Hordeum vulgare* (Cho and Kasha 1989) and flower bud formation from explants of *Nicotiana tabacum* (Smulders *et al.* 1990). On the other hand, ethylene has been reported to be involved in the regulation of cell recalcitrance with respect to caulogenesis (shoot formation) and somatic embryogenesis (Huxter *et al.* 1981, Paterson-Robinson and Adams 1987, Chi *et al.*1991, Biddington and Robinson 1991). The use of ethylene antagonists like $AgNO_3$, norbornadiene and $CoCl_2$ have been reported to enhance organogenesis and embryogenesis in *Nicotiana plumbaginifolia*, *Triticum aestivum* and *Daucus carota* (Purnhauser *et al* . 1987, Roustan *et al.* 1989).

Although the mechanism of hormone action is poorly understood, it is generally realised that the endogenous levels of the various groups of hormones vary at different developmental stages and further, in different tissues at the same developmental stage. Genes governing the manner in which tissues or organs grow or exhibit morphogenesis *in vitro* were obviously not evolved for this purpose; their influence on tissue culture systems is only a secondary effect of their normal function (pleiotrophy). Baroncelli *et al.* (1974) have suggested that there might be groups of genes capable of shifting plant development in different directions; the effect of such genes *in vitro* might be to influence the competing processes of cell proliferation and growth. Bayliss and Dunn (1977) have suggested that such

genes may exert their influence by regulating the effective cellular levels of growth substances. On the other hand, Borkird *et al.* (1988) have isolated two genes that are regulated during somatic embryo development in carrot. These genes were found to be controlled by the developmental programme of embryogenesis, but were not directly regulated by the auxin 2,4-D.

Genetic studies have also indicated that the major genes controlling or affecting the tissue culture response are dominant or partially dominant and are simply inherited. Reisch and Bingham (1980) have found that bud differentiation from callus of alfalfa is controlled by two dominant genes - both of which must be expressed for regeneration. In wheat, genes that control regeneration from anther cultures have been shown to be independent of those that control regeneration in somatic cell or tissue cultures (Henry and DeBuyser 1987). Similarly, in rye, Lazar *et al.* (1987) have reported that chromosome 4 contains genes that control organogenesis in anther cultures, while chromosomes 6 and 7 contain genes that control organogenesis in immature embryos. It is thus probable that the requirements for the desired *in vitro* response from different explants of the same plant may be different.

In the following chapter, an in depth investigation for the establishment of *in vitro* systems from immature inflorescences, mature embryos and root tips is presented. As mentioned in Chapter IV, immature embryos are not easily available throughout the year, and hence the need to tap other explants as sources of initiating embryogenic cultures.

5.2 RESULTS AND DISCUSSION

5.2.1 Immature Inflorescences

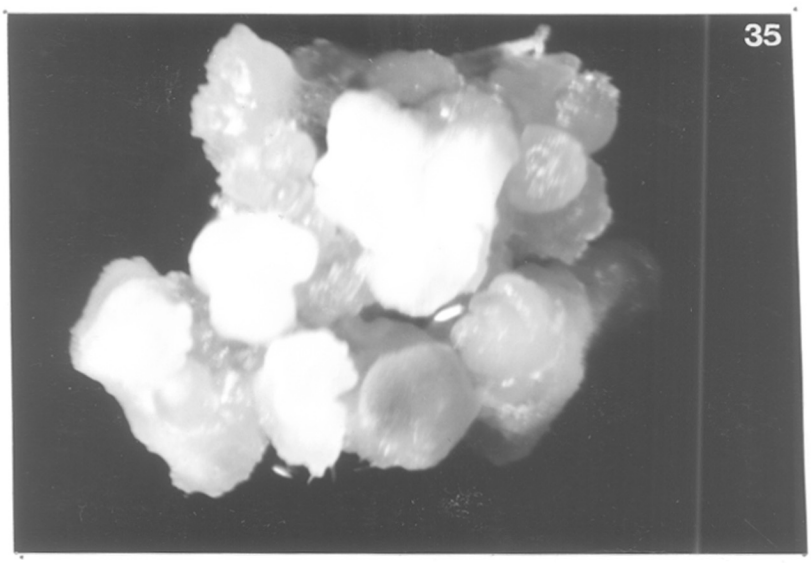
5.2.1.1 Genotype Screening :

Young, immature inflorescences of the five selected genotypes (NI-5439, NI-9272, VL-614, CPAN-2005 and Raj-1777) were collected from field - grown plants. After removing the outer leaves, the inflorescences were surface sterilized according to the protocol outlined in Chapter II. Each inflorescence was cut into pieces, 1 - 2 mm long and cultured in petri dishes (55 mm diameter). The medium used for culture was MS medium supplemented with 2 mg/l 2,4-D. Incubation was carried out in the dark at 27^{o}C for 15 - 20 days.

Within 2 - 3 days of culture on the above medium, swelling of the inflorescences was evident and the explants gradually lost their green pigmentation and became white in color. After another 15 - 20 days, compact, yellowish calli were visible, on the surface of which somatic embryos could be seen under the stereomicroscope (Fig. 35). As was the case with the somatic embryos derived from immature embryos, maturation of the somatic embryos induced from immature inflorescences too, occurred on the induction medium (MS + 2 mg/l 2,4-D) itself.

For conversion of embryoids into plantlets, embryogenic calli were shifted onto MS medium supplemented with 1 mg/l Ki. Cultures were further maintained under a 16h photoperiod. On this medium, germination of the embryoids occurred within 7 - 10 days after transfer to the regeneration medium. For shoot and root elongation, regenerating calli were transferred onto filter paper

FIG. 35 : Immature inflorescence derived embryogenic callus of genotype CPAN-2005.


35

supports in liquid MS medium containing half the normal concentration of MS salts.

The response of the cultured inflorescence segments in the present study is faster than that reported earlier. Ozias-Akins & Vasil (1982) and Rajyalakshmi *et al* (1988) have reported an extended lag period of 10 - 14 days before swelling of the cultured inflorescence segments of wheat became evident. This fast reactivity with the medium was probably a consequence of selecting the best genotypes for *in vitro* culture on the basis of their embryogenic potential using immature embryo explants (Chapter II).

5.2.1.2 Histological Studies

Once the occurrence of somatic embryogenesis was established, its nature was delineated further by histological procedures (described in Chapter II). Tissues of the genotype Raj - 1777 were harvested at the following stages for histological purposes -

(i) Immature inflorescences prior to culture, within a size range of 10 - 30 mm,

(ii) Immature inflorescences cultured for 5 days,

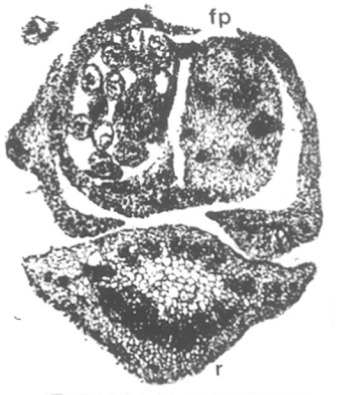
(iii) Immature inflorescences cultured for 10 days,

(iv) Immature inflorescences cultured for 15 days and

(v) Regenerating calli.

Chromosome counts were also done in root tip preparations (as described in Chapter II) for determining the cytological status of the regenerated plants. The inflorescence segments cultured for histological purposes were seen to be at a very early stage of development (Fig. 36). After 4 - 5 days, the peripheral cells of the rachis underwent enlargement. Further, within another 4 - 5 days, distinct meristematic zones were formed in these cells (Fig.

FIG. 36 : Section through inflorescence (2.5 cm long) at time of cuture (fp-floral primordia,r-rachis). Magnification = 50X.


fp
r

37). At this stage, rapid callus formation and proliferation was also evident.

Divisions occurred mainly in the epidermal and sub-epidermal cells of the rachis. Ozias-Akins & Vasil (1982) also reported degeneration of the floral primordia in wheat inflorescences and attributed the embryogenic potential to the rachis and glumes. In the present study, however, the glumes remained inert and failed to give rise to embryoids under similar experimental conditions. The same has been reported in *Sorghastrum nutans* (Chen *et al.* 1977) and *Pennisetum purpureum* (Wang and Vasil 1982) where only the rachis produces embryogenic calli.

The earliest indication of embryoid initiation were the formation of lateral prot uberances (Fig. 38) which rapidly increased in size, and by the 13^{th} - 15^{th} day of culture, bipolar embryoid-like structures could be detected. During conversion, leafy structures associated with shoot and root primordia were evident in sections (Figs. 39 & 40).

5.2.1.3 Effect of age of the inflorescence, the type, concentration and combination of hormones in the medium

Since age and developmental stage of the inflorescence is a function of its size, inflorescences belonging to several size ranges were cultured on MS medium supplemented with 2 mg/l 2,4-D.

These size ranges included :

(i) 7.5 $\pm$ 2.5 mm,

(ii) 15 $\pm$ 5 mm,

(iii) 25 $\pm$ 5 mm and

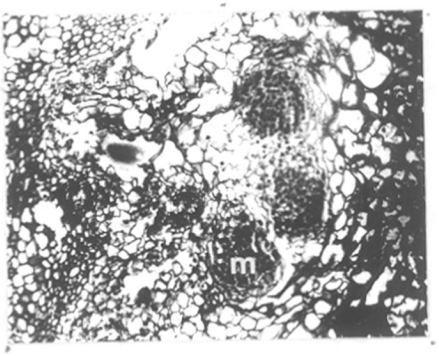
(iv) 35 $\pm$ 5 mm.

Further, inflorescences within the size range (10 - 30 mm) were used to study the effect of type, concentration and

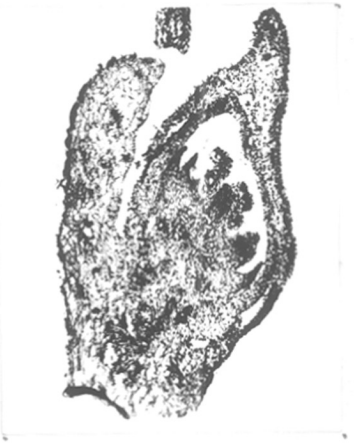
FIG. 37 : Formation of meristematic zones (m) in the rachis. Magnification = 75X.

FIG. 38 : Formation of protuberances from meristematic zones in the rachis. Magnification = 50X.

FIGS. 39 & 40 : Formation of leafy, bipolar structures.
Magnification (Fig.39) = 50X.
(Fig.40) = 75X..


m


p



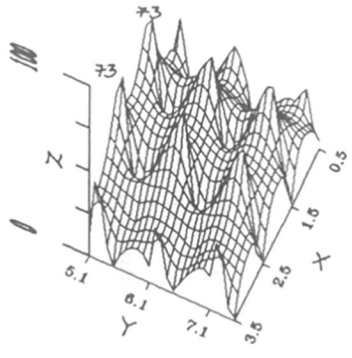
combination of hormones in the medium. Media numbers 1, 2 and 3 (listed in Table 14; Chapter IV) were tested for the induction of somatic embryogenesis. The basal medium used was MS salts and vitamins (Tables 3a, 3b and 3c ; Chapter II). The auxin used was 2,4-D while the cytokinins used were kinetin and BAP.

The results shown in Fig. 41 depict the embryogenic response of the five selected genotypes at the size ranges and media described above. Thus the genotype Raj-1777 exhibited maximum embryogenecity (92%) at a developmental stage corresponding with the size range 25 $\pm$ 5 mm on medium 1.2 containing 2 mg/l 2,4-D while the genotype NI-9272 exhibited the least embryogenic potential at any size range and medium permutation.

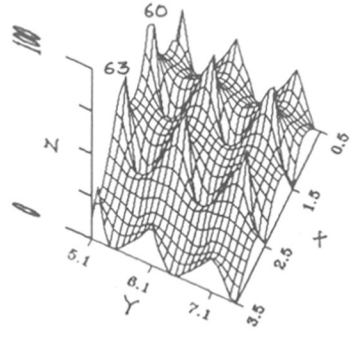
Although the immature inflorescences permitted callus formation and somatic embryogenesis at all the size ranges tested, maximum frequency of embryogenesis was observed in the size ranges of either 15 $\pm$ 5 mm or 25 $\pm$ 5 mm in all the genotypes. In younger inflorescences (size range 75 $\pm$ 25 mm), the formation of E callus was restricted to the central region of the inflorescence, which is comparatively at an advanced state of development than either the lower or upper halves. In the older inflorescences (size range 35 $\pm$ 5 mm), swelling of the spikelets occurred which further led to the formation of friable callus. However, E callus formation was restricted only to the rachis. Thus, although an increased number of explants within this size range gave rise to callus, the frequency of somatic embryogenesis was seen to be relative to the corresponding developmental stage.

FIG. 41 : INDIRECT SOMATIC EMBRYOGENESIS FROM IMMATURE INFLORESCENCES AS AN INTERPLAY BETWEEN GENOTYPE, SIZE (AGE) AND MEDIUM COMPOSITION

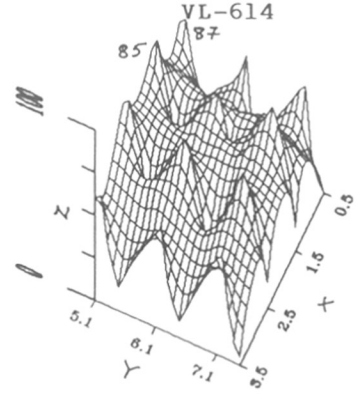
NI-5439



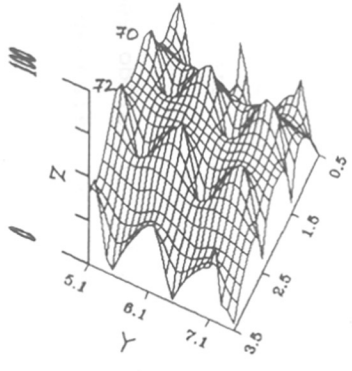
NI-9272



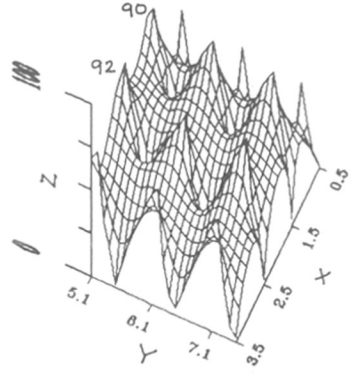
VL-614



CPAN-2005



Raj-1777



LEGENDS TO FIGURES

X-axis : Size of Inflorescences.

Y-axis : Medium Used.

Z-axis : Percentage of Somatic Embryogenesis.

Rhodes *et al.* (1986) have suggested that the correlation between the size of inflorescence and the embryogenic response may be obscured because size is only an approximate measure of the developmental stage. The actual degree of developmental overlap of somatic tissues is however, subject to the genotype, individual plant and environmental variation.

Several media were used to asses the response of the explants to various hormones. The best response was observed at a concentration of 2 mg/l (medium no. 1.2 - Table 14; Chapter IV) in all the genotypes (Fig. 41). Subgroups of medium no. 2 included 2,4-D (2 mg/l) in combination with several concentrations of the cytokinin kinetin (Table 14; Chapter IV). Although the frequencies of somatic embryogenesis in these media were lower than those on media nos. 1.2 or 1.3, the plants produced from somatic embryos induced in the subgroups of medium no. 2 (containing 2,4-D as the auxin and kinetin as the cytokinin) appeared to be healthier (on a qualitative basis) and survived better than those produced on medium no. 1 (which contained no cytokinin in addition to the auxin 2,4-D). Media listed under no. 3 (Table 14, Chapter IV) contained the cytokinin BAP in addition to 2 mg/l 2,4-D and induced lower frequencies of somatic embryogenesis than either of those under nos. 1 or 2. Moreover, on the BAP containing medium, an increased amount of friable callus was produced, which rapidly outgrew the E callus. Thus, E calli produced on this set of media could not be maintained for a period longer than two months.

In the present study, genotypes obviously differed in the frequencis of somatic embryogenesis under the same

cultural conditions. However, the morphological and histological nature of the process of somatic embryogenesis and the basic growth behavior patterns were more or less similar between the five genotypes (as seen in Fig. 41). Moreover, the embryogenic tissue formed was morphologically similar to the immature embryo derived cultures. Such a likeness between the immature inflorescence and immature embryo derived cultures has also been described earlier by Armstrong and Green (1982) and Rhodes *et al.*(1986). This is a direct indication that immature inflorescences are capable of displaying the same *in vitro* response as immature embryos.

Somatic embryogenesis as described here for immature inflorescences strongly resembles that induced from immature embryos. The process is seen to be of the indirect type (involving a callus interphase) and the somatic embryos formed were atypical in nature i.e. they showed certain differences from the zygotic embryos - as described in Chapter III. However, the phenomenon of precocious germination was not observed with the embryoids derived from the immature inflorescence. Hence, this embryogenic callus could be maintained for upto 12 months with very little loss of regeneration potential.

5.2.2 Mature Embryos

Surface sterilized seeds were soaked overnight in sterile distilled water. Embryos were dissected out under the stereomicroscope and cultured with two aims : to induce somatic embryogenesis and to induce microtillering.

5.2.2.1 Induction of somatic embryogenesis

Experiments were carried out in petri dishes (55 mm diameter), each containing 10 embryos. Embryos were inoculated with the scutellum facing downwards on the medium and the embryo axis facing upwards. Two replicates (petri dishes) were made for each medium. The media tested included media numbers 1, 2, 3, 5, 6 and 7 (from Table 14; Chapter IV). Incubation was carried out in the dark at 27^{o}C for 25-30 days. Calli showing indications of morphogenesis were maintained by subculturing at 15 day intervals to media containing lower hormone concentrations (1mg/l auxin and 1mg/l cytokinin corresponding to that in the induction medium).

No special maturation medium was used. For conversion, calli were shifted onto MS medium supplemented with 1mg/l kinetin and incubated under a 16h photoperiod regime. For shoot and root elongation, regenerating calli were transferred onto filter paper supports in liquid MS medium containing half the normal concentration of MS salts.

Mature embryos inoculated on the auxin (2,4-D or dicamba) containing media (Nos. 1 and 5 respectively - Table14; Chapter IV) produced two types of calli. The first type, which was more common, was derived exclusively from the root region of the embryo axis and could be distinguished by its friable and watery nature. This callus was non - embryogenic in nature and was associated with the

TABLE 17 : GENOTYPIC INFLUENCE ON THE *IN VITRO* RESPONSE OF MATURE EMBRYOS OF WHEAT

Media - MS + 2mg/l 2,4-D (Medium no. 1.2)
- MS + 2mg/l dicamba (Medium no.5.2)
Temperature - 27 ± 2°C
Photoperiod - 16h
Light Intensity - 11.7 uE $m^{-2}s^{-1}$

Genotype	Number Of Embryos Inoculated	Number Of Embryos Showing Precocious Germination		Number Of Embryos Showing Response		Number Of NE Calli Formed		Number Of SE/E Calli Formed		Percentage Of Regeneration From E Calli	
	Medium No.->	1.2	5.2	1.2	5.2	1.2	5.2	1.2	5.2	1.2	5.2
1.NI-5439	150	50	46	53	50	48	45	5	5	40.0	60.0
2.NI-9272	150	25	30	41	38	35	30	6	8	33.3	50.0
3.VL-614	150	80	82	85	56	80	82	5	4	40.0	50.0
4.CPAN-2005	150	87	80	90	92	82	85	8	7	62.5	55.5
5.Raj-1777	150	90	87	95	95	88	80	7	15	42.8	66.6

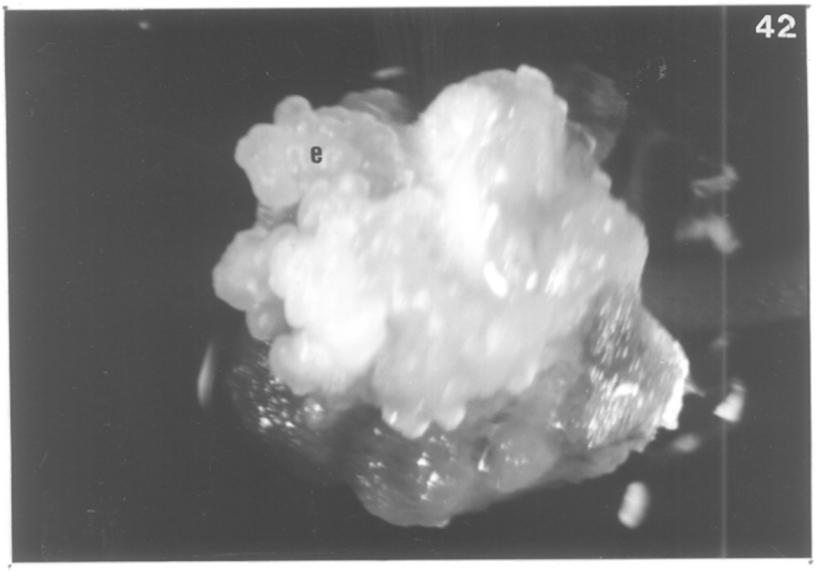
original shoot apex which either remained intact upto two months of culture or underwent precocious germination. The viability of the intact shoot apex could be demonstrated on transfer to the basal medium where the shoot elongated while the friable callus underwent rhizogenesis. No plantlet regeneration via organogenesis or somatic embryogenesis could be demonstrated from this type of callus, although it had a high capacity for rhizogenesis. Such a type of callus has been reported in wheat by Ozias - Akins and Vasil (1983) and McKinnon *et al.* (1987); in the former report, this was the only type of callus that could be initiated from mature embryos.

The second type of callus was derived from the shoot region and could be characterized by its smooth and compact (SE) appearance. This callus, after 2 - 3 subcultures, gave rise to embryogenic (E) callus which was recognized by the presence of embryoids on its surface (Fig. 42). However, the E/SE callus was invariably found to be associated with NE callus which arose from the root regions of the same embryo axis. The scutellum of mature embryos could never give rise to callus, irrespective of the medium used or the orientation of the explant on the medium.

The above described response has been observed exclusively on media nos. 1.2 and 5.2 (Table 14; Chapter IV) containing 2,4-D (2 mg/l) and dicamba (2 mg/l) respectively.

Genotypic variation in the *in vitro* response was also evident in the present study (Table 17). The percentages of precocious germination of the mature zygotic embryos of the five selected genotypes were much higher than those observed earlier in the experiments with immature embryos (Chapter

FIG. 42 : Mature embryo derived callus of genotype Raj-1777.


42
e

III). However, surprisingly, precocious germination of the somatic embryos was not evident in the embryogenic callus derived from mature embryos and hence, such callus could be maintained for upto 14 months with subculturing. The superiority of either auxin in the formation of E callus cannot be stated, since in genotypes VL-614 and CPAN-2005, 2,4-D (medium no. 1.2) induced a higher number of calli (Table 17), while in genotypes NI-9272 and Raj-1777, dicamba (medium no. 5.2) proved to be better. In the genotype NI-5439, the response towards E callus formation was identical with the use of either 2,4-D or dicamba *viz.* either medium no. 1.2 or 5.2 respectively. However, the frequency of plantlet regeneration was higher in all the genotypes from the E calli initiated on medium no. 5.2 (containing dicamba). Plantlets elongated within 10 - 15 days in the liquid, half - strength MS medium and were then hardened and finally potted.

On media containing lowered concentrations of the auxin (media nos. 1.1 and 5.1 - Table 14; Chapter IV), the frequencies of precocious germination of the inoculated embryos increased to 100% in all the genotypes. Correspondingly, the percentage of embryogenesis decreased to 1 - 4% . This was also seen to be the case in media containing a combination of an auxin (either 2,4-D or dicamba at 2 mg/l concentration) with a cytokinin (either BAP or kinetin at varying concentrations), *viz.* all the media under nos. 2,3,6 and 7 (Table 14, Chapter IV). Hence these media were not used in further experiments.

On the other hand, on media containing higher concentrations of the auxin - either dicamba or 2,4-D (media nos. 1.3,1.4,1.5,5.3,5.4 and 5.5), the percentage of

responsive embryos decreased since either auxin (2,4-D or dicamba) at higher concentrations inhibited any response from the shoot-root regions of the embryo axis. In fact, in media nos. 1.4 and 5.4 (10 mg/l of 2,4-D and dicamba respectively) and 1.5 and 5.5 (20 mg/l of 2,4-D and dicamba respectively), the number of responsive embryos was reduced to nil. These results established the superiority of media nos. 1.2 and 5.2 (containing 2 mg/l of 2,4-D and dicamba respectively) for eliciting an embryogenic response from mature embryos of wheat.

5.2.2.2 Induction of microtillering

Experiments were carried out in 250ml conical flasks containing 25ml liquid medium. Several combination of cytokinins were tested as follows :

[Medium M1] : MS + kinetin (1mg/l)

[Medium M2] : MS + BAP (1mg/l)

[Medium M3] : MS + kinetin (1mg/l) + BAP (1mg/l)

[Medium M4] : MS + kinetin (2mg/l) + BAP (2mg/l)

[Medium M5] : MS + kinetin (3mg/l) + BAP (3mg/l)

[Medium M6] : MS + kinetin (4mg/l) + BAP (4mg/l)

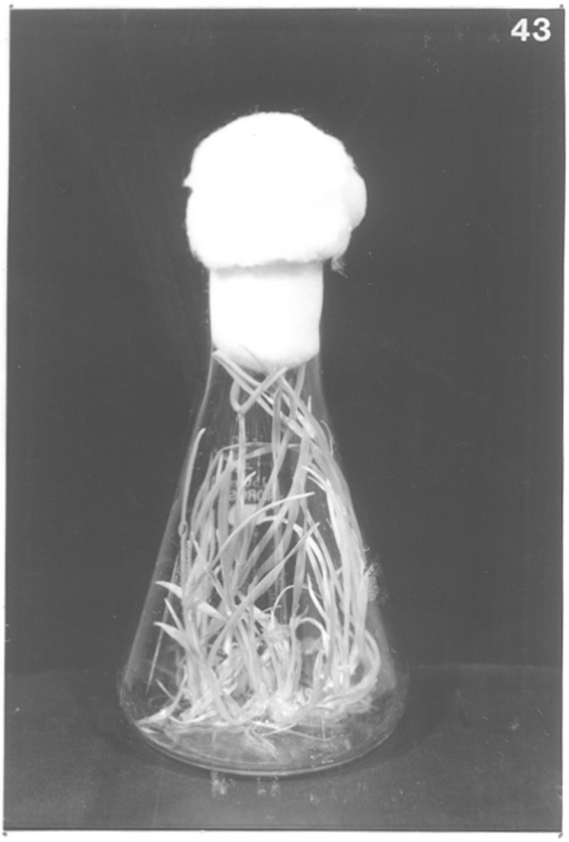
[Medium M7] : MS + kinetin (5mg/l) + BAP (5mg/l)

All the five selected genotypes were screened for microtillering *in vitro*. Five zygotic embryos were inoculated in each flask and these were incubated at 27^{o}C on a rotary shaker at a speed of 70 rpm. During subculture, the original shoot base (which is associated with several tillers), was dissected into clumps of 2 - 3 shoots and each clump was inoculated into fresh liquid medium after trimming the apical tips.

On inoculating mature embryos into the liquid medium, elongation of the shoot axis occurred within 2 - 3 days in all the media used. Tillering from the basal shoot node began after 7 - 10 days. This tillering is believed to be initiated by the derepression of the previous shoot meristems (King *et al.* 1978, Green 1982), which results due to the presence of cytokinins in the medium. At this stage, 2 - 5 tillers were produced, depending on the genotype X medium combination. Maximum number of shoots were produced from the 6th upto the 15th subculture (Fig. 43). The average number of shoots from three flasks of each medium and each genotype are indicated in Fig. 44. As seen in the

FIG. 43 : Microtillering in genotype Raj-1777.

FIG. 44 : Genotypes :
A : NI-5439 C : VL-614 E - Raj-1777
B : NI-9272 D : CPAN-2005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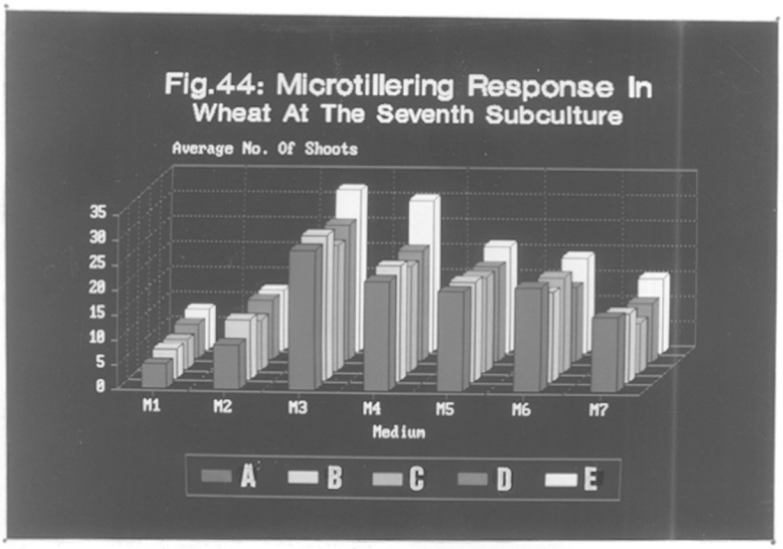


Fig.44: Microtillering Response In
Wheat At The Seventh Subculture
Average No. Of Shoots
35
30
25
20
15
10
5
0
M1
M2
M3
M4
M5
M6
M7
Medium
A
B
C
D
E

figure, the maximum number of shoots were produced on medium M3 containing kinetin and BAP at a concentration of 1 mg/l each. Each cytokinin on its own (Media M1 and M2) was not as effective as in combination. However, at higher concentrations of the cytokinins (Media M4,M5,M6 and M7), not only was the rate of axillary bud proliferation lowered, but occasionally also led to the formation of albino shoots with wavy margins and mottled laminae.

Tanzarella and Greco (1985) have reported the formation of a single sterile spikelet in wheat cultures derived from shoot base explants. In the present study, such spike differentiation has not been observed. However, this system of microtillering can be of immense use for the large scale propagation of interspecific hybrids, aneuploids, haploids, male-sterile lines, beneficial somaclonal variants, transformed, etc. which cannot be easily propagated by seed or simply because multiplication *in vitro* is much faster and more efficient than that by seed.

5.2.3 Root Tips

Seeds of the five selected genotypes were germinated as described for leaf bases. About 8 - 10 root tips were cultured per petri dish (55mm diameter). Incubation was carried out in the dark at 27^{o}C for 25-30 days. Three replicates (petri dishes) were made for each parameter. Three main sources of variation were investigated *viz.* age of the root, genotype and the type, concentration and combination of the hormone used in the medium.

5.2.3.1 Age of the root

Root tips from 3, 4, 5, 6 and 7 day old seedlings were cultured on MS medium supplemented with 2mg/l 2,4-D for their callus forming capacities. The response of the root tips was more or less constant at the different ages (data not shown since the differences in the response were not significant). This response possibly could be due to the fact that at all the chosen plant ages, the physical state of the root tips is similar i.e. the cells were at a highly meristematic state of rapid division and hence the interaction of the explants at all ages with the medium constituents was similar.

On inoculating root sections on the above medium, it was observed that only the lateral root tips (5mm in length) could initiate callus formation after 12 - 15 days, while the older differentiated regions would not produce callus even on prolonged culture at any concentration of the auxin used. This indicated that as in the case of cereal leaves (Wernicke *et al.*1982), a distinct gradient exists in roots with the root tip being the most meristematic region of the root and thereby exhibiting a high sensitivity towards the auxin in the medium. To the best of my knowledge, such a

gradient towards auxin has not been described in cereal roots, although organogenesis and somatic embryogenesis has been reported in rice root tips (Abe and Futsuhara 1984, Kavi Kishor and Reddy 1986, Zimny and Lorz 1986). Thus, a limited amount of work has been carried out with root tips - this despite the fact that they may possibly be the best sources of meristematic tissues due to their physical isolation from other organs of the plant, and thereby ruling out the carry over of other tissues along with the explant.

5.2.3.2 Effect of genotype and type, concentration and combination of hormones in the induction medium

4 day old root tips were used for this purpose. The media tested were numbers 1, 2, 3, 5, 6 and 7 (Table 14, Chapter IV) containing combinations of auxins (2,4-D and dicamba) and cytokinins (kinetin and BAP). Calli developing in media numbers 1.2 and 5.2 (containing 2 mg/l 2,4-D and dicamba respectively) were maintained by subculturing every 15 days. For regeneration, the media used were the same as those described for leaf base cultures *viz.* media R_1, R_2 and R_3.

The response of the root tips differed on the various media used. On media containing combination of an auxin (2,4-D/dicamba) with a cytokinin (kinetin /BAP) viz. on media nos. 2,3,6 and 7, the explant rapidly underwent necrosis, turned brown in color and could not callus even on prolonged culture. On media containing only auxins viz. either 2,4-D or dicamba (media nos. 1 and 5), callusing was evident only at low concentrations of the auxin i.e. only in media nos. 1.1 and 5.1 (1 mg/l of dicamba and 2,4-D respectively) and 1.2 and 5.2 (2 mg/l of dicamba and 2,4-D respectively). However, at 1 mg/l concentration of either

auxin (media nos. 1.1 and 5.1), some of the root tips rapidly underwent elongation without callusing. Hence media nos. 1.2 and 5.2 were identified as being ideal for callus formation.

A distinct genotypic influence on the callusing response was evident on media nos. 1.2 and 5.2 (Fig. 45), with the genotype Raj - 1777 exhibiting maximum callusing capacity (72% and 68%) on media nos. 1.2 and 5.2 respectively and the genotype NI - 5439 showing the least frequencies (50% and 45% respectively on the corresponding media). However, the superiority of dicamba over 2,4-D or vice versa could not be established because genotypes NI-5439, VL-614 and Raj-1777 elicited a better response with the use of 2,4-D; while the remaining two genotypes NI-9272 and CPAN-2005 exhibited a higher frequency in the medium containing dicamba.

5.2.3.3 Nature of callus formed

The callus formed on media 1.2 and 5.2 was highly mucilaginous, watery and translucent during the initial stages of culture (Fig. 46). After 2-3 subcultures on the same medium, the callus grew to 5-7 mm in diameter, turned pale yellow in color and consisted of nodular outgrowths (Fig. 47). However, these could not be stated with certainty to be somatic embryos since they did not form plantlets on transfer to the regeneration medium. The regeneration media used were R_1, R_2 and R_3 viz. the same as those used for leaf base cultures (Table 16; Chapter IV). On transfer to these media, some of the calli underwent complete browning, yet others browned partially, while the rest grew initially, forming more callus. After 10 - 12 days of culture, the last set of calli occasionally

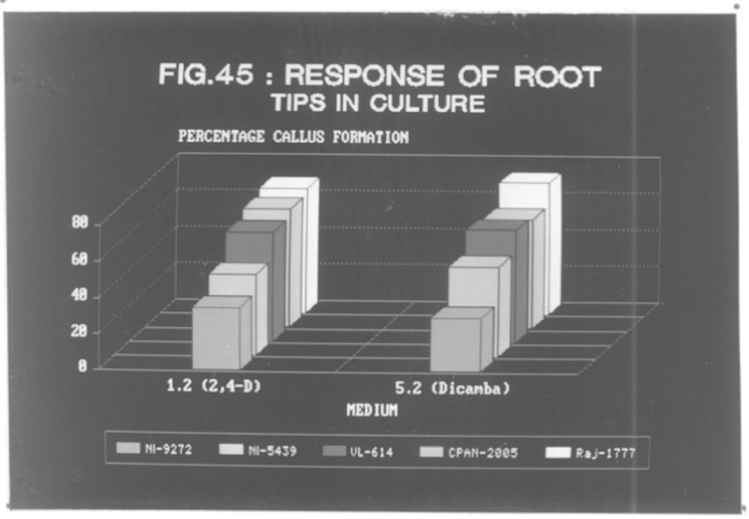
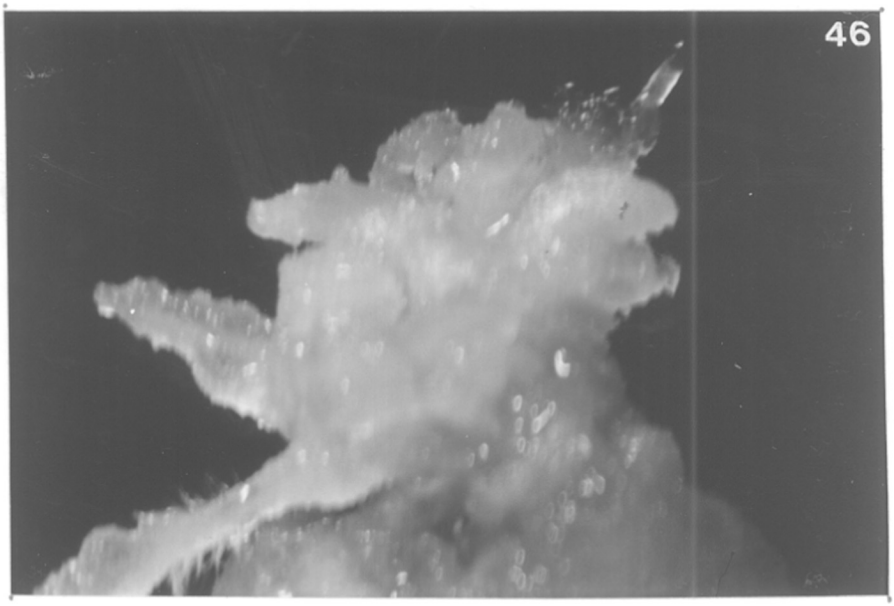


FIG.45 : RESPONSE OF ROOT
TIPS IN CULTURE
PERCENTAGE CALLUS FORMATION
80
60
40
20
0
1.2 (2,4-D)
5.2 (Dicamba)
MEDIUM
NI-9272
NI-5439
VL-614
CPAN-2005
Raj-1777

FIG. 46 : Mucilaginous, root tip derived callus of genotype Raj-1777.

FIG. 47 : Nodular, root tip derived callus of genotype Raj-1777.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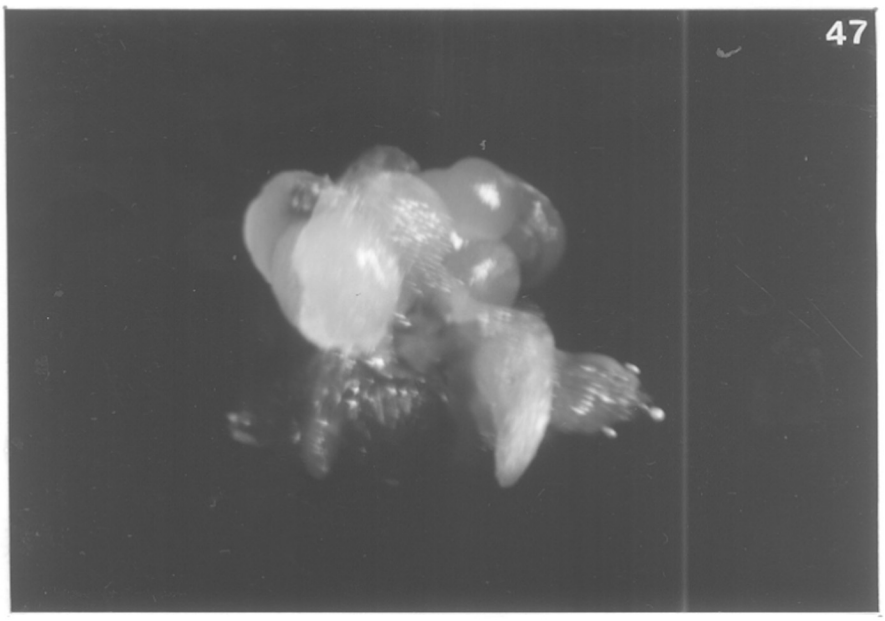
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developed green pigmented spots on their surface. Root initiation without any association with shoots or shoot primordia was frequent on the regeneration media. The response was similar on all the regeneration media.

Thus, in the present study on root tips, although the putative early stages of somatic embryogenesis were observed (as manifested by the formation of nodular structures on the surface of the callus), no complete plantlet regeneration via somatic embryogenesis, or further , by organogenesis could be achieved. Moreover, the presented observations suggest that most of the calli obtained in the presence of the auxin were merely abnormally proliferating root primordia which expressed themselves via rhizogenesis on transfer to the auxin - free regeneration media. Similar patterns have been described earlier for a number of cereal cultures like maize, wheat and sorghum (Cure and Mott 1978, Wernicke *et al.* 1982).

5.4 Conclusion

From the ongoing discussion, it is realized that embryogenic competence of the different explants i.e. the ability to respond to embryogenic induction, depends largely upon the degree of meristem commitment to organ formation and on the degree of differentiation that has occurred. This probably may be because the developmental stage of the cells in a plant is controlled by a set of signals from the surrounding tissues (Maksymowyck 1973).

Immature embryos are the most frequently used explants for initiation of wheat callus cultures. However, the use of immature embryos is limited, because, this explant is available in field only over limited periods of the year, or the donor plants have to be grown in growth chambers or greenhouses with extra effort. Also, as has been described earlier, immature embryos exhibit high frequency embryogenesis only at a specific development stage, which further narrows down the sampling period. Moreover, in cereals, embryogenic cultures cannot be maintained over long periods of time without loss in embryogenic potential. Hence, the importance of explants other than the immature embryo for establishing reproducible *in vitro* regenerative systems cannot be underestimated.

CHAPTER VI : ESTABLISHMENT OF CELL SUSPENSION CULTURES

6.1 INTRODUCTION

The development of *in vitro* systems in cereals from partially or fully differentiated cells has been made a possibility in the last ten to fifteen years. For this realisation, the fact exploited has been the totipotency of differentiated cells to revert back to their meristematic state. However, this reversion potential to undergo "dedifferentiation" is often seen to be repressed due to one of the following reasons:

1. Genetic - this involves a total lack of totipotency. This is the reason underlying the differential response of various genotypes in culture and high degree of variability involved in regeneration (Christianson 1987).
2. Epigenetic - this involves constraints on the functioning of genes required for organ/embryo formation and may be due to nuclear changes (polyploidy, aneuploidy, mutation, etc.) which commonly occur in cultured cells (Smith and Street 1979).
3. Physiological - this involves either a lack of environmental signals e.g. the absence of hormones in optimum ratios and concentrations, presence of inhibitors, etc., or a high degree of sensitivity of cultured cells to exogenous growth substances (Fridborg *et al.* 1978, Drew 1979). The last type of repression *viz.* that of a physiological block is the basic assumption that researchers make when faced with recalcitrant material. Further, it is just a matter of finding the right combination to bring out its capacity. However, callus cultures are believed to harbour mixtures of cell types at different levels of

totipotency. This fact often creates problems when a uniform source of cells is required e.g. in somatic hybridisation or genetic transformation studies.

Plant cell suspension cultures provide the biochemist with a relatively homogenous population of cells readily accessible to exogenously applied chemicals and growing under defined, aseptic conditions. Further, the lack of chlorophyll and carotenoid pigments in most plant cell suspensions is of great benefit for work involving isolation of enzymes or secondary products (Scragg and Fowler 1984).

Although it had been hoped that the growth of plant cells on liquid cultures could be carried out in as simple a manner as microbial cultures, it is not the case, because of the differences in structural and physiological properties. Plant cells are much larger than microbial cells, have much lower metabolic rates and are surrounded by a cellulose based cell wall. The latter is considered to have a high tensile strength but low shear resistance. Moreover, plant suspension cultures are, strictly speaking, not "single" cell cultures, but more often, consist of small clumps or aggregates of rapidly dividing cells.

Anatomical studies of tissue growth in suspension cultures have revealed that a culture comprises of two cell types :

(a) large, highly vacuolated cells, freely dispersed in the medium either singly or in clusters and normally lacking embryogenic potential.

(b) fairly small and densely cytoplasmic cells, capable of forming somatic embryos. These always occur in clusters. These cell clusters capable of embryoid formation have been termed as "proembryogenic masses" by Halperin (1966) and "embryogenic clumps

(EC)" by McWilliam *et al.*(1974).

In the auxin containing medium which does not allow the development of mature embryos, the EC's perpetuate by cell proliferation and fragmentation. The central cells have a single large vacuole, small and compact nucleus, faintly staining nucleolus, low population of mitochondria, little or no spherome - like vescicles, low dehydrogenase activity and a reduced number of amyloplasts. On the periphery of the EC, groups of highly meristematic cells are present. In contrast to the central cells, these are characterised by having several small vacuoles, a large diffusely staining nucleus, a single prominently staining nucleolus, high density of ribosomes, numerous endoplasmic reticulum profiles, normal mitochondria, spherome-like vescicles, high dehydrogenase activity and prominent amyloplasts.

On the disintegration of an EC, which occurs due to enlargement and separation of the central cells, the meristematic periferal cells dissociate into groups and each of them develops into a new EC. However, if these groups of meristematic cells or EC's are sieved off and transferred to an auxin-free medium, numerous embryos arise from their perifery. Each embryo is probably derived from a single cell. Kohlenbach (1978) suggests that EC's in carrot may be regarded as homologous to a single disorganised embryo, and those arising from it as adventitious embryos. Somatic embryos are delimited very early from the parent tissues of the EC by the cutinisation of its outer surface, but they are released from the clumps at various stages of development - generally after the globular stage (McWilliam *et al.*1974). Their further development occurs as free floating structures.

A limited amount of work has been reported in cereal suspension cultures (reviewed earlier in Chapter I). The major limiting factor has been the compact and highly organised nature of the E calli of these species which makes it extremely difficult to establish finely dispersed cell suspensions. The use of sugarcane cell suspension cultures has been of immense value in developmental, biochemical, cytological, mutational and pathological studies (Heinz *et al.* 1977), as well as for the propagation and improvement of the species by mutant selection (Heinz 1973, Liu *et al.* 1977). Further, it is speculated that such applications of suspension cultures may be extended to all the major cereals.

In the following chapter, the establishment and characterisation of suspension cultures in wheat was undertaken to obtain a better understanding of the competence of the genotype Raj - 1777, to *in vitro* conditions, since this genotype had consistently given a better response over the others.

6.2 RESULTS AND DISCUSSION

6.2.1 MEDIA

The basal medium used during the course of studies on suspension cultures was the MS medium (Chapter II). Various additions to this basal medium at different stages *viz.* during induction, maintenence, callus regeneration and plantlet regeneration were as listed in Table 18.

6.2.2 Primary culture initiation and maintenence :

The most often source cited in literature for the initiation of cereal suspension cultures is in friable embryogenic callus (as in sorghum - Brar *et al.* 1979; pearl millet - Vasil & Vasil 1981; wheat - Ahuja *et al* . 1982; and maize - Vasil & Vasil 1986), nodular embryogenic callus (pearl millet - Vasil & Vasil 1982; sugarcane - Ho & Vasil 1983; napier grass - Chandler & Vasil 1984; orchard grass - Gray *et al.* 1984 and wheat - Inagaki *et al.* 1988). Occasionally, suspension cultures have also been initiated directly from explants such as embryos (orchard grass - Gray *et al.* 1984 and wheat - Wang *et al.* 1988). Recently, the use of aged (by repeated subculturing), embryogenic calli has been reported in wheat (Wang & Nguyen 1990; Redway *et al.* 1990), as an excellent source for initiating embryogenic suspension cultures).

In the present study, the potential of several sources of inoculum for initiating suspension cultures was checked. These included the following :

1. Friable callus obtained from immature embryos, leaf bases and root tips on Medium 1.2 (Table 14 - Chapter IV).
2. Aged, compact, nodular, embryogenic callus obtained from immature embryos.

TABLE 18 : MEDIA USED FOR THE ESTABLISHMENT OF SUSPENSION CULTURES

MEDIA Nos.	ADDITIVES						
	2,4-D	Ki	CAA	AgNO3	ABA	Sucrose	Agar
I1	2mg/l	-	100mg/l	-	-	3%	-
M1	0.5mg/l	2mg/l	25mg/l	-	-	3%	-
CR1	2mg/l	-	100mg/l	-	-	3%	0.4%
CR2	2mg/l	-	100mg/l	10mg/l	-	3%	0.4%
CR3	2mg/l	-	100mg/l	-	10ug/l	3%	0.4%
PL1		1mg/l	-	-	-	2%	0.4%
PL2		1mg/l	-	-	-	2%	0.4%
PL3	0.5mg/l	1mg/l	-	-	-	2%	0.4%

Cultural conditions during incubation at different stages of culture are described in the text.

3. Explants used directly included immature embryos, immature inflorescences, leaf bases and root tips. Approximately 15 days after culture initiation on medium I-1 (Table 18), 10 ml of the supernatent medium was removed and replaced with an equal volume of fresh medium of the same composition. This procedure was regularly repeated every week for 30 - 60 days to reduce the size of cell aggregates and also, to reduce the percentage of non-dividing cells. Further, mechanical separation by sieving (through 250 µm sieves) was applied to obtain a more homogeneous suspension. After 75 - 90 days of culturing in the I-1 medium, cultures were maintained by adding an equal amount of fresh maintenence medium (M-1 : Table 18), to the suspension, once every week.

6.2.2.1 From friable callus initiated from immature embryos, leaf bases and root tips :

Calli were cut up finely and 2 - 3g tissue was inoculated into 100 ml flasks containing 15 ml I-1 medium (Table 18). The subculturing protocols for these suspensions were as described above.

The friable calli from immature embryos and root tips were slightly mucilaginous in nature. This mucilage is believed to form a semi-permeable barrier between the cell clumps and the medium, while at the same time, promoting the survival of cells and cell division (Abe and Futsuhara 1986). The presence of mucilage, in the present study, however, was a hindrance in the dispersion of cell clusters, which were thus propagated as large cell clumps. Similar mucilaginous substances have been described in *Pennisetum purpureum* (Haydu and Vasil 1981), *Pennisetum*

americanum (Vasil and Vasil 1982), *Avena sativa* (Heyser and Nabors 1982), *Sorghum bicolor* L. (Wernicke *et al.* 1982) and *Dactylis glomerata* (Gray *et al.*1984). The production of this mucilaginous substance has been correlated with decreased embryogenic potential by these workers. However, Gray *et al.* (1984) suggest that the mucilaginous callus in *Dactylis glomerata* is related with latent embryogenecity, which is expressed after several subcultures. Although the exact composition of this mucilage is unknown, it is believed to contain pectinaceous substances (Conrad *et al.* 1982), as well as polysaccharide (Gray *et al.* 1984).

In the present study, repeated subculturing gave rise to two types of cells in suspension - large, elongated, highly vacuolated cells which were present either singly or in aggregates of 12-16 cells; and small, compact and densely cytoplasmic cells which were present as large clumps (Fig. 48). The size of these clumps could not be decreased significantly even by repeated subculture, while sieving through sieves (250 µm diameter) completely eliminated them from culture.

The friable callus derived from 3 day old leaf bases was of the non-embryogenic type. The suspension cultures initiated from this callus were seen to consist of both the types of cells described above. However, the smaller, richly cytoplasmic cells did not form very large clusters (as in the previous case), but clumps of 8-10 cells were produced. On repeated subculture and application of mechanical sieving for 3-4 months, the suspension could be enriched with this type of cell clumps.

FIG. 48 : Large clumps of embryogenic cells derived from friable callus initiated from immature embryos. Magnification = 650X.

6.2.2.2 From aged, compact, nodular, embryogenic calli obtained from immature embryos :

The calli selected for initiating suspension cultures were those which did not exhibit precocious germination (as has been described in detail in Chapter III). These had been maintained by repeated subculture on a lowered auxin medium (basal MS medium containing 0.5 mg/l 2,4-D) for 4-6 months. The calli were inoculated in a manner similar to that employed with friable calli in I-1 medium. Further subculture protocols were as described in section 6.2.2.1.

Aging of calli prior to the initiation of suspensions has been described recently in wheat (Redway *et al* 1990). In the present study, the use of callus cultures aged by repeated subculture for 4-6 months on a low auxin - containing medium gave rise to two types of responses in suspension after 30 - 60 days :

1. In addition to the larger calli pieces (carried over from the calli used for culture initiation), the two types of cells described above (in case of friable leaf base calli) were also observed. However, in the present case, the size of the cell aggregates was reduced to clumps of 4-6 cells. The larger callus pieces could be eliminated by mechanical sieving. On repeated subculture for 3-4 months, the suspension could be enriched with this type of cell clusters.
2. Some calli failed to dissociate in liquid culture, and sloughed off only large, elongated, vacuolated and non-dividing cells. On repeated subculture, the calli pieces in these cultures either turned brown and necrotic within 60 - 75 days of culture initiation or occasionally gave rise to shoots. No improvement in

the quality of the suspension ensued on repeated subculture for 4 months; hence, after this period, these type of cell suspensions were discarded.

6.2.2.3 From explants directly inoculated into liquid medium for initiation :

The explants were cut up finely and inoculated into 100 ml flasks containing 15 ml culture initiating medium (I-1 : Table 18). The ages of the explants selected for culture were as follows :

Immature embryos : 12-14 days after anthesis : 10-15 embryos per 100 ml flask.

Immature inflorescences : within a size range of 11 - 30 mm 2-3 inflorescences were inoculated per 100 ml flask.

Leaf bases : from 3 day old seedlings : 10-15 leaf bases per 100 ml flask.

Root tips : from 3 day old seedlings : 25-30 root tips per 100 ml flask.

The process of culture initiation as well as the nature of the final suspension formed varied considerably in all these cases as follows :

[I] Immature embryos -

Within 15 days of culture initiation, callus developed from all the embryos inoculated in the liquid I-1 medium. This callus was seen to arise from the scutellum as well as the embryo axis, and by the first subculture, such calli were almost 5-8 mm in diameter. These calli consisted of a firm and hard centre around which, several layers of loosely packed cells were present. These layers of cells gave the calli a loose and friable appearence. On further subculturing in M-1 medium, only these surface layers were sloughed off. However, these consisted only of the large, elongated, highly vacuolated, non-embryogenic cells.

Thus, the cultures derived from immature embryos consisted mainly of large calli, at the core of which lies the embryonic region. Surrounding this initial explant material, are the large non-embryogenic cells described above, which either occur singly, or in clusters of 4-6 cells each, and which undergo irregular divisions. Rarely, an occasional cluster of embryogenic (small, round and densely cytoplasmic) cells were also seen. Repeated subculturing could not succeed in enriching the latter type, which is desired. Hence, after 6-7 months, these suspension cultures were discarded.

[II] Immature inflorescences and leaf bases :

Both these explants exhibited a similar type of response *in vitro*. Most of the smaller pieces (obtained after cutting up the explant finely) turned brown within 7-10 days and necrosed in culture. These pieces were removed from the liquid medium during the first subculture in M-1 medium. The remaining relatively larger pieces turned white in color and produced a compact callus after 3-4 subcultures (45 - 60 days). The rate of growth of these calli was very slow as compared to those initiated in liquid from immature embryos. After four months of subculture, these calli rarely exceeded 7-8 mm in diameter. Moreover, they failed to dissociate in the liquid medium and sloughed off limited amounts of the cell layers, which were, almost always, of the non-embryogenic type. Hence, finely dispersed cell suspensions could not be initiated from both these explants. On observing that even repeated subculturing in M-1 medium for six months did not increase the biomass in culture significantly, these cultures were discarded.

[III] Root tips :

Initially, the development of root tips in liquid media occurred in a manner similar to that in immature embryos. Within 15-20 days of culture, callus developed from 70-80% of the inoculated explants, while the remaining explants turned brown and necrosed. Upto the 5th - 6th subculture, (approximately two months), the calli consisted of a firm and hard centre around which a loose, friable callus was formed. This friable callus rapidly dissociated into the liquid medium. With repeated subculturing for 3-4 months and mechanical separation of the larger calli, a finely dispersed cell suspension was produced.

The cell suspension thus produced from root tips was a mixture of three types of cells -

(i) The non - embryogenic cells described earlier.

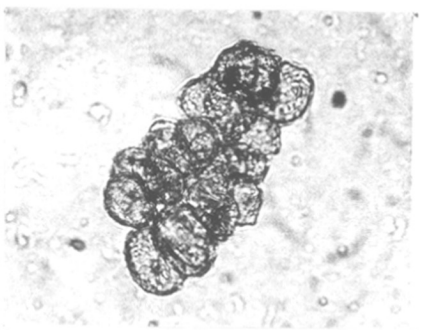
(ii) Clusters of 6 - 10 embryogenic cells (Fig. 49).

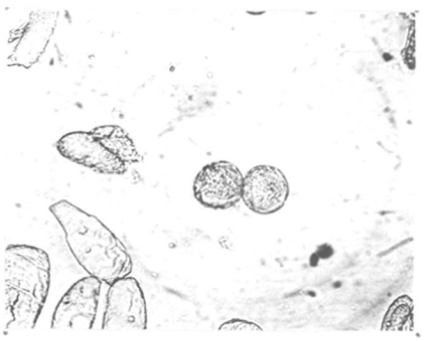
(iii) Occasionally observed single or paired, spherical, thick - walled cells (Fig. 50).

The last group of cells possibly represent an intermediate stage between the conversion of the embryogenic type of cells which may escape from the cell clumps, into the non-embryogenic cell type. These single - celled "escapees" later turn into non-embryogenic cells by rapid elongation, formation of thick walls, gradual loss of starch and irregularities in cell division. Such conversions of embryogenic to non-embryogenic cells is also indicated from the fact that a suspension free of the slow growing non-embryogenic cells and containing only the rapidly multiplying embryogenic ones could not be achieved by any changes in the subculturing frequencies or manipulating the

Fig. 49: Clusters of embryogenic cells in root tip derived cell suspensions.
Magnification = 650X

Fig. 50: Cells at on intermediate stage during reversion to non-embryogenic type.
Magnification = 400X





culture medium. Vasil and Vasil (1986) have also shown that in maize suspension cultures, 10 - 20% of the embryogenic cells in culture become non - embryogenic. The ratio of conversion of the embryogenic cells to non - embryogenic ones also reflects on the growth and rate of biomass increase in the cell suspensions.

6.2.3 Characterisation of cell suspensions :

Cultures were regularly inspected for changes in morphology of the cells/cell aggregates. In the root tip - derived suspension cultures, occasional somatic embryo - like structures appeared after the 10^{th} subculture (Fig. 51). The formation of such embryoid - like structures have been reported earlier in several cereals like pearl millet (Vasil & Vasil 1981), sugarcane (Ho & Vasil 1983), orchard grass (Gray *et al.* 1984), rice (Abe & Futsuhara 1986) and wheat (Wang & Nguyen 1990) suspension cultures. However, further maturation of the embryoid - like structures formed in the present study, did not occur, nor did their number increase on further subculture and maintenence.

Corresponding to changes in the morphology of the cultured cells, one flask was sacrificed at each subculture to monitor the changes in fresh weight at that particular time period. Cells were initially collected from 10 ml of a thoroughly agitated suspension and centrifuged at 1000 rpm for 5 minutes. The pellet was then blotted on a preweighed Whatman filter paper and weighed again. This data has been reported as fresh weight in grams.

As seen in Fig. 52, the rate of increase in fresh weight was slowest in case of the aged embryogenic calli derived from immature embryos (doubling in fresh weight was not evident even at the end of eight weeks - No. D in Figure). On the other hand, the rate of increase in the fresh weight was maximum in suspensions initiated from root tips (No. E in figure). In this case, the increase in fresh weight was almost 4 times by the end of the eight week. Suspensions derived from friable callus derived from leaf bases (No. C in figure) underwent a doubling in fresh weight

Fig. 51: Somatic embryo -like bipolar structure in root tip derived cell suspensions. Magnification = 150X

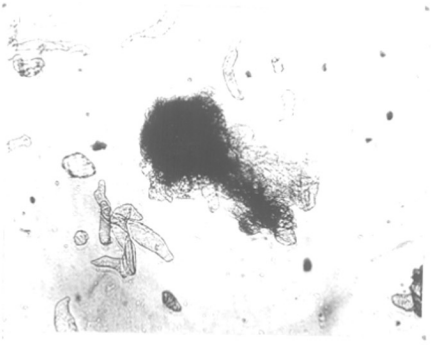
Fig. 52: A : Suspenssion cultures initiated from root tip derived friable callus.

B : Suspenion cultures initiated from immature embryo derived friable callus.

C : Suspension cultures initiated from leaf-base derived friable callus

D : Aged, embryogenic colli derived suspension cultures.

E : Root tip derived cell suspension



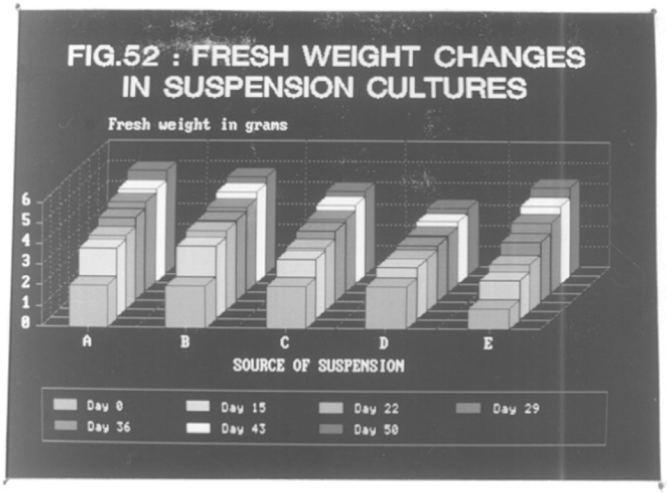


FIG.52 : FRESH WEIGHT CHANGES
IN SUSPENSION CULTURES
Fresh weight in grams
6
5
4
3
2
1
0
A
B
C
D
E
SOURCE OF SUSPENSION
Day 0
Day 15
Day 22
Day 29
Day 36
Day 43
Day 50

after eight weeks of culture initiation. The high rates of cell division in cultures initiated with friable callus derived from root tips and immature embryos (Nos. A & B respectively in figure) were caused by the use of inocula from stock cultures at the exponential phase of growth (day 3-4 after subculture). Such a rapid subculture regime is essential for preventing the formation of non - embryogenic cells (Vasil and Vasil 1982, 1984).

6.2.4 Regeneration from suspension cultures :

Prior to attempting regeneration, the subculturing frequency (using M - 1 medium) was increased to biweekly transfers, as compared to the weekly maintenance transfers. For callus regeneration, 1 ml of the suspension was either directly plated onto solid medium in petri dishes (55 mm diameter), or mixed with 10 ml molten agar medium (45-50^{o}C) and poured just prior to solidifying. Cultures were incubated in the dark at 27^{o}C for 4-5 weeks. Once calli were formed on this medium, they were transferred to various media for further growth. Here, cultures were incubated under a 16h light regime.

Thus, on plating suspensions initiated from leaf bases and aged embryogenic calli from immature embryos, within a period of 3-4 weeks, a lawn of soft, friable callus was formed on all the three plating media used (CR-1, CR-2 and CR-3 : Table 18) . The formation of such soft callus from suspension cultures has been described earlier in wheat by Redway *et al.* (1990). In the present study, no variation in the amount or nature of callus formed was obvious either on plating the suspension directly or mixing it with molten agar medium prior to plating. Further, no organisation of meristems to yield either organs or somatic embryos occurred even on transfer to the three plantlet regeneration media (PL-1, PL-2 and PL-3).

Suspension cultures initiated with the friable callus derived from immature embryos and root tips did not form an uniform lawn of soft callus on plating, as in described above. On the CR-2 medium, mini-calli (3-4 mm in diameter) were formed all over the inoculated plate. Morphologically, these were similar to the non-embryogenic calli described in

the earlier chapters. Further, these calli did not increase significantly in size nor did they undergo any further differentiation on transfer to the plantlet regenerating medium PL-1 (Table 18). Alterations in this medium by the addition of activated charcoal at 0.25% concentration to Medium PL-2) or inclusion of 2,4-D in lowered amounts (Medium PL-3) improved neither the nature of callus formed nor regeneration from the mini calli formed.

On plating suspensions derived directly from root tips after 2-3 weeks, in addition to the uniform lawn of soft and friable callus, mini calli developed on all the three callus regenerating media. However, only on CR-3 medium (containing 10 ug/l ABA), these mini calli increased in size and formed white, crystalline calli, while on the other two media, no significant differences in callus size or nature were observed. Transfer of these calli developed on the CR-3 medium to the plantlet regeneration media gave rise to three types of responses : 1. On PR-1 medium, the callus turned green in color. However, no further organisation of meristems to yield either shoot buds or a somatic embryo occurred.

2. On PR-2 medium, a majority of the calli underwent rhizogenesis and upto 20 roots per callus were formed. However, no caulogenesis was seen to occur.

3. On PR-3 medium containing 2,4-D and kinetin, an increase in callus accompanied by greening of the calli was observed. No further differentiation, however ensued, nor was there any change in the callus morphology. The somatic embryos observed occasionally in the root tip - derived suspension cultures were transferred (with a pair of forceps) onto the plantlet regeneration media. Approximately five somatic embryos were transferred to each

plate (55mm diameter containing 10 ml medium). Although these structures turned green in color within a week of culture, no plantlet regeneration was achieved.

6.3 CONCLUSION

In the present study, differentiation from suspension cultures was achieved upto the callus stage. Occasionally, somatic embryo - like structures were also formed in the root tip - derived suspensions. However, plantlet regeneration could not be achieved. In conclusion, this approach offers some general principles and strategies for the effective initiation and maintenence of competent cells. Further extending such a system upto the plantlet regeneration stage will increase its application in genetic transformation, mutant selection, protoplast fusion, etc. However, a fuller understanding of the control of cellular differentiation towards organogenesis or somatic embryogenesis *in vitro* as well as the factors necessary for initiation and maturation of differentiated structures is necessary before the full potential of the suspension culture system can be exploited.

CHAPTER VII : ISOENZYME ANALYSIS DURING THE COURSE OF SOMATIC EMBRYOGENESIS

7.1 INTRODUCTION

In the earlier chapters, we have seen that the process of somatic embryogenesis in wheat is largely an interplay of several factors like the genotype of the donor plant, the explant, environmental factors etc. However, it must be realised that for a particular tissue to elicit a favorable *in vitro* response, the cells and/or tissues must first be competent to respond to the organogenetic properties of the medium. This developmental state of competence has been construed to be much like the notion of dedifferentiation in plants (Gautheret 1966), in that it may be triggered off by exogenous stimuli such as wounding. Once competent, the tissue responds to the phytohormone balance in the medium and undergoes induction. Thus, competence or determination i.e the decision to do a piece of morphogenesis, necessarily precedes the actual morphogenesis (Waddington 1966) - be it organogenesis or somatic embryogenesis.

Such states of competence and induction however, cannot be measured by direct inspection of the tissue, but may be demonstrated by means of experimental manipulations (Meins and Binns 1979). For such manipulations to ultimately demonstrate the several developmental stages of totipotency, the dependence of a tissue on external physical and chemical factors may be exploited.

Carrot embryogenesis is one of the best characterised differentiative systems *in vitro* (LoSchiavo *et al.* 1989). Cell cultures can be initiated on a simple medium containing low concentrations of an auxin and a cytokinin; a proliferating cell culture can be made to differentiate by simply removing the hormones from the medium. Consequently, embryogenesis, after its start, can be blocked by the

addition of an auxin (Sung *et al.* 1984). Thus, due to its ability to be easily manipulated *in vitro*, the carrot system has been considered as one of the "model" systems and much of the currently available information on gene expression during somatic embryogenesis is based on such systems. However, it has been realised that what pertains to carrot or other model systems need not apply to other plants (Ammirato 1987). Hence, the need to inspect other examples to integrate the information on the molecular and synthetic activities of embryos of each of the embryogenic classes into a general concept of gene regulation.

In cereals, the formation of somatic embryos and shoots cannot be ascribed to any plant growth substance with certainty (Vasil and Vasil 1979). In general, it is recognised that high concentrations of auxins like 2,4-D, dicamba etc. trigger off the embryogenic response *in vitro* which is manifested by the production of embryogenic (E) callus. Further, shoot differentiation follows the transfer of such E calli to a medium devoid of the auxin or containing it at lowered concentrations. However, once embryogenesis ensues, it cannot be easily blocked and the cells remain faithfully commited to the embryogenic pathway. Moreover, embryogenic cereal cultures gradually show a decrease in their regeneration potential on repeated subculture (Schaeffer *et al.* 1984). Thus, it is obvious that cereals differ from the model systems in their requirements for, and their pliability in *in vitro* culture - a fact that has been stressed often in cereal literature (Hahne *et al.* 1988, Vasil 1987). These differences may be mere manifestations of the major differences in the species at the genetic levels. Hence, studies of these crop species are

essential to elucidate the several cellular and molecular processes associated therewith.

During somatic embryogenesis, transition to somatic embryos from unorganised cells encompasses the organisation of actively dividing meristematic cells into different developmental stages. For this, specific genes must be activated at predetermined times to ensure the progressive development of the embryo from somatic cells. Tangible evidence of differential gene regulation and expression at different developmental stages is reflected by biochemical changes that occur during somatic embryogenesis. Changes in the isoenzyme patterns i.e. synthesis of new isoforms or repression of the existing ones thus, may be a part of the functional specialization of different cell populations during somatic embryo formation. This is logical because while progressive development of the embryogenic system is manifested by morphological changes, functional activity of the newly formed structures is essential for triggering off further changes in organisation and development (Scandalios 1974).

In maize, specific isoforms of esterase and glutamate dehydrogenase have been reported in scutellum-derived embryogenic calli (Everett *et al.* 1985), while two isoperoxidases and one isozyme of malate dehydrogenase have been reported in embryogenic calli derived from immature glumes (Rao *et al.* 1990). Franz *et al.* (1989) have also reported differences in the zymograms of glutamate dehydrogenase and peroxidase in embryogenic and non - embryogenic calli. Three isozymes of esterases and two of peroxidases have been shown to be associated with differentiating wheat calli (Chawla 1988). Again, in wheat,

stage - specific peptide hydrolase isozymes have also been identified during differentiation and dedifferentiation (Moreira *et al.* 1990). In barley, two isoesterases and two isoperoxidases were associated with differentiating calli, while one specific peroxidase was associated with non - differentiating calli (Chawla 1988); specific esterases may be used as markers of embryogenesis while peroxidases for detection of leaf and shoot primordia during morphogenesis (Coppens and Dewitte 1990). In rice, peroxidase patterns were reported to be markedly different between regenerating and non - regenerating calli (Abe and Futsuhara 1990). Tissue and developmental stage specific isoenzymes have also been identified in sugarcane (Rawal and Mehta 1990). To further understand the biochemistry of organised development in a narrow and directed perspective, specific metabolic pathways have been investigated e.g. nitrogen assimilation (Dwivedi *et al.* 1984), nitrate reductase activity (Khan *et al.* 1984), carbohydrate metabolism (Rawal *et al.* 1985), vitamin B_6 synthesis (Miyata *et al.* 1988) and aromatic amino acid metabolism (Kavi Kishor 1988).

In the present study, changes in the isoenzyme profiles in wheat were characterised during the induction of embryogenic callus, in the embryogenic and non-embryogenic calli, and also during germination of the embryos. This type of characterisation by subtractive analysis can go a long way in the elucidation of differential gene expression during the development of somatic embryos especially in cereals on account of their economic importance.

7.2 RESULTS AND DISCUSSION

7.2.1 CALLUS ESTABLISHMENT AND REGENERATION

Callus cultures were established from immature embryos of the genotype Raj - 1777, which was identified as exhibiting maximum embryogenic potential (Chapter 3). These immature embryos were cultured on Murashige and Skoog's (MS) basal medium supplemented with 2 mg/l 2,4 - dichlorophenoxyacetic acid (2,4-D) and 3% sucrose as described earlier . Embryogenic and non-embryogenic calli were maintained by subculture on the same medium at 30 day intervals. For regeneration, calli were transferred to MS basal medium supplemented with 1 mg/l kinetin and 2% sucrose.

Tissues were harvested at five developmental stages which were histologically identified. These were :

I. Immature embryos - collected 12-14 days after anthesis.

II. Inductive phase - This stage was evident 5-7 days after initial culture and histology revealed meristematic activity in the scutellar epidermis and subepidermis. This is the period when cells develop embryogenic competence and become committed.

III. Embryogenic (E) callus - This hard, compact and nodular callus was formed from the scutellum after about two weeks of culture and histological examination revealed embryoids at different stages of development. It was in the E calli that embryogenesis in morphological terms occurred.

IV. Non-embryogenic (NE) callus - which was continuously segregated from the E callus, was soft, friable and apparently unorganised in nature. The NE callus served as a reference and a negative control for the process of somatic embryogenesis.

V. Germinating somatic embryos in embryogenic callus - this was the stage when the somatic embryos germinated.

All the samples were liquid nitrogen powdered and stored at -70^{o}C till further use.

7.2.2 SAMPLE PREPARATION, ELECTROPHORETIC PROCEDURES AND ACTIVITY STAINING

About 100 mg of the liquid nitrogen powdered tissue was homogenised with two volumes of 50 mM Tris-HCl buffer (pH 7.5) containing 2% Noidet P -40 (v/v). The homogenate was centrifuged at 18,000 x g for 20 minutes at 4^{o}C, and the supernatent was used for isozyme analysis. Isozymes were resolved on 1.5 mm thick native polyacrylamide gels (5% T, 2.1% C - stacking gel ; 12% T, 2.1% C -separating gel) at 10^{o} C, using 25 mM Tris and 192 mM Glycine (pH 8.3) as the electrode buffer. Each well was loaded with the equivalent of 100-125 µg protein. Protein estimation was done after Bradford (1976) method. Electrophoresis was carried out at 15 mA constant current till the bromophenol blue dye front reached the bottom anode end of the separating gel. The reaction mixtures used for visualization of enzyme activities in the gels were as follows : .pa

PEROXIDASE (E.C.1.11.1.7) :

1.61% benzidine hydrocholoride, 4.83% ammonium chloride and 0.013% hydrogen peroxide in 100 mM sodium phosphate buffer, pH 6.5 (Smith 1972).

ESTERASE (E.C.3.1.1.2) :

0.02% Fast Blue RR and 0.02% α-naphthyl acetate in 100 mM sodium phosphate buffer, pH 6.5 (Paynee and Koszykowski 1978).

GLUTAMATE DEHYDROGENASE (GDH) - E.C.1.4.1.2 :

100 mM $MgCl_2$, 200 mM glutamic acid, 10 mM NAD, 1.6 mg nitrotetrazolium blue (NBT) and 0.4 mg N-methyl phenazonium methosulfate (PMS) in 8 ml of 20 mM Tris-HCl, pH 7.5 (Vallejos 1983).

HEXOKINASE - E.C. 2.7.1.1 :

100 mM $MgCl_2$, 75 mM glucose, 5 mM adenosine triphosphate, 15 mM nicotinamide adenine dinucleotide phosphate (NADP), 2 units of G6PDH, 1.6 mg NBT and 0.4 mg PMS in 8 ml of 100 mM Tris HCl, pH 8.4 (Vallejos 1983).

PHOSPHO GLUCOISOMERASE (PGI) - E.C. 5.3.1.9 :

100 mM $MgCl_2$, 40 mM fructose-6-phosphate, 15 mM NADP, 3.84 mg NBT, 0.6 mg PMS in 8 ml of 20 mM Tris HCl, pH 7.5 (Vallejos 1983).

6-PHOSPHOGLUCONATE DEHYDROGENASE (6PgDH) - E.C. 1.1.1.44 :

100 mM $MgCl_2$, 40 mM 6-phosphogluconate, 15 mM NADP, 3.84 mg NBT and 0.6 mg PMS in 8 ml of 20 mM Tris HCl, pH 7.5 (Vallejos 1983).

GLUCOSE 6 PHOSPHATE DEHYDROGENAASE (G6PDH) - E.C. 1.1.1.49 :

100 mM $MgCl_2$, 100 mM glucose-6-phosphate, 15 mM NADP, 3.84 mg NBT and 0.6 mg PMS in 8 ml 20 mM Tris HCl, pH 7.5 (Vallejos 1983).

MALATE DEHYDROGENASE (MDH) - E.C. 1.1.1.37 :

100 mM $MgCl_2$, 200 mM malate,10 mM NAD, 3.84 mg NBT and 0.6 mg PMS in 8 ml 20 mM Tris HCl, pH 7.5 (Vallejos 1983).

All reactions except that for peroxidase were carried out in dark at $37^{o}C$. The gels after staining for the respective enzymes were scanned on a Perkin - Elmer Lambda 15 UV - VIS double beam spectrophotometer at 590 nm, excepting those for esterase which were scanned at 470 nm.

The isoenzyme profiles of all the enzymes studied showed marked differences among the five samples (Table 1). Peroxidases exhibited a total of 19 isozyme forms (Fig. 53). Isoperoxidase 1(Rm 0.94), the fastest migrating peroxidase, was expressed at all stages of development. Isoperoxidase 11 (Rm 0.43) was expressed only during the germination phase,but isoforms 9,10,12,15,16,18 and 19 (Rms 0.44, 0.43, 0.40, 0.28, 0.26, 0.17 and 0.15 respectively) were expressed at all stages except during the germination phase. Isoperoxidases 4,6 and 8 (Rms 0.61,0.58 and 0.47) were expressed only in the E and NE calli.

Five isoesterases 1,5,12,16 and 24 (Rms 0.97,0.86,0.67,0.50 and 0.21 respectively) were expressed at all the stages of development (Fig. 54). Isoesterase 6 (Rm 0.83) was expressed only during germination of the somatic embryos while isoform 17 (Rm 0.44) was associated with all the developmental stages except germination. Isoesterases 11(Rm 0.72) and 13(Rm 0.64) were detected only in the E and NE calli.

GDH isoenzyme bands 3(Rm 0.57),11 (Rm 0.28) and 14 (Rm 0.13) were constitutively expressed (Fig. 55), while the expression of band 7 (Rm 0.78) was repressed during the germination of embryoids. No isoforms were identified as being mutually exclusive to the E and NE calli.

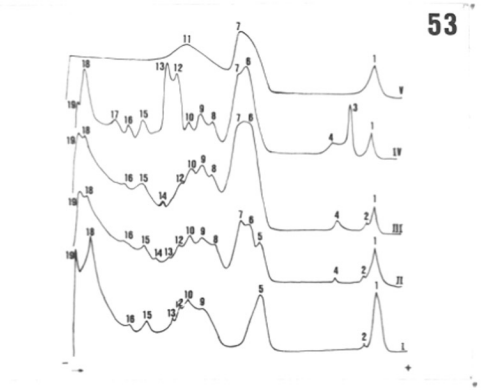
Isohexokinases 7,19 and 23 (Rm's 0.68, 0.23 and 0.14 respectively) were expressed at all the stages of development (Fig. 56). Isohexokinase 27 was exclusive to the germination phase, whereas isoform 13 (Rm 0.42)was repressed during this period. Isoforms 6 (Rm 0.71) were expressed both in the E and NE calli .

Fig. 53: Superimposed gel scans of Peroxidase

Fig. 54: Superimposed gel scans of Esterase

Fig. 55: Superimposed gel scans of GDH

Fig. 56: Superimposed gel scans of Hexokinase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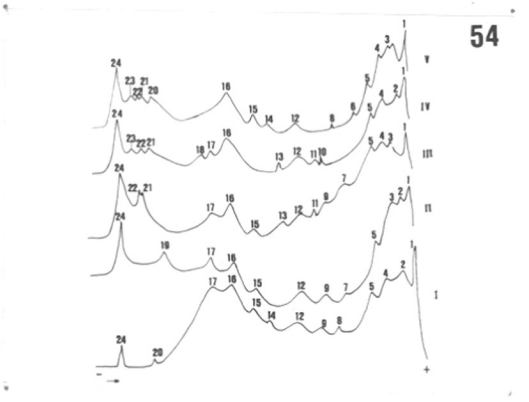
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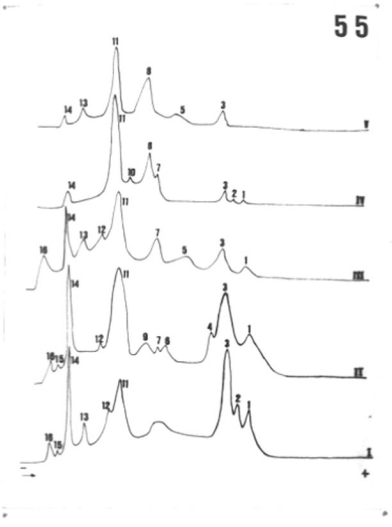
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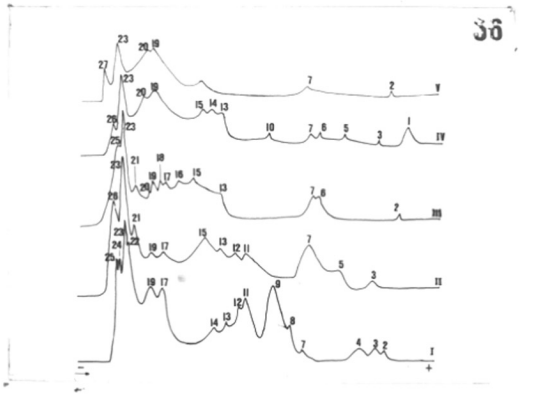
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PGI isoforms 14 (Rm 0.37),18 (Rm 0.24) and 20 (Rm 0.21) were constitutively expressed (Fig. 57). IsoPGI 11 (Rm 0.44) was exclusive to the germination phase while isoforms 10 (Rm 0.46) and 17 (Rm 0.26 respectively) which were expressed at all other stages were however, repressed during the germination phase. Isoform 22 (Rm 0.16) was found to be callus specific.

Three G6PDH isoenzymes were constitutively expressed (Fig. 58). These were 13 (Rm 0.44),19 (Rm 0.22) and 21 (Rm 0.15). Isoform 4 (Rm 0.91) was associated with all the stages except the germination phase. Seven G6PDH isoforms were identified as being common and exclusive to the E and NE calli. These include isoforms 5 (Rm 0.89),8 (Rm 0.74),10 (Rm 0.65), 11 (Rm 0.54), 14 (Rm 0.38), 17 (Rm 0.29) and 24 (Rm 0.10).

6PgDH isoforms 5, 12 and 15 (Rms 0.49, 0.24 and 0.13) were constitutively expressed (Fig. 59). No isoforms were found to be associated exclusively with the germination phase. Only one isoform *viz.* band 11 (Rm 0.26) could be identified as being mutually exclusive to the E and NE calli.

MDH isoforms 3 (Rm 0.86), 14 (Rm 0.43) and 15 (Rm 0.38) were constitutively expressed (Fig. 60). Isoforms 2 (Rm 0.88) and 9 (Rm 0.66) were expressed at all the stages except during germination. No isoforms could be identified to be mutually exclusive to the E and NE calli.

On the basis of the isoenzyme analyses during somatic embryogenesis, the embryogenic pathway could be divided into three major events (Table 16). These are :

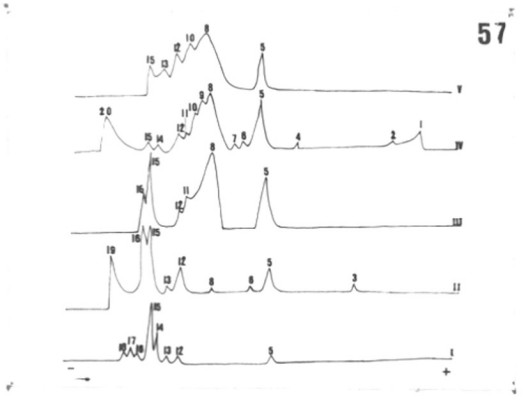
(1) *the early events* - these would encompass the metabolic and molecular changes that occur during the inductive

Fig. 57: Superimposed gel scans of PGI

Fig. 58: Superimposed gel scans of G6PDH

Fig. 59: Superimposed gel scans of 6PgDH

Fig. 60: Superimposed gel scans of MDH



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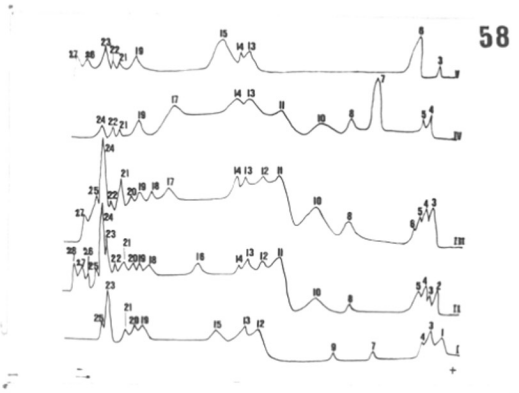

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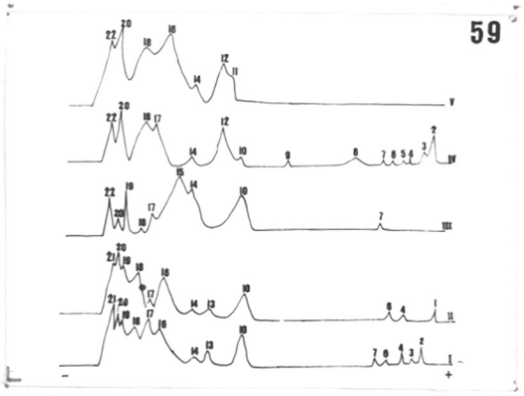

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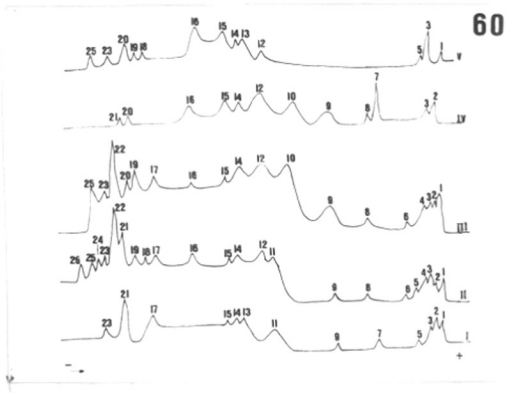

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Table 19 : Developmental Stage Dependent Expression Of Enzyme Isoforms

Enzyme	Constitutively Expressed Isoforms	Early Event Isoforms	Mid-term Event Isoforms	Late Event Isoforms	Callus Specific Isoforms
Peroxidase	1(0.94)	14(0.32	-	-	4(0.61) 6(0.58) 8(0.87)
Esterase	1(0.97) 5(0.86) 12(0.67) 16(0.50) 24(0.21)	3(0.93) 7(0.80)	-	15(0.57)	11(0.72) 13(0.64)
GDH	3(0.57) 11(0.28) 14(0.1)	-	-	13(0.18)	-
Hexokinase	7(0.68) 19(0.23) 23(0.14)	21(0.17)	16(0.32) 18(0.24)	2(0.91)	6(0.71)
PGI	14(0.37) 18(0.24) 20(0.07)	-	15(0.34)	-	22(0.16)
G6PDH	13(0.44) 19(0.22) 21(0.15)	18(0.23)	-	3(0.94) 5(0.36)	5(0.89),8(0.74), 10(0.65),11(0.54) 14(0.38),17(0.29) 24(0.10)
6PgDH	5(0.49) 12(0.24) 15(0.13)	-	-	-	11(0.26)
MDH	3(0.86) 14(0.43) 15(0.38)	4(0.85) 6(0.82) 22(0.15)	-	-	-

Figures in brackets represent Rm values

phase and in the E calli at a stage when cells develop competence and become commited towards embryogenesis. Specific enzyme isoforms which were expressed during this stage were isoperoxidase 14 (Rm 0.32), isoesterases 3 and 7 (Rm's 0.93 and 0.80 respectively),isohexokinase 21 (Rm 0.17), isoG6PDH 18 (Rm 0.23) and isoMDH's 4,6 and 22 (Rms 0.85, 0.82 and 0.15 respectively). GDH , PGI and 6PgDH do not show any participation during the early events of embryogenesis (Table 18) ;

(2) *the midterm events* - these would occur exclusively in the E calli, since it is here that committed or determined cells organise to form the proembryos which developed further into morphologically fully developed bipolar embryos. Novel midterm event enzyme isoforms were hexokinase isoforms 16 and 18 (Rms 0.32 and 0.24 respectively) and PGI isoform 15 (Rm 0.34);

(3) *the late events* - these would occur in morphologically fully developed somatic embryos, be these the ones formed in E calli or the germinating embryos regenerated from the E calli or the immature embryo explant. In all these three instances the embryos are in an advanced state of development and can form entire plants under permissive conditions. Isoesterase 15 (Rm 0.57) ,isoGDH 13 (Rm 0.18), isohexokinase 2 (Rm 0.91) and isoG6PDH's 3 and 15 (Rms 0.91 and 0.36 respectively) were associated with this stage.

Surprisingly, 6PgDH does not show participation in any of the three events listed. Peroxidase and MDH are involved only in the early events. Esterase and G6PDH are not associated with mid-term events, while PGI shows only mid-

term event association and GDH is involved only during the late events. Hexokinase shows differential gene activity at all the different developmental stages.

On comparison of the enzyme profiles in E and NE calli it was observed that these had many isoforms common to each other. The only common denominator among these two tissue types was the formation of callus and its growth. Hence this set of exclusive isoenzymes were termed as callus specific (Table 18). These included isoperoxidases 4,6 and 8 (Rm's 0.61,0.58 and 0.47 respectively), isoesterases 11 and 13 (Rm's 0.72 and 0.64 respectively), isoGDH 7 (Rm 0.38), isohexokinase 6 (Rm 0.71), isoPGI 22 (Rm 0.16), isoG6PDH's 5, 8, 10, 14, 17 and 24 (Rms 0.89, 0.74, 0.65, 0.54, 0.38, 0.29 and 0.10 respectively) and iso6PgDH 11 (Rm 0.26).

On further comparison of the isoenzyme profiles in the E and NE calli, it was seen that several enzyme isoforms are expressed in the E calli but are not so in the NE calli , and vice versa. These novel isoforms discriminate, on a biochemical level, between the E calli which regenerate embryos, and the NE calli which remain unorganised, when both are transferred to the same regeneration medium. This group of isoforms includes isoperoxidases 2 (Rm 0.59) and 14 (Rm 0.37), which were expressed in the E calli but remained suppressed in the NE calli, and isoperoxidases 3 (Rm 0.88), 13 (Rm 0.40) and 17(Rm 0.26) were expressed in the NE calli while remaining suppressed in the E calli. Similarly, isoesterases 3,7,9 and 15 (Rms 0.93, 0.80,0.75 and 0.57) were associated with the E calli but were conspicuously absent in the NE calli while isoforms 2,10,18 and 23 (Rms 0.95,0.73,0.43 and 0.25 respectively) which were associated with the NE calli were absent in the E calli. IsoGDH's 5, 12, 13 and 16(Rms 0.46,0.22,0.18 and 0.09

respectively) were synthesized in the NE calli and not so in the E calli. No isoform of GDH was detected to be exclusive to the E calli. Hexokinase isoforms 2,16,17,18,21 and 25 (Rms 0.91,0.32,0.25,0.24, 0.17 and 0.12 respectively) were expressed in the embryogenic callus (E callus) but not in the NE callus. Isohexokinases associated with the NE calli but not with the E calli included 1,3,5,10,14 and 26 (Rms 0.97,0.87,0.78,0.52, 0.41 and 0.10 respectively). PGI isoforms 15 and 19 (Rms 0.34 and 0.22 respectively) were expressed in the E calli but not in the NE calli whereas isoforms 2,3,4,5,6,8,9 and 12 (Rms 0.94,0.93,0.91,0.90,0.88,0.75,0.58 and 0.42 respectively) were expressed in the NE calli but not in the E calli. IsoG6PDH bands 1,3,6,12,18,20,25 and 27 (Rms 0.94,0.88, 0.47, 0.23, 0.20, 0.09 and 0.05 respectively) were expressed in the E calli but remained suppressed in the NE calli. The G6PDH isoform 7 (Rm 0.80) was synthesized in large amounts in the NE calli while its synthesis was totally abolished in the E calli. The 6PgDH isoform 16 (Rm 0.12) was expressed in the E calli but not in the NE calli, on the contrary isoforms 1,2,4,6,7,9,10,14 and 20 (Rms 0.93, 0.86, 0.63,0.45, 0.42, 0.32, 0.30, 0.17 and 0.07 respectively) were expressed in the NE calli but not in the E calli. IsoMDH's expressed in the E calli but repressed in the NE calli include isoforms 1,4,6,17,19,22,23 and 25 (Rms 0.90, 0.85, 0.82,0.24, 0.19, 0.14, 0.12 and 0.09 respectively), while isoMDH's 7 (Rm 0.75) and 21 (Rm 0.16) expressed in the NE calli were not detected in the E calli.

It is thus suggested that the presence of certain exclusive isoforms in the E calli is manifested by specific gene activities which are essential for establishing and

maintaining an embryogenic state. The reverse could also hold true. Thus, it is possible that, depending on which of the genes are triggered on or off or possible post-translational modification of the existing isozymes, the fate of a cell towards embryogenecity or non-embryogenecity is determined.

7.2.3 CONCLUSION

The present biochemical studies have revealed several facts concerning the process of somatic embryogenesis :

1. Each developmental stage during somatic embryogenesis is associated with a definite isoenzyme profile. As a result of this differential expression and repression of isoforms of enzymes, it was possible to catagorise the entire process of somatic embryogenesis into three classes of events viz. the early events, the midterm events and the late events, which have been described in detail earlier.

2. Callus specific isoforms of peroxidase, esterase, GDH, hexokinase, G6PDH and 6PgDH were detected. On a similar line of thought, callus specific proteins have been reported in carrot (Sung and Okimoto 1983), rice (Chen and Luthe 1987) and orchard grass (Hahne *et al* 1988). The last group of authors have concluded that since callus is apparently a tissue type without a direct equivalent in whole plants, its development would naturally be associated with the formation of new proteins. This theory appears to hold true with the present wheat system with respect to specific isoforms.

3. Isoforms of enzymes which were exclusive to the E calli, but were repressed in the NE calli, and *vice versa* were detected. This finding is of significance in view of the fact that although embryogenic cells often can be recognised by their small size and isodiametric form, in contrast to the highly vacuolated, elongated non - embryogenic cells, this is not always valid because small cells occasionally do appear in non - embryogenic tissues (Franz *et al*. 1989). The detection

of biochemical differences between the two cell types, as presented here, therefore provides an additional method of confirming the embryogenic pathway.

Given the phenomenon of co-dominant gene expression with respect to the isoforms of any enzyme, these observations reflect on the basic changes occurring during the process of dedifferentiation and redifferentiation. Though the mechanisms underlying such changes remain as yet rather obscure, nevertheless, differential gene expression and regulation is indicated by the present study. During the period of induction, the cells undergo dedifferentiation and hence try to recover a maximum capacity of gene expression (Moreira *et al.*1990). Conversely, during germination, some of the genes may be switched off upon the attaining of a differentiated state of a higher magnitude. This is manifested, in the current study by an increased order of magnitude in complexity of the isoenzyme patterns during the early periods of induction and commitment of calli towards embryogenesis (which represent histologically dedifferentiated stages) and a simplification of the patterns during germination of the somatic embryos.

Thus, the study of isozyme profiles during development of somatic embryos gives an insight into differential gene activity associated with the phenomenon of embryogenesis and thereby provides biochemical markers to follow the process. The application of such markers for embryogenesis has been demonstrated by Schou and Pederson (1988) in barley. These workers raised monoclonal antibodies against the plasma membranes of zygotic embryos, somatic embryos and embryogenic callus and isolated specific antibodies for

each type of membrane. Further, they succeeded in identifying a regeneration associated antigen which is a protein with a molecular weight of approximately 20 kD. However, it is obvious from the present study that the induction of somatic embryogenesis and the ultimate morphplogical manifestations of a somatic embryo is not controlled by a limited number of gene products as described by Sung and Okimoto (1983). Recently, Gartenbach - Scharrer *et al.* (1990) have determined that the sequential activation of around 130 proteins occurred during the 18h experimental period prior to the onset of cell division activity during induction in cultured carrot cells. Further, we still do not know the functions of gene products in the formation of plant organs and their role in the developmental hierarchies. Until such questions are resolved, the process of embryogenesis will remain an enigma to researchers of developmental biology.

RESUME

General strategies for the regeneration of cereals have evolved. Earlier, the focus was on varying components in the nutrient medium for regeneration. In the late 1970's, there were the first reports of regenerable cultures from cereals on simple media supplemented with 2,4-D. The 1980's showed a proliferation of reports of regeneration in most of the important cereals. In part, this was due to the screening of several genotypes for regeneration potential. Paralleling was the concept that the immature embryo at a critical stage of development was the most appropriate explant for inducing somatic embryogenesis.

The work presented in this thesis succeeds in the advancement of knowledge in the tissue culture of cereals with respect to -

1. Screening of several indigeneous genotypes of wheat in view of identifying genotypes highly responsive to *in vitro* culture especially with reference to somatic embryogenesis.
2. The first report on genotype dependency of precocious germination of zygotic and somatic embryos is presented.
3. Use of the immature embryo explant to optimise the medium parameters for enhancing somatic embryogenesis, plantlet regeneration, and further, for the maintenence of embryogenic cultures. 4. The establishment of direct somatic embryogenesis in cultured leaf bases also constitutes a first report of such a system in this species. Further, indirect somatic embryogenesis could also be achieved from this explant.
5. Embryogenic systems from immature inflorescences of wheat were also established.

6. Somatic embryogenesis was also induced from mature embryos of wheat, albeit at lower frequencies than either immature embryos, inflorescences or leaf bases.
7. Callus cultures from root tissues were established. However, plantlet regeneration from these calli could not be achieved.
8. Suspension cultures capable of regenerating upto the callus stage were established.
9. A detailed report on the isoenzyme profiles of several key enzymes at five developmental stages during somatic embryogenesis is also presented.

To initiate any studies on Indian wheat, the first step was to establish reproducible, regenerative systems with the indigeneous genotypes. Initially, a screening program using the immature embryo explant was carried out. This resulted in the identification of five highly responsive genotypes out of a total of thirty three screened for their *in vitro* embryogenic potential. The phenomenon of precocious germination of somatic embryos was observed to occur in several of these genotypes, and this resulted in a detailed, genotype related study of precocious germination with respect to somatic embryogenesis and plantlet regeneration. Further, using these five genotypes, several components in the nutrient medium were varied to establish an optimum system for embryogenesis and plantlet regeneration. Maintenance of the embryogenic cultures by further manipulations of the media components as well as visual identifiation of the embryogenic calli and monitoring a proper subculture schedule was also achieved.

A major disadvantage of using immature embryos is that they are responsive only at a specific developmental stage.

Moreover, in India, wheat being a seasonal crop is available only once a year. This limits the sampling period for the explant. In order to identify other sources of inoculum for *in vitro* culture, explants such as immature inflorescences, mature embryos, leaf bases and root tips were also screened for their embryogenic potential. Immature inflorescences were comparable with immature embryos in their embryogenic potential. However, the frequencies of plantlet regeneration from these cultures was slightly lower than those in case of immature embryo derived cultures.

The establishment of *in vitro* cultures from leaf bases proved to be slightly more tedious than those from immature embryos and inflorescences. Wernicke and Milkovits (1986) have conceded that the ability of the shoot meristem to give rise to regenerative cultures in many cereal genotypes may be lost within a fraction of a millimeter distance from the apical meristem. The present study constitutes the first ever report on direct morphogenesis (formation of shoot buds and occasionally, somatic embryos) from leaf tissues of wheat. Albeit its low regeneration frequency, this system holds great potential in further studies in protoplast and transformation work.

Many publications have established that mature stem, embryos and leaf tissues from most cereals are not satisfactory explants for embryogenic callus induction. In the present study too, mature embryos exhibited a poor response as compared to either immature emryos inflorescences or leaf bases. However, the use of mature embryos for micropropagation studies proved to be fruitful to a certain extent.

Root tissue as a source of inoculum for initiating embryogenic cultures has been greatly overlooked in cereals. There are very few reports of plantlet regeneration from cereal root tissues. In the present study, a nodular callus was developed from root tips. Although the callus failed to develop somatic embryos and no further differentiation was achieved, this represents a beginning, and the resistance faced in regeneration are possibly, a physiological block which may be eliminated by further manipulating cultural conditions.

Although embryogenic callus cultures have proved to be suitable for the rapid clonal propagation of cereals, it is clear that even more enhanced levels of growth, embryogenesis and plantlet regeneration could be achieved from embryogenic suspension cultures. Such suspension cultures are often preferred because the cells can be effectively exposed to varying nutrient milieu because of their dispersed state. Non-morphogenic suspension cultures have been known in the Gramineae for a very long time. Such suspensions were readily established in the present study from friable callus derived from leaf bases as well as from aged, embryogenic calli from immature embryos. Reports on plantlet regeneration from cell suspensions began in the early 1980's, and by the late 1980's, suspension cultures have been established in most of the major cereals. More recently, plantlet regeneration from cell suspensions of wheat have also been reported (Redway *et al.* 1990). However, such results could not be reproduced in the present study. Regeneration in suspension cultures initiated from explants of the genotype Raj-1777 was restricted to callus formation in plated cultures.

What exactly are the intracellular events following the commitment of a cell towards somatic embryogenesis? In each of the above systems, a detailed histological study has been performed, yet it is difficult to describe a general strategy or sequence of events that occur universally in all the embryogenic cells and which leads to the formation of the somatic embryo. The universal point of agreement concerning embryogenesis, however, begins and ends with the morphology of the embryogenic cells (described earlier). The topics involved in much of the discussion and controversy are:

1. The origin of the somatic embryo.
2. The extent of similarity to zygotic embryogenesis.

The controversy concerning the origin of the somatic embryo is inextricably linked with the theories put forth by various workers to explain somatic embryogenesis. There are two major schools of thought for the origin of somatic embryos. The first of these is the cell isolation theory, which suggests that a cell removed from the constraints and influences of its neighbouring cells can aquire embryogenic competence (Williams and Maheshwaran 1986). However, in a majority of cases, there is not enough convincing proof for such an occurence.

The second theory viz. the predetermination theory which suggests that direct embryogenesis in cultures proceeds from pre-detrmined embryogenic cells (PEDC's), which require only favourable external stimuli to allow the expression of the embryogenic programme. On the other hand, indirect embryogenesis involves induced embryogenically determined cells (IEDC's). The re-entry of these cells into mitosis as a step towards embryogenesis requires the

redetermination of differentiated cells, callus proliferation and development of the embryogenic state. However, once cells are determined for embryogenesis, there are no fundamental differences between the PEDC's and the IEDC's.

Thus somatic embryogenesis appears to be directly related to the coordinated behaviour of the neighbouring cells as a morphogenetic group. Haccius (1978) defined somatic embryos as "new individuals arising from single cells and having no vascular connections with the maternal tissues". However, a single cell origin cannot be unequivocally demonstrated in several cases. If a cell is alone in its state of readiness, it may act as a single entity. On the other hand, if the cell is surrounded by neighbouring cells in the same state of induction, the group together, may behave as a single entity and form the somatic embryo.

It is, thus, now indicated that single and multiple cell proliferation simply represents varying expressions of the same underlying phenomenon. Franz and Schel (1991b) have suggested that not the origin, but the transition from an unorganised embryogenic unit to a more organised group of cells with polarity is of much importance in somatic embryogenesis. Such a transition unit may be considered as homologous to the proembryo during somatic embryogenesis. This transition unit differs from the embryogenic unit in possessing a definite polarity. These authors have also described ultrastructural details of this transition unit as proof of its existance. They further suggested that the morphogenetic change from an embryogenic unit to the transition unit may be a result of mechanical forces caused

by the proliferating callus, creating a polarised structure within the embryogenic unit. Once polarity is established, gradients of nutrients, hormones and possibly, ionic currents are built up, that reinforce this polarity and activate the process of somatic embryogenesis.

The similarity between somatic and zygotic embryony lies mainly in their end-product. However, there are distinct morphogenetic differences underlying the morphogenetic events during the two processes. In zygotic embryony, cell division and destiny follow a predictable precision. All the cells derived from the zygote together constitute the embryo. Contrarily, somatic embryony is extremely plastic - the planes of cell divisions during early somatic embryogenesis are extremely irregular and unpredictable (Rangaswamy 1986), and a large number of embryos fail to acquire bilateral symmetry (McWilliam *et al.* 1986). In fact, there has been no report of an isolated cell *in vitro* that has faithfully duplicated the embryological events occuring in the zygotic embryo with specific reference to the planes of divisions and orientations of actively dividing cells which ultimately give rise to different parts of the embryo. Moreover, in instances where somatic embryos are formed in the callus, a lot of cellular material is wasted - which is not seen in zygotic embryony.

As a step towards identifying the causative biochemical events during somatic embryogenesis, the isoenzyme profiles of several key enzymes involved in plant metabolism were investigated at different developmental stages during somatic embryogenesis. This study indicated that each developmental stage is associated with a definite isoenzyme

profile. Embryogenesis and callus specific isoforms were detected, and their presence could be used as an indication of the onset of somatic embryogenesis. Thus, the process of somatic embryogenesis may be the result of sequential gene expression which is manifested at the biochemical and morphological levels.

Until a decade ago, the process of *in vitro* embryogenesis in cereals was far from being understood. However, many approaches are now being used to elucidate the process of somatic embryogenesis and the expression of totipotency. In the near future, it is anticipated that further detailed information at the molecular level will also be revealed.

Thus, it is envisaged that practical application of somatic embryogenesis in cereals will follow if it is possible to establish embryogenic systems and manipulate their growth and differentiation. Also, an understanding of the process at the cellular and molecular levels may facilitate the transfer of genes into these crop species for the improvement of agronomic characters. Further, clonal propagation of identified transformants in batch cultures may facilitate faster growth of an improved plant. In addition, artificial dormancy may be induced in the somatic embryos, which may then be stored or incorporated into artificial seeds as reported in barley (Datta and Potrykus 1989). These are intriguing and potentially valuable prospects.

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Plant Cell Reports

Occurrence and frequency of precocious germination of somatic embryos is a genotpye – dependent phenomenon in wheat*

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ABSTRACT

Immature embryos of thirty-three genotypes of wheat were cultured on 2,4-D containing medium. Occurrence of precocious germination of the zygotic and somatic embryos simultaneously on the same medium was a striking feature observed during the course of work. The percentage of precocious germination was seen to vary extensively from 0-88% and 0-84% for zygotic and somatic embryos respectively. In the genotypes NI-5439 and NI-5643 which are characterized by a high tillering capacity, the phenomenon of precocious germination seems to take a different path from that observed in the other genotypes. This is evident since these two genotypes require total absence of hormone for shoot elongation although multiple shoot primordia are formed on auxin containing medium.

Precocious germination also seems to be relevant to somatic embryogenesis and plantlet regeneration. This conclusion stems from the observation that a majority of the genotypes that show precocious germination of zygotic embryos have greater embryogenic potential. Consecutively, most of the genotypes that show precocious germination of somatic embryos exhibit a higher frequency and faster rate of plantlet regeneration.

Abbreviations:

2,4-D	-2,4-dichlorophenoxyacetic acid
Ki	-Kinetin
thi - HCl	-Thiamine hydrochloride
E calli	-Embryogenic calli

INTRODUCTION

It is now a well established fact that when immature cereal embryos are excised from the grain and cultured, they develop into seedlings prematurely instead of forming mature embryos. This phenomenon, known as precocious germination has long been recognized (Norstog, 1972). The terminology has since then been extended to include the premature germination of somatic embryos that develop from in vitro cultures.

Ozias-Akins and Vasil (1982) were the first to report precocious germination of somatic embryos formed in calli derived from the scutelli of immature embryos of wheat. Since then, precocious germination has been reported in several cereals such as maize (Lu et al. 1982; Novak and Dolezelova, 1983), sugarcane (Ho and Vasil, 1983), sudan grass (Boyes and Vasil, 1984) and rye (Lu et al. 1984). However the frequency of precocious germination has never been reported, nor has its significance been highlighted.

In this report, we describe the induction of somatic embryogenesis and plantlet regeneration in several genotypes of wheat, using immature embryos as explants. Our objectives were to examine genotypic influences on somatic embryogenesis and precocious germination, and to determine the relationship between precocious germination of somatic and zygotic embryos. Furthermore, the effect of the synthetic auxin 2,4-D in preventing precocious germination was also studied in one genotype viz. NI-8629.

MATERIALS AND METHODS

Experimental material consisted of thirty-three genotypes of field-grown wheat plants. Of these, three genotypes (viz. N-59, MACS-1967 and HD-2278) were Triticum durum (2n=28) while the rest were Triticum aestivum (2n=42).

Dehusked, immature caryopses (12-15 days after anthesis) were first thoroughly washed with a commercial detergent solution diluted 1:1 with water, exposed to 70% alcohol for 30 seconds, 0.1% mercuric chloride solution for 15 minutes, and finally rinsed 3-4 times in sterile distilled water. Immature embryos (1.5 - 2.5 mm in length) were excised aseptically under a stereomicroscope and were cultured with the embryo axis in contact with the medium. Approximately 100 or more embryos of each genotype were cultured except for NI-8729, MP-845 and MP-847 wherein around 50 embryos were cultured. The callus-inducing medium used, for all the genotypes, was Murashige and Skoog's medium (1962), modified by elimination of edamine, kinetin and indole acetic acid, and supplemented with 0.4 mg/l thi-HCl; 2 mg/l 2,4-D 30 g/l sucrose and 0.4% agar (Glaxo), (henceforth referred to as MMS). The cultures were maintained at 27°C with a 16 h photoperiod under illumination by cool, white fluorescent light of intensity 11.67 $\mu E\ m^{-2}\ s^{-1}$. After 20-25 days, somatic embryos

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were transferred to the regeneration medium which was identical to the MMS medium described above, except that 2,4-D was replaced by 1 mg/l Ki. Regenerating plantlets were further transferred to filter paper supports in liquid MMS medium containing half-strength MMS salts, full-strength MMS vitamins, and 20 g/l sucrose.

RESULTS AND DISCUSSION

Callus developed from the scutellum as well as the embryo axis, 5-7 days after placing on the callus induction medium. However as observed earlier (Joshi, C.P. and Joshi, R.C., personal communication) only the scutellar calli showed the presence of somatic embryos on their surface. The morphological nature of the scutellar proliferations and development of somatic embryos was similar to that observed in Triticum aestivum by Ozias-Akins and Vasil (1982) and Joshi, C.P. and Joshi, R.C. (Personal communication). Therefore the pathway by which the somatic embryos are formed can be reasonably interpreted as being an atypical one, in which the somatic embryos when transferred on a cytokinin containing medium, form a green, leafy scutellum from the base of which arise multiple shoot primordia (Fig.1).

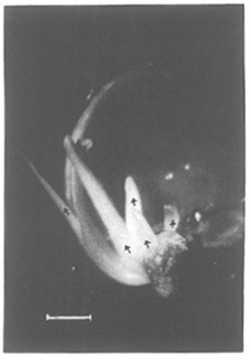

Figure 1 : Atypical Somatic Embryogenesis. The curved leaf-like structure is the scutellum from the base of which arise multiple shoot primordia (indicated by arrows). Scale indicates 2 mm.

The extent of somatic embryogenesis was seen to vary extensively with the genotype (Table 1). Twenty out of the thirty-three genotypes cultured showed an embryogenic response. Of these, five genotypes (NI-5439, NI-9272, CPAN-2005, VL-614 and Raj-1777) exhibited a high frequency of embryogenesis (70-83%), five other genotypes (NI-917, NI-8188, HD-2278, NI-8629 and MACS-1967) gave a medium frequency of embryogenesis (50-60%), ten genotypes (NI-9075, NI-5643, NI-4, MACS-2067, MP-845, HI-977, Kite, Kalyan Sona and Sonalika) were poorly embryogenic (4-49% frequency), while the remaining genotypes (NI-8611, NI-9065, NI-8289, NI-747-19, CPAN-1994, CC-464, N-59, HD-4502, MP-847 and Hy-65 were totally non-embryogenic.

It has been earlier reported that auxin depletion is an absolute necessity for the development of root and shoot primordia (Ozias-Akins and Vasil, 1983). Our observations however indicate that the somatic embryos from sixteen of the twenty embryogenic genotypes displayed the phenomenon of precocious germination, i.e. they develop scutelli and multiple root and shoot primordia while on the 2,4-D containing medium itself. Our data also shows that the frequency of precocious germination of the somatic embryos varies extensively (5-84%) across the genotypes tested.

Some relation between precocious germination of the zygotic embryos and that of the somatic embryos is evident. This was concluded from our studies where thirteen out of sixteen genotypes (81%) exhibited precocious germination of somatic as well as zygotic embryos. However, five genotypes showed either only zygotic or somatic embryo precocious germination (Table 1). Furthermore, two genotypes showed precocious germination of neither zygotic nor somatic embryos. These results suggest that precocious germination is a genotype dependent phenomenon.

Embryogenic calli showing precocious germination of both zygotic as well as somatic embryos, produced shoots within 4-5 days after transfer to the regeneration medium. On the other hand, calli not showing any precocious germination took longer to develop shoot primordia (10-15 days). The percentage of such calli giving rise to shoots was also lower (Table 1). This suggests that precocious germination is favorable for regeneration of shoots from the somatic embryos. However, once precocious germination is initiated, the somatic embryos cannot multiply to give rise to secondary embryogenic tissues. This may limit the long-term morphogenic potential of the cultures.

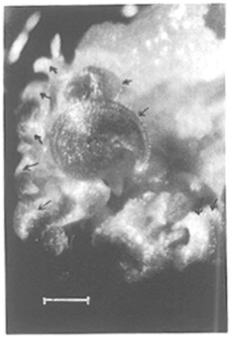

Figure 2 : Scutellar Multiplication Phenomenon. All arrows indicate scutelli, scale indicates 500 μm.

A peculiar phenomenon was observed with the genotypes NI-5439 and NI-5643. Both these genotypes showed precocious germination of the somatic embryos

TABLE 1 : Genotypic Variation In Percentages of E Calli Formed, Precocious Germination of Zygotic and Somatic Embryos, and Shoot-forming Calli.

Genotype	Percentage E Calli Formed	Percentage Precocious Germination of zygotic Embryos on 2,4-D medium.[a]	Percentage Precocious Germination of Somatic Embryos on 2,4-D medium.[a]	Ratio of no. of shoot-forming calli to no. of calli transferred to Ki medium.[b]	Percentage shoot-forming calli on Ki medium[b].
1. NI-9075	17.0	-	-	7/16	43.8
2. MACS-2067	9.3	-	-	9/20	45.0
3. NI-8796	20.5	10.3	-	8/20	40.0
4. KITE	29.0	2.0	-	2/11	18.2
5. NI-5439	70.0	-	69.6	18/18	100.0
6. NI-5643	29.0	-	51.7	12/14	85.7
7. NI-4	38.8	-	48.4	13/29	44.8
8. NI-8188	63.2	61.6	52.4	53/60	88.3
9. NI-9272	71.0	1.0	7.0	5/21	23.8
10.NI-8629	63.0	77.8	56.9	42/42	100.0
11.NI-345	51.0	87.4	42.3	18/18	100.0
12.CPAN-2005	71.4	14.3	84.3	45/45	100.0
13.MACS-1967	52.1	0.6	14.8	19/63	30.2
14.MP-845	35.7	42.9	52.9	18/18	100.0
15.VL-614	70.3	40.3	51.3	43/44	97.7
16.HI-977	15.7	1.5	9.5	7/16	43.8
17.KALYAN SONA	41.0	1.0	4.9	7/41	17.1
18.SONALIKA	25.0	20.0	17.0	2/11	18.2
19.RAJ-1777	83.0	43.2	67.5	27/27	100.0
20.HD-2278	54.5	87.9	67.8	17/17	100.0

a - Measured 15-17 days after inoculation on 2,4-D medium.

b - Measured 10-12 days after transfer to Ki medium.

on the callus initiating medium resulting in the formation of multiple scutelli without the formation of shoot primordia (Fig. 2). Transfer of these embryos to the regeneration medium did not result in shoot primordia development although the scutelli multiplied extensively. However, after transfer to a hormone free medium, shoot and root primordia developed and plantlets were regenerated. These two genotypes are characteristically high tillering and whether the unique phenomenon of scutellar multiplication is related to this character will require further investigation.

Several workers have reported that 2,4-D may be effective in preventing precocious germination (Ozais-Akins and Vasil, 1983; Novak et al., 1983; Carman et al., 1987). To confirm this observation, immature embryos of the genotypes NI-8629, (which exhibits precocious germination of zygotic as well as somatic embryos), were placed on media containing various concentrations of 2,4-D (0, 1.0, 2.0, 4.0, 8.0,10.0 mg/l; Fig. 3). With an increase in the levels of 2,4-D, precocious germination of both - zygotic and somatic embryos was effectively checked. However, the percentage of E calli formed also decreased gradually from 65% to 44% at 2,4-D concentrations from 2 mg/l to 10 mg/l respectively. This indicates that the change in percentage precocious germination is more significantly affected by increasing 2,4-D, than percentage embryogenic

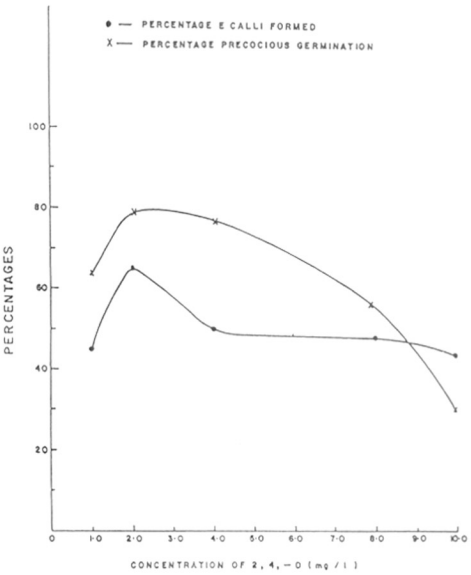

FIG 3 – EFFECT OF 2, 4 – D ON E CALLI FORMATION AND PRECOCIOUS GERMINATION OF SOMATIC EMBRYOS OF NI – 8629

calli formed. Thus increased concentrations of 2,4-D provide a partial solution to preventing precocious germination, and at the same time, maintaining the embryogenic potential of the tissue.

These results indicate a need to study the phenomenon of precocious germination in greater detail. At present, it seems that precocious germination is favorable from the point of fast regeneration of shoots from somatic embryos. However, the phenomenon needs to be suppressed if a long-term morphogenic culture is to be established. With this in view, the effect of organic additives like abscisic acid (Quatrano, 1986), osmolarity effects by varying sucrose concentrations (Ozias-Akins and Vasil, 1982; Joshi, C.P. and Joshi, R.C., personal communication), manipulating the length of the photoperiod and intensity of light supplied, and several other parameters will be investigated.

ACKNOWLEDGEMENTS

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Studies On Somatic Embryogenesis And Transformation In *Triticum aestivum*

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SUMMARY

Plant improvement by transfer of specific desired genes is being visualised to overcome the random genetic variability generated by conventional plant breeding programmes. To make this feasible, it is essential to establish systems in which genes can be readily transferred. Tissue culture methods offer such systems which may be further manipulated with the aim of achieving gene transfer, we have established reproducible, highly regenerative, embryogenic systems in five genotypes of Triticum aestivum using immature embryos, immature inflorescences and leaf bases as expalnts. The effect of genotype on somatic embryogenesis and plantlet regeneration, which has been documented in many plant species, is evident in our experiments.

Attempts have also been made with naked DNA transformations using the NPT II gene in 2 plasmid constructs viz. pRT 103 neo and pASK 10. Our results indicate successful transfer of this trait to a few plants.

INTRODUCTION

The ability to culture plant tissues *in vitro* is now, several decades old. One of the applications of such *in vitro* culture systems is to extend the horizons of genetic variability generated via conventional plant breeding programmes. In cereals, tissue culture systems have been established in several species, with somatic embryogenesis as the favoured route of plant regeneration. Several factors such as genotype, explant, conditions of culture, etc. are known to be of importance in the induction of somatic embryogenesis.

Earlier, 33 genotypes of *Triticum aestivum* were screened for their embryogenic response and from these 5 highly embryogenic genotypes were identified (Bapat *et al.*,1988). In the following paper, a comparative account of induction of somatic embryogenesis in these 5 genotypes, using three explants viz. immature embryos, immature inflorescences and leaf bases is reported. Furthermore, preliminary results of transformation via DNA imbibition by mature embryos of *T. aestivum* has been discussed.

Abbreviations Used:

2, 4-D	2, 4-dichlorophenoxy acetic acid
Ki	Kinetin
thi-HCl	Thiamine hydrochloride
E callus	Embryogenic callus

MATERIALS AND METHODS

Young, unemerged inflorescences (size range 1.0 - 3.0 cm) were collected from field grown plants. Immature embryos selected for culture were at a developmental stage corresponding to 10-14 days after anthesis. These embryos were cultured with the embryo axis in contact with the medium. Mature seeds were germinated on filter paper supports and leaf bases were taken when the seedlings were approximately 1.5 cm long. The explants were surface sterilised as described earlier (Bapat *et al.*,1988). These were cultured on MS (Murashige and Skoog's medium, 1962) modified by the elimination of edamine, Ki and IAA and supplemented with 0.4 mg/l thi-HCl, 2mg/l 2,4-D, 30g/l sucrose and 0.4% agar (henceforth referred to MMS meduim). Cultures were maintained at 27 C in the dark. For regeneration, cultures were transferred to MMS without 2,4-D, but with 1 mg/l Ki and were shifted to a 16 h photoperiod at an illumination of 11.67 μ E m^{-2} s^{-1}.

Two plasmid constructs viz. pRT 103 neo and pASK 10, which contained the NPT II gene, were used in the transformation experiments. Plasmid DNA was extracted using the standard methods described by Maniatis *et al.*(1982). Wheat embryos of the genotype VL-614 were dissected out from soaked seeds and were further kept for imbibition in DNA solution (50 μ g/ml) for 48 hrs. These were then thoroughly washed with sterile distilled water and placed on MMS medium without hormones, but supplemented with 200 mg/l kanamycin monosulfate. Cultures wre maintained at 27 C with a 16 h photoperiod.

RESULTS AND DISCUSSION

Somatic embryogenesis was observed in all 3 explants of the 5 genotypes tested. A common feature encountered with all the explants was the genotypic effect on embryogenesis and plantlet regeneration (Table).

Table : Comparative account of embryogenesis and plantlet regeneration

Genotype	Immature embryos		Immature Infloresences		Leaf Bases	
	E	R	E	R	E	R
NI-5439	70.0	100.0	73.3	68.0	30.0	24.0
NI-9272	71.0	83.0	60.0	67.8	18.2	12.0
Raj-1777	83.3	100.0	91.0	84.0	42.0	31.0
VL-614	70.3	97.7	86.67	81.1	17.0	14.8
CPAN-2005	71.4	100.0	70.0	65.0	32.0	23.3

E = Percentage somatic embryogenesis

R = Percentage regeneration

Somatic embryogenesis from leaf bases has been reported earlier (Wernicke and Milkovits, 1984). However, the frequency reported by these authors is extremely low (less than 10%). The relatively higher frequency of embryogenesis observed in our experiments (17 - 42%) may be attributed to the selection of highly embryogenic genotypes. As compared to immature embryos and inflorescences, the percentages of embryogenesis and plantlet regeneration with leaf bases are lower; however, it would be interesting to develop the latter system as a potential source of totipotent cells for protoplast isolation and regeneration.

In the immature inflorescences cultured, almost all the E callus formed originated from the rachis while the floral primordia only showed swelling upto 30 days of culture on 2,4-D containing medium.

On the other hand, in case of the immature embryos, the scutellar callus was embryogenic while that derived from the embryo axis was non embryogenic. The E callus, on transfer to regeneration medium, rapidly gave rise to plantlets. In the genotypes NI-5439, Raj-1777 and VL-614, the percentage embryogenesis was higher when immature inflorescences rather than immature embryos were used as explants. However, considering their respective regeneration capacities, the immature embryos provide a better system for embryogenesis and regeneration.

Mature embryos treated with DNA started germinating on the kanamycin - containing medium within 3-4 days of transfer. After two weeks of germination, cultures were scored for expression of kanamycin resistance. Sensitive plants could be readily recognised by virtue of their bleached appearance. On the other hand, the putative transformants, which remained green, were slow growing. In case of the pASK 10 plasmid, 2 transformed plants out of 307 seed imbibed, while in case of the pRT 103 neo plasmid 3 transformed plants out of 348 imbibed, were obtained. The transformation frequency using these plasmid constructs are fairly high as compared with earlier reports using other methods (Lorz *et al.*,1985; Potrykuset *et al.,1985).* The transformants are being maintained *in vitro* for further studies.

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Isozyme profiles during ontogeny of somatic embryos in wheat (*Triticum aestivum* L.)

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The developmental patterns of isozymes of some key enzymes in wheat were investigated. The transition of the immature embryos to the formation of embryogenic (E) or non-embryogenic (NE) callus and finally to germination of somatic embryos was associated with selective expression or repression of isoforms of peroxidase, esterase, glutamate dehydrogenase (GDH), hexokinase, phosphogluco-isomerase (PGI), 6-phosphogluconate dehydrogenase (6PgDH), glucose-6-phosphate dehydrogenase (G6PDH), and malate dehydrogenase (MDH). An isoperoxidase (Rm 0.32), isoesterases of Rm values 0.93 and 0.80, an isohexokinase (Rm 0.17), an isoG6PDH (Rm 0.23) and isoMDHs of Rm values 0.85, 0.82 and 0.15 were expressed during the early inductive phase of embryogenesis and in embryogenic (E) callus. Isohexokinases of Rm values 0.32 and 0.24 and an isoPGI (Rm 0.34) were expressed exclusively in the E calli. Callus specific isoforms which were expressed in the E and NE calli included three isoperoxidases (Rm values 0.61, 0.58 and 0.47), two isoesterases (Rm values 0.72 and 0.64), two isohexokinases (Rm 0.71 and 0.38), an isoPGI (Rm 0.16), an iso6PgDH (Rm 0.26) and isoG6PDHs of Rm values 0.89, 0.74, 0.65, 0.54, 0.38, 0.29 and 0.10. Each developmental stage is associated with a definite isozyme profile.

Key words: Triticum aestivum; somatic embryogenesis; isozyme analysis

Introduction

A major pathway for plant regeneration in tissue cultures is through somatic embryogenesis. The process of somatic embryogenesis has been reported in several cereals like rice, wheat, sorghum, millets, etc. [1]. Though the culture conditions required are well defined, the molecular and biochemical events underlying the phenomenon of somatic embryogenesis in cereals are ill defined. An understanding of these events would help overcome difficulties like genotype dependent embryogenic responses [2].

Transition to somatic embryos from unorganised cells encompases the organisation of actively dividing meristematic cells into different developmental stages. Changes in the isoenzyme patterns, i.e. synthesis of new isoforms or repression of the existing ones may be a part of the functional specialization of different cell populations during somatic embryo formation. Thus, enzyme isoforms have been shown to be involved in the regeneration process of somatic embryos in cereals [3–7]. Given the fact that isoenzymes are co-dominantly expressed and developmentally regulated, their study and analysis is an important step towards establishing the molecular events which trigger the induction and subsequent development of the somatic embryo and thereby establish the presence of causative agents at molecular and macromolecular levels.

Earlier, we had demonstrated genotype dependence of somatic embryogensis in wheat [2]. A total of 33 genotypes of *Triticum aestivum* were screened for their embryogenic potential. The genotype Raj-1777 was identified as exhibiting the highest frequency of somatic embryogenesis (83%) and at the same time the embyrogenic callus could continously segregate sectors of non-embryogenic calli. In the present study, we have characterised changes in the isoenzyme profiles during the in-

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duction of embryogenic callus, in the embryogenic and non-embryogenic calli, and also during germination of the embryos. This type of characterisation is essential for the elucidation of differential gene expression during the development of somatic embryos especially in cereals on account of their economic importance.

Materials and Methods

Callus establishment and regeneration

Callus cultures were established from immature embryos (harvested 12–14 days after anthesis) on Murashige and Skoog's (MS) basal medium [8] supplemented with 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 3% (w/v) sucrose as described earlier [2]. Embryogenic and non-embryogenic calli were maintained by subculture on the same medium at 30 day intervals. For regeneration, calli were transferred to the MS basal medium supplemented with 1 mg/l kinetin and 3% sucrose.

Sample collection

Tissues were harvested at five developmental stages which were histologically identified. These were: (I) Immature embryos — collected 12–14 days after anthesis (Fig. 1). (II) Inductive phase — this stage was 5–7 days after initial culture and histology revealed meristematic activity in the scutellar epidermis and subepidermis. This is the period when cells develop embryogenic competence and become committed (Fig. 2). (III) Embryogenic (E) callus — this hard, compact and nodular callus was formed from the scutellum after about two weeks of culture and histological examination revealed embryoids at different stages of development. It was in the E calli that embryogenesis in morphological terms occurred (Fig. 3). (IV) Non-embryogenic (NE) callus — which was continuously segregated from the E callus and was soft, friable and appareantly unorganised in nature. (V) Germinating somatic embryos in embryogenic callus — this was the stage when the somatic embryos germinate (Fig. 4). All the samples were liquid nitrogen powdered and stored at −70°C till further use.

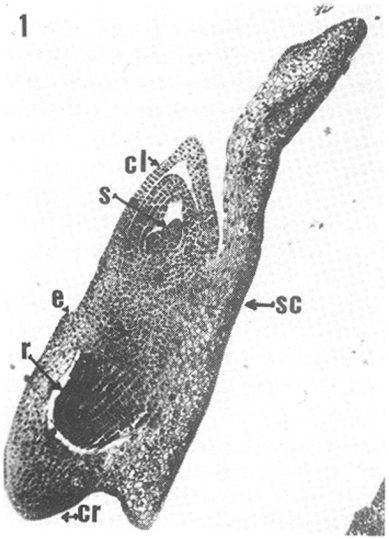


Fig. 1. An immature wheat embryo at the time of culture. (sc, scutellum; e, epiblast; cl, coleoptile; cr, coleorhiza; s, shoot; r, root).

Sample preparation, electrophoretic procedures and activity staining

About 100 mg of liquid nitrogen powdered tissue was homogenised with two volumes of 50 mM Tris–HCl buffer (pH 7.5) containing 2% (v/v) Nonidet p-40. The homogenate was centrifuged at 18 000 × *g* for 20 min at 4°C, and the supernatant fraction was used for isozyme analysis. Isozymes

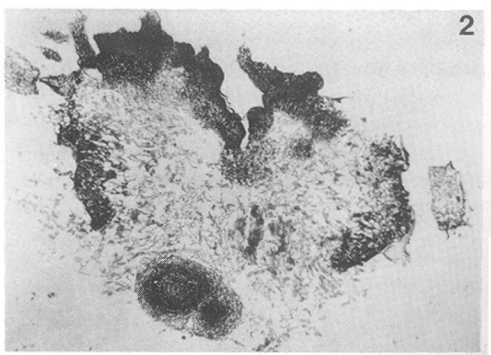
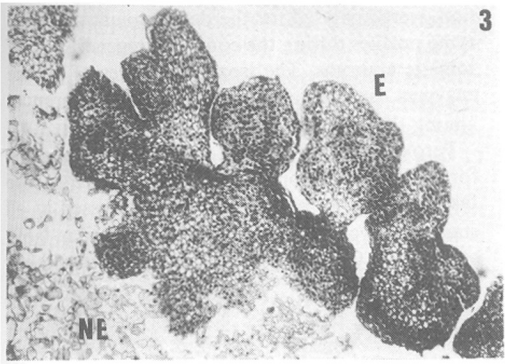


Fig. 2. Inductive phase during wheat somatic embryogenesis.



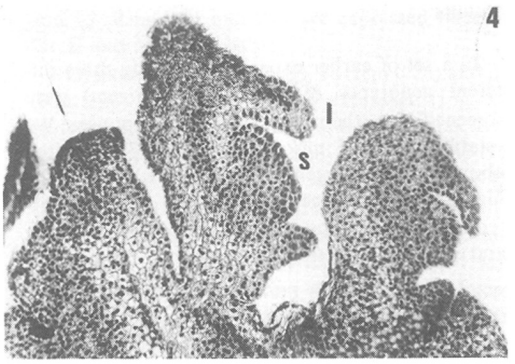


Fig. 3. Embryogenic (E) and non-embryogenic (NE) calli.

Fig. 4. Germinating wheat somatic embryos (l-leaf, s-shoot). The roman numerals in Figures 5–12 indicate: I — Immature embryos, II — Induction phase, III — E calli, IV — NE calli, V — Germinating somatic embryos.

were resolved on 1.5 mm thich native polyacrylamide gels [5% T (total acrylamide), 2.1% C (extent of cross-linking) for stacking gel and 12% T, 2.1% C for separating gel] at 10°C, using a solution containing 25 mM Tris and 192 mM glycine (pH 8.3) as the electrode buffer. Each well was loaded with the equivalent of 100–125 μg protein. Protein estimation was done after the Bradford [9] method. Electrophoresis was carried out at 15 mA constant current till the bromophenol blue dye front reached the bottom (anode) end of the separating gel. The reaction mixtures used for visualization of enzyme activities in the gels were as follows:

Peroxidase (EC 1.11.1.7). 1.61% benzidine hydrochloride, 4.83% ammonium chloride and 0.013% hydrogen peroxide in 100 mM sodium phosphate buffer (pH 6.5) [10].

Esterase (EC 3.1.1.2). 0.02% Fast Blue RR and 0.2% α-naphthyl acetate in 100 mM sodium phosphate buffer (pH 6.5) [11].

GDH (EC 1.4.1.2). 100 mM $MgCl_2$, 200 mM glutamic acid, 10 mM nicotinamide adenine dinucleotide (NAD), 1.6 mg nitrotetrazolium blue (NBT), 0.4 mg *N*-methyl phenazonium methosulfate (PMS) in 8 ml of 20 mM Tris–HCl (pH 7.5) [12].

Hexokinase (EC 2.7.1.1). 100 mM $MgCl_2$, 75 mM glucose, 5 mM adenosine triphosphate, 15 mM nicotinamide adenine dinucleotide phosphate (NADP), 2 units of G6PDH, 1.6 mg NBT and 0.4 mg PMS in 8 ml of 100 mM Tris–HCl (pH 8.4) [12].

PGI (EC 5.3.1.9). 100 mM $MgCl_2$, 40 mM fructose-6-phosphate, 15 mM NADP, 3.84 mg NBT, 0.6 mg PMS in 8 ml of 20 mM Tris–HCl (pH 7.5) [12].

6PgDH (EC 1.1.1.44). 100 mM $MgCl_2$, 40 mM 6-phosphogluconate, 15 mM NADP, 3.84 mg NBT and 0.6 mg PMS in 8 ml of 20 mM Tris–HCl (pH 7.5) [12].

G6PDH (EC 1.1.1.49). 100 mM $MgCl_2$, 100 mM glucose-6-phosphate, 15 mM NADP, 3.84 mg NBT and 0.6 mg PMS in 8 ml 20 mM Tris–HCl (pH 7.5) [12].

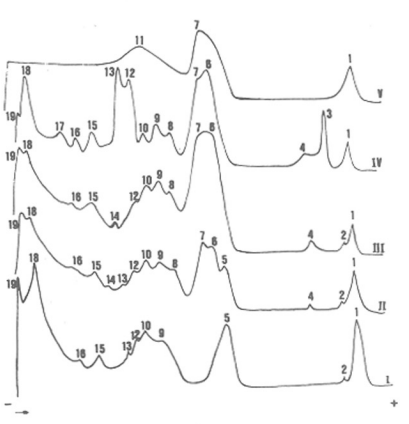
MDH (EC 1.1.1.37). 100 mM $MgCl_2$, 20 mM malate, 10 mM NAD, 3.84 mg NBT and 0.6 mg PMS in 8 ml 20 mM Tris–HCl (pH 7.5) [12].

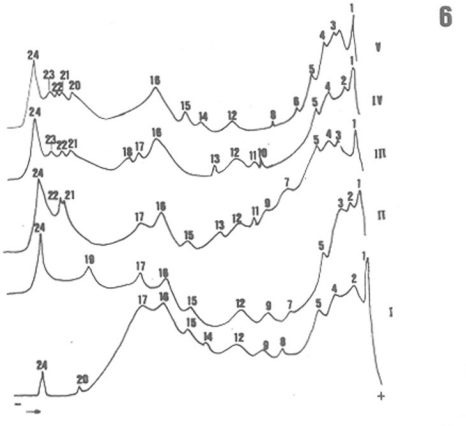
All reactions except that for peroxidases were carried out in dark at 37°C. The gels after staining for the respective enzymes were scanned on a Perkin-Elmer Lambda 15 UV-VIS double beam spectrophotometer at 590 nm, excepting those for esterase which were scanned at 470 nm.

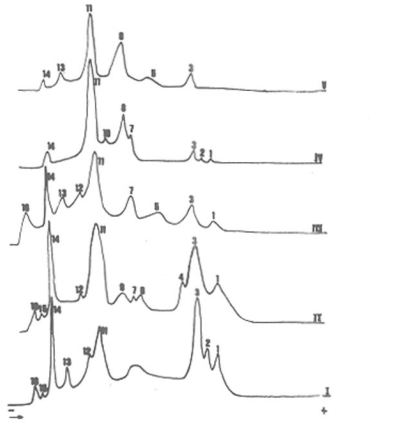
Results

In a set of earlier experiments, thirty-three different genotypes of wheat (*T. aestivum*) were screened for their embryogenic response. We established that *T. aestivum* cv. Raj-1777 exhibited about 83% embryogenic response — which was the highest among the genotypes tested [2]. Five different developmental stages, verified histologically and enumerated in the Materials and Methods section, were analysed for the development of isoenzyme profiles during the course of regeneration of somatic embryos. The isozyme profiles of all the enzymes studied showed marked differences among the five samples.

Peroxidases exhibited a total of 19 isozyme forms (Fig. 5). Isoperoxidase 1 (Rm 0.94), the fastest migrating peroxidase, was expressed at all stages of development. Isoperoxidases 2 (Rm 0.59) and 14 (Rm 0.37), were expressed in the E calli but







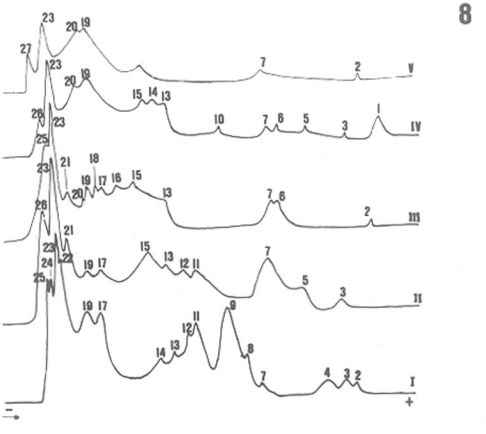


Fig. 5. Superimposed gel scans of wheat isoperoxidases.
Fig. 6. Superimposed gel scans of wheat isoesterases.
Fig. 7. Superimposed gel scans of wheat isoGDH.
Fig. 8. Superimposed gel scans of wheat isohexokinases.

remain suppressed in the NE calli. On the contrary isoperoxidases 3 (Rm 0.88), 13 (Rm 0.40) and 17 (Rm 0.26) were expressed in the NE calli while remaining suppressed in the E calli. Isoperoxidase 11 (Rm 0.43) was expressed only during the germination phase, but isoforms 9,10,12,15,16,18 and 19 (Rms 0.44, 0.43, 0.40, 0.28, 0.26, 0.17 and 0.15 respectively) were expressed at all stages except during the germination phase. Isoperoxidases 4, 6 and 8 (Rms 0.61, 0.58 and 0.47) were expressed only in the E and NE calli.

Five isoesterases 1, 5, 12, 16 and 24 (Rms 0.97, 0.86, 0.67, 0.50 and 0.21, respectively) were expressed at all the stages of development (Fig. 6). Isoesterases 3, 7, 9 and 15 (Rms 0.93, 0.80, 0.75 and 0.57) which were associated with the E calli were conspicuously absent in the NE calli while isoforms 2, 10, 18 and 23 (Rms 0.95, 0.73, 0.43 and 0.25 respectively) which were associated with the NE calli were absent in the E calli. Isoesterase 6 (Rm 0.83) was found to be expressed only during germination of the somatic embryos while isoform 17 (Rm 0.44) was associated with all the developmental stages except germination. Isoesterases 11 (Rm 0.72) and 13 (Rm 0.64) were detected only in the E and NE calli.

GDH isoenzyme bands 3 (Rm 0.57), 11 (Rm 0.28) and 14 (Rm 0.13) were constitutively expressed (Fig. 7). IsoGDHs 5, 12, 13 and 16 (Rms 0.46, 0.22, 0.18 and 0.09, respectively) were synthesized in the NE calli and not in the E calli. No isoform was found to be exclusive in the E. calli. The expression of band 7 (Rm 0.78) was abolished during the germination of embryoids. No isoforms were identified which were found to be exclusive to either the E or NE calli.

Isohexokinases 7, 19 and 23 (Rm's 0.68, 0.23 and 0.14, respectively) were expressed at all the stages of development (Fig. 8). Isoforms 2, 16, 17, 18, 21 and 25 (Rms 0.91, 0.32, 0.25, 0.24, 0.17 and 0.12 respectively) were expressed in the embryogenic callus (E callus) but not in the NE callus. Isoforms associated with the NE calli but not with the E calli included 1, 3, 5, 10, 14 and 26 (Rms 0.97, 0.87, 0.78, 0.52, 0.41 and 0.10, respectively). Isohexokinase 27 was exclusively associated with the germination phase whereas isoform 13 (Rm 0.42) was repressed during the period. Isoforms 6 and 15 (Rms 0.71 and 0) were expressed both in the E and NE calli only.

PGI isoforms 14 (Rm 0.37), 18 (Rm 0.24) and 20 (Rm 0.21) were constitutively expressed (Fig. 9). Isoforms 15 and 19 (Rms 0.34 and 0.22, respectively) were expressed in the E calli but not in the NE calli whereas isoforms 2, 3, 4, 5, 6, 8, 9 and 12 (Rms 0.94, 0.93, 0.91, 0.90, 0.88, 0.75, 0.58 and 0.42, respectively) were expressed in the NE calli but not in the E calli. IsoPGI 11 (Rm 0.44) was specific for the germination phase while isoforms 10 (Rm 0.46) and 17 (Rm 0.26, respectively) were expressed at all other stages but were absent in the germination phase. Isoform 22 (Rm 0.16) was found to be callus specific.

Three G6PDH isoenzymes were constitutively expressed (Fig. 10). These were 13 (Rm 0.44), 19 (Rm 0.22) and 21 (Rm 0.15). IsoG6PDH bands 1, 3, 6, 12, 18, 20, 25 and 27 (Rms 0.94, 0.88, 0.47, 0.23, 0.20, 0.09 and 0.05, respectively) were expressed in the E calli but remained suppressed in the NE calli. The G6PDH isoform 7 (Rm 0.80) was synthesized in large amounts in the NE calli while its synthesis was totally abolished in the E calli. Isoform 4 (Rm 0.91) was associated with all the stages except the germination phase. Seven G6PDH isoforms expressed were exclusively found in both the E and NE calli. These include isoforms 5 (Rm 0.89), 8 (Rm 0.74), 10 (Rm 0.65), 11 (Rm 0.54), 14 (Rm 0.38), 17 (Rm 0.29) and 24 (Rm 0.10).

6PgDH isoforms 5, 12 and 15 (Rms 0.49, 0.24 and 0.13) were constitutively expressed (Fig. 11). Isoform 16 (Rm 0.12) was expressed in the E calli but not in the NE calli. On the contrary isoforms 1, 2, 4, 6, 7, 9, 10, 14 and 20 (Rms 0.93, 0.86, 0.63, 0.45, 0.42, 0.32, 0.30, 0.17 and 0.07, respectively) were expressed in the NE calli but not in the E calli. No isoforms were found to be associated exclusively with the germination phase. Only one isoform viz. band 11 (Rm 0.26) could be identified as being exclusively found in both the E and NE calli.

MDH isoforms 3 (Rm 0.86), 14 (Rm 0.43) and 15 (Rm 0.38) were constitutively expressed (Fig. 12). IsoMDHs expressed in the E calli but repressed in the NE calli include isoforms 1, 4, 6, 17, 19, 22, 23 and 25 (Rms 0.90, 0.85, 0.82, 0.24, 0.19,

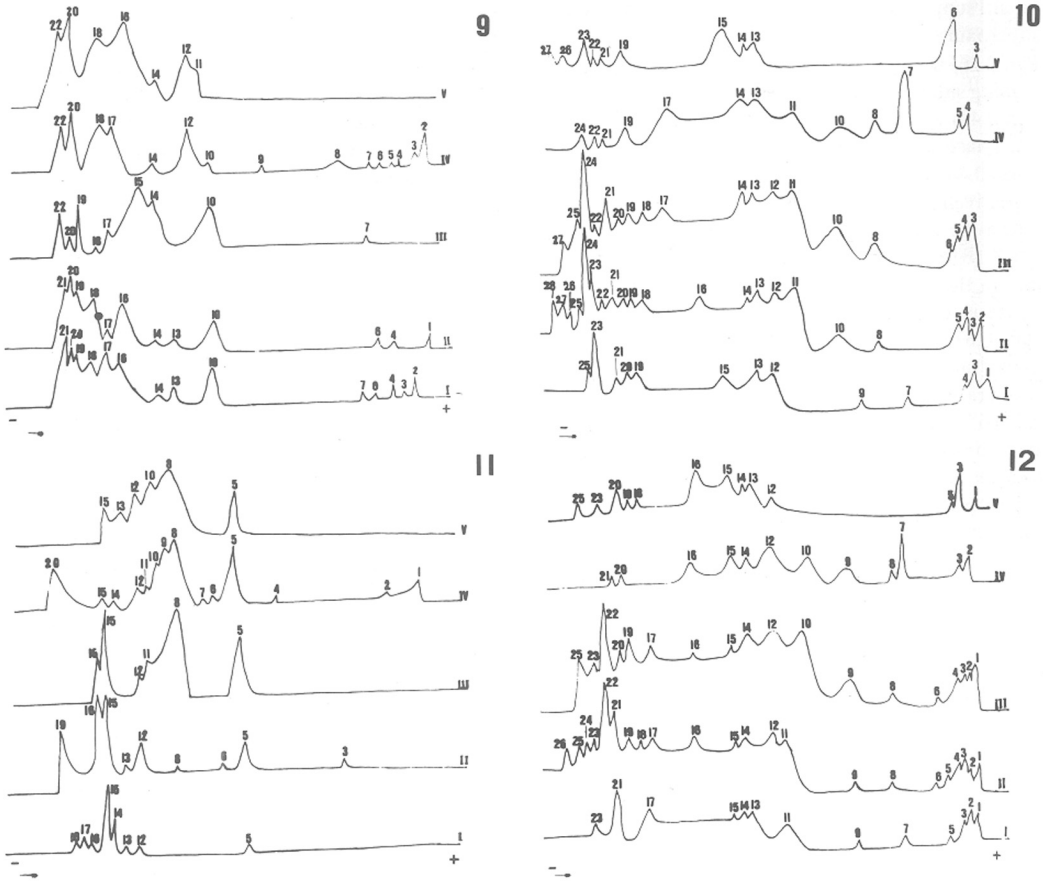


Fig. 9. Superimposed gel scans of wheat isoPGI.
Fig. 10. Superimposed gel scans of wheat isoG6PDH.
Fig. 11. Superimposed gel scans of wheat iso6PgDH.
Fig. 12. Superimposed gel scans of wheat isoMDH.

0.14, 0.12 and 0.09, respectively). IsoMDHs 7 (Rm 0.75) and 21 (Rm 0.16) expressed in the NE calli were not detected in the E calli. Isoforms 2 (Rm 0.88) and 9 (Rm 0.66) were expressed at all the stages except during germination. No isoforms could be identified as being exclusive to both the E and NE calli.

Discussion

Somatic embryogenesis has been considered to occur in a sequential manner [13]. The target cells are first thought to develop competence to undergo embryogenesis. These then become committed to or determined for the pathway of

somatic embryogenesis. These determined cells now undergo sequential divisions leading to the formation of proembryos and fully developed embryos in the embryogenic (E) calli. This E callus is recognised on the strength of histological examination and its regenerative potential. Based on histological analyses, four stages in the development of somatic embryos could be recognised in wheat viz. the immature embryo, the inductive phase of embryogenesis, the E calli and the germinating somatic embryos. It is likely that only a certain percentage of the total cell mass undergoes embryogenesis. It is obvious that specific biochemical changes would accompany the differentiation into different cell types during development. Hence, to gain an insight into the process, we have resorted to subtractive analysis of isoforms of key enzymes.

Due to some genetic instability within the E calli, certain sectors turned into a soft, friable and unorganised callus — the NE (non-embryogenic) callus which in our hands could never be differentiated. Similar observations have been made earlier with rice [14], maize [15] and orchard grass [16]. This NE callus served as a reference and a negative control for the process of somatic embryogenesis.

On the basis of the isoenzyme analyses during somatic embryogenesis, we can divide the embryogenic pathway into three major events. These are: (1) *the early events* — these would include the metabolic and molecular changes that occur during the inductive phase and in the E calli at a stage when the cells develop competence and become committed towards embryogenesis. Specific enzyme isoforms which were expressed during this stage were isoperoxidase 14 (Rm 0.32), isoesterases 3 and 7 (Rms 0.93 and 0.80, respectively), isohexokinase 21 (Rm 0.17), isoG6PDH 18 (Rm 0.23) and isoMDHs 4, 6 and 22 (Rms 0.85, 0.82 and 0.15, respectively). GDH, PGI and 6PgDH do not show any participation here; (2) *the midterm events* — these would occur exclusively in the E calli, since it is here that committed or determined cells organise to form the proembryos which develop further into morphologically fully developed bipolar embryos. Novel midterm event enzyme isoforms were hexokinase isoforms 16 and 18 (Rms 0.32 and 0.24, respectively) and PGI isoform 15 (Rm 0.34); (3) *the late events* — these would occur in morphologically fully developed somatic embryos, be these the ones formed in E calli or the germinating embryos regenerated from the E calli or the immature embryo explant. In all of these three instances the embryos are in an advanced state of development and can form entire plants under permissive conditions. Isoesterase 15 (Rm 0.57), isoGDH 13 (Rm 0.18), isohexokinase 2 (Rm 0.91) and isoG6PDHs 3 and 15 (Rms 0.91 and 0.36, respectively) were associated with this stage; Surprisingly, 6PgDH does not show participation in any of the three events listed. Peroxidase and MDH are involved only in the early events. Esterase and G6PDH are not associated with midterm events, while PGI shows only midterm event association and GDH is involved only during the late events. Hexokinase shows differential expression at all the different developmental stages.

On comparison of the enzyme profiles in E and NE calli we observed that these had many isoforms in common but had also a few which were exclusively expressed at these 2 stages. The only common denominator among these two tissue types was the formation of callus and its growth. Hence this set of exclusive isoenzymes were considered to be callus-specific. These included isoperoxidases 4, 6 and 8 (Rms 0.61, 0.58 and 0.47, respectively), isoesterases 11 and 13 (Rms 0.72 and 0.64, respectively), isoGDH 7 (Rm 0.38), isohexokinase 6 (Rm 0.71), isoPGI 22 (Rm 0.16), isoG6PDHs 5, 8, 10, 14, 17 and 24 (Rms 0.89, 0.74, 0.65, 0.54, 0.38, 0.29 and 0.10, respectively) and iso6PgDH 11 (Rm 0.26).

On further comparison of the isoenzyme profiles in the E and NE calli, we found that several enzyme isoforms are expressed in the E calli but are not in the NE calli, and vice versa. These novel isoforms discriminate, on a biochemical level, between the E calli which regenerate embryos, and the NE calli which remain unorganised, when both are transferred to the same regeneration medium. The molecular biological basis for selective stage-specific accumulation of isozymes, however, remains to be elucidated.

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