1

PM-1 3 1/2"x4" PHOTOGRAPHIC MICROCOPY TARGET
NBS 1010a ANSI/ISO #2 EQUIVALENT

PRECISION^{SM} RESOLUTION TARGETS

PIONEERS IN METHYLENE BLUE TESTING SINCE 1974

MICROD
SOLUTIONS

1800 COUNTRY ROAD & SUITE 100, BURNING WOOD, OHIO 44027 USA
TEL: 512 426 7887 FAX: 512 426 7887 TLF: 510000400

National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file - Votre référence

Our file - Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

BIOCHEMICAL AND DEVELOPMENTAL MARKERS OF
INDUCTION OF
SOMATIC EMBRYOGENESIS IN ALFALFA
TISSUE CULTURE

by

KIRSTEN IRENE FINSTAD, B.Sc., M.Sc.

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of
the requirements for the degree of

Doctor of Philosophy

Department of Biology

Carleton University

Ottawa, Ontario

16 June 1992

© copyright

1992, Kirsten Finstad

National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Votre titre / Votre référence

Votre titre / Votre référence

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-315-79887-4

The undersigned hereby recommend to
the Faculty of Graduate Studies and Research
acceptance of the thesis,

"BIOCHEMICAL AND DEVELOPMENTAL MARKERS OF
INDUCTION OF
SOMATIC EMBRYOGENESIS IN ALFALFA
TISSUE CULTURE"

submitted by
KIRSTEN IRENE FINSTAD, B.Sc., M.Sc.

in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

_____
Chair, Department of _____

_____
Thesis Co-supervisor

_____
Thesis Co-supervisor

_____
External Examiner

Carleton University

ABSTRACT

Induction of somatic embryogenesis was studied in well-defined callus and suspension cultures of alfalfa (*Medicago sativa* L.).

A competence phase, during which a state of receptiveness to the inductive signal is acquired, and the induction phase itself, were clearly defined. A published (Meijer EGM and Brown DCW 1987, *Physiol. Plant.* 69:591-596) protocol for somatic embryogenesis in callus culture was optimized and used as an experimental system. Through the use of a non-embryogenic auxin (phenylacetic acid), competence was demonstrated in the initial three to four days of callus culture from petiole segments. Induction occurred during subsequent culture in the presence of the embryogenic auxin, 2,4-dichlorophenoxyacetic acid (2,4-D). Once competent, the tissue could be induced by very short exposures to 2,4-D.

Capacity for somatic embryogenesis in alfalfa was shown to be influenced by genotype. Intermediate degrees of response were observed, suggesting either additive effects or involvement of more than one locus.

Glutamate dehydrogenase isozyme pattern, glutamate dehydrogenase and glutamine synthetase activities, amino

acid contents and tissue ammonium levels were studied in suspension cultures to examine the relationship between ammonium assimilation and induction of embryogenesis.

While the isozyme banding pattern of glutamate dehydrogenase was very variable among genotypes, it was not related to embryogenic capacity. This suggests that previous reports of correlations between isozyme patterns and embryogenesis might be attributable to genotype differences other than embryogenic capacity. Treatment with the non-embryogenic auxin phenylacetic acid tended to increase the occurrence and intensity of faster-migrating bands.

In the presence of 2,4-D, which induces embryogenesis, glutamate dehydrogenase activity increased sharply. Exposure to phenylacetic acid did not induce embryogenesis but slightly enhanced glutamine synthetase and greatly increased accumulation of asparagine. It is suggested that auxin type influences the routing of ammonium assimilation, and that this could play a role in embryogenesis induction.

ACKNOWLEDGEMENTS

I would like to thank my supervisors Dr. D.C.W. Brown and Dr. K.W. Joy for their interest, advice and assistance. I am grateful for the use of laboratory and computer facilities at the Plant Research Centre. My sincere thanks are also due to the staff of Building 21 of the Plant Research Centre for their help and encouragement.

These studies were partially funded by an Ontario Graduate Scholarship award.

TABLE OF CONTENTS

	Page
Acceptance sheet	ii
Abstract	iii
Acknowledgements	v
Table of contents	vi
List of tables	ix
List of figures	xi
List of abbreviations	xiii
List of media	xiv
 Chapter 1. Introduction	 1
 Chapter 2. Callus culture manipulations and demonstration of competence and induction phases	 4
2.1 Literature review	4
2.1.1 Factors affecting in vitro somatic embryogenesis	4
2.1.1.1 Genotype	5
2.1.1.2 Media and protocol	7
2.1.2 The concept of competence	10
2.2 Materials and methods	14
2.2.1 Donor plant material	14
2.2.1 Tissue culture protocol	15
2.3 Results and discussion	18
2.3.1 Effect of genotype on embryogenesis	18
2.3.2 Effect of auxin choice and concentration on embryogenesis	22
2.3.3 Effect of kinetin on embryogenesis	28
2.3.4 Optimization of protocol	30
2.3.5 Time of induction and demonstration of competence phase within the induction period	33
2.3.6 Perception of the inductive signal	39
2.3.6.1 Immersion of calli in liquid medium	40
2.3.6.2 Minimum MS1 exposure required to induce embryogenesis in competent tissue	43
2.3.7 Effects of PAA on competence and differentiation	47
2.3.7.1 Relative effectiveness of PAA and 2,4-D in the acquisition of competence	47
2.3.7.2 Effect of PAA following embryogenesis induction	50

Chapter 3. Development of experimental system	52
3.1 General alfalfa cell suspension method	54
3.2 Development of non-inductive liquid suspension culture	56
3.2.1 Effect of mechanical homogenization	57
3.2.2 Effect of auxin and cytokinin type and concentration in suspension medium	59
3.2.3 Effect of auxin and cytokinin type and concentration in callus initiation medium	60
3.2.4 Non-embryogenic suspensions from calli cultured on MS1 and B5h	61
3.3 Selection of embryogenic and non-embryogenic genotypes of 'Rangelander'	66
Chapter 4. Ammonium assimilation in embryogenic and nonembryogenic cultures and genotypes	69
4.1 Literature review	69
4.1.1 Nitrogen requirements of somatic embryogenesis	69
4.1.2 Ammonium assimilation in plants	71
4.1.2.1 The role of glutamate dehydrogenase	74
4.1.3 Isozymes and embryogenesis	78
4.1.3.1 Glutamate dehydrogenase isozymes and embryogenesis	81
4.2 Materials and methods	84
4.2.1 Suspension cultures	84
4.2.1.1 Embryogenic and non-embryogenic suspension cultures of 'Rangelander' genotypes	84
4.2.1.2 Nitrogen source experiments	84
4.2.2 Determination of embryogenic capacity of suspensions	87
4.2.3 Tissue extraction	87
4.2.3.1 Extraction buffers for ammonium and GS assays	88
4.2.3.2 Extraction buffers for GDH gels and assays	89
4.2.3.3 Protein assay	90
4.2.4 Glutamate dehydrogenase assay	92
4.2.5 Glutamate dehydrogenase activity-stained gels for isozyme visualization	94
4.2.6 Glutamine synthetase assay	97
4.2.7 Amino acid determination	100
4.2.8 Ammonium assay	101

4.3	Results and discussion	104
4.3.1	Comparison of embryogenic and non-embryogenic cultures and genotypes	104
4.3.1.1	Glutamate dehydrogenase activity	104
4.3.1.2	Glutamate dehydrogenase isozyme pattern	107
4.3.1.3	Glutamine synthetase activity	116
4.3.1.4	Amino acid profile	118
4.3.1.5	Ammonium content	126
4.3.2	Effect of varying ratio of $\text{NO}_3^-:\text{NH}_4^+$ in medium	129
4.3.2.1	Degree of embryogenesis	129
4.3.2.2	Glutamate dehydrogenase activity	132
4.3.2.3	Glutamate dehydrogenase isozyme pattern	134
4.3.2.4	Glutamine synthetase activity	137
4.3.2.5	Ammonium content	139
4.3.3	Relation to embryogenesis	141
Chapter 5.	Summary and conclusions	154
	References	159
Appendix I.	Recipes for discontinuous, non-denaturing electrophoresis gels and buffers	178
Appendix II.	Recipes for experimental extraction buffers	179
Appendix III.	Amino acid contents of various suspensions at various subcultures	180

LIST OF TABLES

	Page
2.1 Sources of <i>Medicago</i> selections used in the genotype experiment	16
2.2 Callus rating at day 10, number of days to appearance of first embryo (DTFE), percentage of calli producing embryos and number of embryos per callus at day 30 in eighteen <i>Medicago</i> selections	20
2.3 Mean number of embryos per callus at day 30, callus rating (CF) at day 10 and number of days to appearance of first embryo (DTFE) in A70.34, cultured with different auxin types and concentrations and with and without kinetin	23
2.4 Mean number of embryos per callus at day 26, callus rating at day 26 and number of days to appearance of first embryo (DTFE) in A70.34 following exposure to liquid or solid MS1	41
2.5 Mean number of embryos per callus at day 26, callus rating at day 6 and number of days to appearance of first embryo (DTFE) in A70.34 following various exposures to MSPAA and MS1	44
3.1 Initial packed cell volume ratio (PCRI) and embryogenic capacity in A70.34 suspension	

cultures in various media	63
4.1 Composition of media for nitrogen source experiment	85
4.2 "Vegetative extraction buffer II" of Cheliak and Pitel (1984)	91
4.3 Reaction mixture for glutamate dehydrogenase assay, final concentrations	93
4.4 Glutamate dehydrogenase activity in μ units mg^{-1} protein	105
4.5 Glutamine synthetase activity in μ units mg^{-1} protein	117
4.6 Probabilities of non-significant effects due to genotype and medium, from two-way analyses of variance performed on each of 20 amino acids	120
4.7 Total amino acids in picomoles mg^{-1} fresh weight of samples for which simultaneous measurements in 2,4-D- and PAA-containing media were made	124
4.8 Ammonium content in nmol mg^{-1} protein	127
4.9 Summary of data from genotype/medium experiments	142
4.10 Summary of data from nitrogen source experiments	144

LIST OF FIGURES

	Page
2.1 Optimization of induction phase. Effect of varying length of exposure to MS1 on number of embryos after 30 d in culture	31
2.2 Optimization of induction phase. Effect of varying length of exposure to MS1 on number of embryos 20 d after transfer to MS2	32
2.3 Demonstration of competence phase. Effect of varying length of exposure to MSPAA prior to exposure to MS1 on number of embryos 20 d after transfer to MS2	34
2.4 Acquisition of competence in presence of various growth regulator combinations	37
2.5 Minimum exposure to MS1 required for induction of embryogenesis	45
2.6 Relative effectiveness of MSPAA and MS1 in the acquisition of competence, and effect of MSPAA on embryogenesis	49
 4.1 The GS/GOGAT pathway of ammonium assimilation (after Miflin and Lea 1982)	 73
4.2 Example of glutamate dehydrogenase assay spectrophotometric output	95
4.3 Standard curve for glutamine synthetase assay	99

4.4	Standard curve for ammonium assay	103
4.5	Zymograms of glutamate dehydrogenase activity-stained gels. Extracts from various genotypes in B5mod and 95PAA suspensions and from in vitro shoots ..	108
4.6	Effect of varying ammonium level in the suspension medium on embryogenesis	131
4.7	Effect of varying ammonium level in the suspension medium on glutamate dehydrogenase activity	133
4.8	Zymograms of glutamate dehydrogenase activity-stained gels. Extracts from A70.34 suspensions with varying ammonium level in the suspension medium	135
4.9	Effect of varying ammonium level in the suspension medium on glutamine synthetase activity	138
4.10	Effect of varying ammonium level in the suspension medium on tissue ammonium content	140
4.11	Proposed routes of ammonium assimilation in suspensions under different growth regulator treatments	150

LIST OF ABBREVIATIONS

AS	asparagine synthetase
BAP	benzylamino purine
CF	subjective rating of callus formation from 0 (no callus) to 7 (maximum callus)
4-Cl-IAA	4-chloro-indole-3-acetic acid
4-CPA	4-chlorophenoxyacetic acid
2,4-D	2,4-dichlorophenoxyacetic acid
DTFE	days to appearance of first embryo
EDTA	ethylenediaminetetracetic acid
GDH	glutamate dehydrogenase
GOGAT	glutamate synthase
GS	glutamine synthetase
IAA	indole-3-acetic acid
IPA	indole-3-propionic acid
NAA	1-naphthaleneacetic acid
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide, reduced form
NADP	nicotinamide adenine dinucleotide phosphate
NADPH ₂	nicotinamide adenine dinucleotide phosphate, reduced form
PAA	phenylacetic acid
PCRI	initial packed cell volume ratio
2,4,5-T	2,4,5-trichlorophenoxyacetic acid
TEMED	N, N, N', N'-tetramethylethylenediamine

LIST OF MEDIA

MS media - based on basal medium of Murashige and Skoog (1962), supplemented as follows:

MS1	22.6 μ M 2,4-D, 4.7 μ M kinetin
MS2	no additions
MS3	4.7 μ M kinetin
MSIAA	22.6 μ M IAA, 4.7 μ M kinetin
MSPAA	22.6 μ M PAA, 4.7 μ M kinetin

B5 media - based on basal medium of Gamborg et al. (1968), supplemented as follows:

B5h	3% sucrose, 5.5 mM glutamine, 32.5 μ M glutathione, 0.95 mM serine, 7.4 μ M adenine, 4.5 μ M 2,4-D, 0.9 μ M kinetin, 0.9% agar
B5mod	4.1 μ M nicotinic acid, 2.4 μ M pyridoxine HCl, 3.0 μ M thiamine HCl, 0.55 mM myo-inositol, 4.5 μ M 2,4-D, 0.54 μ M NAA
B5IAA	as B5mod but with 5.06 μ M IAA in place of 2,4-D and NAA
B5PAA-1	as B5mod but with 5.06 μ M PAA in place of 2,4-D and NAA
B5PAA	as B5mod but with 50.6 μ M PAA in place of 2,4-D and NAA

BOi2Y Basal medium of Blaydes (1966) plus 0.55 mM inositol and 0.2% yeast extract (Bingham et al. 1975)

Chapter 1. Introduction

Plant somatic embryogenesis is the process of development of embryos directly from non-zygotic, somatic plant cells without a preceding gametogenesis and fertilization. In vitro somatic embryogenesis was first reported in 1958 independently by Steward et al. (1958) and Reinert (1958) in carrot culture. Since then the process has been described in over a hundred different plant species (Terzi and LoSchiavo 1990), including monocots, herbaceous and woody dicots and gymnosperms (Ammirato 1989).

In recent years interest in somatic embryogenesis has been great due to the potential benefits to agriculture, forestry and horticulture (Terzi and LoSchiavo 1990, Sharp et al. 1982, Ammirato 1985). As a method of micropropagation it has applications for crops with poor seed set, inefficient vegetatively propagated crops, crops requiring virus eradication and others (Sharp et al. 1982). The potential for industrial scale-up (Ammirato 1989) and production of synthetic seeds is already being explored. Somatic embryos may be useful for making and propagating novel hybrids. They are particularly well suited as a system for crop improvement through genetic engineering (Ammirato 1989, Terzi and LoSchiavo 1990). Finally, because of similarities with zygotic embryogenesis, somatic embryogenesis can

provide a useful experimental system for the study of seed development.

The actual process of somatic embryogenesis, particularly its induction, is poorly understood. Induction has classically been achieved by transferring cells or tissues from one medium to another, usually involving a change or changes in exposure to growth regulators. Whether these changes permit the expression of a natural or innate totipotency by removing constraints or whether the embryogenesis pathway is specifically triggered is not known (Henshaw et al. 1982). In alfalfa and in many other somatic embryogenesis systems including the model system of carrot, exposure to and/or removal from auxin is absolutely required. How auxin treatment leads to the initiation of the embryogenesis pathway is the focus of much research.

A commonly accepted view of induction of morphogenesis *in vitro* is that cells or tissue must first become competent for induction and then are induced by a specific morphogenic signal. The induction, and possibly also the acquisition of competence, are thought to be regulated by auxin. Several studies have shown that a treatment required for morphogenic induction can be subdivided into at least two phases, one of competence and one of induction, each having different requirements or sensitivities (Carman 1990, Smulders et al.

1990, Coleman and Ernst 1990, Christianson and Warnick 1983, 1984, 1985, and Walker et al. 1979). Many attempts have been made to characterize competence and/or induction from biochemical, molecular and developmental viewpoints by comparing embryogenic and non-embryogenic treatments or comparing genotypes of varying embryogenic capacity.

The present work investigates the induction of somatic embryogenesis in alfalfa by the auxin 2,4-dichlorophenoxyacetic acid. Competence and induction are demonstrated and studied in callus culture (Chapter 2). Biochemical changes associated with inductive treatments are studied in suspension cultures of both embryogenic and non-embryogenic alfalfa genotypes (Chapters 3 and 4). Specifically, parameters of nitrogen metabolism are examined, following indications in the literature of an involvement of ammonium assimilation in embryogenesis. A general model is presented to explain the interrelationship of auxin treatment, ammonium assimilation and somatic embryogenesis in alfalfa.

Chapter 2. Callus culture manipulations and demonstration of competence and induction phases

2.1 Literature Review

2.1.1 Factors affecting in vitro somatic embryogenesis

There have been numerous reviews discussing somatic embryogenesis and the main factors controlling the embryogenic response (e.g. Ammirato 1983, Ammirato 1984, Tisserat 1985, Terzi and Loschiavo 1990). These factors fall into three general categories: those relating to the donor plant, the media and protocol followed and the physical conditions during culture. The most important donor plant factors include genotype, growth conditions, growth stage and choice of explant. Media and protocol are the factors most commonly manipulated to induce and control embryogenesis and to investigate the processes involved. Physical culture conditions which may influence embryogenesis include such factors as light, temperature, humidity, osmotic environment and O_2 tension.

Previous studies had identified genotype and protocol manipulations as key factors influencing somatic embryogenesis in *Medicago sativa*, and these were focused on in this chapter. The objectives were to optimize and

improve the existing system developed by Meijer and Brown (1987a) and, for future study of induction-specific processes, to develop a manipulable controlled system and to identify the actual time of induction.

2.1.1.1 Genotype

Genotype is well-established as an important factor influencing the embryogenic response. Among herbaceous dicots, variability in both the occurrence and frequency of somatic embryogenesis has been observed among species (e.g. legumes, Oelck and Schieder 1983; *Cucumis* spp., Punja et al. 1990; *Glycine* spp., Komatsuda and Ko 1990; *Lycopersicon* spp., Koornneef et al. 1986; *Manihot* spp., Szabados et al. 1987), and among cultivars (soybean, Parrot et al. 1989, Komatsuda and Ohyama 1988, Komatsuda and Ko 1990; cotton, Trolinder and Chen 1989, Gawel and Robacker 1990; alfalfa, Matheson et al. 1990, Brown and Atanassov 1985, Brown 1988, Takamizo et al. 1990, Bianchi et al. 1988, Hernandez-Fernandez and Christie 1989). Variation in degree of embryogenesis has also been noted among individuals of a given cultivar (alfalfa, Bingham et al. 1975, Seitzkris and Bingham 1988, Chen et al. 1987, Bianchi et al. 1988, Matheson et al. 1990; potato, Sonnino et al. 1989; red clover, Keyes et al. 1980).

Genotype effects may interact with media or other culture effects. The ranking of 26 soybean cultivars according to embryogenic response changed when the auxin in the embryogenesis medium was changed (Komatsuda and Ohyama 1988). Seitzkris and Bingham (1988) observed an interaction between tissue culture protocol and genotype on embryogenesis in alfalfa. Chen et al. (1987) found that medium and explant source affected the response of less embryogenic alfalfa genotypes but not that of the highly embryogenic ones. Similarly, Brown (1988) found that while medium choice modulated the absolute level of embryogenesis, highly regenerating alfalfa cultivars were consistently so while the relative response of lower regenerating cultivars was more subject to medium.

Evidence for the genetic basis of the embryogenic response comes partly from the fact that highly embryogenic cultivars can be traced to common origin(s). Brown and Atanassov (1985) screened 76 alfalfa cultivars, finding that those showing the highest frequency of somatic embryogenesis had either *Medicago falcata* or 'Ladak' germplasm in their background, or both. Of 33 soybean cultivars, all those showing high levels of embryogenesis were descended from one or both of two ancestral germplasm sources (Parrot et al. 1989).

Inheritance studies have shown that capacity for somatic embryogenesis is heritable and probably controlled by at least two loci. Variations in the degree of embryogenesis expressed in cotton (Gawel and Robacker 1990) and in potato (Sonnino et al. 1989) is taken as evidence for the involvement of more than one gene. F_1 hybrids from parents of different embryogenic capacity may show heterosis (Sonnino et al. 1989) or embryogenesis at less than the midparent value but more than the non-embryogenic parent (Parrot et al. 1989). Hernandez-Fernandez and Christie (1989) selfed and intercrossed three alfalfa genotypes of differing embryogenic capacities and concluded that the embryogenesis trait is controlled by two complementary loci. In F_1BC_1 families descended from a cross between an embryogenic and a non-embryogenic potato clone, two non-embryogenic parents sometimes give rise to embryogenic offspring, suggesting either that the embryogenic alleles are recessive or that there is more than one complementary factor (Sonnino et al. 1989).

2.1.1.2 Media and protocol

The history of plant tissue culture, and somatic embryogenesis in particular, is to a large extent the story of media development. Although the first success in *in vitro* somatic embryogenesis, the classic studies in carrot

by Steward (1958) and Reinert (1959), used White's (1943) basal medium supplemented with coconut milk, the widespread extension of somatic embryogenesis to other angiosperms did not begin until the introduction of Murashige and Skoog's (1962) formulation, commonly referred to as MS medium (Kohlenbach 1977).

Culture media for embryogenesis should provide:

1. a carbon and energy source. Sucrose is by far the most frequently used; glucose, sorbitol and a few other sugars are rarely used (Ammirato 1983).
2. the inorganic macro- and micronutrients essential for growth and metabolism. For embryogenesis, potassium and iron EDTA have been shown to be absolutely required in the medium (Ammirato 1983). Most protocols use the salts of Murashige and Skoog's (1962) MS medium, or occasionally Gamborg et al.'s (1968) B5 medium. The more dilute White's (1943) medium is still used, but rarely.
3. some level of reduced nitrogen. The presence of either ammonium ion or one or more amino acids is a virtual requirement for embryogenesis (Vasil and Vasil 1972, Kohlenbach 1977, Ammirato 1983). The success of MS medium in somatic embryogenesis has been attributed to its high levels of reduced nitrogen (Kohlenbach 1977).
4. organic additives of often unidentified function which have been shown empirically to stimulate growth and/or

embryogenesis. MS and B5 media contain nicotinic acid, thiamine, pyridoxine and myo-inositol. Other vitamin supplements are sometimes used. Amino acids are often added singly or in a mixture such as casein hydrolysate or yeast extract. Coconut milk is occasionally added, but with the modern trend towards fully-defined media it is often replaced with myo-inositol (Street 1979) and/or cytokinin (Wareing and Phillips 1978).

5. growth regulators, primarily auxin and cytokinin. Sequences of at least two media are very common, with exogenous growth regulators present in initial "induction" media, and reduced or removed in subsequent "differentiation" media where their continued presence appears to inhibit embryo development. The induction medium virtually always contains an auxin, the "prime controlling factor" of embryogenesis (Kohlenbach 1978). This is usually 2,4-dichlorophenoxyacetic acid (2,4-D), though indole-3-acetic acid and 2-naphthaleneacetic acid are not uncommon. A cytokinin is also usually present in the induction medium, normally either kinetin, benzylaminopurine or 2-isopentenyl adenine. Other growth regulator classes such as gibberellins and ABA are rare in these media, but may be included in embryo development media.

Media are frequently optimized for a given family, species, cultivar or even genotype. George and Sherrington (1984)

list the compositions of over 260 published media used in plant tissue culture. Systematic methods for optimizing media composition to the specific material and objective have been outlined (Bhojwani and Razdan 1983, George and Sherrington 1984, Albrecht 1985). Starting with one of a few basal media such as MS or B5 is always recommended. Sometimes composite media are made by adding or replacing the vitamins or micronutrients from another formulation; for example MS salts with B5 levels of vitamins are used occasionally. Then type, and concentrations of auxin and cytokinin are optimized, starting with levels suggested in the literature for closely-related taxa. Optimal lengths of exposure to induction and differentiation media, respectively, must be determined. Finally, pH, osmotic potential (largely determined by sugar concentration), and type and concentration of solidifying agent (when used) should be adjusted to optimal levels (Bhojwani and Razdan 1983, George and Sherrington 1984).

2.1.2 The concept of competence

In the early usage of the term, competence was defined as the ability to express inherent totipotency or morphogenic capacity (Halperin 1967, Street 1979). What this ability consists of and how some cells or tissues have or come to have it while others do not was left for speculation.

Steward et al. (1964) suggested that cellular isolation from surrounding tissue is required for expression of totipotency. Halperin (1973) proposed that epigenetic factors which comprise the differentiated state of the cells of the original explant tissues may be lost during callus formation. In this classical view, the callus cells are competent because of their lack of differentiation. Street's view, on the contrary, was that cells are competent for a particular morphogenetic pathway at the time of culture initiation and that this competence may appear to diminish or change due to intercellular competition during culture (Street 1979).

Recently, competence has been defined somewhat more specifically as the ability to receive and/or respond to a developmental signal (Choi and Sung 1989, Carman 1990). Thus competence is viewed as a necessary physiological, cellular or molecular state of readiness for the next step of the particular morphogenetic pathway. This definition is more compatible with Halperin's (1973) view of competence as requiring a cellular change than with Street's (1979) concept that competence is predetermined in the explant tissues. Recent work in morphogenetic competence and induction is generally interpreted in terms of the acquisition of competence during culture. For example, Christianson and Warnick (1983), Coleman and Ernst (1990)

and Ammirato (1985) all show evidence for a preculture requirement before morphogenetic induction or determination can take place. Competence, as the ability to respond to the inductive signal (usually exposure to certain growth regulators or their removal), is in these cases not present at the time of culture initiation but is acquired sometime after.

Though competence can be defined both in terms of a time period and as a concept, there is virtually no data available to describe its nature, either in terms of physiology, biochemistry, cytology or molecular biology. In fact, a competent culture or tissue can only be recognized by its later behaviour following an inductive treatment, although sometimes competent subpopulations of suspension cultures can be distinguished morphologically (Meins 1986, Thorpe 1988). A recent review (Carman 1990) lists many factors influencing degree of competence such as genotype, cultural environment of both donor and explant, and tissue "memory" of the developmental pathways underway physically or ontogenetically proximal to the original explant. At present, however, it is not possible to distinguish effects on and variation in degree of competence from that of the subsequent differentiation, the only measure of competence available.

The question of what constitutes this competence remains untackled. It is tempting to interpret competence as simply the triggering of mitosis in culture, which may facilitate the interaction of the inductive signal with the DNA. The fact that the auxin requirements of the competence and induction stages are often distinct suggests a possible change in auxin receptors.

The work described in this chapter explores the effects of genotype and protocol on the embryogenic response in 'Rangelander' alfalfa callus, and demonstrates a competence phase in these cultures prior to induction of embryogenesis.

2.2 Materials and methods

2.2.1 Donor plant material

The starting plant material was from vegetatively-propagated greenhouse-grown clones. For micropropagation, stem segments were cut about 2-3 cm long such that each included one of the uppermost two or three nodes at its upper end. These were sterilized by immersion in Lipshaw baskets in 70% ethanol containing 1 drop Tween-80 per 100 mL for 30 seconds, then in 7% calcium hypochlorite containing 1 drop Tween-80 per 100 mL for 20 minutes, followed by three rinses in sterile distilled water. Each end was trimmed by 1-2 mm to remove the whitened tissue, and then the nodal segments were placed upright in culture boxes (GA7, Magenta Corp.; hereafter referred to as Magenta boxes) containing 75 mL sterile MS medium (Murashige and Skoog 1962) lacking growth regulators, containing 3% sucrose and solidified with 0.8% agar (Difco Bacto). Shoots and roots were sufficiently developed from these explants in three to eight weeks. Sterile nodes from these plantlets were excised and subcultured in the same medium and in the same manner for up to five cycles.

A previously-identified (Brown and Atanassov 1985) highly-embryogenic genotype of *Medicago sativa* L. cv.

'Rangelander', known as A70.34 (or RL34 in Meijer and Brown 1987b), was used for most experiments. Other cultivars and selections of *Medicago sativa* and *M. falcata* were used in the genotype experiments as indicated. The original sources of these selections, provided by D.C.W. Brown, Agriculture Canada, Plant Research Centre, Ottawa, are given in Table 2.1.

2.2.2 Tissue culture protocol

For each experiment, sterile 1 cm petiole segments were excised from the nodally-propagated *in vitro* plantlets. On day 0 of each experiment, these were laid lengthwise in sterile 60 mm x 20 mm plastic petri dishes containing 15 mL of sterile solidified medium. Each treatment of each experiment consisted of four dishes containing five petiole segments. Dishes were sealed with Parafilm and incubated at 25C with a 16 h photoperiod ($25 \mu\text{mol m}^{-2}\text{s}^{-1}$; Sylvania 'Cool-White' fluorescent tubes). At specific times as required by the experimental design, calli were transferred under sterile conditions to new plates containing different media, which were sealed and incubated as before.

The media employed were based on that of Murashige and Skoog (1962) with added growth regulators as follows: MS1, 22.6 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 4.7 μM

Table 2.1 Sources of *Medicago* selections used in genotype experiment.

Species	Cultivar	Material	Source
<i>M. sativa</i>	Rangelander	seed	G. Lees, Saskatoon Research Station, Agriculture Canada
	Regen Y	seed	E.T. Bingham, University of Wisconsin
	Regen S A53	seed	E.T. Bingham, University of Wisconsin
	Regen S RA3.3 (Walker et al. 1978)	clone	K. Redenbaugh, Plant Genetics Inc.
	Rambler	seed	B. Murray, Ottawa Research Station, Agriculture Canada
	Europa	seed	P. Devaux, Maison Florimond Desprez, Templeuve, France
	WL 210	seed	R. Loiselle, Plant Genetic Resources, Agriculture Canada
<i>M. falcata</i>	Wild accession	seed	K. Armstrong, Plant Research Centre, Agriculture Canada

kinetin; MS2, no growth regulators; MS3, 4.7 μ M kinetin only; MSPAA, 22.6 μ M phenylacetic acid (PAA) and 4.7 μ M kinetin; and others as indicated. All were supplemented with 3% sucrose and solidified with 0.8% agar (Difco Bacto).

Subjective ratings of callus proliferation were determined at the time of transfer to MS2. These were judged visually on a scale of 0 (no proliferation) to 7 (maximum proliferation).

The number of embryos per callus was usually determined 20 days after transfer to MS2 or as indicated. Anything clearly resembling at least a globular stage embryo was counted; clumps of green cells appearing as promontories on the callus mass and not showing physical discontinuity from it were not included in the embryo count. Secondary embryos, those appearing to have differentiated from the tissue of a previously differentiated embryo, were counted as were their 'mother' primary embryos.

2.3 Results and discussion

Culture manipulation experiments centred around the investigation of factors affecting somatic embryogenesis in alfalfa, specifically genotype, kinetin, auxin, and length and method of exposure to 2,4-dichlorophenoxyacetic acid, and in the mapping of competence and determination phases in the induction of embryogenesis.

2.3.1 Effect of genotype on embryogenesis

Ten selections from cv. Rangeland and eight single selections from eight other cultivars (Table 2.1) were run through the standard embryogenesis protocol as given in Meijer and Brown (1987a). The 1 cm sterile petiole segments were plated on MS1 for 10 days and then transferred to MS2. After a further 20 days (day 30) embryo counts were made. This procedure is illustrated in Appendix IV.

The ten genotypes of cv. Rangeland had been previously selected on the basis of their ability to produce embryos. The cultivars 'Regen S' and 'Regen Y' are synthetic "lab" cultivars composed of genotypes showing embryogenic response to another protocol (Bingham et al. 1975). Thus the genotypes listed in Table 2.1 are not a random sample of *Medicago* selections. Yet even among these mostly

embryogenic clones there is clearly a high degree of variability in all the parameters measured, both within cv. 'Rangelander' and among the six cultivars and one wild accession (Table 2.2). Whether mean number of embryos per callus is calculated on the basis of all 20 calli cultured per treatment or only those calli producing embryos, there is unquestionably a wide range of response among the genotypes selected. The results support the conclusion of Brown and Atanassov (1985) that the embryogenic response is associated with high contributions of *M. falcata* and 'Ladak' germplasm. The wild *M. falcata* accession and the two alfalfa cultivars 'Rangelander' and 'Rambler', both with major proportions of *M. falcata* and 'Ladak' parental germplasm (Brown and Atanassov 1985, Brown 1988), showed the highest levels of embryogenesis in this system (Table 2.2). 'Europa' has no germplasm from either of these sources while 'Regen S', 'Regen Y' and 'WL 210' have only low contributions from *M. falcata* and Ladak (Brown 1988). The clones that were surveyed of these latter cultivars showed no or very little embryogenesis (Table 2.2).

Embryogenesis is clearly not an all-or-none response. This is evident when comparing the genotypes of cv. 'Rangelander'. There is a 73-fold difference between the mean number of embryos per callus of the highest and lowest embryo-producers of that cultivar, with an even

Table 2.2 Callus rating at day 10, number of days to appearance of first embryo (DTFE), percentage of calli producing embryos and number of embryos per callus at day 30 of eighteen *Medicago* selections.

Species	Cultivar	Genotype	Callus rating at day 10	DTFE	% of calli producing embryos	Mean no. embryos $\pm$ S.E., all calli ¹	Mean no. embryos $\pm$ S.E., emb. calli only ²
<i>M. sativa</i>	Rangelander	A70.4	1	20	55	1.25 $\pm$ 3.04	2.27 $\pm$ 0.54
		A70.6	1	23	15	0.15 $\pm$ 0.08	1.00 $\pm$ 0.00
		A70.10	2	29	50	1.55 $\pm$ 0.46	3.10 $\pm$ 0.59
		A70.11	3	18	30	0.85 $\pm$ 0.35	1.83 $\pm$ 0.65
		A70.27	2	18	100	9.55 $\pm$ 1.25	9.55 $\pm$ 1.25
		A70.34	3	14	100	11.00 $\pm$ 1.44	11.00 $\pm$ 1.44
		A70.40	6	21	55	6.30 $\pm$ 2.03	11.45 $\pm$ 2.88
		A70.43	5	20	80	5.55 $\pm$ 1.29	6.93 $\pm$ 1.41
		A70.45	3	18	70	2.60 $\pm$ 0.79	3.71 $\pm$ 1.00
		A70.51	2	15	15	9.05 $\pm$ 1.00	9.05 $\pm$ 1.00
		RA3.3	4	21	10	0.10 $\pm$ 0.07	1.00 $\pm$ 0.00
		A53.24	1	-	0	0.00	-
			3.5	29	15	0.20 $\pm$ 0.14	2.00 $\pm$ 0.00
			4	-	0	0.00	-
<i>M. falcata</i>	Wild accession		1	-	0	0.00	-
			7	22	100	19.20 $\pm$ 2.27	19.20 $\pm$ 2.27
			2	17	90	9.65 $\pm$ 1.56	10.72 $\pm$ 1.56

¹Mean number of embryos per callus at day 30 based on all calli, $\pm$ standard error.

²Mean number of embryos per callus at day 30 based only on calli producing embryos, $\pm$ standard error.

representation of intermediate values (Table 2.2).

Similarly the percentage of calli producing embryos is fairly evenly distributed between 15 and 100% among these genotypes (Table 2.2). These points, and the fact that the number of days from the beginning of culture to the appearance of the first embryo also shows considerable but evenly-distributed variation within 'Rangelander' (Table 2.2), suggest that embryogenesis is not simply determined, in 'Rangelander' at least. The embryogenic response is modified by other genotype-dependent factors.

One obvious possibility for such a factor is the degree of callus proliferation: the bigger the callus the more embryos can potentially differentiate from it. The 'Rangelander' genotypes shown in Table 2.2 do vary considerably in callus production. Regression analysis showed a correlation in Rangelander genotypes between callus rating and mean number of embryos per callus (based on all calli) of only $R^2 = 0.28$ ($\alpha = 0.42$). However, when all 18 genotypes are considered the R^2 value is 0.51 ($\alpha = 0.03$). The degree of callus proliferation appears to have an overall influence on number of embryos produced, but clearly other factors also affect the response.

These results may be taken as further evidence for additivity effects and/or the involvement of more than one

locus in the expression of embryogenesis. Of particular relevance is the work of Hernandez-Fernandez and Christie (1989), in which the highly-embryogenic 'Rangelander' clone A70.34 was intercrossed with two non-embryogenic genotypes and the inheritance of the embryogenesis trait analyzed. They concluded that embryogenesis is controlled by two complementary loci with additivity within each locus.

2.3.2 Effect of auxin choice and concentration on embryogenesis

Again based on the standard protocol of Meijer and Brown (1987a), a selection of eight auxins, at five concentrations encompassing biologically-active levels, were used to replace 2,4-D in the induction medium MS1 (Table 2.3). Only the embryogenic 'Rangelander' genotype A70.34 was used. The choice of auxins was based on 1) availability, and 2) diversity of both synthetic and naturally-occurring auxins. To my knowledge the use of the natural auxin 4-chloro-indole-3-acetic acid (4-Cl-IAA) has not been previously reported in tissue culture. It was felt to be of interest here, however, because it is found in pea seeds and might have an involvement in embryogenesis in legumes. 2,4-D and two of its analogues, 4-chlorophenoxyacetic acid (4-CPA) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) were used. These three synthetic auxins represent a series of

Table 2.3 Mean number of embryos per callus at day 30, callus rating (CF) at day 20 and number of days to appearance of first embryo (DTFE) of A70.34. Calli were cultured 10 d on MS media supplemented with auxin and kinetin as indicated, then transferred to MS2. *Embryo counts taken after 25 d on MS2. **As no embryos were visible on any of the cultures without kinetin after 25 d or 45 d on MS2, embryo counts shown were taken after 65 d on MS2. Means of 20 calli $\pm$ standard error.

		With 4.7 μ M kinetin*			Without kinetin**
		-----			-----
		No. embryos	CF	DTFE	No. embryos
NATURAL AUXINS					
Indole-3-acetic acid (IAA)	10 ⁻⁴ M	0.0	2.5		0.1 $\pm$ 0.1
	10 ⁻⁵ M	0.0	2.5		0.0
	10 ⁻⁶ M	0.0	2		0.0
	10 ⁻⁷ M	0.0	2		0.0
	10 ⁻⁸ M	0.0	1.5		0.0
4-Chloro-indole-3-acetic acid	10 ⁻⁴ M	0.6 $\pm$ 0.3	1.5	15	0.0
	10 ⁻⁵ M	0.4 $\pm$ 0.2	3	17	0.0
	10 ⁻⁶ M	0.0	1.5		0.0
	10 ⁻⁷ M	0.0	1.5		0.0
	10 ⁻⁸ M	0.0	1.5		0.0
Indole-3-propionic acid	10 ⁻⁴ M	0.2 $\pm$ 0.0	2.5	27	0.0
	10 ⁻⁵ M	0.0	2.5		0.0
	10 ⁻⁶ M	0.0	2		0.0
	10 ⁻⁷ M	0.0	1		0.0
	10 ⁻⁸ M	0.0	1		0.0
Phenylacetic acid (PAA)	10 ⁻⁴ M	0.0	3		0.0
	10 ⁻⁵ M	0.0	2		0.0
	10 ⁻⁶ M	0.0	1		0.0
	10 ⁻⁷ M	0.0	1.5		0.0
	10 ⁻⁸ M	0.0	1.5		0.0
SYNTHETIC AUXINS					
4-chloro-phenoxyacetic acid	10 ⁻⁴ M	4.6 $\pm$ 1.2	2	17	0.9 $\pm$ 0.3
	10 ⁻⁵ M	2.0 $\pm$ 0.5	4	19	0.5 $\pm$ 0.3
	10 ⁻⁶ M	0.0	3		0.0
	10 ⁻⁷ M	0.0	2		0.0
	10 ⁻⁸ M	0.0	1		0.0
2,4-dichloro-phenoxyacetic acid	10 ⁻⁴ M	4.4 $\pm$ 1.3	1	15	0.0
	10 ⁻⁵ M	8.0 $\pm$ 1.4	3	15	1.2 $\pm$ 0.1
	10 ⁻⁶ M	0.3 $\pm$ 0.2	3	25	0.2 $\pm$ 0.2
	10 ⁻⁷ M	0.0	3	26	0.1 $\pm$ 0.1
	10 ⁻⁸ M	0.0	2.5		0.0

Table 2.3 continued.

		With 4.7 μ M kinetin*			Without kinetin**
		-----			-----
		No. embryos	CF	DTFE	No. embryos
2,4,5-trichloro- phenoxyacetic acid	10 ⁻⁴ M	2.3 $\pm$ 0.7	1	17	0.1 $\pm$ 0.1
	10 ⁻⁵ M	11.3 $\pm$ 1.4	2	15	0.0
	10 ⁻⁶ M	1.4 $\pm$ 0.4	3	15	0.3 $\pm$ 0.2
	10 ⁻⁷ M	0.7 $\pm$ 0.2	2.5	21	0.0
	10 ⁻⁸ M	0.0	1.5		0.0
1-Naphthalene acetic acid	10 ⁻⁴ M	1.1 $\pm$ 0.3	2.5	21	0.0
	10 ⁻⁵ M	0.3 $\pm$ 0.1	3	23	0.0
	10 ⁻⁶ M	0.0	2		0.0
	10 ⁻⁷ M	0.0	2		0.0
	10 ⁻⁸ M	0.0	1		0.0

phenoxyacetic acids with one, two or three chlorine atoms substituted on the phenoxy ring. Indole-3-acetic acid (IAA) and 1-naphthaleneacetic acid (NAA) are both commonly used in *in vitro* morphogenesis systems. Indole-3-propionic acid (IPA) is an analogue of IAA which is considered to have auxin activity without being first converted to IAA, as is the case with other IAA analogues (Audus 1972). Finally, phenylacetic acid (PAA) is a simple and naturally-occurring auxin which was available.

Clearly both auxin choice and concentration affect the occurrence and degree of embryogenesis, the rate at which it occurs as reflected by time to appearance of first embryo, and the extent of callus formation (Table 2.3). None of the auxins induced embryogenesis when present at 10^{-6} M, and most were effective only at 10^{-4} M and/or 10^{-5} M. However, the two most embryogenic auxins, 2,4-D and 2,4,5-T, showed maximum callus development at intermediate concentrations with proliferation being reduced at both higher and lower levels. Interestingly, there was a slight negative correlation of $R^2 = -0.26$ ($\alpha = 0.34$) between mean number of embryos (when embryogenesis was observed) and callus formation, whereas the previously-made comparison of genotypes suggested a positive relationship between these two parameters (see section 2.3.1). Petiole segments of A70.34 plated directly on growth regulator-free medium (MS2) or on medium

supplemented with kinetin only and no auxin (MS3) showed essentially no callus proliferation and died in 10 to 15 days. While some cell division is obviously necessary for embryogenesis and more callus proliferation theoretically permits more embryos to be formed, auxins and auxin concentrations which are most effective at stimulating callus formation are not necessarily the same as those most effective at inducing embryogenesis. This suggests that the two processes, while interrelated, are distinct and represent separate auxin effects. The identification of an auxin which can support growth of a culture while not inducing embryogenesis may then permit a study of processes unique to embryogenesis or its induction.

The results in Table 2.3 suggest that either PAA or IAA may be such an auxin. Since embryos were rarely observed in later IAA cultures, particularly cell suspensions (to be discussed later), it was felt that the ineffectiveness of IAA in inducing embryogenesis in this experiment may have been due to its photo-oxidation in the agar-solidified medium (Audus 1972). Of the eight auxins shown in Table 2.3 only PAA was never observed to induce embryogenesis.

Although 2,4-D, IAA and NAA are commonly used in induction media for embryogenesis (Bhojwani and Razdan 1983), only 2,4-D proved usefully effective in this system. Two other

natural auxins, 4-Cl-IAA and IPA induced only very low levels of embryogenesis. All three phenoxyacetic acids were more or less effective, but degree of embryogenesis increased as the number of chlorine atoms in the auxin molecule increased, with 2,4,5-T being the most embryogenic auxin of the eight tested. Similarly, Suprasanna et al. (1989) found 2,4,5-T to be more stimulating to maize callus growth than 2,4-D.

Where most embryo counts are taken on day 30, some of the variation in number of embryos is likely due to variation in number of days to the appearance of the first embryo. Cultures appear to respond slower to some treatments than to others. This could be due to differences in rates of uptake of the auxins from the medium, rates of metabolism of the auxin in the cells, effectiveness of triggering of the theoretical transduction pathway or perhaps even different transduction pathways. It is even possible that different auxins have different target cells or tissues within the explant. Wenzel and Brown (1991) demonstrated histological evidence that embryogenesis is initiated at different times in two different cell types in 2,4-D-treated alfalfa petiole-derived calli. Similarly, Ammirato (1985) showed evidence for two populations of cells in carrot calli having different time responses to embryogenesis-inducing treatment.

2.3.3 Effect of kinetin on embryogenesis

To elucidate the individual roles of kinetin and 2,4-D in the embryogenesis protocol of Meijer and Brown (1987a), five petiole segments of A70.34 were plated on each of four plates of solid MS media supplemented with both 22.6 μM 2,4-D and 4.7 μM kinetin (MS1), four plates with only 22.6 μM 2,4-D and four with only 4.7 μM kinetin (MS3). After six days all calli were transferred to solid basal MS medium (MS2), and embryos were counted after an additional twenty days.

Those calli which had been cultured initially on kinetin and no 2,4-D were hard, shrivelled and browned or blackened, showing no or little sign of callus formation and no embryos. The auxin is then concluded to be an absolute requirement for embryogenesis and probably for cell division. However, it cannot be ruled out that the effect of 2,4-D on embryogenesis is through its effect on cell division.

The controls, calli cultured initially on MS1 with both 2,4-D and kinetin, produced in this experiment a mean of 4.94 embryos per callus with a standard error of 0.99. This is about half the level usually observed in this same control treatment in other experiments. Nevertheless such

unexplained variations were not uncommon throughout the studies and were probably due to differences in subculturing technique, fluctuations in the growth chamber environment, water quality or age and strength of growth regulator stocks. Calli cultured initially on medium containing 2,4-D only and no kinetin produced a mean of 0.53 embryos per callus with a standard error of 0.27. Even with the unusually low control embryo production in this experiment, the number of embryos in the no-kinetin treatment was still about one-tenth the control level.

In another experiment previously discussed, eight auxins at each of five concentrations were tested in the induction medium, both with and without kinetin (Table 2.3). Again, embryogenesis induction still occurs in the absence of kinetin but much later and at a much reduced level than that observed in its presence.

Thus, kinetin is apparently not absolutely required for embryo induction and differentiation, but its inclusion in the induction medium greatly enhances both the degree of embryogenic response and the rate of its occurrence. For this reason, kinetin was included in the induction medium in nearly all the experiments reported here. Both Saunders and Bingham (1975) and Walker et al. (1979) also found that kinetin was not required for but considerably increased

morphogenesis in the alfalfa cultivar 'Saranac'.

2.3.4 Optimization of protocol

In order to further define and optimize the induction period of the embryogenesis system developed by Meijer and Brown (1987a), the initial culture period on the induction medium MS1 (the "induction period") was varied from 0 to 22 days. The number of embryos per callus was determined both on the 30th day of culture (Fig. 2.1) and 20 days after transfer to MS2 (Fig. 2.2). Similar trends were observed in each of the two sets of measurements. There was considerable variation between experiments in maximum embryo number as has been commonly observed (see section 2.3.3). However, there was a very consistent minimum requirement of four days' exposure to the kinetin and 2,4-D-containing MS1 medium for embryogenesis to occur. Walker et al. (1979) also used a four-day induction period for morphogenesis in alfalfa. Embryo number reached a maximum after about 8 or 10 days' exposure to MS1. Ammirato (1985) also observed that increasing length of exposure to 2,4-D led to increased embryogenesis in carrot.

Embryo formation was generally reduced by exposures of longer than 12 days. Meijer and Brown (1987a) also reported a reduction in embryo numbers following 2,4-D exposure in

Fig. 2.1 Optimization of induction phase. Effect of varying length of exposure to MS1 on number of embryos after 30 d in culture. Embryo counts per callus after culture for varying periods on MS1 as indicated, followed by transfer to MS2. Each curve represents one of three experiments; each point represents a mean of 20 calli $\pm$ standard error.

Fig. 2.2 Optimization of induction phase. Effect of varying length of exposure to MS₁ on number of embryos 20 d after transfer to MS₂. Embryo counts per callus after culture for varying periods on MS₁ as indicated, followed by transfer to MS₂. Each curve represents one of three experiments; each point represents a mean of 20 calli $\pm$ standard error.

alfalfa callus of longer than ten days. A slight increase in embryo number was observed on calli cultured on MS1 for about 20 days. This later increase may represent secondary embryogenesis, or a second population of embryos arising from cells or tissues requiring longer MS1 exposure. Similarly, in petiole-derived carrot calli Ammirato (1985) proposed that there are two populations of cells having different courses and time responses to the inductive treatment.

For simplification most subsequent experiments were based on six-day induction periods, which give rise to optimal or nearly optimal levels of embryogenesis.

2.3.5 Time of induction and demonstration of competence phase within the induction period

To further determine when embryogenic induction actually takes place, petiole explants were plated first on the non-inductive PAA-containing medium, MSPAA, and then transferred to the inductive 2,4-D-containing MS1 medium. The number of embryos 20 days after transfer to MS2 decreased as the first part of a six-day exposure to MS1 is replaced by exposure to MSPAA, with greater decreases seen as MSPAA exposure increases (Fig. 2.3). Embryo numbers were nearly constant when the first one to four days of a six-day induction

Fig. 2.3 Demonstration of competence phase. Effect of varying length of exposure to MSPAA prior to exposure to MS1 on number of embryos 20 d after transfer to MS2. Number of embryos per callus after culture for 6 d on MSPAA and/or MS1 followed by 20 d on MS2. Horizontal bars represent sequential exposure in days to MSPAA or MS1, from left to right, before transfer to MS2. Figures are means of 60 calli ± standard error. Figures followed by the same letter are not significantly different ($\alpha=0.05$) by Duncan's Multiple Range test.

period is replaced by an exposure to MSPAA, though they were reduced to roughly 40-55% that of the six-day continuous exposure to MS1 (control) (Fig. 2.3).

Given that embryos appear on petiole-derived calli after they were cultured on MS1 and do not appear on calli after they were cultured on MSPAA (Table 2.2), the assumption can be made that induction of embryogenesis occurred during the MS1 exposure and did not occur during the MSPAA exposure. On this assumption the data in Figs. 2.1 and 2.3 present evidence for a competence phase, within the six-day induction period but preceding the actual induction event(s). When petiole segments are first excised, a minimum of four days of continuous exposure to MS1 is required for embryogenesis, with an optimum of eight to ten days (Fig. 2.1). The fact that MS1 can be replaced with MSPAA for the first one to three or four days of a six-day induction period without diminishing the number of embryos appearing beyond an initial reduction (Fig. 2.3) suggests that induction does not actually take place until after the third or fourth day. In a six-day induction period the induction event(s) are thus localized to the last two or three days. If petioles are plated directly onto MS1 for a three-day, exposure no embryogenesis results (Fig. 2.1). This implies that during those first three days, though induction itself is not taking place, events nevertheless

occur which are necessary for embryogenesis. This is what is referred to here as the competence phase.

Knowing that competence can be acquired by culture in the presence of the non-embryogenic PAA plus kinetin, an experiment was performed to determine if competence can arise in the absence of any auxin, or without either auxin or kinetin (Fig. 2.4). Competent callus is defined here as that which forms embryos following a three-day exposure to 2,4-D and kinetin, a treatment which without preculture is non-inductive (treatment 1, Fig. 2.4). Three days of culture on an auxin-less MS medium with or without kinetin gave rise to competence (treatments 3 and 4, Fig. 2.4). However, the number of embryos was at least doubled when PAA was included in the initial medium (treatment 5, Fig. 2.4). Calli cultured continually on MS2 showed no visible sign of callus formation and were apparently dead in 10 to 15 days. Calli cultured continuously on MS3 (kinetin alone) showed very little callus formation and appeared to be dead within 15 days.

It is difficult to propose an explanation of the nature of competence based on this data. Since PAA can induce competence and not embryogenesis it is reasonable to suppose that different auxin receptors such as the membrane-bound auxin-binding proteins discussed by Klamt (1990) are

Fig. 2.4 Acquisition of competence in presence of various growth regulator combinations. Number of embryos per callus after culture for 3 d or 6 d on MS1, MS2, MS3 and/or MSPAA, followed by 20 d on MS2. Horizontal bars represent sequential exposure in days to the various media, from left to right, before transfer to MS2. Figures are means of 20 calli ± standard error. Figures followed by the same letter are not significantly different ($\alpha=0.05$) by Duncan's Multiple Range test.

sequentially expressed. However, neither PAA nor 2,4-D, nor in fact the cytokinin kinetin, are absolutely required for competence by our definition (Fig. 2.4).

The relationship between callus proliferation and competence is ambiguous. It seems intuitively obvious that cell division and callus formation is necessary for embryogenesis in alfalfa since the embryos differentiate from the callus and not from the tissues of the original explant. It might be suggested that competence is callus proliferation. However, the case for a relationship between degree of callus formation and degree of embryogenesis, while not necessarily directly related to competence, is not a strong one (see sections 2.3.1 and 2.3.2). Furthermore, Wenzel and Brown (1991) concluded that somatic embryos probably arise from subepidermal and epidermal tissues of alfalfa petiole segments which do not appear to divide until after three to five days of 2,4-D exposure. This is almost after the acquisition of competence. Finally, cell division is not likely nor was it discernible after three days' culture in the absence of auxin, though such tissue showed a low level of competence (Fig. 2.4).

The acquisition of competence may not be so much the result of specific culture conditions as of the excision of the explant. Removal from neighbouring tissues and organs may

release the explant from external controls regulating its developmental state. Physiological or physical isolation has long been considered a prerequisite for somatic embryogenesis (Brown and Thorpe 1986, Meins 1986). Although Steward did not use the term competence, this idea is very reminiscent of the view (Steward et al. 1964) that isolation of cells from their maternal tissue allows them to express their inherent totipotency.

2.3.6 Perception of the inductive signal

The preceding discussion provided evidence for a relationship between length of exposure time to 2,4-D and the number of embryos formed. The way in which exposure time limits embryogenesis induction is likely through the number of 2,4-D molecules that can reach their target, in which case a linear relationship between exposure time and embryogenesis would be expected. If the internal 2,4-D concentration must reach some minimum level to induce embryogenesis a threshold response would be expected, and a minimum exposure time might be expected. Our interpretation of Fig. 2.1 tells us that after competence is acquired through 4 days' exposure to MS1, embryogenesis induction increases rapidly to a maximum over the next 2 to 4 days. A number of experimental treatments were set up to evaluate 1) if uptake of 2,4-D is limiting to embryogenesis induction,

and 2) if there is a minimum exposure to 2,4-D required for induction in competent tissue; in other words, is there a linear or a threshold relationship between 2,4-D exposure of competent tissue and degree of embryogenesis.

2.3.6.1 Immersion of calli in liquid medium

It is assumed that the uptake of 2,4-D into calli would be greater from a liquid medium in which they are immersed than from the agar-solidified medium on which they are normally cultured. In the latter case, only the lower surface of the calli is in physical contact with the medium whereas in the former, the entire surface area is exposed; and secondly, diffusion of 2,4-D within the agar matrix might be expected to be restricted relative to a liquid medium. Therefore, 30 calli cultured for 5.75 d on solid MSPAA were submersed for 6 h in liquid MS1 for induction, while another 30 were transferred to solid MS1 for 6 h induction. Embryo counts were determined after a subsequent culture for 20 d on MS2.

Again, while the control level of embryogenesis was lower than average in experiment 1, it was considerably higher than average in experiment 2 (Table 2.4). Despite the variation in absolute levels, there is about a 2-fold increase in number of embryos at day 26 when liquid induction treatment replaces an agar-based one in both

Table 2.4 Mean number of embryos per callus at day 26, callus rating at day 26 and number of days to appearance of first embryo (DTFE) of A70.34. All calli cultured a total of 6 d on auxin- and kinetin-containing media: control treatments cultured the full 6 d on MS1, others cultured 6 d on MSPAA, or 5.75 d on MSPAA followed by 6 h exposure to liquid or agar MS1 (see text). Means of 30 calli $\pm$ standard error.

	Treatment	Mean no. embryos/callus	Callus rating	DTFE
Experiment 1	Control	4.63 $\pm$ 1.24	4	13
	Liquid	3.80 $\pm$ 0.86	3	15
	Agar	1.90 $\pm$ 0.82	2	15
Experiment 2	Control	23.67 $\pm$ 3.54	4.5	-
	Liquid	5.40 $\pm$ 1.04	4	-
	Agar	2.27 $\pm$ 0.47	4	-
	6 d MSPAA	0.00 $\pm$ 0.00	0	-

experiments. In experiment 1 the liquid treatment led to detectably greater callus formation as well. This data might support the idea that shorter 2,4-D treatments lead to fewer embryos and less callus (e.g. Fig. 2.1), and that lower auxin concentrations give rise to fewer embryos and less callus (Table 2.2) because fewer 2,4-D molecules reach the target cells.

In the one experiment in which it was recorded, the number of days to the appearance of the first embryo was not reduced by a liquid induction treatment relative to an agar one (Table 2.4). However, the first embryo did appear sooner on calli cultured for a full six days on induction medium rather than only six hours. It may be expected that the rate of diffusion of 2,4-D within the callus, and/or the rate of the transduction process, would be independent of the number of 2,4-D molecules taken up. Time since first exposure to 2,4-D might be more relevant to time of appearance of embryos than total uptake. More complete data on the rate of appearance of embryos, rather than just the date of appearance of the first embryo among 30 calli, might provide more solid evidence. Counter to this hypothesis is the data given in Table 2.3, which shows that appearance of the first embryo is delayed as auxin concentration is reduced. This may suggest there is a minimum internal auxin concentration required for embryogenesis, which may take

longer to accumulate when the external concentration is lower.

2.3.6.2 Minimum MS1 exposure required to induce embryogenesis in competent tissue

The experiments discussed in section 2.3.5 showed that a single day's exposure to 2,4-D could induce embryogenesis in calli cultured for five days in the presence of the non-embryogenic auxin PAA. In the following experiments calli were transferred from MSPAA to MS1 at various times within a six-day total auxin exposure with particular attention to very short MS1 treatments ranging from 30 min to 24 h. Data from three experiments is given in Table 2.5 and graphed in Fig. 2.5. The control treatment of six days' continuous exposure to MS1 was again variable among treatments. (Reasons for this not-infrequent observation have been discussed in section 2.3.3). For this reason the data is expressed in terms of percent of control in Fig. 2.5.

All MS1 treatments, no matter how short, induced a detectable level of embryogenesis. Even 30 min culture on the inductive MS1 treatment gave rise to one embryo among 20 calli. However the data suggests there may be a threshold limit of 5 or 6 h MS1 exposure, after which the degree of embryogenesis induced increases at a steeper rate (Fig.

Table 2.5 Mean number of embryos per callus at day 26, callus rating at day 6 and number of days to appearance of first embryo (DTFE) of A70.34. Calli cultured a total of 6 d on auxin- and kinetin-containing media, initially on MSPAA then transferred to MS1 for the final period of various times as indicated. Means of 20 calli $\pm$ standard error.

	Exposure to MS1	Mean no. embryos/callus	Callus rating	DTFE
Experiment 1	nil	0.00 $\pm$ 0.00		
	6 h	1.44 $\pm$ 0.71		
	12 h	0.75 $\pm$ 0.29		
	18 h	2.20 $\pm$ 0.91		
	24 h	2.57 $\pm$ 0.84		
	6 d	7.10 $\pm$ 1.46		
Experiment 2	nil	0.00 $\pm$ 0.00	1	-
	1 h	0.13 $\pm$ 0.13	2.25	19
	2 h	0.27 $\pm$ 0.10	2.5	18
	3 h	0.50 $\pm$ 0.34	2.5	18
	4 h	0.50 $\pm$ 0.17	2.5	18
	5 h	1.73 $\pm$ 0.65	2.75	18
	6 d	21.10 $\pm$ 2.44	5.5	14
Experiment 3	nil	0.00 $\pm$ 0.00		
	0.5 h	0.05 $\pm$ 0.05		
	1 h	0.05 $\pm$ 0.05		
	3 h	0.05 $\pm$ 0.05		
	6 h	0.10 $\pm$ 0.10		
	12 h	1.35 $\pm$ 0.36		
	18 h	0.55 $\pm$ 0.25		
	24 h	1.15 $\pm$ 0.44		
	6 d	7.10 $\pm$ 1.19		

Fig. 2.5 Minimum exposure to MS1 required for induction of embryogenesis. Mean number of embryos per callus at day 26, genotype A70.34. Calli cultured a total of 6 d on auxin- and kinetin-containing media, initially on MSPAA then transferred to MS1 for the final period of various times as indicated. Data from Table 2.4 expressed as percent of control treatment (6 d continuous exposure to MS1). Means of 30 calli $\pm$ standard error.

2.5). Given the large standard errors and the variability among experiments, it is not possible to deduce from this data whether the rate of embryogenesis induction with time of MS1 exposure is linear or changing beyond 6 h. However, it is safe to conclude that competent tissue requires at least 6 h exposure to MS1 to reliably induce embryogenesis and that a higher degree of embryogenesis can be induced by longer exposures.

Callus proliferation too shows a relationship with time of MS1 treatment over very short exposures (Table 2.5). This experiment illustrates the significant difference in the relative abilities of PAA and 2,4-D to stimulate callus formation. When exposed to MS1 for a single hour the increase in callus proliferation was visually distinct. Further increases were detectable as MS1 exposure increased up to 5 h, presumably due to increasing total uptake of 2,4-D.

The length of time to the appearance of the first embryo did not appear to be affected by time of MS1 exposure between 1 h and 5 h. However, embryos appeared four to five days earlier on calli cultured for 6 d on MS1 than on those exposed to short MS1 treatments after nearly 6 d on MSPAA. This is in keeping with the hypothesis that time to first appearance of embryos is related to time since first

exposure to MS1 (see section 2.3.6.1). It should be borne in mind that induction is not considered to be occurring during the first three to four days of MS1 exposure (see section 2.3.5). Nevertheless, calli cultured 6 d on MS1 have had nearly 6 d longer than the other treatments for diffusion of 2,4-D to the target cells for embryogenesis, or for the action of the transduction pathway to proceed. It is also conceivable that 2,4-D, its metabolite or a subsequent transducing factor can be stored in the tissue until competence is acquired.

2.3.7 Effects of PAA on competence and differentiation

A number of experimental treatments were evaluated (Fig. 2.6) to further define the effect of PAA on competence and embryogenesis, specifically to determine 1) is PAA equally or more or less effective at inducing competence than 2,4-D? Are the minimum exposures required for competence different for PAA and 2,4-D?; and 2) does PAA exposure enhance or inhibit differentiation of embryos when it follows 2,4-D exposure?

2.3.7.1 Relative effectiveness of PAA and 2,4-D in the acquisition of competence

The greater embryogenesis observed in treatment 12 relative

to treatment 4 (Fig. 2.6) suggests that competence in the presence of PAA is enhanced by the early exposure to 2,4-D. That this early exposure is not embryogenesis-inducing is suggested by the lack of embryos seen in treatments 14 and 15 (Fig. 2.6). Furthermore, a four to five day exposure to MSPAA is needed to acquire competence for induction by a subsequent one day exposure to MS1 (comparing treatments 2 through 5, Fig. 2.6), whereas both competence and induction occurred within four to five days on MS1 (Fig. 2.6).

Although competence can be acquired during exposure to either auxin, PAA is apparently less effective than 2,4-D. The darker, drier, harder and slower-growing callus grown on PAA compared to that grown on 2,4-D may be evidence that PAA initiates and maintains callus growth less efficiently; it seems likely that this is related to a difference in competence or differentiation.

2.3.7.2 Effect of PAA following embryogenesis induction

PAA has been shown to inhibit endogenous IAA transport (Morris and Johnson 1987). Given that PAA was the only one of eight auxins surveyed which failed to induce embryogenesis (Table 2.3) it seems plausible that PAA might be actually inhibiting differentiation. However, a one-day exposure to PAA following three, four or five days' exposure to 2,4-D had no consistent effect on embryogenesis (cf.

treatments 6 vs. 9, 7 vs. 10 and 8 vs. 11, Fig. 2.6). Subsequent studies using PAA cultures as non-embryogenic controls should take into account the possibility that PAA may in fact induce embryogenesis and inhibit embryo development concomitantly. In somatic embryogenesis in carrot cell suspensions, 2,4-D is both inductive to embryogenesis and inhibitory to differentiation (Choi and Sung 1989). The findings of Meijer and Brown (1987a) indicating that long exposures to 2,4-D may be inhibitory to embryogenesis in alfalfa calli are also supported here in Figs. 2.1 and 2.2. It is possible that PAA, like 2,4-D, also exerts self-opposing influences in embryogenic induction and inhibition of development.

Chapter 3. Development of experimental system

An objective was set to study changes in biochemistry, particularly nitrogen metabolism, associated with somatic embryogenesis in alfalfa. The plan was to use this approach to tackle the question of what determines the embryogenic capacity of individual 'Rangelander' genotypes, and the question of what changes when 2,4-D induces somatic embryogenesis. Therefore an experimental system had to be developed which would facilitate the necessary comparisons towards answering these questions. The development of such a system is described in this chapter, while the studies themselves are discussed in Chapter 4.

Most cultures are not composed of uniform populations of equipotent cells (Meins 1986). The detection of biochemical responses resulting from induction of embryogenesis requires that induced cells comprise a reasonable proportion of the total cells sampled. "Dilution" effects of induction-specific elements by non-induced material is at least a theoretical obstacle to detection (Thorpe 1978). By the callus method of embryogenesis described in Chapter 2, a single callus from a 1 cm petiole segment of A70.34 gives rise to an average of 9 - 11 embryos. It was reasoned that no more than 9 - 11 cells might actually be induced of the many thousands that make up that single callus. A

suspension culture method was felt to provide a higher proportion of induced cells, since preliminary studies had shown that a 500 μ L aliquot of alfalfa suspension containing 200 μ L of packed cells could produce in the range of 50 embryos. Furthermore, use of petiole-derived calli complicates biochemical analysis because many tissue systems (vascular tissue, ground tissue, epidermis and possibly several types of callus) are extracted at once. Cell suspensions, on the other hand, provide a more homogeneous material of more or less one cell type. Therefore suspension cultures were selected as the preferred experimental system for biochemical studies.

A model system for studying characteristics associated with induction of embryogenesis in suspension culture necessitates the use of a scientifically sound non-embryogenic control treatment. This chapter discusses a number of trials which were carried out during the development of appropriate control suspensions.

Two main approaches were taken. One was to develop a non-inductive suspension method such that even embryogenic types could be grown and maintained without triggering embryogenesis by an embryogenic auxin such as 2,4-D (Section 3.2). This would permit the detection of changes specific to the inductive treatment. The other approach was to

establish suspensions using a range of embryogenic and non-embryogenic genotypes so that biochemical changes might be correlated with embryogenic capacity (Section 3.3).

3.1 General alfalfa cell suspension method

The suspension culture method previously developed for alfalfa (Senaratna et al. 1989) was based on the B5 media of Samborg et al. (1968), and is illustrated in Appendix V. Sterile 1-cm petiole segments were plated on a B5 medium supplemented by 3% sucrose, 5.5 mM glutamine, 32.5 μ M glutathione, 0.95 mM serine, 7.4 μ M adenine, 1.0 mg L⁻¹ (4.5 μ M) 2,4-D and 0.2 mg L⁻¹ (0.9 μ M) kinetin (B5h medium), solidified with 0.9% agar. After two to three weeks, five calli (approximately 1.4 g) were used to inoculate 25 mL of sterile liquid B5mod medium in 125 mL Erlenmeyer flasks. This is a B5-based medium modified to contain 4.1 μ M nicotinic acid, 2.4 μ M pyrodoxine HCl, 3.0 μ M thiamine HCl, 0.55 mM myo-inositol, 4.5 μ M (1.0 mg L⁻¹) 2,4-D and 0.54 μ M (0.1 mg L⁻¹) naphthaleneacetic acid (NAA). The flasks were stoppered with sterile foam plugs and placed on a G10 Gyrotory shaker (New Brunswick Scientific) at 140 revolutions per minute, 25 C, under a 16 h photoperiod (25 μ mol m⁻² s⁻¹).

Once a week these initial suspension were subcultured in

the same B5mod medium. Flasks were removed from the shakers and the suspensions allowed to settle for approximately twenty minutes. The clear supernatant medium was poured off under sterile conditions. The resultant thick slurry was then divided into two, or occasionally three new flasks. Care was taken to ensure the remnants of the initial explants were not transferred. The new flasks were prepared ahead of time to contain 25 mL sterile B5mod medium.

For embryo development, aliquots were removed at the time of subculture. Sterility was maintained throughout. About 4-5 mL of homogenous suspension (prior to settling) was carefully poured into a sterile 15-mL Falcon Blue Max tube. This was tightly sealed and centrifuged at 1600 rpm (400xg) for 5 min in a bench-top centrifuge. The packed cell volume ratio (volume of "pellet" of packed cells divided by total volume of pellet plus supernatant) was determined and recorded, then adjusted to 0.4 by removal of excess supernatant with a sterile Pasteur pipette. The packed cells were resuspended by inversion or by gentle vortexing of resealed tubes. Then, 0.5 mL of homogeneous suspension was pipetted onto embryo development medium (solid BOi2Y, Bingham et al. 1975) in 60 x 20 mm petri dishes. Because the suspensions often contained cell clumps which were too big to pass through disposable blue pipette tips, these latter were first modified by cutting off the end 5-10 mm at

a diagonal with a sharp razor blade prior to autoclaving. The plates were sealed with Parafilm and placed in a controlled environment growth chamber set at 25 C with a 16 h photoperiod at $25 \mu\text{mol m}^{-2}\text{s}^{-1}$. Normally three replicate plates were prepared from each suspension at each sampling. After 14 days the number of embryos formed in each plate was recorded.

Another method studied was to inoculate MS liquid medium with 10 d-old MS-grown calli. The suspensions were cultured, subcultured and plated on BOi2Y for embryo development in the same way as the B5-based cultures.

3.2 Development of non-inductive liquid suspension cultures

The basis of this approach is the use of the non-embryogenic auxin phenylacetic acid (PAA). PAA does support some callus growth but does not induce embryogenesis in A70.34 (Section 2.3.2). Calli could be grown from petiole segments on a solid medium modified to contain PAA instead of the inductive 2,4-D. Suspension cultures established from these in a medium in which the embryogenic auxins 2,4-D and naphthaleneacetic acid were replaced by PAA would theoretically maintain cells in a non-induced state. However, a major limitation in developing PAA-based cultures is the fact that alfalfa tissue proliferates to a much

lesser extent in the presence of PAA relative to 2,4-D. In particular, calli grown on solid PAA-containing medium tend to be small and hard, and it is rarely possible to establish suspension cultures from them. Attempts to overcome this obstacle included mechanical homogenization of calli prior to suspension establishment, varying PAA concentration as well as type and concentration of cytokinin in both the callus initiation medium and the suspension medium, and the use of 2,4-D-grown calli for establishment of PAA-supported suspension cultures.

3.2.1 Effect of mechanical homogenization

A Wareing blender was used to test the potential of mechanically homogenizing MS-grown calli of A70.34 in the initiation of suspensions in MS liquid media. In this experiment IAA was used as the non-embryogenic auxin, although it was later shown to induce embryogenesis to a low degree (Section 2.3.2). (At the time the experiments discussed in this chapter were performed, evidence to date indicated that both IAA and PAA were non-inductive for embryogenesis). However, calli cultured 10 d on an MS medium containing 22.6 μ M IAA and 4.65 μ M kinetin (MSIAA) followed by 11 d on MS2 were comparable to PAA-cultured calli in their size and hardness. Approximately 2.1 g or 32-33 of these three-week-old MSIAA-initiated calli were

homogenized with 15 mL sterile MSIAA liquid in a small, sterilized Wareing blender cup and poured into a sterile 50 mL Erlenmeyer flask. An equivalent quantity of calli were also added directly to 15 mL MSIAA liquid in the same size flask. Two flasks of each treatment were prepared. The flasks were placed on a shaker at 140 rpm, under the same conditions described earlier (Section 3.1).

After only 20 h, the homogenized treatments showed a thicker and more homogenous consistency than the controls, as well as a more pronounced "ring" of callus material adhering to the inner neck of the flask. However, after 6 d there was no noticeable difference in thickness or consistency between the two treatments, though the non-homogenized suspensions tended to have bigger solid pieces settling at the bottom of the flask. One of the two flasks of homogenized material showed a wider heavier callus "ring" than the other flasks.

Homogenization apparently speeds up the establishment of a suspension from hard calli but appeared to have no persistent advantage. Taking into consideration the difficulty in maintaining sterility throughout the homogenization process, the method was not pursued as part of a routine suspension culture protocol.

3.2.2 Effect of auxin and cytokinin type and concentration in suspension media

A preliminary experiment studied the effect of varying the growth regulator composition of MS liquid media on the establishment of non-embryogenic suspensions from A70.34 calli cultured 20 d on MS1. IAA was used at either 10 μ M or 50 μ M in combination with either benzylamino purine (BAP), zeatin or kinetin as cytokinin at either 1 μ M or 5 μ M. The results were somewhat variable and will not be presented here in full because they were only qualitatively assessed as visual thickness of suspension; because MS suspensions were subsequently found to be much slower-growing than B5-based ones and thus later abandoned; and because IAA was later found to be somewhat embryogenic. Nevertheless, two useful observations were made. First, zeatin-containing media resulted in poorer performance overall than either kinetin- or BAP-containing media, which were not consistently different from each other. Secondly, no single level of IAA was optimum at all cytokinin treatments, nor was any cytokinin treatment consistently optimal at both IAA levels. Further to that, no single growth regulator combination resulted in significant improvement in or even acceptable levels of growth. Thus, it was concluded that there was little to be gained from adjusting the growth regulator composition of the suspension medium.

3.2.3 Effect of auxin and cytokinin type and concentration in callus initiation media

The object of these trials was to produce non-induced callus of sufficiently soft, friable texture for successful establishment of suspensions. Two tests were done. In the first, a number of solid MS media were used to replace MS1 for a 14 d culture of calli from A70.34 petiole segments. IAA was used at 10 μ M, 50 μ M and 100 μ M in combination with 2 μ M, 5 μ M or 8 μ M kinetin, BAP or zeatin (27 treatments in all). After 10 d there were no significant differences among the treatments nor any noticeable trends in either extent of callus formation or callus type.

These calli were used to inoculate an MS liquid medium containing 22.6 μ M IAA and 1 μ M kinetin, at approximately 1.0 g callus per 25 mL liquid medium. The failure of any of these treatments to establish as healthy, proliferating suspensions might be partly due to low inoculation density or to the use of MS- rather than B5-based media. However, the calli showed very low tendency to break up and appeared to stay as discrete, single pieces. Despite the variation in concentration and accompanying cytokinin, none of the callus initiated on IAA-containing MS medium was sufficiently friable for suspension initiation.

In the second trial, both IAA and PAA were tested in B5 callus initiation media. Three concentrations of auxin (25 μM , 50 μM and 75 μM) were used with each of 1 μM , 2 μM and 3 μM kinetin and with 3 μM , 5 μM and 7 μM BAP. These growth regulators replaced the 2,4-D and kinetin in the B5h medium described in Section 3.1. After three weeks, when suspensions would normally be initiated, all calli were small, very hard and showing considerable browning. There were no detectable trends with auxin or cytokinin type or concentration, except that 75 μM PAA resulted in slightly more callus growth than the lower PAA levels and that calli grown with BAP were slightly bigger on average than calli grown with kinetin calli. While the 2,4-D-containing B5h medium produced much more prolific, softer callus than any MS-based medium including MS1 with 2,4-D, B5 media containing IAA or PAA produced the smallest, hardest calli of all. These were thus discarded without attempting to inoculate suspensions.

3.2.4 Non-embryogenic suspensions from calli cultured on MS1 and B5h

Previous attempts (Sections 3.2.1, 3.2.2 and 3.2.3) determined that initiating callus on non-inductive media shows little promise for successful establishment of suspension cultures. Only B5h medium appears to produce

callus soft and friable enough for routine suspension initiation. MS1 calli are more firm and less prolific than B5h calli but do break up in suspension more than any calli cultured on MS or B5h media containing PAA or IAA. Strictly speaking, callus initiated on B5h or MS1 cannot be considered non-induced because of the presence of 2,4-D in those media. However, any 2,4-D effect might theoretically be lost through a few cell generations if the calli are subsequently cultured in a liquid media lacking 2,4-D. The following experiment examined actual embryogenic capacity of suspensions in non-inductive media inoculated with potentially induced callus grown on B5h or MS1.

A70.34 calli were initiated either 10 d on MS1 or 21 d on B5h (Section 2.2.2). MS liquid media were inoculated with MS1-cultured calli and B5 media with B5h-cultured calli, at approximately 1.0 - 1.4 g per 25 mL. MS liquid media included MS1, MS2, MSIAA (22.6 μ M IAA and 4.65 μ M kinetin), and MSPAA (22.6 μ M PAA and 4.65 μ M kinetin). B5 media were based on the B5mod formulation (Section 3.1) but with the 2,4-D and NAA replaced by 5.06 μ M IAA (B5IAA) or 5.06 μ M PAA (B5PAA-1). The suspensions were subcultured twice with a subculture interval of 11 days. Initial packed cell volume ratio (PCRi) and number of embryos developed per 0.5 mL aliquot plated on BOi2Y (Section 3.1) were determined at the time of subculturing (Table 3.1).

Table 3.1 Initial packed cell volume ratio (PCRi) and embryogenic capacity of A70.34 suspension cultures in various media. *denotes cultures lost to contamination.

Medium	Replicate	1st subculture		2nd subculture	
		PCRi	Number of embryos	PCRi	Number of embryos
B5mod	1	0.29	14, 17	0.70	3, 8
	2	0.47	9, 6		11, 10
	3	0.45	6, 6		3, 4
	Mean±S.E.	0.40±0.06	9.67±1.94	0.70	6.50±1.15
B5IAA	1	0.10	4, 2	0.05	0, 0
	2	0.15	0, 1		0, 0
	3	0.18	9, 4		0, 0
	Mean±S.E.	0.14±0.02	3.33±1.31	0.05	0.00±0.00
B5PAL-1	1	0.48	1, 0	0.04	0, 0
	2	0.30	1, 1		0
	3	0.22	20, 26		0, 0
	Mean±S.E.	0.33±0.08	8.17±4.76	0.04	0.00±0.00
MS1	-	0.16	*	*	*
MS2	-	0.20	*	*	*
MSIAA	-	0.21	*	*	*
MSPAA	-	0.18	*	*	*

A bacterial contamination in all four MS cultures led to a loss of both B0i2Y plates and suspension flasks after the first subculture (Table 3.1). However, these and other MS cultures were clearly and consistently less vigorous than the B5 suspensions. It was decided to abandon MS liquid media as a useful suspension culture system in favour of B5-based systems.

Among B5 suspensions, then, any embryogenic inductive effects of the B5h callus initiation medium are quickly lost upon culture in liquid B5 media lacking 2,4-D (Table 3.1). Even by the first subculture B5IAA and B5PAA-1 media were less embryogenic than was B5mod. By the second subculture B5IAA and B5PAA-1 induced no embryos at all. Thus inoculation of B5PAA-1 liquid medium with callus initiated on B5h solid medium was adopted as an acceptable non-embryogenic control suspension culture method.

Three cautionary points must be drawn from this data. First, the drastic drop in growth rate between first and second subcultures in both B5IAA and B5PAA-1 cultures must be examined. This may have been due to subculturing at too low a ratio of old culture to new media; care was thus taken in subsequent cultures to inoculate subcultures with larger volumes. In addition the subculture interval of 11 days may have been too long, resulting in loss of vigour.

In subsequent practise, the interval was reduced to 7 days, conforming with the standard method of Senaratna et al. (1989).

Secondly, B5mod cultures did not show this same drop in growth rate, and in fact showed higher growth rates than B5IAA and B5PAA-1 even in the first subculture. At the concentrations used, 2,4-D supports much more growth in cell suspension than either IAA or PAA. To compensate for this, the concentration of PAA in subsequent B5 suspension media was increased tenfold to 50.6 μM ($5.06 \times 10^{-5} \text{ M}$). This medium is referred to as B5PAA to distinguish it from B5PAA-1, which contains the lower PAA level. In calli cultured on solid MS media, growth was noticeably enhanced by increasing the PAA concentration from 10^{-6} M to 10^{-5} M or 10^{-4} M (Table 2.3).

Finally, the reduction of embryogenic capacity in B5mod cultures between the first and second subcultures is unexpected. Continuous culture in the inductive B5mod medium was hoped to provide a steady source of embryogenic material for biochemical analyses. Such suspensions might in fact only be optimally embryogenic in very early subcultures, whereas control B5PAA-1 cultures may only become truly non-embryogenic in somewhat later subcultures. Again, the long subculture interval may have led to a

partial loss of embryogenic capacity. Other possible explanations of similar observations are discussed in Section 4.3.3.

3.3 Selection of embryogenic and non-embryogenic genotypes of 'Rangelander'

Another means by which biochemical processes might be related to embryogenesis is to compare them in a range of genotypes which vary in their embryogenic capacity. A number of 'Rangelander' seeds were screened for their ability to produce embryos. Suspension cultures of these embryogenic and non-embryogenic genotypes were used as the basis for the ammonium assimilation experiments discussed in Sections 4.2.1.1 and 4.3.1.

Two lots of 'Rangelander' seeds were screened on two different occasions, one of 87 and one of 84 seeds. The seeds were first scarified by rubbing between two sheets of 120 grit sandpaper for about 10 s. They were surface-sterilized 10 min in Lipshaw baskets suspended in 0.2% mercuric chloride, followed by three rinses in sterile distilled water. Seeds were then laid, under sterile conditions, on solid MS2 medium in either 60 mm x 20 mm plates or Magenta boxes (Section 2.2.2). Germination counts were made after 10 to 14 days, the first lot showing a 65.5%

germination rate, and the second having 80.5% germination.

Of those germinated, 20 or 21 seedlings in each lot were considered suitable for screening, exhibiting two normal cotyledons, a hypocotyl, some root elongation and, in most cases, a first leaf and petiole. These seedlings were removed and cut one at a time under sterile conditions. The cotyledons were excised and "feathered" by parallel cuts about 1 mm apart extending across the cotyledon to within 1-2 mm of one edge. These and 1-cm segments of root and petiole were laid on a sterile 60 mm x 20 mm MS1 plate (Section 2.2.1). A 10-15 mm segment of hypocotyl including the cotyledonary node at the upper end was placed upright in a Magenta box containing MS2. The plate and the Magenta box were labelled alike to identify the source seedling, which was arbitrarily assigned a number from K1 through K41.

Plates and Magentas were incubated at 25 C with a 16 h photoperiod ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$; Sylvania 'Cool-White' fluorescent tubes). After 3 to 5 weeks most of the hypocotyls developed into plantlets which were further propagated as described in Section 2.2.1. Calli developed from cotyledons, root and petiole segments were transferred under sterile conditions after 10 days to MS2 agar-solidified medium in 60 mm x 20 mm plates (Section 2.2.2). Callus formation ratings (Section 2.2.2) and embryo counts

were made at day 30, 20 days after transfer. About 15 genotypes that were high callus producers, had healthy corresponding plantlets and which represented a range of embryogenic and non-embryogenic types were selected for further propagation and study.

Chapter 4. Ammonium assimilation in embryogenic and non-embryogenic cultures and genotypes

4.1 Literature review

There are two lines of evidence suggesting that a unique change in ammonium assimilation is associated with the induction of somatic embryogenesis in plants. One is the frequently-observed requirement for reduced nitrogen in embryogenesis induction. Second is the reports of changes of activity or isozyme pattern of the enzyme glutamate dehydrogenase associated with embryogenesis. This evidence is outlined in the following sections.

4.1.1 Nitrogen requirements of somatic embryogenesis

The requirement of somatic embryogenesis for reduced nitrogen in the form of ammonium or amino acids in the inductive medium has been observed for many years in many species (e.g. Kohlenbach 1977, Murashige and Huang 1985, Grimes and Hodges 1990). In 'Regen S' alfalfa both an absolute requirement and stimulation by reduced nitrogen has been demonstrated for embryogenesis (Walker and Sato 1981, Stuart and Strickland 1984a,b, Skokut et al. 1985, Meijer and Brown 1987b, Bingham et al. 1988). Generally induction of embryogenesis cannot proceed without some ammonium ion

present, while amino acid preparations such as casein hydrolysate or certain single amino acids enhance the degree of the embryogenic response (Stuart and Strickland 1984a,b, Meijer and Brown 1987b, Bingham et al. 1988, Skokut et al. 1985, Halperin 1966, Kamada and Harada 1979, Wetherell and Dougall 1976). Commonly proline, alanine and glutamine are found to stimulate embryogenesis, while Stuart and Strickland (1984a) also showed some enhancing effect of arginine, lysine, serine and asparagine. Glutamate and glycine were observed to be inhibitory or neutral to embryogenesis (Skokut et al. 1985, Stuart and Strickland 1984a). Stuart and Strickland (1984a) also showed no effect of aspartate, cysteine, histidine, isoleucine, leucine, methionine, phenylalanine, threonine, tryptophan, tyrosine or valine.

The role of reduced nitrogen in embryogenesis is not understood. Skokut et al. (1985) studied the metabolism of ¹⁵N-labelled amino acids in alfalfa embryogenesis. They found that the incorporation into protein of exogenous proline and alanine, which stimulate embryogenesis, was not different from that of glutamate and glycine, which have no effect on embryogenesis. Nor was there a difference in the incorporation of these four amino acids into protein in a regenerating compared to a non-regenerating line of 'Regen S' alfalfa. This supports the suggestion of Bingham et al.

(1988) that amino acid additions are effective only by providing ammonium ion upon catabolism.

Stuart and Strickland (1984b) also concluded that stimulation of embryogenesis by glutamine could be fully substituted by ammonium supplement. They suggest that the amide nitrogen of glutamine is labile and that glutamine increases embryogenesis by providing a ready source of reduced nitrogen to the culture. However, the enhancing effect of proline and alanine could not be explained as an ammonium source but in fact required additional ammonium present (Stuart and Strickland 1984b).

4.1.2 Ammonium assimilation in plants

All nitrogen utilized in the metabolism of higher plants is ultimately incorporated only as ammonium (Srivastava and Singh 1987). Inorganic nitrate taken up by roots is converted to ammonium through nitrate reductase and nitrite reductase. Cellular ammonium also arises from a number of other processes such as lignin biosynthesis, the deamidation of nitrogen transport compounds, catabolism of amino acids, photorespiration and nitrogen fixation (Joy 1988, Kumar and Abrol 1990). The major source in photosynthetic tissue (unlike alfalfa suspension callus) is considered to be photorespiratory release of NH_4^+ from glycine (Kumar and

Abrol 1990). This released ammonium must then be assimilated into amino acids and nucleotides, both to support cellular growth and to avoid the toxic effects of excess ammonium (Mifflin and Lea 1982, Joy 1988).

There are a few possible pathways by which ammonium is assimilated in plants but it is now widely accepted that by far the most important path is the glutamate synthase cycle, or GS/GOGAT pathway (Mifflin and Lea 1982, Joy 1988, Srivastava and Singh 1987). This cycle is represented in Fig. 4.1. The two major enzymes involved, glutamine synthetase or GS (EC 6.3.1.2) and glutamate synthase or GOGAT (EC 1.4.1.13 or EC 1.4.7.1), are thought to be found mostly in chloroplasts or in plastids in non-green tissue and to a lesser extent in the cytoplasm (Mifflin and Lea 1982, Joy 1988). Glutamine synthetase has a very high affinity for ammonium with a K_m of 10^{-5} to 10^{-4} M (Mifflin and Lea 1982). The GS/GOGAT system efficiently and irreversibly incorporates ammonium into glutamate to form glutamine, and then transfers the amide group of glutamine to 2-oxoglutarate resulting in formation of two glutamate molecules. One glutamate is recycled back to receive more ammonium while the second can act as an amino source for amino acid biosynthesis through aminotransferases (see Fig. 4.1) or as a source for synthesis of other nitrogen compounds (Joy 1988).

Fig. 4.1 The GS/GOGAT pathway of ammonium assimilation (after Mifflin and Lea 1982).

4.1.2.1 The role of glutamate dehydrogenase

Enzyme systems other than GS/GOGAT may be capable of assimilating ammonium. Asparagine synthetase, carbamoyl phosphate synthetase, alanine dehydrogenase and aspartate dehydrogenase all may catalyze the incorporation of ammonium into organic compounds but there is no evidence that they are effective *in vivo* (Mifflin and Lea 1982, Joy 1988). However, there are indications that the widely-occurring and abundant enzyme glutamate dehydrogenase may operate in ammonium assimilation under certain conditions.

Glutamate dehydrogenase or GDH (EC 1.4.1.3) catalyzes the reversible incorporation of ammonium directly into 2-oxoglutarate to form glutamate as follows:

GDH

GDH has a much lower affinity for ammonium than does GS, with a K_m in the range of 10^{-2} M (Stewart et al. 1980). However, the K_m value is reportedly dependent on pH and light and may be somewhat lower under certain conditions (Srivastava and Singh 1987). It has also been proposed that its true substrate is NH_3 rather than NH_4^+ and that GDH may have a much higher affinity for ammonia within the lipids of the mitochondrial membrane where it cannot combine with

water to form ammonium ion (Davies and Teixeira 1975).

The reaction equilibrium favours the aminating direction for nearly all GDH enzymes (Stewart et al. 1980, Srivastava and Singh 1987). However, the enzyme may be assayed in either direction (Pahlich and Joy 1971).

GDH has been detected in nearly all living organisms including almost all higher plants (Stewart et al. 1980, Srivastava and Singh 1987). Joy (1988) reviewed a number of lines of evidence to suggest that GDH is not important in ammonium assimilation. One is the low affinity of GDH for ammonium. Second are observations of arrested ammonium assimilation when either GS or GOGAT are specifically inhibited, even when cellular ammonium levels are high enough for GDH activity. Similarly, ammonium accumulates in photorespiratory mutants lacking either GS or GOGAT activity. Finally isolated mitochondria, which contain most of the cell's GDH, assimilate very little photorespiratory ammonium into glutamate but rather release it into the medium (Yamaya et al. 1986, reviewed by Joy 1988). Thus many workers have concluded that GDH functions *in vivo* in the deaminating direction in amino acid catabolism such as in senescing tissue (Mifflin and Lea 1982, Srivastava and Singh 1987, Joy 1988).

By contrast, Oaks and Yamaya (1990) summarized evidence that mitochondrial GDH can assimilate ammonium *in vivo*. They point out that ammonium levels in mitochondria are sufficiently high (1 mM or more) to drive GDH in the aminating direction, while O_2 uptake and electron transport in mitochondria is relatively resistant to the high NH_4^+ . Furthermore, GDH is thought to be loosely associated with the mitochondrial membrane where photorespiratory NH_4^+ is released (Oaks and Yamaya 1990). Isolated mitochondria of corn and pea do assimilate at least some ammonium into glutamate (Yamaya et al. 1986, Hartmann and Ehmke 1980, Davies and Teixeira 1975). This can be presumed to be catalyzed by GDH since that enzyme is primarily located in mitochondria while GS is not (Stewart et al. 1980, Kumar and Abrol 1990. Oaks and Yamaya (1990) also cite ^{15}N -feeding studies (Bielawski and Kaczkowski 1984) that show that tobacco leaf protoplasts and rye leaves incorporate ^{15}N from glycine or ammonium into the α -amino group of glutamate and glutamine, suggesting that both GS and aminating GDH are active.

Most workers suggest that GDH serves to assimilate excess ammonium during stress and senescence (Kumar and Abrol 1990, Laurière and Daussant 1983, Mifflin and Lea 1982, Cammaerts and Jacobs 1985, Srivastava and Singh 1987, Joy 1988). There is some evidence that GDH is more stable under stress

conditions than GS and GOGAT (Srivastava and Singh 1987). Yamaya and Oaks (1987) propose an anaplerotic function for GDH, whereby it replenishes glutamate (and ultimately other 5-carbon amino acids) lost from the system through export or protein synthesis. There is as yet no solid evidence for this view.

Factors affecting GDH activity

Glutamate dehydrogenase activity is known to be modulated by many factors, admirably reviewed by Srivastava and Singh (1987). Exogenous ammonium usually increases GDH activity, though the response depends on duration of exposure, tissue, age, and the presence of other salts and metabolites (Srivastava and Singh 1987, Laurière and Daussant 1983, Loyola-Vargas and de Jimenez 1984). Nitrate may inhibit or have no effect on GDH in the short-term though longer exposures may enhance it, possibly through reduction to ammonium (Srivastava and Singh 1987). Amino acids, particularly glutamate and glutamine, have variable effects on GDH activity in different systems (Srivastava and Singh 1987). Exogenous carbohydrates frequently inhibit GDH in a way which is not counteracted by addition of ammonium (Srivastava and Singh 1987) and which is distinct from the considerable enhancement of GDH activity by carbon and energy starvation (Maestri et al. 1991). Light intensity

and quality, photoperiod, temperature, water stress, salinity, pollution and exposure to disease organisms all may influence GDH activity (Srivastava and Singh 1987). Generally conditions causing stress stimulate GDH activity (possibly through increasing carbohydrate levels, or cellular ammonium; Laurière and Daussant 1983).

There is one account of an increase in GDH activity associated with somatic embryogenesis. Fransz et al. (1989) performed *in situ* GDH activity staining of maize callus, observing that the small, embryogenic cells stained intensely while the non-embryogenic cells remained unstained. Auxins such as IAA may stimulate GDH (Srivastava and Singh 1987). Low concentrations of 2,4-D (2×10^{-6} M to 2×10^{-5} M) stimulate GDH of pea and corn roots while higher concentrations are inhibitory; it was suggested that 2,4-D increases ammonium which in turn enhances GDH activity (Tkemaladze 1981, cited by Srivastava and Singh 1987).

4.1.3 Isozymes and embryogenesis

Isozymes are variant forms of an enzyme all catalyzing the same reaction but with structural and/or amino acid sequence differences which may result in different isoelectric points, electrophoretic mobilities, pH or temperature optima, substrate affinities or kinetics. There are two

classes of isozymes, those arising from genetic mechanisms and those generated by non-genetic modifications (Scandalios 1983). Genetic isozymes may be generated by different genes (multilocus isozymes) or by different alleles at the same locus (allelic isozymes or allozymes), or, in the case of polymeric enzymes, by random association of different subunits of either multilocus or allelic origin (hybrid isozymes) (Scandalios 1983). Isozymes or isozyme subunits coded for by different loci may be differently regulated (Markert 1983) and so isozyme patterns or profiles may vary among phenological stages, tissues, organs or physiological states.

Changes in the isozyme profiles of various enzyme systems have been associated with morphogenesis in culture in a number of species. In most of these cases, though not all, specific isozymes are found to be present in morphogenic cultures or differentiated tissues that are absent in non-morphogenic cultures or undifferentiated callus. In other cases a specific enzyme form may be associated with the non-morphogenic state. In yet still other cases an isozyme pattern is suggested to be related to the embryogenic capacity of different genotypes.

Changes in isoesterases and isoperoxidases are perhaps most common. Chawla (1989) found three bands on esterase

activity-stained gels appearing specifically in extracts from wheat callus induced for root or shoot formation and not in non-induced callus. These esterase bands as well as two peroxidase bands were also found in regenerated shoots but not in the surrounding callus from which they formed (Chawla 1989). Embryogenic and organogenic maize cultures could be distinguished on the basis of their esterase isozyme pattern (Everett et al. 1985). Both esterases and peroxidases showed varying isozyme patterns among tissues of immature embryos and seedlings of barley (Coppens and Gillis 1987). Chibbar et al. (1988) identified one esterase isozyme specific to an embryogenic carrot cell line and another exclusive to a non-embryogenic cell line. The same group also found yet another esterase isozyme which appeared only in the medium of the non-embryogenic line (Chibbar et al. 1989). Similarly, Lee and Dougall (1973) identified an esterase band which appeared in embryogenic carrot cell cultures lacking 2,4-D and not in vegetative cultures maintained in the presence of 2,4-D. An isoperoxidase also appeared in an embryogenic carrot cell line when transferred to 2,4-D-free medium, stimulating embryogenesis (Joersbo et al. 1989). Wochok and Burleson (1974) observed several isoperoxidases appearing in somatic embryo tissues from carrot cultures that were not present in the suspensions from which they developed. Two peroxidases were found in embryogenic callus of maize which were not detected in non-

embryogenic calli (Fransz et al. 1989). Finally, Del Grosso et al. (1987) found that tissue-specific isoperoxidases disappeared when *Phaseolus vulgaris* seedlings were induced to form callus, but that genotype-specific bands persisted.

Other isozyme systems showing changes associated with morphogenesis are acid phosphatase (Coppens and Gillis 1987, Lee and Dougall 1973, Chawla 1989, Fransz et al. 1989), alcohol dehydrogenase (Everett et al. 1985), malate dehydrogenase (Lee and Dougall 1973), aspartate aminotransferase (Lee and Dougall 1973), gamma-glutamyl transferase (Lee and Dougall 1973), and glutamate dehydrogenase (see section 4.1.3.1).

Most authors do not speculate on the physiological significance of morphogenic-specific isozymes (Lee and Dougall 1973), but many suggest rather that they might be useful as markers of the developmental processes involved (e.g. Chisbar et al. 1988, Coppens and Gillis 1987, Everett et al. 1985, Fransz et al. 1989).

4.1.3.1 Glutamate dehydrogenase isozymes and embryogenesis

The isozyme system of glutamate dehydrogenases may be one of very few for which a functional link with morphogenesis could be suggested, given the well-established requirement

2

PM-1 3½"x4" PHOTOGRAPHIC MICROCOPY TARGET
NBS 1010a ANSI/ISO #2 EQUIVALENT

PRECISION^{SM} RESOLUTION TARGETS

PIONEERS IN METHYLENE BLUE TESTING SINCE 1971

MicroD

15800 COUNTRY ROAD 6, BURNHILL, PA 19001 USA
TEL: 610-426-1957 FAX: 610-426-1957 TLE 870000000

for ammonium in embryogenesis (Lee and Dougall 1973). Close associations of GDH isozyme changes with embryogenesis have been documented. Lee and Dougall (1973) observed a loss of three to four GDH bands when carrot cell cultures were transferred to 2,4-D-free medium, stimulating embryogenesis. However in maize tissues two GDH bands, more or less specific to zygotic embryos both in planta and cultured in vitro, were also seen in embryogenic cultures in the presence of 2,4-D and not in the absence of 2,4-D (Everett et al. 1985). Fransz et al. (1989) also found more GDH isozymes in embryogenic than in non-embryogenic maize callus.

Certain other conditions can induce changes in plant GDH isozyme patterns (reviewed by Srivastava and Singh 1987). Maestri et al. (1991) showed that GDH zymograms from *Nicotiana plumbaginifolia* callus varied with the level and type of carbon source. Several workers have reported isozyme changes with changes in nitrogen source, particularly increases in isozyme number upon exposure to ammonium or amino acids (Srivastava and Singh 1987, Mifflin and Lea 1982, Stewart et al. 1980, Loyola-Vargas and de Jimenez 1984, and citations). Laurière and Daussant (1983) showed that one specific GDH isozyme in wheat leaves was inducible by darkness stress, senescence and exposure to ammonium. Tissue- and organ-specific isozymes of glutamate

dehydrogenase are well-documented (Everett et al. 1985, Srivastava and Singh 1987, Hartmann et al. 1973).

This chapter is a discussion of evidence for a specific involvement of nitrogen metabolism in the induction of somatic embryogenesis in alfalfa tissue culture.

4.2 Materials and methods

4.2.1 Suspension cultures

4.2.1.1 Embryogenic and non-embryogenic suspension cultures of 'Rangelander' genotypes

As described in chapter 3, callus of A70.34 and other embryogenic and non-embryogenic genotypes of 'Rangelander' was initiated on B5h medium. Flasks containing either B5mod or B5PAA liquid media were inoculated with three-week-old calli. The suspensions were subcultured every seven days and were sampled for biochemical studies at the time of the second, third and fourth subculture.

4.2.1.2 Nitrogen source experiment

To test the effect of the form of nitrogen in suspension medium on embryogenesis and ammonium assimilation, a number of experimental cultures were set up. The total molar concentration of nitrogen was kept at 31.72 mM as it is in the liquid B5mod medium normally used for suspension cultures. The respective levels of nitrate and ammonium were varied from 21 mM nitrate-N and 11 mM ammonium-N to 32 mM nitrate-N and no ammonium-N. The treatments and their composition from stock solutions are given in Table 4.1.

Table 4.1 Composition of media for nitrogen source experiment.

$[\text{NO}_3^-]:[\text{NH}_4^+]$ (mM:mM)	$(\text{NH}_4)_2\text{SO}_4$ 50.5 mM	NH_4Cl 214 mM	KCl 214 mM	K_2SO_4 50.5 mM	KNO_3 750 mM	NH_4 citrate 214 mM	K citrate 214 mM	distilled water
21.15:10.57 (33% NH_4^+)	1.0 mL	-	-	-	705 μL	1.0 mL	1.0 mL	13.795 mL
21.15:10.57 (33% NH_4^+)	1.0	1.0 mL	1.0 mL	-	705	-	-	13.795
28.84:2.88 (9% NH_4^+)	1.0	-	-	-	961	-	-	15.339
28.84:2.88 (9% NH_4^+)	1.0	0.1	0.1	-	961	-	-	15.339
29.70:2.02 (6% NH_4^+)	1.0	-	-	-	990	-	-	15.510
30.20:1.52 (5% NH_4^+)	752 μL	-	-	248 μL	1007	-	-	15.493
31.72:0 (0% NH_4^+)	-	-	-	1.0 mL	1057	-	-	15.443

For treatments containing more than 2.02 mM ammonium (i.e. more ammonium than that normally used in B5mod medium) the addition of ammonium was unavoidably accompanied by up to 13.8 mM additional anion. Also, K^+ concentrations were kept constant by addition of an alternate potassium salt when KNO_3 was decreased, and this resulted in a further addition of up to 13.8 mM excess anion. In an attempt to identify any possible effects of the excess anion, these treatments were repeated, using either citrate or chloride as the additional anion.

Treatments containing less ammonium than B5mod would have up to 2.02 mM less sulphate, so this was kept constant by addition of potassium sulphate. This increased the K^+ concentration, but only by a maximum of 7.3% over the level in B5mod.

Callus of genotype A70.34 was initiated on B5h medium as described in chapter 3. After 21 days the calli were used to inoculate one flask of each treatment prepared according to Table 4.1. The suspensions thus established were subcultured weekly (Section 3.1). Suspension callus was sampled at the time of the second, third and fourth subculture and used for biochemical studies.

4.2.2 Determination of embryogenic capacity and growth rates of suspensions

In order to relate biochemical observations of various treatments to degree of embryogenesis, the embryogenic capacity of each suspension was monitored at the time of sampling. This was done according to the method given in Section 3.1. At the same time the initial packed cell volume ratio (prior to adjustment) was determined and recorded as a measure of suspension growth rate.

4.2.3 Tissue extraction

As mentioned, aliquots of the described 'Rangelander' suspensions were removed and set aside at the time of the second, third and fourth weekly subculture. Approximately 15-20 mL were gravity filtered through 44 μ nylon mesh. The collected callus was weighed into 1.7 mL Eppendorf microfuge tubes for small samples (about 130-250 mg fresh weight) or into a 5 mL glass homogenizer for larger samples (300-450 mg fresh weight). These were kept on ice.

For homogenization in Eppendorf tubes a few grains of sand (approximately 20 μ L) were sprinkled on the callus. The chilled buffer was measured directly into the Eppendorf tube or glass homogenizer at a ratio of 1:1 or 2:1 volume

(μ L):fresh weight (mg). The extractions were carried out on ice and continued until the material appeared homogeneous, using either the glass plunger made to fit the glass homogenizer or a disposable plastic Eppendorf pestle made to fit the Eppendorf tubes. For cell disruption the extracts were then sonicated on ice for 11 s with a Vibra-cell Sonicator (Sonics and Materials Inc.) using a microtip. Extracts were centrifuged 25 min (14,000xg) at 4 C in a BHG Hermle microcentrifuge.

4.2.3.1 Extraction buffers for ammonium and GS assays

Samples intended for either ammonium assay or glutamine synthetase assay were extracted in a simple buffer consisting of 0.2 M Tris-HCl (pH 8.5) and 56 mM 2-mercaptoethanol. This is a modification of the buffer used by Wetter and Dyck (1983) excluding the sucrose, which presumably served only to increase the density of the extract for gel loading. Samples extracted in the sucrose-containing buffer showed GDH activities roughly equal or somewhat less than that of samples extracted in buffer without sucrose and thus sucrose was omitted. Two other simple buffers recommended for isozyme studies were also tried, those of Chibbar et al. (1988) and Everett et al. (1985) (see Appendix I). However, extracts made with these buffers showed less GDH activity than those made with the

modified Wetter and Dyck's (1983) buffer.

4.2.3.2 Extraction buffers for GDH gels and assays

Problems with streakiness of GDH activity-stained gels led to the trial of various other extraction buffers or buffer additives. It was supposed that some component of the plant extract was responsible for the streaks. To effectively bind any potentially interfering substances a number of additions to the basic extraction buffer were systematically tested. Bovine serum albumin was added to 0.1% to bind free fatty acids, and 0.6% soluble polyvinylpyrrolidone was used to bind phenolics; neither showed any effectiveness in reducing streakiness. Triton X100 was added to 0.1% in the extraction buffer in the hope that such a concentration would not denature the enzyme but would dissociate it from other components of the extract which may cause it to streak. However, both control and Triton-treated samples gave streaky bands. Finally, NADH was added to 0.08% in the extraction buffer so that any NADH-binding sites on the enzymes might be fully occupied, making the entire enzyme population of a uniform electrophoretic mobility. This treatment also had no effect in reducing streakiness.

More complex extraction buffer formulations from other sources were then tried. Extracts were made in the buffer

used for isozyme work by Dr. S. Warwick, Biosystematics Research Centre, Agriculture Canada (see Appendix I), and in the "Vegetative extraction buffer II" of Cheliak and Pitel (1984) (see Table 4.2); these were used for GDH isozyme stained gels. The latter buffer resulted in much less streaky bands (though still not perfectly clean), as well as protein contents two to three times higher than did the modified buffer of Wetter and Dyck (1983) described above. Thus the buffer of Cheliak and Pitel (1984) was used in all subsequent gels and GDH assays. However, this buffer interfered with measurements of NH_4^+ content and GS activity, causing cloudy precipitates in both those assays. Therefore separate extractions were carried out in the modified Wetter and Dyck (1983) buffer for the NH_4^+ and GS assays.

4.2.3.3 Protein assay

Protein contents of the extracts were determined by the Bio-Rad protein assay kit (Bio-Rad Laboratories) following the Standard Assay procedure and reading optical density with a Beckman DU-8 spectrophotometer. A standard curve was prepared each time using 20, 35, 50, 65 and 80 μg of bovine serum albumin. Sample protein contents were calculated using the regression coefficients from the standard curve.

Table 4.2 Vegetative extraction buffer II of Cheliak and Pitel (1984).

2% Tergitol 15-S-9
2% polyethylene glycol 20,000
8% polyvinylpyrrolidone 7:1 40M:360M
50 mM ascorbic acid
0.4 mM NAD
0.1% bovine serum albumin
0.2 mM pyridoxal 5'-phosphate
0.3 M sucrose
12 mM cysteine-HCl
0.05 M boric acid, pH 7.1
0.66% 2-mercaptoethanol (add just before using)
Adjust final pH to 7.1

4.2.4 Glutamate dehydrogenase assay

Glutamate dehydrogenase activity was assayed in the aminating direction following the method of Pahlich and Joy (1971). For each sample two matched 1.0 mL quartz cuvettes were used, one without substrates as a control and one experimental. In preparation for the assay all reagents were brought to 25 C in a water bath and the Beckman DU-8 spectrophotometer was warmed up 30 min with the Kinetics II program module in place and the cuvette holder programmed to 25 C.

It was determined through experimentation that with 65 μ g protein per cuvette the GDH activity was in a measurable range. The volume of sample extract required to give 65 μ g protein was calculated from the Bio-Rad protein contents. Into each of control and experimental cuvettes was pipetted 134 μ L of freshly-made 1 mM NADH as well as a volume of 0.2 M Tris-HCl pH 8.5 equal to 560 μ L less the calculated sample volume. To the control cuvette was added 106 μ L distilled water, and to the experimental cuvette 53 μ L of 3.2 M $(\text{NH}_4)_2\text{SO}_4$ and 53 μ L of 0.2 M 2-oxoglutarate. Final concentrations are given in Table 4.3.

Each cuvette was closed with a square of Parafilm and mixed by inversion. Then the calculated volume of sample extract,

Table 4.3 Reaction mixture for glutamate dehydrogenase assay, final concentrations.

0.135 M Tris-HCl, pH 8.5

13.25 mM 2-oxoglutarate

212 mM $(\text{NH}_4)_2\text{SO}_4$

168 μM NADH

approx. 81 $\mu\text{g mL}^{-1}$ protein

maintained on ice, was quickly pipetted into each cuvette. These were capped with new squares of Parafilm and again rapidly inverted. The outer and inner faces of the cuvettes were wiped with a tissue moistened with ethanol and the cuvettes immediately placed in the spectrophotometer. The program was started as soon as possible in order that the initial reaction rate could most accurately be recorded. The program made repeated OD340nm readings of each cuvette and subtracted the control measurements from the experimental. Normally 40 cycles of 16 s each were recorded. These were then plotted, and the range of points felt to represent the initial reaction rate were selected (see Fig. 4.2 as an example). The slope of this range of points was determined by regression, expressed as OD340nm min⁻¹. This value was divided by 6.22 to convert to $\mu\text{mol NADH mL}^{-1}$ for a 1 cm light path, multiplied by the total reaction volume of 0.8 mL and divided by the total protein assayed in mg. The results are then expressed as $\mu\text{mol NADH min}^{-1} \text{mg}^{-1}$ protein, or m units mg⁻¹ protein.

4.2.5 Glutamate dehydrogenase activity-stained gels for isozyme visualization

The method followed for GDH isozyme staining was basically that of Wetter and Dyck (1983). Both full-size and mini non-denaturing, discontinuous gels were run, usually 6.25%

Fig. 4.2 Example of spectrophotometric results of glutamate dehydrogenase assay. Absorbance at 340 nm was monitored every 16 s, cycling between sample and blank; points shown are $(OD_{340nm_{sample}} - OD_{340nm_{blank}})$. Generally the first six to twelve points of each curve were used to calculate the initial reaction rate. In this case the first six points were used to calculate a regression with an R^2 value of 0.995481 and a slope of $-0.01409 \text{ OD}_{340nm} \text{ cycle}^{-1}$. Initial reaction rate was calculated as follows (see text):

$$\begin{aligned}
 & \frac{-0.01409 \text{ OD}_{340nm}}{16 \text{ s}} \times \frac{60 \text{ s}}{\text{min}} \times \frac{1}{0.0659 \text{ mg protein}} \\
 & \times \frac{1}{6.22 \text{ OD}_{340nm} \cdot \text{mL} \cdot \mu\text{mol}^{-1} \text{ NADH}} \times 0.8 \text{ mL} \\
 & = -0.802 \frac{\mu\text{mol NADH}}{\text{min} \cdot \text{mg protein}} = -0.802 \frac{\text{m units}}{\text{mg protein}}
 \end{aligned}$$

polyacrylamide but occasionally 5.0% and 5.5%. Hoefer electrophoresis equipment was used. Gels and tank buffers were prepared according to the Hoefer Scientific Instruments catalogue; those recipes and procedures are given in Appendix II. Tank buffers were maintained at 4 C by the circulating water cooling system. Extracts were kept on ice. Supernatants were removed from pellets and sucrose-dye solution (Appendix II) was added to 20%. Experience showed that visualization was optimal when 50 μ g protein were loaded per 8 mm-wide well; proportionately lesser quantities were loaded for narrower wells. Extracts were mixed and loaded with a Hamilton syringe.

Gels were run at 4 C, at 7.5 watts constant power per full-sized gel or at 200 V constant voltage for mini-gels. The run was stopped when the dye front just reached the bottom of the gel, about 5.5 h for full-sized gels and about 2 h for mini-gels.

The staining solution was made fresh each time. Into a plastic tray just bigger than the gel was measured 40 mg NAD, 1 mg phenazine methosulfate, 20 mg nitro blue tetrazolium, and 46 mL 0.1 M Tris-HCl pH 8.5. The tray was gently agitated until the chemicals were dissolved and the solution was homogeneously coloured. The tray was kept covered with aluminum foil. At the last moment, just when

the gel had been removed from the electrophoresis equipment and notched in the upper right corner, 4 mL of 1 M sodium glutamate in 0.1 M Tris-HCl pH 8.5 was added to the tray and shaken to mix. The gel was carefully slid into the tray and the tray recovered with foil. Incubation was at room temperature on a gentle orbital shaker. Bands usually appeared within 10-15 min but occasionally gels were incubated up to 30-40 min to intensify weak bands. Gels were then carefully drained, and rinsed and stored in 7% acetic acid at room temperature. Clear gels were either photographed and/or dried. Many gels were too streaky to be saved, but zymograms were transcribed on paper from the discernible bands.

4.2.6 Glutamine synthetase assay

The GS activity assay was based on the transferase assays of Donn et al. (1984) and Ishida et al. (1989). A reaction mixture was made up fresh each time, by first mixing 2 mL 0.4 M imidazole, 2 mL 0.6 M NH_2OH , 2 mL 0.2 M glutamine, 2 mL 0.2 M sodium arsenate, 0.2 mL 50 mM ADP (sodium salt) and 11.74 mL distilled water. This was brought to pH 6.8-6.9 with NaOH. Then 0.06 mL 0.1 M MnCl_2 was added and the pH adjusted to 6.90.

For each extract a sample tube and a control tube were

prepared. In each labelled glass test tube was placed 500 μL reaction mixture; these were brought to 30 C in a test tube rack in a water bath. Experimentation showed that extract volumes providing 350 μg protein gave activity in a measurable range. Calculated volumes of extract supernatants were thus diluted with buffer to give 400 μL containing 700 μg protein. Following a stopwatch, 200 μL of diluted extract were added to each sample tube. After exactly 10 min 700 μL of stopping mixture (3.3% FeCl_3 , 2% trichloroacetic acid, 1.25 M HCl) were pipetted into each sample tube, and then to each control tube. Equivalent quantities of extract were then added to the "stopped" control tubes. The tubes were then removed from the water bath and the contents transferred to labelled microfuge tubes. After 15 min centrifugation the absorbance of the supernatants at 540 nm was determined.

The results were interpreted according to a standard curve (Fig. 4.3). A range of 0.1 μmol to 2.0 μmol gamma-glutamylhydroxamate in 200 μL distilled water was added to tubes containing 500 μL reaction mixture. After exactly 10 min, 700 μL stopping mixture was pipetted into each tube. Absorbance at 540 nm was determined following centrifugation as above, and a regression equation ($R^2=0.997941$) calculated between μmol standard and OD540 nm as follows:

Fig. 4.3 Standard curve for glutamine synthetase assay. Absorbance at 540 nm of reaction mixtures containing varying concentrations of gamma-glutamylhydroxamate standard. Means of five measurements $\pm$ standard error.

$$\mu\text{mol } \gamma\text{-glutamylhydroxamate} = \frac{\text{OD}_{540\text{nm}} - 0.010459}{0.211022}$$

GS activity was expressed as $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein = m units mg^{-1} protein.

4.2.7 Amino acid determination

Amino acid analysis was performed by technicians at the Department of Biology, Carleton University on a Waters HPLC amino acid analyzer system. Samples were first deproteinized and neutralized. About 300-500 mg fresh weight callus was measured into a glass homogenizer with 3:1 water ($\mu\text{L}:\text{mg}$ fresh weight). After partial homogenization the same volume of sulphosalicylic acid solution (0.7 g/12 mL) was added and the tissue was thoroughly ground. This was transferred to a microfuge tube and spun 3 min at 10,000 rpm. Then 400 μL were pipetted into a clean glass vial and neutralized with 50 μL 1 N NaOH. At this point the samples were usually frozen until just before analysis. Upon thawing samples were mixed with 500 μL 20% methanol and 50 μL 2 mM α -amino adipic acid (internal standard). Finally, 150 μL of prepared sample were measured into labelled minicentrifuge tubes for analysis.

The output provided picomoles per sample of up to 20 amino

acids. Results are expressed in picomoles per mg fresh weight as well as molar proportion of each amino acid of total amino acids.

4.2.8 Ammonium assay

Endogenous tissue ammonium levels were determined by the microdiffusion method given by Shelp et al. (1985) and in more detail by Sieciechowicz (1986). This involved the preparation of Nessler's reagent, which was made in a 500 mL graduated cylinder on a magnetic stirrer in a fume hood. Mercuric iodide (22.75 g) and potassium iodide (17.45 g) were dissolved in 10-15 mL distilled water in the cylinder. A potassium hydroxide solution was prepared in advance by adding 56 g KOH to 70 mL distilled water and allowing to cool to room temperature. This was added to the stirring mixture in the cylinder, very slowly to avoid precipitation. Distilled water was then added carefully to 500 mL total volume. The solution was left standing for one week to allow any precipitate to settle, and was stored in a brown bottle in the fume hood. All assays were carried out using a single batch.

The assay requires clean glass scintillation vials specially fitted with rubber stoppers with fire-polished glass rods inserted such that the rounded ends projected 10-15 mm below

the stopper. Into each vial was pipetted 1.0 mL saturated K_2CO_3 solution ($pH > 11.0$). The rounded end of the glass rod was dipped sparingly in concentrated H_2SO_4 such that no drop could form and fall off the tip. Immediately upon adding 200 μL of extract each vial was sealed with the stopper and dipped glass rod. The vials were gently swirled to mix the extract and K_2CO_3 without disturbing the H_2SO_4 . After incubation for 1 h at room temperature the NH_3 volatilized from the extract is theoretically dissolved in the H_2SO_4 (Sieciechowicz 1986). The stopper was then carefully removed and the rod stirred into a spectrophotometer cuvette containing 0.5 mL Nessler's reagent and 1.0 mL distilled water.

After five minutes and before 1 h the absorbance at 420 nm was determined. The absorbance of a blank, incubated with 200 μL buffer in place of a sample extract, was subtracted from each reading.

A standard curve was prepared using an NH_4Cl stock solution (Fig. 4.4). Three to five replicate vials containing each of 50, 100, 150, 200, 300 and 400 nmol NH_4Cl were run through the assay procedure. A regression equation ($R^2 = 0.997986$) was calculated to convert OD420nm to nmol NH_4^+ :

$$\text{nmol } NH_4^+ = \frac{OD420nm + 0.2913}{0.002176}$$

Ammonium contents are expressed as nmol mg^{-1} protein.

Fig. 4.4. Standard curve for ammonium assay. Absorbance at 420 nm of reaction mixtures containing varying concentrations of ammonium chloride.

4.3 Results and discussion

The two sets of ammonium assimilation experiments are discussed in two sections: those comparing embryogenic and non-embryogenic cultures and genotypes in Section 4.3.1, and those performed on the embryogenic genotype A70.34 cultured under inductive conditions with varying ratios of $\text{NO}_3^-:\text{NH}_4^+$ in Section 4.3.2.

4.3.1 Comparison of embryogenic and non-embryogenic cultures and genotypes

These experiments were carried out on the cultures described in 4.2.1.1.

4.3.1.1 Glutamate dehydrogenase activity

Table 4.4 shows GDH specific activity (μ units mg^{-1} protein) of various genotypes in embryogenic B5mod suspensions and in non-embryogenic B5PAA suspensions, as well as some measurements from extracts of the *in vitro* shoot material from which the suspension callus was initiated. An analysis of variance of the suspension data revealed a significant difference due to medium ($\alpha = 0.0148^*$) with B5mod suspensions showing higher activity than B5PAA. This is in accordance with the findings of Fransz et al. (1989) in

Table 4.4 Glutamate dehydrogenase activity in μ units mg^{-1} protein. Means of one to four experiments $\pm$ standard error.

	<u>B5mod</u>		<u>B5PAA</u>		<u>In vitro</u> <u>shoots</u>	
		n		n		n
Embryogenic genotypes						
A70.34	14.5 $\pm$ 4.4	3	15.7 $\pm$ 4.5	3	11.4 $\pm$ 2.8	4
K25	138.3	1	96.0	1		
K28	126.2 $\pm$ 24.3	2	43.8 $\pm$ 8.7	2		
K29	27.0	1	3.0	1	26.2	1
Mean	76.5 $\pm$ 32.4		39.6 $\pm$ 20.6			
Non-embryogenic genotypes						
K4	72.0 $\pm$ 50.3	4	15.8 $\pm$ 6.7	4	28.8	1
K10	61.6 $\pm$ 25.5	3	12.5 $\pm$ 3.8	3	25.2 $\pm$ 11.7	3
K21	38.3 $\pm$ 13.6	3	30.5 $\pm$ 16.0	3		
Mean	57.3 $\pm$ 10.0		19.6 $\pm$ 5.5			

which GDH activity was detected *in situ* in embryogenic and not in non-embryogenic callus tissue of maize. Tkemaladze (1981; cited by Srivastava and Singh 1987) also reported increased GDH activity upon 2,4-D treatment, and Srivastava and Singh (1987) review other examples of GDH enhancement by auxins. While these results show a clear effect of auxin on GDH, it cannot be related except circumstantially to the induction of embryogenesis, nor can it be discerned if 2,4-D enhances or PAA represses GDH activity or both.

The effect of genotype on GDH and its interaction with medium is less clear. Analysis of variance showed significant differences due to genotype ($\alpha = 0.017^*$). Genotypes with low activity in one medium tend to have low activity in the other medium and, when measured, in the *in vitro* shoot material. This suggests that there is a genotype-specific "base" level of activity. While individual genotypes appear to vary in their "base" GDH activity, there is not enough data to confirm that a capacity for embryogenesis is associated with higher base activity, or with a greater magnitude of difference between B5mod and B5PAA suspensions. This is perhaps not surprising as these selected non-embryogenic genotypes might be blocked at any one of several stages throughout competence, induction or development of embryogenesis. It would be quite coincidental if they were all blocked in a process

which involved GDH activity.

Both embryogenic and non-embryogenic genotypes respond in the same direction to the change of medium, suggesting that the effect is specific to the auxin treatment and independent of inherent embryogenic capacity. The mean increase in GDH activity in B5mod over B5PAA cultures was 3.59-fold for embryogenic and 3.16-fold for non-embryogenic genotypes. Differences in GDH activity between embryogenic and non-embryogenic genotypes were not significant by t-test, though both B5mod and B5PAA suspensions tended to show higher GDH activity in embryogenic than in non-embryogenic lines.

4.3.1.2 Glutamate dehydrogenase isozyme pattern

Isozyme patterns were studied from hand-drawn zymograms transcribed from the original GDH activity-stained gels (see Appendix VI). Fig. 4.5 gives zymograms from various subcultures of three series of suspensions. Mitochondrial, plastid and cytoplasmic enzymes are all theoretically represented since the extraction procedure did not protect plastids or mitochondria from disruption. Enzyme forms that are strictly NADP-dependent will not be represented since only NAD was used as a cofactor in the gel staining. However, most higher plant GDH enzymes apparently show

Subculture 2, Series 1

Subculture 3, Series 1

Subculture 4, Series 1

Fig. 4.5 Zymograms of glutamate dehydrogenase activity-stained gels. Extracts from various genotypes in B5mod and B5PAA suspensions and from in vitro-grown shoot material (IVS).

Subculture 1, Series 2

Subculture 2, Series 3

Subculture 3, Series 3

Fig. 4.5 continued

either dual specificity for NAD and NADP or are NAD-specific, though chloroplastic forms may show higher affinity for NADP (Stewart et al. 1980).

Another point worthy of consideration is a possible interaction with the extraction buffer. Results from another set of experiments (see Section 4.3.2.3) suggest that GDH isozyme banding pattern changes with extraction buffer. Though all the extracts in these gels were made in the same buffer and should be comparable to each other, it is important to remember that the banding patterns are not necessarily invariable.

Several isozymes of alfalfa GDH were found in most extracts. Bands were mostly in one group of evenly-spaced bands, occasionally with one, two or three others more distantly spaced. One to nine bands may be seen in any given lane. Given that GDH is generally considered to be a hexameric (or possibly tetrameric) enzyme (Srivastava and Singh 1987) the isoforms probably result from different combinations of two or more monomer forms (de Vienne 1980). If there were two monomers of different molecular weights combining randomly, seven possible hexamers would be expected. In fact seven is a common number of GDH isozymes, although more have also been observed (Srivastava and Singh 1987). Three monomers could give rise to 28 possible hexamer isoforms. Hexamers

would vary in their frequency if the monomers were not present in equal frequency. Thus, for example, if the heavier of two different-sized monomers was less frequent than the smaller form, polyacrylamide gel electrophoresis would be expected to show up to seven isozyme bands of gradually decreasing intensity towards the top of the gel. The heaviest hexamers might be so rare as to be undetectable. Generally, the major group of isozymes found have consisted of seven or fewer bands, and directional gradation of intensity was frequently observed. Two of the genotypes, A70.34 and K29, sometimes show more than seven bands, with an additional one or two faster bands being present. If all the isozymes do in fact arise from the combination of different monomers, at least three monomeric forms would be required to explain this pattern. These monomers might be coded by different loci or by different alleles at the same locus. De Vienne (1980) also concluded that three different subunits were responsible for the complex GDH zymogram observed in alfalfa pollen.

All gels showed one isozyme band (marked with an arrow) common to all or most of the lanes in that gel (Fig. 4.5). It is reasonable to assume that this "common" band is at the same molecular weight in all gels and that it can be used to relate one gel to another. The following analyses are based on this assumption.

The common band was usually the most intense band in any given lane, with other bands becoming gradually fainter with distance either above or below it. This suggests that the common band represents a "pure" hexamer made of six identical monomers of high frequency. Bands above and below would be mixtures of the high-frequency monomer with less-frequent monomers of lesser and greater electrophoretic mobility, respectively. Interestingly, the common band was often absent or fainter than other bands in extracts from *in vitro* shoots. Monomer frequencies may be quite different in differentiated tissue.

Alternatively, some bands might represent isozymes from different subcellular compartments. For example, a group of up to seven evenly-spaced isoforms might be mitochondrial in origin, while more distantly-spaced bands might come from cytoplasmic or plastid locations.

It would be interesting to determine if and how the monomeric composition of GDH affects the regulation and activity of the whole enzyme. GDH isozymes have been shown to vary in their efficiencies in aminating and deaminating activity (Srivastava and Singh 1987). Aminating and deaminating forms may show different tissue distribution or subcellular localization (Srivastava and Singh 1987). Differential regulation of GDH isozymes has been proposed as

a means of responding to different nitrogen metabolism requirements (Laurière and Daussant 1983, Loyola-Vargas and de Jimenez 1984, Maestri et al. 1991).

Differences between embryogenic and non-embryogenic genotypes

Genotypes A70.34, K25, K28 and K29 are embryogenic, while K4, K10 and K21 are not. There are clearly differences in GDH isozyme pattern among these selected 'Rangelander' genotypes (Fig. 4.5). However, there are no specific bands consistently associated with either embryogenic or non-embryogenic genotypes. It cannot be concluded that the GDH isozyme profile in 'Rangelander' can be used as a marker for embryogenic capacity. Published correlations between appearance of specific isozyme bands and embryogenic capacity (e.g. Chibbar et al. 1988, 1989) might be criticized for the narrowness of the investigation. The present work demonstrates that genotype-specific patterns might erroneously appear to be related to embryogenesis if only a few genotypes are examined.

Comparing any given genotype across different gels shows some differences (Fig. 4.5). Usually the "common" band is always present and often those closest to it; when bands are faint or absent they are often the most distant from the

common band, supporting the suggestion that isozymes are formed from different monomers of varying frequency as discussed above. Why these distant bands are occasionally faint or absent could be related to subculture number, extraction efficiency, total GDH activity, gel running conditions or staining efficiency. It is possible that an association between an isozyme band or bands and embryogenic capacity would not be recognized if the bands were always or occasionally faint or undetected under these conditions.

Differences between B5mod and B5PAA suspensions

Comparing GDH isozymes of individual genotypes in B5mod and B5PAA cultures does reveal a noticeable though not completely consistent trend (Fig. 4.5). Frequently one, two or three of the fastest-migrating bands occur only, or more intensely, in the PAA-containing suspensions relative to the 2,4-D-containing ones. These are always faster-migrating than the common band. Again, a gradual decrease in intensity is observed with distance away from the common band. This might suggest that culture in PAA medium is associated with an increase in frequency of a monomer of greater electrophoretic mobility. A general increase of total GDH activity might conceivably reveal otherwise faint or absent bands. However, it has already been shown that GDH activity, at least as assayed in the aminating

direction, is actually less in the presence of PAA than in 2,4-D (see Section 4.3.1.1).

This occasional effect of auxin type on GDH isozyme pattern is independent of genotype, occurring sometimes in both embryogenic and non-embryogenic lines. GDH isozyme profiles have previously been shown to change with changes in auxin treatment. Lee and Dougall (1973) found more GDH isozyme bands in carrot cells cultured with 2,4-D (the non-embryogenic state) than without 2,4-D (the embryogenesis-permissive state). As with the present work, no direct relation with embryogenesis can be deduced. However, Everett et al. (1985) observed two GDH bands, one faster- and one slower-migrating than the basic four or five bands, which appeared both in zygotic embryos and in differentiating embryogenic, minus-2,4-D cultures of maize, which suggests that these bands may be associated with embryo development.

In each of these published accounts, extra bands were found in embryogenic cultures. In the present work the additional bands are seen in B5PAA, the non-embryogenic treatment. There is no evidence to distinguish whether these bands are induced by PAA or repressed by 2,4-D. A change in auxin type may shift the frequencies of monomers resulting in a shift in the population of GDH enzymes, perhaps with

different efficiencies in amination and deamination. A change in the importance of either aminating or deaminating GDH might well be related to embryogenesis. This seems more likely than a repression or induction of embryogenesis by the presence or absence of specific isozyme bands.

4.3.1.3 Glutamine synthetase activity

Because the glutamine synthetase assay procedure requires relatively large volumes and/or relatively concentrated extracts, there were few extractions for which an assay was possible. Assays carried out on extracts made with Cheliak and Pitel's (1984) extraction buffer showed a precipitate in the reaction mixture and results from these cannot be considered valid. Table 4.5 summarizes the useable GS activity data from two experiments representing the 2nd and 3rd subcultures of two different series of cultures. In addition some extracts made from *in vitro* shoot material were assayed for comparison with the cultures made from them.

No conclusions can be made from this data regarding differences in GS activity between embryogenic and non-embryogenic genotypes. There is clearly a wide variation among genotypes, with the non-embryogenic line showing considerably less than the two embryogenic types. However,

Table 4.5 Glutamine synthetase activity in μ units mg^{-1} protein. Means of one or two experiments $\pm$ standard error.

	B5mod	B5PAA	<i>In vitro</i> shoots
Embryogenic genotypes			
A70.34	355 $\pm$ 181	408 $\pm$ 86	433
K29	301 $\pm$ 136	331 $\pm$ 122	225
Non-embryogenic genotypes			
K4			147
K10		403 $\pm$ 150	
K21	152	169	

an analysis of variance based on the data from the two types of suspensions showed a non-significant difference due to genotype ($\alpha = 0.145$). The *in vitro* shoots show a wide range of GS activities but a more extensive survey of genotypes would be necessary to identify any correlation with embryogenic capacity. A tendency towards higher GS activities in the presence of B5PAA can be seen, but an analysis of variance showed a non-significant difference due to medium ($\alpha = 0.302$). Interestingly, an assay performed on extracts made with the buffer of S. Warwick did not detect any GS activity in B5mod cultures of either K29 or K10, but activities of 21 and 626 μ units mg^{-1} protein, respectively, were measured in B5PAA cultures. While the precipitate in the reaction mixture renders these data questionable, they do serve to re-enforce the suggestion that GS may be to some degree either repressed by the presence of 2,4-D or enhanced by PAA. Further experimentation on the same and additional genotypes would confirm this.

4.3.1.4 Amino acid profile

The levels of 20 amino acids in picomoles per mg fresh weight and as proportion of total amino acids are given in Appendix III for suspension callus of several genotypes in B5mod, B5PAA, MS1 or MSPAA liquid culture at various subcultures. Concentration of total free amino acids varies

considerably among samples, possibly due to inconsistencies in extraction efficiency or to variation in culture growth rates or protein contents. Because of this variation it was felt that analysis of data for individual amino acids was better made using the proportion of total amino acids represented by each amino acid. These comparisons were made among genotypes, between embryogenic and non-embryogenic genotypes, and between B5mod and B5PAA suspensions. There is not enough data on MS suspensions to make a comparison with B5 suspensions since the slower-growing MS cultures were soon abandoned. However, the little data that is available does not indicate that MS cultures respond differently than B5 cultures. Overall the data is not extensive enough to make conclusions regarding changes over consecutive subcultures, but there appears to be a slight tendency towards decreasing total amino acids with increasing subculture number.

For each amino acid an analysis of variance was calculated using only data from B5 suspensions and pooling results from first, second and, in two cases, fourth subcultures (Table 4.6). Since any given genotype can only be either embryogenic or non-embryogenic, embryogenic capacity could not be randomized as a factor in the analysis of variance. Thus genotype effects reflect variation among all genotypes considered equally and regardless of their embryogenic

Table 4.6 Probabilities of non-significant effects due to genotype and medium, from two-way analyses of variance performed on each of 20 amino acids.

Amino acid	Genotype	Medium
aspartate	0.20	0.20
glutamate	0.02*	0.54
asparagine	0.27	0.00**
serine	0.22	0.37
glutamine	0.00**	0.09
histidine	0.57	0.28
homoserine	0.44	0.85
glycine	0.12	0.69
threonine	0.15	0.86
arginine	0.75	0.60
alanine	0.43	0.37
γ -aminobutyric acid	0.04*	0.14
tyrosine	0.70	0.49
α -aminobutyric acid	0.35	0.94
methionine	0.20	0.20
valine	0.32	0.48
phenylalanine	0.11	1.00
isoleucine	0.02*	0.62
leucine	0.34	0.48
lysine	0.58	0.50

capacity. To detect effects associated specifically with embryogenic capacity, comparisons were made between means of embryogenic and non-embryogenic genotypes by t-test, pooling B5mod and B5PAA data together.

While variation among the proportion of total amino acids by any given amino acid is quite extensive, there are very few consistent trends. Table 4.6 shows that the only statistically significant trends are an effect of medium on asparagine (asn) and genotype effects on glutamate, glutamine, gamma-aminobutyric acid and isoleucine.

The accumulation of asparagine in B5PAA cultures is in several cases very dramatic, though not totally consistent - two of 12 samples showed less asn in B5PAA than in B5mod. One of these two was the genotype K4 sampled at its fourth subculture, another culture of which actually showed over three times as much asn in B5PAA than in B5mod when sampled at its first subculture. The only paired measurements from MS suspensions, being A70.34 at the time of first subculture, showed a very remarkable 66-fold increase in asn in MS1 over MSPAA cultures.

A very important observation is that the effect of medium on asparagine accumulation is independent of genotype. That is, both embryogenic and non-embryogenic genotypes respond

to 2,4-D by producing less, or to PAA by producing more asn, whereas only embryogenic genotypes respond to 2,4-D by producing embryos. Thus, the differential accumulation of asparagine is clearly and strictly a growth regulator effect and may be totally unrelated to embryogenesis.

Genotype effects on glutamate, glutamine, gamma-aminobutyric acid and isoleucine are difficult to interpret. The mean glutamate level of the non-embryogenic genotypes given in Table 4.6 was somewhat higher than that of the embryogenic genotypes (0.109 and 0.076 respectively, as proportion of total amino acids when B5mod and B5PAA data were pooled). This was not significant by t-test ($\alpha = 0.111$) and it is possible that glutamate levels may reflect genotype-specific differences in amino acid metabolism unrelated to embryogenic capacity. Nevertheless, the possible association between increased glutamate level and non-embryogenic genotypes will be discussed later.

When embryogenic and non-embryogenic genotypes were compared for glutamine, gamma-aminobutyric acid and isoleucine levels, no differences were seen at all. T-tests based on pooled B5mod and B5PAA data gave high probabilities of non-significant differences ($\alpha = 0.50, 0.52$ and 0.57 respectively). Significant genotype effects among these amino acids are apparently unrelated to embryogenic

capacity.

The absence of striking or statistically significant differences between embryogenic and non-embryogenic genotypes in levels of aspartate, asparagine, glutamate or glutamine, the amino acids most involved in primary assimilation of ammonium might be unexpected. It should be remembered, however, that any given non-embryogenic genotype selected from an embryogenic cultivar, such as 'Rangelander', might theoretically be blocked at one of several stages of competence, induction or development of the embryogenesis pathway. Though certain parameters of ammonium assimilation may be associated with embryogenic treatment, a culture "impaired" in embryogenesis might well not show any differences in these parameters.

Table 4.7 shows paired measurements of total amino acids from suspensions of various genotypes. The high variability of these non-replicated measurements may be due in part to differences in extraction efficiency. Nevertheless, the non-embryogenic treatment of culture in B5PAA tends to lead to somewhat higher levels of total amino acids than the embryogenic B5mod, though the difference is non-significant ($\alpha = 0.114$ by paired t-test). However, when embryogenic and non-embryogenic genotypes were compared, a highly significant difference was found among B5mod cultures ($\alpha =$

Table 4.7 Total amino acids in picomoles mg⁻¹ fresh weight of samples for which simultaneous measurements in 2,4-D- and PAA-containing media were made.

Genotype	Subculture	Embryogenic capacity ¹	Medium	
			MS1	MSPAA
A70.34	2	+	119	1494
			B5mod	B5PAA
A70.34	1 [*]	+	765	385
K4	1 ^{**}	-	131	520
K21	2	-	142	248
K21	2	-	147	265
K22	2	+	123	150
K25	2	-	157	269
K29	2	+	152	122
K30	2	+	642	1242
K34	2	-	283	870
K36	2	+	200	83
K39	2	-	102	188
A70.34	3 [*]	+	85	180
K4	3 ^{**}	-	139	52

^{*}, ^{**} Those samples followed by the same symbols are part of the same series of subcultures.

¹'+' = embryogenic genotype; '-' = non-embryogenic genotype.

0.001"), with embryogenic lines showing over two-fold higher mean total amino acid levels than the non-embryogenic ones (328 vs. 157 picomoles mg^{-1} fresh weight, respectively). This is a specific response to the inductive medium B5mod, as there was no significant difference between the two classes of genotypes in B5PAA. Because it appears only in inductive conditions, this response might be a consequence of the onset of embryogenesis in embryogenic genotypes and not reflect an inherent difference associated with the embryogenic capacity of the genotypes. Again, no clear conclusion can be made regarding consistent, detectable differences between embryogenic and non-embryogenic genotypes of 'Rangelander'. However, this interpretation contrasts with the non-significant tendency towards higher amino acids in B5PAA than in B5mod in paired measurements, discussed above.

Also in contrast with these results are the findings of Skokut et al. (1985) in which higher amino acid levels were observed in a non-regenerating vs. two regenerating lines of alfalfa. The genotypes they used were a selection from *M. sativa* cv. 'Regen S' and two hybrids from 'Regen S' and 'Rangelander'. Their analysis was not statistical but based only on these one non-embryogenic and two embryogenic lines. These workers also showed higher amino acid levels under conditions which suppress or do not increase embryogenesis

(Skokut et al. 1985). This confirms the observed tendency towards higher amino acid levels in the non-embryogenic B5PAA than in B5mod (Table 4.7). Higher free amino acids might reflect lower rates of incorporation into proteins as might be expected in non-embryogenic conditions, or possibly higher rates of amino acid biosynthesis as might be expected in ammonium-stimulated embryogenesis.

4.3.1.5 Ammonium content

Tissue ammonium levels are shown in Table 4.8. An analysis of variance of the means showed no significant differences due to genotype ($\alpha = 0.165$) or to medium ($\alpha = 0.096$). However, two trends can be observed. Non-embryogenic genotypes tend to show more ammonium accumulation than embryogenic genotypes. Secondly and perhaps more striking, the ammonium content of any given genotype tends to be higher in B5mod than in B5PAA, and most especially in non-embryogenic genotypes. A paired t-test on all the raw data for which simultaneous B5mod and B5PAA measurements are available indicated a significant ammonium accumulation in B5mod ($\alpha = 0.018'$). The same test performed on data from non-embryogenic genotypes only showed an even more significant difference ($\alpha = 0.003''$). The two classes of genotypes may respond somewhat differently to auxin treatment in terms of ammonium metabolism. It also follows

Table 4.8 Ammonium content in nmol mg⁻¹ protein. Means of one to three experiments $\pm$ standard error.

	<u>B5mod</u>		<u>B5PAA</u>	
		n		n
Embryogenic genotypes				
A70.34	106.6 ± 29.1	3	116.4 ± 58.9	3
K29	37.0 ± 15.4	3	37.4 ± 37.4	2
Mean	71.8 ± 34.8		76.9 ± 39.8	
Non-embryogenic genotypes				
K4	402.5 ± 161.4	3	166.2 ± 113.2	2
K10	411.2 ± 145.0	3	141.5 ± 32.5	2
K21	181.3 ± 48.7	2	48.0	1
Mean	331.7 ± 75.2		118.6 ± 36.0	

that, since there is both a genotype-dependent and a genotype-independent effect, choice of auxin has at least two consequences, the one influencing induction of embryogenesis being distinct from the one involving ammonium metabolism.

An increase in ammonium levels could have any of three theoretical causes: greater uptake of ammonium from the medium, greater release of ammonium by cellular processes (discussed in Section 4.1.2), or reduced ammonium assimilation might be reduced. Furthermore, the auxin effect might be explained as either a 2,4-D-enhanced accumulation of NH_4^+ in B5mod or as a PAA-mediated repression of accumulation in B5PAA cultures.

In my view there are only two facts available as evidence to distinguish among these possibilities. Given the established requirement of embryogenesis for ammonium one might guess that the lower levels of NH_4^+ in embryogenic types results from a greater NH_4^+ consumption under embryogenic conditions, rather than a repression of processes that cause NH_4^+ accumulation. Secondly, asparagine, a probable ultimate endpoint of excess ammonium, accumulates significantly in B5PAA, suggesting much less incorporation of NH_4^+ into asn in B5mod (see Section 4.3.1.4). Thus it is proposed that ammonium from the

medium, normally assimilated into asn under non-embryogenic conditions (B5PAA), is re-directed in the presence of 2,4-D (B5mod) (in some way possibly involving GDH; see Section 4.3.1.1). In embryogenic genotypes, NH_4^+ is somehow consumed in the process of embryogenesis. In non-embryogenic genotypes in the presence of 2,4-D, NH_4^+ tends to accumulate.

It cannot be assumed that GDH acts in the aminating direction. The tendency to higher ammonium contents in B5mod cultures could be a result of the observed increased GDH activity in those suspensions (Section 4.3.1.1) if the enzyme were acting in the deaminating direction. However, this does not explain the accumulation of asparagine in B5PAA cultures.

4.3.2 Effect of varying ratio of $\text{NO}_3^-:\text{NH}_4^+$ in the medium

These experiments were carried out on the cultures discussed in Section 4.2.1.2.

4.3.2.1 Degree of embryogenesis

Monitoring the number of embryos produced from aliquots removed at each subculture showed a rapid decline in embryogenesis with increasing subculture number in each

suspension series (Fig. 4.6). The reason for this change is not known; possible explanations are discussed in Section 4.3.3. With this in mind it seems reasonable to discuss the embryogenesis data only from the second subculture when comparing the different $\text{NO}_3^-:\text{NH}_4^+$ treatments, since embryo numbers are clearly reduced in the third and fourth subcultures. Second-subculture embryo numbers generally increase as the proportion of total nitrogen as ammonium increases up to 33% (10.6 mM ammonium). This affirms other work showing ammonium-stimulation of embryogenesis (see Section 4.1.1). For example, Walker and Sato (1981) found a requirement for 12.5 mM ammonium for optimal embryogenesis in alfalfa. A surprising observation is that the zero ammonium treatment gave rise to relatively large numbers of embryos, while none would be expected. One possibility is that the tissue had accumulated ammonium during its culture on solid, nitrogen-rich B5h medium prior to suspension inoculation, and that some of this ammonium would persist particularly during the first few subcultures. It is more likely, though, that the 5.5 mM glutamine and 0.95 mM serine present in both solid and liquid B5 media can support embryogenesis as a source of reduced nitrogen (Section 4.1.1).

Fig. 4.6 Effect of varying ammonium level in the suspension medium on embryogenesis. Number of embryos per plate from aliquots taken from suspension of A70.34 at different subcultures. Means of three plates $\pm$ standard error.

4.3.2.2 Glutamate dehydrogenase activity

Fig. 4.7 shows the results of a GDH assay carried out on suspension callus cultured in a range of $\text{NO}_3:\text{NH}_4^+$ ratios. A trend towards higher GDH activities at higher ammonium levels is apparent. This might be consistent with the view that GDH serves in the aminating direction as a "mop" for excess ammonium. However, GDH is clearly not fully effective in that capacity, since tissue ammonium levels do increase with increasing ammonium in the medium (Section 4.3.2.5).

Evidence previously discussed (Section 4.3.1.1) demonstrated a correlation between increased GDH activity and culture in the 2,4-D-containing medium B5mod. This medium also induces embryogenesis. If there is a relationship between GDH and induction of embryogenesis, an increase in GDH activity might be expected in association with other conditions which enhance embryogenesis. In the present experiment, all suspensions were cultured with the inductive B5mod but the degree of embryogenesis varies with the $\text{NO}_3:\text{NH}_4^+$ ratio in the medium (Section 4.3.2.1). The increase in GDH activity with increasing ammonium parallels the increase in embryogenesis with increasing ammonium. The $\text{NO}_3:\text{NH}_4^+$ treatments which give rise to the highest numbers of embryos (at least in the second subculture; Fig. 4.6) also give the

Fig. 4.7 Effect of varying ammonium level in the suspension medium on glutamate dehydrogenase activity. Means of two or three measurements from second, third and fourth subcultures of A70.34 $\pm$ standard error.

highest GDH activity. However there is no proof of a causal relationship between GDH activity and embryogenesis, and GDH may be independently stimulated by elevated ammonium directly. Glutamate dehydrogenase activity has been generally observed to be induced by NH_4^+ in a wide range of species and tissue types (Srivastava and Singh 1987). Magalhães and Huber (1989) found higher rates of GDH activity in roots of tomato, rice and corn when nitrogen was provided in the culture solution in the form of ammonium rather than nitrate.

4.3.2.3 Glutamate dehydrogenase isozyme pattern

Fig. 4.8 shows zymograms from GDH activity-stained gels in which extracts from cultures of different $\text{NO}_3^-:\text{NH}_4^+$ ratios were electrophoresed. An immediate observation is that, in almost every case, these extracts from A70.34 suspension callus show only three bands. In the gels discussed in Section 4.3.1.2, A70.34 callus extracts occasionally gave up to four bands above the common band and as many as three below (see Fig. 4.5). This does not appear to correlate with differences in total GDH activity as A70.34 extracts in the two sets of experiments showed comparable GDH activities (cf. Table 4.4 and Fig. 4.7). It might have been expected that more bands would become visible when overall GDH activity of an extract is higher. The staining reaction

Subculture 2

Subculture 3

Fig. 4.8 Zymograms of glutamate dehydrogenase activity-stained gels. Extracts from A70.34 suspensions with varying ammonium level in the suspension medium, and from *in vitro* grown shoot material (IVS). Some media formulations used either chlorine (Cl) or citrate (citrate) as the counter anion, as indicated. Extracts made in buffer of Wetter and Dick (1983) were indicated (W+D); all others made in buffer of Cheliak and Pitel (1984) (C+P).

would not likely have been limiting to the number of bands appearing, since the reagents were always prepared fresh, and since the staining was always allowed to continue until band intensity ceased increasing.

Another striking point is the near constancy of the pattern throughout the range of nitrogen-source combinations (Fig. 4.8). All culture extracts showed the same three bands, the common band and two immediately above it. Only one extract on one occasion, that from a culture in a $\text{NO}_3^-:\text{NH}_4^+$ ratio of 30.2 mM:1.5 mM, showed two extremely faint additional bands below the common band (Fig. 4.8). Again, these extra bands do not correlate with increased GDH activity of the extract. At least one if not both of these faint bands probably corresponds to a lower band appearing in the extract made from *in vitro* shoots of A70.34. More gels would be necessary to determine if there is any significance to the one-time, faint appearance of these two extra bands. If their occurrence was related to the high NO_3^- levels in that medium, they would also be expected to appear in the 100% NO_3^- medium. However, they were undetectable in any other extract. There was no sign of ammonium-inducible GDH isozymes as observed by Laurière and Daussant (1983) and other workers cited by Srivastava and Singh (1987).

Finally, a difference can be observed between the banding

patterns of extracts of *in vitro* shoots made in two different extraction buffers. The need for caution in interpreting these GDH zymograms has already been stressed on this account (see Section 4.3.1.2). It is not clear whether some isoforms appear while others disappear when a different buffer is used, or whether the electrophoretic mobility of the same isoforms is altered by the change in buffer. The latter seems more likely since the total number of bands in extracts from each buffer is approximately the same but their location on the gel is different.

4.3.2.4 Glutamine synthetase activity

Although the data is not extensive enough to establish a clear relationship between GS activity and $\text{NO}_3^-:\text{NH}_4^+$ ratio, a trend can be discerned (Fig. 4.9). Glutamine synthetase activity seems to be maximal at moderately low levels of ammonium (1.5 mM; $\text{NO}_3^-:\text{NH}_4^+ = 30.2 \text{ mM}: 1.5 \text{ mM}$) and to decrease at higher and lower levels of ammonium (Fig. 4.9). GS activity is lowest when no NH_4^+ is supplied in the medium, suggesting a substrate activation, but also at high $[\text{NH}_4^+]$ (10.6 mM).

The regulation of glutamine synthetase by nitrogen source is not clear. Rhodes et al. (1975) found that GS activity in *Lemna minor* L. was substantially lower when cultured in 5 mM

Fig. 4.9 Effect of varying ammonium level in the suspension medium on glutamine synthetase activity. Measurements from A70.34 suspensions at time of fourth subculture.

ammonium than in 5 mM nitrate. Conversely, Magalhães and Huber (1989) found a statistically significant increase in GS activity in roots and shoots of rice cultured with 10 mM ammonium rather than 10 mM nitrate. Tomato and corn, however, showed no significant difference in GS activity between the two nitrogen sources (Magalhães and Huber 1989). Mifflin and Lea (1982) suggested that the GS pathway, as the primary route for ammonium assimilation, would most likely be regulated in green plants in such a way as to maintain maximal operation under conditions when ammonium is being generated. Intuitively it might be expected that GS, like GDH, would increase in activity as the demand for ammonium assimilation increased through increased $[\text{NH}_4^+]$ in the medium. The fact that GS actually appears to be repressed at high $[\text{NH}_4^+]$ might be a consequence of ammonium toxicity, to which GDH might be less susceptible. Glutamate dehydrogenase is considered to be a more stable and generally stress-resistant enzyme than glutamine synthetase (Srivastava and Singh 1987).

4.3.2.5 Ammonium content

As the proportion of total nitrogen in the medium available as ammonium increases from 0 to 33%, tissue levels of ammonium also increase (Fig. 4.10). The trend is apparent though the data is not extensive enough to determine the

Fig. 4.10 Effect of varying ammonium level in the suspension medium on tissue ammonium content. Means of two measurements from second and third subcultures of A70.34 suspensions $\pm$ standard error.

shape of the curve. This suggests that any ammonium exclusion mechanisms are at best not fully effective over this range. It also implies that any cellular mechanisms for detoxification through ammonium assimilation are not fully effective. A correlation between the level of ammonium supplied exogenously in the medium and the assayed ammonium levels in the tissue was, not surprisingly, significant ($\alpha = 0.015^*$).

4.3.3 Relation to embryogenesis

The preceding Sections 4.3.1 and 4.3.2 discuss changes in ammonium metabolism associated with treatments that affect embryogenesis. To suggest a direct relationship between embryogenesis and these observed changes requires first a comparison with actual measurements of embryogenesis, and secondly an investigation of other possible explanations of the observations.

Tables 4.9 and 4.10 summarize basic data from the second, third and fourth subculture of one series of the genotype-medium experiment suspensions (Table 4.9) and of the nitrogen source experiment suspensions (Table 4.10). No embryos would be expected in B5PAA suspensions of any genotype at any subculture, nor in non-embryogenic genotypes under any conditions. However, no embryos were observed at

Table 4.9 Summary of data from genotype/medium experiments

Sub-culture:	PCRi ¹			No. embs. ²				Protein ³ (W & D buffer)				Protein ⁴ (C & P buffer)				GDH ⁵	GS ⁶	NH ₄ ⁺ ⁷
	2	3	4	2	3	4	2	3	4	2	3	4	2	3	4			
<u>Treatment</u>																		
A70.34 B5mod	0.21	0.18	0.30	1.7	0.0	0.0	0.28	1.16	1.36	4.40	4.08	3.62	8.91	141	76			
A70.34 B5PAA	0.20	0.20	0.13	0	0	0	0.94	2.26	1.26	5.12	3.06	4.48	24.71	273	190			
K4 B5mod			0.16			0			0.28			3.18			571			
K4 B5PAA		0.15	0.09		0	0			0.62			4.58			53			
K10 B5mod			0.08			0			0.08			4.42			670			
K10 B5PAA			1.25			0	1.66	1.78	0.52	4.32	4.32	4.38	21.25	211	174			
K21 B5mod	0.25	0.24	0.20	0	0	0	0.86	1.70	1.10	3.96	4.10	4.42	3.84	121	230			
K21 B5PAA	0.25	0.31	0.24	0	0	0	1.50	4.66	1.50	4.70	6.00	7.46		137	48			

Table 4.9 continued

	PCRi ¹			No. embs. ²				Protein ³ (W & D buffer)				Protein ⁴ (C & P buffer)				GDH ⁵	GS ⁶	NH ₄ ⁷
	2	3	4	2	3	4	2	3	4	2	3	4	2	3	4			
Sub-culture:	2	3	4	2	3	4	2	3	4	2	3	4	2	3	4			
<u>Treatment</u>																		
K29	0.18	0.21	0.18	0	0	0	0.60	2.56	0.58	3.62	4.20	5.66						
B5mod																		
K29	0.18	0.26	0.13	0	0	0	1.36	3.91	0	4.66	7.04	5.22	25.43	172				
B5PAA																		
K7																		
B5mod																		

¹initial packed cell volume ratio²number of embryos per 0.5 mL aliquot of suspension adjusted to 0.4 packed cell volume ratio, plated on BOi2Y³protein content ($\mu\text{g } \mu\text{L}^{-1}$) of extracts made in a modified buffer of Wetter and Dyck (1983) (Section 4.2.3.1), 1 μL buffer per mg fresh weight callus⁴protein content ($\mu\text{g } \mu\text{L}^{-1}$) of extracts made in buffer of Cheliak and Pitel (1984) (Table 4.2), 1 μL buffer per mg fresh weight callus⁵glutamate dehydrogenase activity, m units mg^{-1} protein⁶glutamine synthetase activity, m units mg^{-1} protein⁷callus ammonium content, nmol mg^{-1} protein

Table 4.10 Summary of data from nitrogen source experiments

Sub-culture:	PCRi ¹				No. embs. ²				Protein ³ (W & D buffer)				Protein ⁴ (C & P buffer)			
	2	3	4		2	3	4		2	3	4		2	3	4	
<u>Treatment</u>																
33% NH ₄ ⁺ (Cl ⁴)	0.14		0.15		0	0	0		0.26	0.42	0.15		4.86	4.00	2.28	
33% NH ₄ ⁺ (cit. ⁹)	0.23	0.20	0.19		68	6	2		0.80	0.96	2.17		5.92	4.28	4.00	
9% NH ₄ ⁺ (Cl ⁸)	0.18	0.24	0.24		18	9	1		1.32	1.30	1.56		6.28	4.30	3.52	
9% NH ₄ ⁺ (cit. ⁸)	0.22	0.21	0.25		45	2	0		0.58	1.38	2.01		6.40	4.52	3.56	
6% NH ₄ ⁺	0.18	0.23	0.30		21	2	0		0.56	0.72	1.56		5.52	4.26	4.00	
5% NH ₄ ⁺	0.19	0.40	0.80		10	1	2		1.46	1.36	1.13		6.66	4.22	3.40	
0% NH ₄ ⁺	0.24	0.22	1.19		24	23	2		0.42	0.56	1.55		6.90	5.04	4.12	

Table 4.10 continued
GDH⁵

Sub- culture:	GDH ⁵			GS ⁶	NH ₄ ⁺⁷		
	2	3	4		2	3	4
<u>Treatment</u> 33% NH ₄ ⁺ (Cl ⁶)	43.89	4.46			862	243	
33% NH ₄ ⁺ (cit. ⁹)	42.44	26.68		131	368	872	
9% NH ₄ ⁺ (Cl ⁶)	18.72	18.45		213	399	113	
9% NH ₄ ⁺ (cit. ⁹)	15.00	12.88	36.66	262	442	234	
6% NH ₄ ⁺	26.72	20.09	27.91	262	579	259	
5% NH ₄ ⁺	10.72	8.41		315	23	37	
0% NH ₄ ⁺	9.27	14.21		132	223		

¹initial packed cell volume ratio

²number of embryos per 0.5 mL aliquot of suspension adjusted to 0.4 packed cell volume ratio, plated on BOi2Y

³protein content (μg μL⁻¹) of extracts made in a modified buffer of Wetter and Dyck (1983) (Section 4.2.3.1), 1 μL buffer per mg fresh weight callus

⁴protein content (μg μL⁻¹) of extracts made in buffer of Cheliak and Pitel (1984) (Table 4.2), 1 μL buffer per mg fresh weight callus

⁵glutamate dehydrogenase activity, m units mg⁻¹ protein

⁶glutamine synthetase activity, m units mg⁻¹ protein

⁷callus ammonium content, nmol mg⁻¹ protein

any time in the embryogenic genotype K29 in embryogenic B5mod medium (Table 4.9). Furthermore, the only other embryogenic genotype in that particular series, A70.34, gave rise to a few embryos only in the first subculture and none at all in the third and fourth subcultures. Similarly, six of the seven nitrogen-source experimental treatments showed a dramatic decline in number of embryos produced at successive subcultures (Table 4.10). The seventh treatment, from which a high number of embryos might have been expected because of the high level of ammonium in the medium, actually produced no embryos at all in any subculture.

The decline in embryogenic capacity could theoretically be due to a loss of vigour of the suspensions with increasing subculture number. However, the initial packed cell volume ratios do not indicate a decline in suspension growth rates (Tables 4.9, 4.10). Protein contents of extracts of the genotype-medium experiment suspensions changed, sometimes considerably, between subcultures but did not show a general decline with time (Table 4.9). Suspensions of the nitrogen source experiment did tend to show a decrease in protein content when extracted in Cheliak and Pitel's (1984) buffer, but not in Wetter and Dyck's (1983) buffer (Table 4.10). Since extractions in Cheliak and Pitel's (1984) buffer are more efficient, yielding more detectable protein, these protein contents are probably more accurate than those

determined in Wetter and Dyck's (1983) buffer. Correlation between number of embryos and protein contents was in fact statistically significant ($\alpha = 0.001$) among the nitrogen source suspensions extracted in Cheliak and Pitel's (1984) buffer, but not in the other buffer. It is possible that a loss of some component of suspension "vigour", reflected by decreasing protein content, is at least partially responsible for the observed decline in embryogenic capacity with successive subcultures. Another possibility is that some selection pressure is acting against embryogenic cells relative to non-embryogenic cells during the suspension culture.

The fact that so few embryos were produced by suspensions of the genotype-medium experiment casts serious doubts on the legitimacy of relating any of the ammonium metabolism parameters to embryogenesis. Any of the trends discussed in Section 4.3.1 are specifically relative to genotype and medium only. In inferring relationships to embryogenesis or induction of embryogenesis, the lack of embryos must be explained. It is possible that embryogenic genotypes in the embryogenic B5mod medium were in fact induced to form embryos but that embryo development was somehow blocked.

By contrast, direct comparisons between number of embryos developing from 0.5 mL aliquots and parameters of ammonium

metabolism can be made using the data from the nitrogen source experiment, the cultures of which were sufficiently embryogenic (Table 4.10). No significant correlations were found between number of embryos and either GDH activity ($\alpha = 0.447$), GS activity ($\alpha = 0.635$) or ammonium content ($\alpha = 0.937$).

While direct relationships with embryogenesis cannot be made, then, still it can be said that number of embryos, GDH activity and tissue ammonium levels tend to increase together as the level of ammonium in the medium increases, while GS activity tends to decrease (above a certain minimum level). Similarly, GDH activity and tissue ammonium levels tend to be higher in suspensions in the embryogenic B5mod medium than in the non-embryogenic B5PAA, while GS activity is somewhat higher and asparagine is much higher in B5PAA than in B5mod. No significant differences were seen between embryogenic and non-embryogenic genotypes, but there tended to be higher tissue ammonium levels in non-embryogenic than in embryogenic types.

An influence of exogenous ammonium on ammonium assimilation is thus likely, and possibly on embryogenesis as well, as is a definite effect of choice of auxin on ammonium assimilation and, simultaneously, on embryogenesis. A possible model which takes into account most of the

available information is represented in Fig. 4.11. It is reasonable to suppose that the primary assimilation of ammonium is affected by the choice of auxin, since auxin type affects the activity of GDH and, to a lesser extent, GS. The fate of the ammonium is clearly different in the two auxin treatments, with considerable accumulation of asparagine, a storage form of reduced nitrogen, in B5PAA relative to B5mod. It is proposed that, under the influence of 2,4-D, ammonium tends to be redirected away from the normal assimilatory route of the glutamine synthetase pathway (Fig. 4.11). In the 2,4-D-containing medium B5mod, GDH becomes significantly more active, probably assimilating ammonium into glutamate which does not accumulate but enters some other pathway. This other pathway need not necessarily be involved in embryogenesis but could be merely an unrelated consequence of 2,4-D treatment. The fact that ammonium tends to accumulate more in non-embryogenic than in embryogenic genotypes suggests that embryogenic capacity may be associated with increased ammonium consumption. Even in non-embryogenic genotypes, though, ammonium tends to accumulate in the presence of 2,4-D. Any redirection of ammonium must be a separate consequence of 2,4-D treatment independent of embryogenesis, whereas the rate of ammonium consumption is influenced both by embryogenesis and by independent factors.

Fig. 4.11 Proposed routes of ammonium assimilation under different growth regulator treatments

Such a scenario does not account for the observed, albeit inconsistent, tendency towards more faster-migrating GDH isozymes appearing in B5PAA suspensions than in B5mod cultures. A higher number of GDH bands in the non-embryogenic treatment does not appear to be consistent with higher GDH activity in the embryogenic treatment. However, it is important to remember that the gel staining procedure actually detects deaminating GDH activity, while the assay detects only aminating activity. It is conceivable that changing the auxin from 2,4-D to PAA causes a shift in isozymes towards an enzyme population less efficient at amination and more efficient at deamination.

The significant accumulation of asparagine as well as the tendency towards higher GS activity in the presence of PAA suggests that ammonium is effectively assimilated into glutamine through GS and then into asparagine. Synthesis of asparagine from glutamine is catalyzed by the enzyme asparagine synthetase (AS):

(Mifflin and Lea 1982). Significant activity of AS may imply that GS is not completely coupled with GOGAT activity. Assays for both AS and GOGAT activity would be very relevant to an investigation of the proposed model.

Another fact to be reconciled with this simple model is the slight tendency towards higher glutamate levels in non-embryogenic genotypes discussed in Section 4.3.1.4. This is perhaps the only characteristic found that might be attributed to embryogenic capacity independent of treatment. The non-significantly higher glutamate levels may be a result of the inability to form embryos or may be a cause of that inability, or may be due to random variation.

Glutamate is generated by either GDH or GOGAT. The simple model presented in Fig. 4.11 proposes that GDH becomes more active in embryogenic conditions and that the glutamate formed is consumed in some further process, possibly related to embryogenesis. Either glutamate consumption is greater or production is less in embryogenic compared to non-embryogenic lines. It is possible that capacity for glutamate utilization, by some unknown biochemical pathway(s), is a determinant of embryogenic capacity.

The nature and significance of the redirection of ammonium in the presence of 2,4-D remains open for speculation and further study. The relationship to embryogenesis, if any, needs to be clarified. The well-established fact of a role for reduced nitrogen in embryogenesis is confirmed in this work. However, there is still no evidence available to tackle the mystery of how changes in the assimilation of ammonium can be related to the triggering of a particular

developmental pathway. Incorporation of reduced nitrogen into amino acids and subsequently into proteins is a necessary process for any living plant tissue. Does induction of embryogenesis simply require even more protein and hence more reduced nitrogen? Or must the ammonium be incorporated, perhaps through GDH, into some regulatory molecule which then plays some crucial role in the induction process? The control of developmental processes in plants is still very poorly understood, but is imagined to be a series of inductions and/or repressions of structural genes or blocks of genes controlled spatially and temporally by regulatory DNA sequences, genes or gene products. Much new knowledge will be required to demonstrate if and how ammonium metabolism might fit into such a model.

Chapter 5. Summary and conclusions

As in other systems, somatic embryogenesis in alfalfa is dependent on genotype. Cultivars and accessions with high proportions of *M. falcata* and 'Ladak' germplasm showed relatively high degrees of embryogenic response, supporting the conclusions of Brown and Atanassov (1985). A wide range in degree of embryogenesis observed among several genotypes of 'Rangelander' indicates that embryogenesis is not determined simply but either exhibits additivity or is controlled by more than one genetic factor. One such factor may be the degree of callus proliferation.

Induction of embryogenesis in the 'Rangelander' genotype A70.34 required the presence of an auxin and was significantly enhanced by 4.7 μ M kinetin. The embryogenic response was also influenced by auxin type and concentration. The occurrence as well as the degree of embryogenesis, the time to the appearance of the first embryo, and the degree of callus formation were all affected. Of four synthetic auxins and four natural auxins tested, 2,4,5-trichlorophenoxyacetic acid induced the highest degree of embryogenesis. Only one of the eight auxins tested, phenylacetic acid or PAA, did not induce embryogenesis under any conditions. Embryogenic auxins were most effective at 10^{-6} to 10^{-4} M; none were inductive at 10^{-8}

M.

The effect of the stress of excision and placement in culture conditions must be considered among the factors influencing induction of embryogenesis. However, this system does not permit assessment of the effects of wounding, physical contact with the agar medium and exposure to high levels of some nutrients, particularly ammonium, on the embryogenic response.

A previously-developed protocol for somatic embryogenesis in alfalfa callus culture (Meijer and Brown 1987) was optimized. Callus from 1 cm petiole segments cultured only six days on a solid MS medium (Murashige and Skoog 1962) containing 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin were induced for embryogenesis. Embryo development proceeded most effectively when these were then transferred to a basal MS medium lacking growth regulators.

The timing of the actual induction event was further delineated by a series of systematic transfers between a non-inductive PAA-containing medium and the inducing 2,4-D-containing medium, during the six-day induction period. It was demonstrated that induction does not occur within the first three or four days of culture. However, events necessary for embryogenesis are occurring during this early

culture phase. This period is analogous to the competence phase prior to morphogenic induction discussed by Christianson and Warnick (1983) and others. Competence in 'Rangelander' alfalfa genotype A70.34 arises in the first three or four days of culture and does not absolutely require either an auxin nor kinetin. It is, however, acquired to a greater extent in the presence of growth regulators, and more in the presence of 2,4-D than PAA. It might be related to the initiation of callus formation, and/or to the isolation of the explant tissue from the donor plant.

Degree of embryogenesis is affected by the method and length of exposure to the inductive signal 2,4-D. Once the tissue is competent, embryogenesis can be induced by as little as 30 min exposure to 2,4-D. However, more embryos are produced by longer exposures, to an optimum of six to ten days. Also, more embryos are produced following a 6 h submersion of calli in liquid 2,4-D-containing medium than after 6 h on the same medium in solid form. Presumably embryogenesis is enhanced by an increased uptake of 2,4-D from the liquid medium.

Suspension cultures are theoretically better suited to biochemical studies than callus cultures because of their more homogeneous nature. Non-embryogenic control

suspensions were established from callus of non-embryogenic 'Rangelander' genotypes. Non-induced controls were also initiated using callus of embryogenic genotypes. Though this callus was cultured on 2,4-D-containing medium and thus potentially induced, the 2,4-D effect was lost within one or two subcultures.

Biochemical studies focused on changes in nitrogen metabolism associated with induction of embryogenesis, with embryogenic capacity and with the form of nitrogen supplied in the medium.

Activity of glutamate dehydrogenase (GDH) was shown to be significantly higher in 2,4-D-containing suspensor cultures than in PAA-containing cultures. There was also a tendency for one, two or three fast-migrating isozyme bands of GDH to appear only or more intensely in PAA relative to 2,4-D-treated cultures. Glutamine synthetase (GS) activities were somewhat higher in PAA than in 2,4-D-treated cultures. PAA-treated cultures showed a slight tendency towards higher total free amino acids than 2,4-D-treated cultures. Finally, ammonium accumulates more in suspensions cultured with 2,4-D than with PAA.

Considerable genotype-dependent variation in GDH isozyme pattern, consistent in both treatments, was observed among

the several genotypes examined. There was, however, no correlation of any bands with the embryogenic capacity of the genotypes. This emphasizes the danger of correlating isozyme pattern with genotype-specific traits (such as embryogenic capacity) based on patterns of only a few lines (Chibbar et al. 1988).

While a few of the measured parameters showed some effects due to genotype in general, only tissue ammonium levels were different when embryogenic genotypes were grouped and compared to non-embryogenic lines. Non-embryogenic genotypes showed a significantly greater sensitivity to auxin type, accumulating ammonium in the presence of 2,4-D more than did embryogenic types.

The literature demonstrates that the form in which nitrogen is supplied in the medium affects embryogenesis. Induction requires reduced nitrogen, either as ammonium or certain amino acids. Keeping the total nitrogen in the suspension medium a constant 31.7 mM, A70.34 callus was cultured in varying nitrogen sources ranging from all nitrate to 67% nitrate and 33% ammonium. The number of embryos produced increased as ammonium level increased. GDH activity and tissue ammonium content increased while GS activity fell with increasing ammonium in the medium. GDH isozyme banding pattern was unaffected by nitrogen source.

Since all these measurements were taken at the second, third and fourth subculture and since the number of embryos produced falls rapidly after the second subculture, the biochemical changes observed cannot be linked directly to embryogenesis but only to medium or to genotype.

A simple model is proposed to explain the observed effects of auxin on ammonium assimilation and possibly on embryogenesis. It is suggested that in normal proliferating, non-embryogenic suspension cultures supported by PAA, ammonium ion is incorporated through the GS assimilatory pathway into amino acids, with any surplus being stored in the transport form asparagine. In the presence of 2,4-D, however, ammonium might also be assimilated by another pathway involving GDH and, possibly, embryogenesis.

REFERENCES

- Albrecht C. 1985. Optimization of tissue culture media. Proc. Int. Plant Prop. Soc. 35:196-199.
- Ammirato PV. 1983. Embryogenesis. In: DA Evans, WR Sharp, PV Ammirato, Y Yamada, eds. Handbook of Plant Cell Culture, Vol. 1. Macmillan, New York.
- Ammirato PV. 1985. Patterns of development in culture. In: RR Henke, KW Hughes, MJ Constantin, A Hollaender, eds. Tissue Culture in Forestry and Agriculture, pp. 9-29. Plenum Press, New York and London.
- Ammirato PV. 1989. Recent progress in somatic embryogenesis. International Association for Plant Tissue Culture Newsletter No. 57, pp. 2-16.
- Audus LJ. 1972. Plant Growth Substances. Vol. 1 Chemistry and Physiology. Leonard Hill, London.
- Bhojwani SS, Razdan MK. 1983. Plant Tissue Culture: Theory and Practice. Developments in Crop Science (5). Elsevier, Amsterdam.
- Bianchi S, Flament P, Dattée. 1988. Embryogenèse somatique

et organogenèse *in vitro* chez la luzerne: évaluation des potentialités de divers génotypes. *Agronomie* 8(2):121-126.

Bielawski W, Kaczkowski J. 1984. Pathways of ammonia assimilation in rye seedlings at different concentrations of NH_4^+ . II. Kinetic studies. *Acta Physiol. Plant.* 6:159-169.

Bingham ET, Hurley LV, Kaatz DM and Saunders JW. 1975. Breeding alfalfa which regenerates from callus tissue in culture. *Crop Sci.* 15:719-721.

Bingham ET, McCoy TJ, Walker KA. 1988. Alfalfa tissue culture. In: AA Hanson, DK Barnes, RR Hill, eds. *Alfalfa and Alfalfa Improvement. Agronomy Monograph No. 29*, pp. 903-929. Amer. Soc. Agron., Crop Sci. Soc. Amer., Soil Sci. Soc. Amer., Madison.

Blaydes DF. 1966. Interaction of kinetin and various inhibitors in the growth of soybean tissue. *Physiol. Plant.* 19:748-753.

Brown DCW. 1988. Germplasm determination of *in vitro* somatic embryogenesis in alfalfa. *HortScience* 23(3):526-531.

Brown DCW, Atanassov A. 1985. Role of genetic background in somatic embryogenesis in *Medicago*. Plant Cell Tissue Organ Culture 4(2):111-122.

Brown DCW, Thorpe TA. 1986. Plant regeneration by organogenesis. In: IK Vasil, ed. Cell Culture and Somatic Cell Genetics of Plants, Vol. 3, pp. 49-65. Academic Press, Orlando.

Cammaerts D, Jacobs M. 1985. A study of the role of glutamate dehydrogenase in the nitrogen metabolism of *Arabidopsis thaliana*. Planta 163:517-526.

Carman JG. 1990. Embryogenic cells in plant tissue cultures: occurrence and behavior. In Vitro Cell. Dev. Biol. 26:746-753.

Chawla HS. 1989. Regeneration responses of callus from different explants and changes in isozymes during morphogenesis in wheat. Biologia Plantarum 31(2):121-125.

Cheliak WM, Pitel JA. 1984. Techniques for starch gel electrophoresis of enzymes from forest tree species. Information Report PI-X-42, Petawawa National Forestry Institute, Canadian Forestry Service.

Chen THH, Marowitch J, Thompson BG. 1987. Genotypic effects on somatic embryogenesis and plant regeneration from callus cultures of alfalfa. *Plant Cell Tissue Organ Culture* 8:73-81.

Chibbar RN, Shyluk J, Georges F, Mallard CS, Constabel F. 1988. Esterase isozymes as markers of somatic embryogenesis in cultured carrot cells. *J. Plant Physiol.* 133:367-370.

Chibbar RN, Polowick PL, Newsted WJ, Shyluk J, Georges F. 1989. Identification and isolation of a unique esterase from the medium of non-embryogenic cell line of cultured carrot cells. *Plant Cell Tissue Organ Culture* 18:47-53.

Choi JH, Sung ZR. 1989. Induction, commitment, and progression of plant embryogenesis. In: S-D Kung, CF Arntzen, eds. *Plant Biotechnology* 11:141-159. Butterworths, Stoneham.

Christianson ML, Warnick DA. 1983. Competence and determination in the process of *in vitro* shoot organogenesis. *Devel. Biol.* 95:288-293.

Christianson ML, Warnick DA. 1984. Phenocritical times in the process of *in vitro* shoot organogenesis. *Devel. Biol.* 101:382-390.

- Christianson ML, Warnick DA. 1985. Temporal requirement for phytohormone balance in the control of organogenesis in vitro. *Devel. Biol.* 112:494-497.
- Coleman GD, Ernst SG. 1990. Shoot induction competence and callus determination in *Populus deltoides*. *Plant Sci.* 71:83-92.
- Coppens L, Gillis E. 1987. Isoenzyme electrofocusing as a biochemical marker system of embryogenesis and organogenesis in callus tissues of *Hordeum vulgare* L. *J. Plant Physiol.* 127: 153-158.
- Davies DD, Teixeira AN. 1975. The synthesis of glutamate and the control of glutamate dehydrogenase in pea mitochondria. *Phytochem.* 14:647-656.
- Del Grosso E, Grazia S, Maraldi AC. 1987. Peroxidase activity in *Phaseolus vulgaris* seedling tissues and callus cultures: a comparison of genotypes and developmental stages. *Develop. Exper. Biol.* 27(4):387-391.
- de Vienne D. 1980. Relation entre la structure et les formes multiples de la glutamate déshydrogenase du pollen du Luzerne (*Medicago sativa* L.). *C R Acad. Sc. Paris* 291:885-888.

Donn G, Tischer E, Smith JA, Goodman HM. 1984. Herbicide-resistant alfalfa cells: an example of gene amplification in plants. *J. Molec. Appl. Genet.* 2:621-635.

Everett NP, Wach MJ, Ashworth DJ. 1985. Biochemical markers of embryogenesis in tissue cultures of the maize inbred B73. *Plant Sci.* 41:133-140.

Fransz PF, de Ruijter NCA, Schel JHN. 1989. Isozymes as biochemical and cytochemical markers in embryogenic callus cultures of maize (*Zea mays* L.). *Plant Cell Reports* 8:67-70.

Gamborg O, Miller R, Ojima K. 1968. Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.* 50:151-158.

Gawel NJ, Robacker CD. 1990. Genetic control of somatic embryogenesis in cotton petiole callus cultures. *Euphytica* 49:249-253.

George EF, Sherrington PD. 1984. *Plant Propagation by Tissue Culture.* Exegetics Ltd., Eversley.

Grimes HD, Hodges TK. 1990. The inorganic $\text{NO}_3^-:\text{NH}_4^+$ ratio influences plant regeneration and auxin sensitivity in

primary callus derived from immature embryos of indica rice (*Oryza sativa* L.). J. Plant Physiol. 136:362-367.

Halperin W. 1966. Alternative morphogenetic events in cell suspensions. Amer. J. Bot. 53:443-453.

Halperin W. 1967. Population density effects on embryogenesis in carrot cell cultures. Exp. Cell Res. 48:170-173.

Halperin W. 1973. The use of cultured tissue in studying developmental problems. Can. J. Bot. 51:1801-1806.

Hartmann T, Ehmke A. 1980. Role of mitochondrial glutamate dehydrogenase in the reassimilation of ammonia produced by glycine serine transformation. Planta 149:207-208.

Hartmann T, Nagel M, Ilert H-I. 1973. Organ-specific multiple forms of glutamic dehydrogenase in *Medicago sativa*. Planta 111:119-123.

Henshaw GG, O'Hara JF, Webb KJ. 1982. Morphogenetic studies in plant tissue cultures. In: MM Yeoman, DES Truman, eds. *Differentiation In Vitro*, pp. 231-251. Cambridge University Press, Cambridge.

Hernández-Fernández MM, Christie BR. 1989. Inheritance of somatic embryogenesis in alfalfa (*Medicago sativa* L.)

Genome 32:318-321.

Ishida Y, Hiyoshi T, Sano M, Kumashiro T. 1989. Selection and characterization of a herbicide-tolerant cell line of tobacco (*Nicotiana tabacum* L.). Plant Sci. 63:227-235.

Joy KW. 1988. Ammonia, glutamine and asparagine: a carbon-nitrogen interface. Can. J. Bot. 66:2103-2109.

Joersbo M, Andersen JM, Okkels FT, Rajagopal R. 1989. Isoperoxidases as markers of somatic embryogenesis in carrot cell suspension cultures. Physiol. Plant. 76:10-16.

Kamada H, Harada H. 1979. Studies on the organogenesis in carrot tissue cultures. II. Effects of amino acids and inorganic nitrogenous compounds on somatic embryogenesis. Z. Pflanzenphysiol. 96:135-141.

Keyes GT, Collins GB, Taylor NL. 1980. Genetic variation in tissue cultures of red clover. Theor. Appl. Genet. 58:265-271.

Klamt D. 1990. A view about the function of auxin-binding proteins at plasma membranes. Plant Molec. Biol.

14(6):1045-1050.

Kohlenbach HW. 1977. Basic aspects of differentiation and plant regeneration from cell and tissue cultures. In: W Barz, E Reinhard, MH Zenk, eds. Plant Tissue Culture and its Biotechnological Applications. Springer-Verlag, Berlin.

Kohlenbach HW. 1978. Comparative somatic embryogenesis In: TA Thorpe, ed. Frontiers of Plant Tissue Culture. University of Calgary, Calgary.

Komatsuda T, Ohyama K. 1988. Genotypes of high competence for somatic embryogenesis and plant regeneration in soybean *Glycine max*. Theor. Appl. Genet. 75:695-700.

Komatsuda T, Ko S-W. 1990. Screening of soybean [*Glycine max* (L.) Merrill] genotypes for somatic embryo production from immature embryo. Japan. J. Breed. 40:249-251.

Koorrneef M, Manhart C, Jongsma M, Toma I, Weide R, Zabel P, Hille J. 1986. Breeding of a tomato genotype readily accessible to genetic manipulation. Plant Sci. 45:201-208.

Kumar PA, Abrol YP. 1990. Ammonia assimilation in higher plants. In: YP Abrol, ed. Nitrogen in Higher Plants, pp. 159-179. John Wiley, New York.

Laurière C, Daussant J. 1983. Identification of the ammonium-dependent isoenzyme of glutamate dehydrogenase as the form induced by senescence or darkness stress in the first leaf of wheat. *Physiol. Plant.* 58:89-92.

Lee DW, Dougal DK. 1973. Electrophoretic variation in glutamate dehydrogenase and other isozymes in wild carrot cells cultured in the presence and absence of 2,4-dichlorophenoxyacetic acid. *In Vitro* 8(5):347-352.

Loyola-Vargas VM, de Jimenez ES. 1984. Differential role of glutamate dehydrogenase in nitrogen metabolism of maize tissues. *Plant Physiol.* 76:536-540.

Maestri E, Restivo FM, Gulli M, Tassi F. 1991. Glutamate dehydrogenase regulation in callus cultures of *Nicotiana plumbaginifolia*: effect of glucose feeding and carbon source starvation on the isoenzymatic pattern. *Plant Cell Envir.* 14:613-618.

Magalhães JR, Huber DM. 1989. Ammonium assimilation in different plant species as affected by nitrogen form and pH control in solution culture. *Fertilizer Research* 21:1-6.

Markert CL. 1985. Isozymes: conceptual history and biological significance. In: MC Rattazzi, JG Scandalios,

GS Whitt, eds. Isozymes. Current Topics in Biological and Medical Research. Vol. 7 Molecular Structure and Regulation, pp. 1-17. Alan R. Liss, New York.

Matheson SL, Nowak J, MacLean NL. 1990. Selection of regenerative genotypes from highly productive cultivars of alfalfa. *Euphytica* 45(2):105-112.

Meijer EGM, Brown DCW. 1987a. A novel system for rapid high frequency somatic embryogenesis in *Medicago sativa*. *Physiol. Plantarum* 69:591-596.

Meijer EGM, Brown DCW. 1987b. Role of exogenous reduced nitrogen and sucrose in rapid high frequency somatic embryogenesis in *Medicago sativa*. *Plant Cell Tissue Organ Culture* 10:11-19.

Meins J Jr. 1986. Determination and morphogenetic competence in plant tissue culture. In: MM Yeoman, ed. Botanical Monographs (Oxford) Vol. 23, Plant Cell Culture Technology. Blackwell Scientific Publications, Oxford.

Mifflin BJ, Lea PJ. 1982. Ammonia assimilation and amino acid metabolism. In: D Boulter, B Parthier, eds. Nucleic Acids and Proteins in Plants I, pp. 5-64. Springer-Verlag, Berlin.

Morris DA, Johnson CF. 1987. Regulation of auxin transport in pea (*Pisum sativum* L.) by phenylacetic acid: inhibition of polar auxin transport in intact plants and stem segments. *Planta* 172:408-416.

Murashige T, Huang L-C. 1985. Organogenesis in vitro: structural, physiological, and biochemical aspects. Proc. of the Inter-Center Seminar on International Agricultural Research Centers (IARCs) and Biotechnology 23-27 April 1984, pp. 227-239.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15:473-497.

Oaks A, Yamaya T. 1990. Nitrogen assimilation in leaves and roots - a role for glutamate dehydrogenase. In: YP Abrol, ed. *Nitrogen in Higher Plants*, pp. 181-194. John Wiley, New York.

Oelck MM, Schieder O. 1983. Genotypic differences in some legume species affecting the redifferentiation ability from callus to plants. *Z. Pflanzenzüchtung.* 91:312-321.

Pahlich E, Joy KW. 1971. Glutamate dehydrogenase from pea roots: purification and properties of the enzyme. *Can. J.*

Biochem. 49:127-138.

Parrot WA, Williams EG, Hildebrand DF, Collins GB. 1989. Effect of genotype on somatic embryogenesis from immature cotyledons of soybean. Plant Cell Tissue Organ Culture 11:111-123.

Punja ZK, Abbas N, Sarmiento GG, Tang FA. 1990. Regeneration of *Cucumis sativus* var. *sativus* and *C. sativus* var. *hardwickii*, *C. melo*, and *C. metuliferus* from explants through somatic embryogenesis and organogenesis. Plant Cell Tissue Organ Culture 21:93-102.

Reinert J. 1958. Morphogenese und ihre Kontrolle an Gewebekulturen aus Karotten. Naturwissenschaften 45:344-345.

Rhodes D, Rendon GA, Stewart GR. 1975. The control of glutamine synthetase level in *Lemna minor* L. Planta 125:201-211.

Saunders JW, Bingham ET. 1975. Growth regulator effects on bud initiation in callus cultures of *Medicago sativa*. Amer. J. Bot. 62(8):850-855.

Scandalios JG. 1983. Molecular varieties of isozymes and

their role in studies of gene regulation and expression during eukaryote development. In: MC Rattazzi, JG Scandalios, GS Whitt, eds. Isozymes. Current Topics in Biological and Medical Research. Vol. 9 Gene Expression and Development, pp. 1-31. Alan R. Liss, New York.

Seitzkris MH, Bingham ET. 1988. Interactions of highly regenerative genotypes of alfalfa (*Medicago sativa*) and tissue culture protocols. In Vitro Cell. Devel. Biol. 24(10):1047-1052.

Senaratna T, McKersie BD, Bowley SR. 1989. Desiccation tolerance of alfalfa (*Medicago sativa* L.) somatic embryos. Influence of abscisic acid, stress pretreatments and drying rates. Plant Sci. 65:253-259.

Sharp WR, Evans DA, Sondahl MR. 1982. Application of somatic embryogenesis to crop improvement. Proc. 5th Intl. Cong. Plant Tissue & Cell Culture, pp. 759-762.

Shelp BJ, Sieciechowicz K, Ireland RJ, Joy KW. 1985. Determination of urea and ammonia in leaf extracts: applications to ureide metabolism. Can. J. Bot. 63:1135-1140.

Sieciechowicz KA. 1986. Diurnal variation of asparaginase

in developing pea leaves. Ph.D. thesis, Carleton University.

Skokut TA, Manchester J, Schaefer J. 1985. Regeneration in alfalfa tissue culture. Stimulation of somatic embryo production by amino acids and N-15 NMR determination of nitrogen utilization. Plant Physiol. 79:579-583.

Smulders MJM, Visser EJW, Van der Kriecken WM, Croes AF, Wullems GJ. 1990. Effects of the developmental state of the tissue on the competence for flower bud regeneration in pedicel explants of tobacco. Plant Physiol. 92:582-586.

Sonnino A, Tanaka S, Iwanaga M, Schilde-Rentschler L. 1989. Genetic control of embryo formation in anther culture of diploid potatoes. Plant Cell Reports 8:105-107.

Srivastava HS, Singh RP. 1987. Role and regulation of L-glutamate dehydrogenase activity in higher plants. Phytochem. 26(3):597-610.

Steward FC, Mapes MO, Mears K. 1958. Growth and organized development of cultured cells. II. Organization in cultures grown from freely suspended cells. Amer. J. Bot. 45:705-708.

Steward FC, Mapes MO, Kent AE, Holsten RD. 1964. Growth and development of cultured plant cells. *Science* 143:20-27.

Stewart GR, Mann AF, Fentem PA. 1980. Enzymes of glutamate formation: glutamate dehydrogenase, glutamine synthetase and glutamate synthase. In: *The Biochemistry of Plants*, Vol. 5, pp. 271-293. Academic Press, New York.

Street HE. 1979. Embryogenesis and chemically-induced organogenesis. In: WR Sharp, PO Larsen, EF Paddock, V Raghavan, eds. *Plant Cell and Tissue Culture*. Ohio State University Press, Columbus.

Stuart DA, Strickland SG. 1984a. Somatic embryogenesis from cell cultures of *Medicago sativa* L. I. The role of amino acid additions to the regeneration medium. *Plant Sci. Lett.* 34:165-174.

Stuart DA, Strickland SG. 1984b. Somatic embryogenesis from cell cultures of *Medicago sativa* L. II. The interaction of amino acids with ammonium. *Plant Sci. Lett.* 34:175-181.

Suprasanna P, Rao KV, Reddy GM. 1989. Effect of 2,4-D analogues on callus cultures in maize. *Current Sci.* 58(1):35-37.

Szabados L, Hoyos R, Roca W. 1987. In vitro somatic embryogenesis and plant regeneration of cassava. *Plant Cell Reports* 6:248-251.

Takamizo T, Sugimoto K, Ohsuga R. 1991. Somatic embryogenesis in a recalcitrant cultivar of alfalfa (*Medicago sativa* L.) in an improved medium. *Bull. Natl. Grassl. Res. Inst.* 44:15-21.

Terzi M, LoSchiavo F. 1990. Somatic embryogenesis In: SS Bhojwani, ed. *Plant Tissue Culture: Applications and Limitations*, pp. 54-65. Elsevier, Amsterdam.

Thorpe TA. 1978. Physiological and biochemical aspects of organogenesis in vitro. In: TA Thorpe, ed. *Frontiers of Plant Tissue Culture*. University of Calgary, Calgary.

Thorpe TA. 1988. In vitro somatic embryogenesis. *ISI Atlas of Science (Animal and Plant Sciences)* 1:81-88.

Tisserat B. 1985. Embryogenesis, organogenesis and plant regeneration. In: RA Dixon, ed. *Plant Cell Culture: A Practical Approach*, pp. 79-105. IRL Press, Oxford.

Tkemaladze GS. 1981. *USSR Plant Physiol.* 28:1013.

Trolinder NL, Chen X. 1989. Genotype specificity of the somatic embryogenesis response in cotton. *Plant Cell Reports* 8:133-136.

Vasil IK, Vasil V. 1972. Totipotency and embryogenesis in plant cell and tissue cultures. *In Vitro* 8(3):117-125.

Walker KA, Yu PC, Sato SJ, Jaworski EG. 1978. The hormonal control of organ formation in callus of *Medicago sativa* L. cultured in vitro. *Amer. J. Bot.* 65(6):654-659.

Walker KA, Wendeln ML, Jaworski EG. 1979. Organogenesis in callus tissue of *Medicago sativa*. The temporal separation of induction processes from differentiation processes. *Plant Sci. Lett.* 16:23-30.

Walker KA, Sato SJ. 1981. Morphogenesis in callus tissue of *Medicago sativa*: the role of ammonium ion in somatic embryogenesis. *Plant Cell Tissue Organ Culture* 1:109-121.

Wareing PF, Phillips IDJ. 1978. The control of growth and differentiation in plants. Pergamon Press, Oxford.

Wenzel CL, Brown DCW. 1991. Histological events leading to somatic embryo formation in cultured petioles of alfalfa. *In Vitro Cell. Dev. Biol.* 27P:190-196.

Wetherell DF, Dougall DK. 1976. Sources of nitrogen supporting growth and embryogenesis in cultured carrot tissue. *Physiol. Plant.* 37:97-103.

Wetter L, Dyck J. 1983. Isoenzyme analysis of cultured cells and somatic hybrids. In: DA Evans, WR Sharp, PV Ammirato, Y Yamada, eds. *Handbook of Plant Tissue Culture*, Vol. 1, pp. 607-628. Macmillan, New York.

White PR. 1943. *A Handbook of Plant Tissue Culture*. Jaques Cattell Press, Tempe.

Wochok ZS, Burleson B. 1974. Isoperoxidase activity and induction in cultured tissues of wild carrot: a comparison of proembryos and embryos. *Physiol. Plant.* 31:73-75.

Yamaya T, Oaks A, Rhodes D, Matsumoto H. 1986. Synthesis of [^{15}N]glutamate from [^{15}N]H $_4^+$ and [^{15}N]glycine by mitochondria isolated from pea and corn shoots. *Plant Physiol.* 81:754-757.

Yamaya T, Oaks A. 1987. Synthesis of glutamate by mitochondria - an anaplerotic function for glutamate dehydrogenase. *Physiol. Plant.* 70:749-756.

3 of/de 3

PM-1 3½"x4" PHOTOGRAPHIC MICROCOPY TARGET
NBS 1010a ANSI/ISO #2 EQUIVALENT

PRECISION^{SM} RESOLUTION TARGETS

PIONEERS IN METHYLENE BLUE TESTING SINCE 1974

MICROD

1480 COUNTY ROAD 6, SUITE 100, MINNAPOLIS, MN 55425 USA
TEL: 612 480 7887 FAX: 612 480 7887 TUL: 8188888888

Appendix I. Recipes for discontinuous, non-denaturing electrophoresis gels and buffers

Lower tank buffer	63 mM Tris 50 mM HCl pH 7.47
Upper tank buffer	37.6 mM Tris 40 mM glycine pH 8.89
4X Separating gel buffer	947 mM Tris 0.289 N HCl pH 8.48
4X Stacking gel buffer	158 mM Tris 0.256 N H_2PO_4 pH 6.90
Monomer solution for separating gel	40%T, 5%C*
Monomer solution for stacking gel	6.25%T, 20%C*
*T=total acrylamides=acrylamide + bisacrylamide C=crosslinker=bisacrylamide/acrylamide	
Catalyst	0.06% ammonium persulphate 0.002% riboflavin phosphate
Sucrose-dye solution	50% sucrose 0.1% bromophenol blue
To make 2 full-size 6.25% separating gels:	
	6.25 mL separating gel monomer solution
	10.00 mL 4X separating gel buffer
	18.75 mL distilled water
	Degas about 10 min
	5.0 mL catalyst
	80 μ L TEMED
	Pour immediately
For stacking gels for 2 full-size gels:	
	5.0 mL stacking gel monomer solution
	2.5 mL 4X stacking gel buffer
	Degas about 10 min
	2.5 mL catalyst
	5 μ L TEMED
	Pour immediately

Appendix II. Recipes for experimental extraction buffers

Buffer of S. Warwick, Biosystematics Research Centre,
Agriculture Canada, Ottawa

0.1 M Trizma Base (Sigma)
1 mM EDTA
10 mM KCl
Adjust pH to 7.5 with HCl
0.4% Triton X
0.1% 2-mercaptoethanol (add just before using)

Buffer of Chibbar et al. 1988, J. Plant Physiol.
133:367-370

1 M sucrose
0.2 M Tris-HCl, pH 8.5
3 mM dithiothreitol

Buffer of Everett et al. 1985, Plant Sci. 41:133-140

16.7% (w/v) sucrose
8.3% (w.v) ascorbate, pH 7.4

Appendix III. Amino acid contents of various suspensions at various subcultures.

Genotype A70.34
Medium MS1
Subculture 2

	Picomoles mg ⁻¹ fresh wt	Proportion of total amino acids
asp	3.3	0.013
glu	4.8	0.019
asn	29.8	0.115
ser	8.4	0.033
gln	9.4	0.036
his	3.2	0.013
hser		
gly	3.3	0.013
thr	3.0	0.012
arg	0.7	0.003
ala	7.1	0.028
gaba	2.7	0.010
tyr	110.7	0.428
aaba	2.2	0.008
met		
val	47.4	0.183
phe	3.3	0.013
ile	3.6	0.014
leu	3.2	0.012
lys	12.4	0.048

Genotype A70.34
Medium MSPAA
Subculture 2

asp	4.0	0.007
glu	43.1	0.071
asn	344.8	0.568
ser	14.6	0.024
gln	23.0	0.038
his	18.9	0.031
hser		
gly	11.6	0.019
thr	7.2	0.012
arg	7.1	0.012
ala	58.1	0.096
gaba	12.2	0.020
tyr	2.2	0.004
met		
val	37.0	0.061
phe	3.0	0.005
ile	3.6	0.006
leu	3.5	0.006
lys	13.6	0.022

Appendix III. Continued.

Genotype K4
Medium MS1
Subculture 2

	Picomoles mg ⁻¹ fresh wt	Proportion of total amino acids
asp	11.3	0.014
glu	21.6	0.027
asn	379.2	0.479
ser	35.8	0.045
gln	24.6	0.031
his	26.1	0.033
hser	0.8	0.001
gly	17.3	0.022
thr	16.4	0.021
arg	8.6	0.011
ala	45.3	0.057
gaba	13.0	0.016
tyr	90.9	0.115
aaba	0.2	0.000
met		
val	53.2	0.067
phe	7.8	0.010
ile	12.4	0.016
leu	10.0	0.013
lys	17.0	0.021

Genotype K4
Medium MSPAA
Subculture 2

asp	5.2	0.001
glu	22.2	0.005
asn	1331.0	0.329
ser	980.8	0.242
gln	868.3	0.214
his		
hser		
gly	45.8	0.011
thr	45.6	0.011
arg	309.2	0.076
ala	214.2	0.053
gaba	87.6	0.022
tyr		
aaba	11.0	0.003
met		
val	66.0	0.016
phe	7.5	0.002
ile	12.3	0.003
leu	14.7	0.004
lys	29.9	0.007

Appendix III. Continued.

Genotype A70.34

Medium MS1

Subculture 2

	Picomoles mg ⁻¹ fresh wt	Proportion of total amino acids
asp		
glu	2.2	0.018
asn	1.4	0.011
ser	6.7	0.056
gln	3.5	0.029
his	3.8	0.032
hser	0.8	0.007
gly	6.4	0.054
thr	0.9	0.008
arg	5.1	0.043
ala	2.8	0.023
gaba	1.9	0.016
tyr	22.7	0.191
aaba	1.9	0.016
met		
val	44.9	0.377
phe	0.9	0.008
ile	4.1	0.034
leu	3.2	0.027
lys	6.1	0.051

Genotype A70.34

Medium MSPAA

Subculture 2

asp	11.6	0.008
glu	19.0	0.013
asn	1079.0	0.722
ser	29.0	0.019
gln	88.7	0.059
his		
hser		
gly	15.3	0.010
thr	24.0	0.016
arg	58.3	0.039
ala	53.6	0.036
gaba	32.6	0.022
tyr	10.3	0.007
aaba	2.6	0.002
met	0.3	0.000
val	37.9	0.025
phe	5.3	0.004
ile	4.8	0.003
leu	7.0	0.005
lys	14.8	0.010

Appendix III. Continued.

Genotype K4
 Medium MSPAA
 Subculture 2

	Picomoles mg ⁻¹ fresh wt	Proportion of total amino acids
asp	12.1	0.010
glu	24.5	0.019
asn	681.2	0.537
ser	49.7	0.039
gln	189.9	0.150
his		
hser	7.1	0.006
gly	20.4	0.016
thr	28.9	0.023
arg	22.2	0.018
ala	18.1	0.014
gaba	12.9	0.010
tyr	2.7	0.002
aaba	21.4	0.017
met	7.2	0.006
val	90.4	0.071
phe	26.5	0.021
ile	21.7	0.017
leu	11.0	0.009
lys	21.4	0.017

Genotype A70.34
 Medium B5mod
 Subculture 1

asp	5.6	0.007
glu	14.1	0.018
asn	31.3	0.041
ser	66.3	0.087
gln	10.9	0.014
his	14.7	0.019
hser	3.6	0.005
gly	19.0	0.025
thr	14.6	0.019
arg	37.8	0.049
ala	170.0	0.222
gaba	273.2	0.357
tyr	26.7	0.035
aaba	0.5	0.001
met		
val	26.1	0.034
phe	6.5	0.009
ile	7.1	0.009
leu	10.8	0.014
lys	26.3	0.034

Appendix III. Continued.

Genotype A70.34

Medium B5PAA

Subculture 1

	Picomoles mg ⁻¹ fresh wt	Proportion of total amino acids
asp	6.9	0.018
glu	10.8	0.028
asn	94.8	0.246
ser	60.8	0.158
gln	8.8	0.023
his	15.1	0.039
hser	2.8	0.007
gly	13.6	0.035
thr	11.8	0.031
arg	9.3	0.024
ala	28.0	0.073
gaba	48.8	0.127
tyr	6.6	0.017
aaba	7.2	0.019
met		
val	16.1	0.042
phe	7.4	0.019
ile	11.9	0.031
leu	8.9	0.023
lys	15.8	0.041

Genotype K4

Medium B5mod

Subculture 1

asp	11.1	0.085
glu	17.2	0.131
asn	10.2	0.078
ser	9.1	0.070
gln	5.5	0.042
his		
hser	0.6	0.005
gly	1.9	0.015
thr	4.3	0.033
arg	2.5	0.019
ala	12.3	0.094
gaba	15.8	0.121
tyr	16.5	0.126
aaba	4.3	0.033
met		
val	7.0	0.054
phe	2.6	0.020
ile	3.7	0.028
leu	2.4	0.018
lys	3.8	0.029

Appendix III. Continued.

Genotype K4
 Medium B5PAA
 Subculture 1

	Picomoles mg ⁻¹ fresh wt	Proportion of total amino acids
asp	12.2	0.024
glu	42.0	0.081
asn	128.8	0.248
ser	40.7	0.078
gln	6.7	0.013
his	18.5	0.036
hser	2.9	0.006
gly	9.4	0.018
thr	15.5	0.030
arg	13.1	0.025
ala	81.7	0.157
gaba	97.7	0.188
tyr	2.3	0.004
aaba	11.2	0.021
met		
val	14.1	0.027
phe	2.9	0.006
ile	5.9	0.011
leu	3.8	0.007
lys	10.3	0.020

Genotype A70.34
 Medium MS1
 Subculture 2

asp	2.7	0.018
glu	3.1	0.020
asn	6.7	0.044
ser	7.7	0.050
gln	11.2	0.074
his		
hser	0.3	0.002
gly	4.5	0.030
thr	3.1	0.021
arg	5.8	0.038
ala	16.2	0.107
gaba		
tyr	20.1	0.133
aaba		
met	0.6	0.004
val	49.5	0.326
phe	6.2	0.041
ile	0.6	0.004
leu	3.0	0.020
lys	10.7	0.070

Appendix III. Continued.

Genotype A70.34

Medium MSPAA

Subculture 3

	Picomoles mg ⁻¹ fresh wt	Proportion of total amino acids
asp	22.6	0.012
glu	10.8	0.006
asn	1136.2	0.623
ser	38.5	0.021
gln		
his	141.4	0.078
hser	5.3	0.003
gly	26.8	0.015
thr	31.4	0.017
arg	83.5	0.046
ala	65.1	0.036
gaba		
tyr	73.4	0.040
aaba	5.0	0.003
met		
val	25.5	0.014
phe	3.5	0.002
ile	39.3	0.022
leu	22.0	0.012
lys	92.0	0.050

Genotype K4

Medium MSPAA

Subculture 3

asp	5.5	0.004
glu	7.7	0.006
asn	831.3	0.684
ser	24.0	0.020
gln	132.1	0.109
his		
hser	4.7	0.004
gly	12.1	0.010
thr	17.6	0.014
arg	48.0	0.040
ala	20.2	0.017
gaba	35.1	0.029
tyr		
aaba	2.4	0.002
met		
val	50.8	0.042
phe	14.8	0.012
ile		
leu	5.7	0.005
lys	3.1	0.003

Appendix III. Continued.

Genotype A70.34

Medium B5mod

Subculture 3

	Picomoles mg ⁻¹ fresh wt	Proportion of total amino acids
asp	9.8	0.116
glu	7.3	0.086
asn	11.3	0.134
ser	4.7	0.056
gln	4.5	0.054
his		
hser	0.9	0.010
gly	4.2	0.050
thr	2.5	0.029
arg	2.3	0.027
ala	8.1	0.096
gaba	2.7	0.032
tyr		
aaba	1.8	0.022
met	0.6	0.008
val	12.6	0.150
phe	7.0	0.083
ile		
leu	2.5	0.030
lys	1.6	0.019

Genotype A70.34

Medium B5PAA

Subculture 3

asp	4.8	0.027
glu	7.7	0.043
asn	32.6	0.181
ser	31.9	0.177
gln	9.3	0.051
his		
hser	1.3	0.007
gly	4.6	0.026
thr	8.2	0.046
arg	4.1	0.023
ala	35.8	0.199
gaba		
tyr	20.9	0.116
aaba	1.1	0.006
met		
val	2.3	0.013
phe	4.8	0.027
ile	0.8	0.005
leu	3.0	0.017
lys	6.7	0.037

Appendix III. Continued.

Genotype K4
 Medium B5mod
 Subculture 2

	Picomoles mg ⁻¹ fresh wt	Proportion of total amino acids
asp	3.0	0.022
glu	15.4	0.111
asn	2.1	0.015
ser	13.9	0.100
gln	3.5	0.025
his		
hser	0.5	0.003
gly	3.2	0.023
thr	3.8	0.028
arg	1.8	0.013
ala	30.5	0.220
gaba	14.0	0.295
tyr		
aaba	0.6	0.004
met		
val	7.3	0.053
phe	3.4	0.024
ile	1.8	0.013
leu	4.0	0.029
lys	3.1	0.022

Genotype K4
 Medium B5PAA
 Subculture 2

asp	0.5	0.010
glu	0.8	0.015
asn	0.6	0.012
ser	2.3	0.044
gln	0.7	0.014
his	29.0	0.556
hser		
gly	1.5	0.028
thr	0.4	0.008
arg	1.9	0.037
ala	1.4	0.027
gaba	2.8	0.053
tyr	0.6	0.012
aaba	2.3	0.045
met	0.4	0.008
val	1.9	0.036
phe	0.8	0.015
ile	0.6	0.011
leu	1.8	0.035
lys	1.8	0.035

Appendix III. Continued.

Genotype K21
 Medium B5mod
 Subculture 2

	Picomoles mg ⁻¹ fresh wt	Proportion of total amino acids
asp	7.9	0.060
glu	13.6	0.103
asn	3.3	0.025
ser	7.3	0.055
gln		
his	24.8	0.188
hser	1.4	0.011
gly	5.2	0.039
thr	4.9	0.037
arg	1.1	0.009
ala	4.3	0.033
gaba	4.0	0.030
tyr	1.2	0.009
aaba		
met		
val	18.5	0.140
phe	23.5	0.178
ile	3.8	0.029
leu	3.8	0.029
lys	3.4	0.026

Genotype K21
 Medium B5PAA
 Subculture 2

asp	10.0	0.040
glu	45.1	0.182
asn	22.9	0.092
ser	17.3	0.069
gln		
his	24.8	0.100
hser	0.9	0.004
gly	16.6	0.067
thr	8.0	0.032
arg	7.9	0.032
ala	15.8	0.064
gaba	9.4	0.038
tyr	2.6	0.010
aaba	1.3	0.005
met		
val	23.3	0.094
phe	17.4	0.070
ile	6.6	0.027
leu	8.9	0.036
lys	9.6	0.039

Appendix III. Continued.

Genotype K22
Medium B5mod
Subculture 2

	Picomoles mg ⁻¹ fresh wt	Proportion of total amino acids
asp	36.4	0.297
glu	12.0	0.098
asn	1.1	0.009
ser	1.9	0.015
gln		
his	8.5	0.069
hser	0.6	0.005
gly	2.1	0.017
thr	2.6	0.021
arg	4.1	0.033
ala	1.0	0.008
gaba	5.0	0.040
tyr	2.2	0.018
aaba	1.6	0.013
met		
val	20.0	0.163
phe	10.1	0.082
ile	4.3	0.035
leu	4.2	0.034
lys	5.3	0.043

Genotype K22
Medium B5PAA
Subculture 2

asp	11.1	0.074
glu	16.2	0.108
asn	29.4	0.196
ser	6.0	0.040
gln		
his	17.3	0.116
hser	0.6	0.004
gly	6.0	0.040
thr	3.7	0.024
arg	3.6	0.024
ala	6.7	0.045
gaba	3.2	0.021
tyr	2.7	0.018
aaba	1.0	0.007
met	6.8	0.046
val	9.0	0.060
phe	17.2	0.115
ile	3.8	0.025
leu	2.6	0.018
lys	2.6	0.017

Appendix III. Continued.

Genotype K25
 Medium B5mod
 Subculture 2

	Picomoles mg ⁻¹ fresh wt	Proportion of total amino acids
asp	13.5	0.086
glu	20.0	0.128
asn	9.8	0.063
ser	8.1	0.052
gln		
his	8.5	0.054
hser	0.4	0.002
gly	8.1	0.052
thr	4.1	0.026
arg	3.9	0.025
ala	14.4	0.092
gaba	13.7	0.088
tyr	1.7	0.011
aaba		
met		
val	18.5	0.118
phe	13.5	0.086
ile	5.1	0.033
leu	5.9	0.038
lys	7.4	0.047

Genotype K25
 Medium B5PAA
 Subculture 2

asp	17.9	0.066
glu	33.1	0.123
asn	19.7	0.073
ser	12.5	0.047
gln		
his	16.9	0.063
hser	3.3	0.012
gly	12.5	0.047
thr	7.4	0.027
arg	5.0	0.018
ala	23.3	0.086
gaba	19.7	0.073
tyr	2.5	0.009
aaba	3.0	0.011
met		
val	38.5	0.143
phe	25.4	0.094
ile	8.7	0.032
leu	9.1	0.034
lys	10.6	0.039

Appendix III. Continued.

Genotype K29
 Medium B5mod
 Subculture 2

	Picomoles mg ⁻¹ fresh wt	Proportion of total amino acids
asp	17.4	0.068
glu	15.3	0.100
asn	5.7	0.038
ser	10.3	0.068
gln	0.7	0.005
his	14.4	0.095
hser	0.5	0.003
gly	8.4	0.055
thr	5.5	0.036
arg	4.6	0.030
ala	9.4	0.061
gaba	10.9	0.072
tyr	2.6	0.017
aaba	1.0	0.007
met		
val	18.4	0.121
phe	19.1	0.125
ile	4.4	0.029
leu	4.6	0.030
lys	6.3	0.041

Genotype K29
 Medium B5PAA
 Subculture 2

asp	8.0	0.065
glu	11.6	0.095
asn	7.7	0.063
ser	8.4	0.069
gln	1.3	0.010
his	10.7	0.087
hser	4.0	0.033
gly	6.4	0.052
thr	5.2	0.043
arg	4.2	0.034
ala	7.0	0.057
gaba	6.3	0.051
tyr	1.6	0.013
aaba	1.1	0.009
met		
val	12.2	0.100
phe	13.4	0.109
ile	3.7	0.030
leu	4.1	0.033
lys	5.6	0.045

Appendix III. Continued.

Genotype K30
Medium B5mod
Subculture 2

	Picomoles mg ⁻¹ fresh wt	Proportion of total amino acids
asp	14.8	0.023
glu	34.9	0.054
asn	12.4	0.019
ser	24.4	0.038
gln		
his	28.9	0.045
hser	3.6	0.006
gly	20.4	0.032
thr	15.6	0.024
arg	16.4	0.026
ala	9.4	0.015
gaba	324.9	0.506
tyr	19.3	0.030
aaba	0.9	0.001
met	12.5	0.019
val	23.1	0.036
phe	20.6	0.032
ile	10.8	0.017
leu	17.4	0.027
lys	31.9	0.050

Genotype K30
Medium B5PAA
Subculture 2

asp	51.1	0.043
glu	89.1	0.076
asn	128.9	0.110
ser	22.0	0.019
gln		
his	58.3	0.050
hser	4.7	0.004
gly	14.7	0.012
thr	16.1	0.014
arg	24.5	0.021
ala	24.0	0.020
gaba	37.2	0.032
tyr	5.3	0.005
aaba	1.4	0.001
met	131.1	0.111
val	144.3	0.123
phe	196.8	0.167
ile	60.1	0.051
leu	166.1	0.141
lys		

Appendix III. Continued.

Genotype K34
 Medium B5mod
 Subculture 2

	Picomoles mg ⁻¹ fresh wt	Proportion of total amino acids
asp	7.4	0.026
glu	13.7	0.048
asn	16.3	0.058
ser	18.5	0.065
gln	22.0	0.078
his		
hser	0.8	0.003
gly	7.3	0.026
thr	6.0	0.021
arg	6.3	0.022
ala	43.1	0.152
gaba	114.4	0.404
tyr		
aaba	3.9	0.014
met	1.0	0.004
val	10.8	0.038
phe	2.8	0.010
ile		
leu	2.7	0.010
lys	6.3	0.022

Genotype K34
 Medium B5PAA
 Subculture 2

asp	10.4	0.012
glu	20.7	0.024
asn	15.4	0.018
ser	66.5	0.076
gln	40.2	0.046
his		
hser	2.9	0.003
gly	19.2	0.022
thr	20.2	0.023
arg	15.1	0.017
ala	166.9	0.192
gaba	447.3	0.514
tyr		
aaba	1.6	0.002
met	7.7	0.009
val	21.4	0.025
phe	2.6	0.003
ile		
leu	10.6	0.012
lys	0.9	0.001

Appendix III. Continued.

Genotype K36
 Medium B5mod
 Subculture 2

	Picomoles mg ⁻¹ fresh wt	Proportion of total amino acids
asp	12.3	0.061
glu	25.6	0.128
asn	12.3	0.062
ser	15.2	0.076
gln	0.7	0.004
his	12.5	0.063
hser	6.6	0.033
gly	16.5	0.082
thr	7.8	0.039
arg	5.5	0.027
ala	18.8	0.094
gaba	5.9	0.030
tyr	3.1	0.015
aaba	1.0	0.005
met	0.3	0.002
val	28.2	0.141
phe	19.0	0.095
ile		
leu	7.9	0.039
lys	0.8	0.004

Genotype K36
 Medium B5PAA
 Subculture 2

asp	9.3	0.112
glu	6.9	0.083
asn	18.6	0.224
ser	4.1	0.050
gln		
his	6.9	0.083
hser	1.0	0.012
gly	2.9	0.036
thr	1.8	0.022
arg	3.8	0.046
ala	4.9	0.059
gaba		
tyr	1.1	0.013
aaba	0.9	0.011
met		
val	10.8	0.131
phe	8.0	0.060
ile		
leu	2.4	0.029
lys	2.3	0.028

Appendix III. Continued.

Genotype K39
 Medium B5mod
 Subculture 2

	Picomoles mg ⁻¹ fresh wt	Proportion of total amino acids
asp	3.3	0.032
glu	16.3	0.159
asn	2.0	0.019
ser	4.7	0.046
gln	1.8	0.018
his	6.9	0.068
hser	1.0	0.010
gly	4.8	0.047
thr		
arg	2.5	0.025
ala	30.9	0.303
gaba		
tyr	18.6	0.183
aaba	1.2	0.012
met	0.7	0.007
val	3.5	0.035
phe		
ile	0.6	0.006
leu	0.5	0.005
lys	2.5	0.025

Genotype K39
 Medium B5PAA
 Subculture 2

asp	10.2	0.054
glu	38.2	0.203
asn	31.6	0.168
ser	9.4	0.050
gln		
his	20.9	0.111
hser	2.3	0.012
gly	9.6	0.051
thr	3.7	0.020
arg	5.7	0.031
ala	12.2	0.065
gaba		
tyr	2.5	0.013
aaba	2.8	0.015
met	0.6	0.003
val	23.3	0.124
phe	10.8	0.058
ile		
leu	2.6	0.014
lys	1.5	0.008

Appendix III. Continued.

Genotype A70.34
 Medium B5mod
 Subculture 3

	Picomoles mg ⁻¹ fresh wt	Proportion of total amino acids
asp	5.9	0.282
glu		
asn	5.8	0.277
ser	3.2	0.152
gln		
his		
hser		
gly	2.9	0.136
thr		
arg		
ala	2.6	0.122
gaba		
tyr		
aaba		
met		
val		
phe		
ile		
leu	0.6	0.030
lys		

Genotype A70.34
 Medium B5PAA
 Subculture 3

asp	1.5	0.188
glu		
asn	4.8	0.605
ser	0.6	0.078
gln		
his		
hser		
gly	0.5	0.063
thr		
arg		
ala		
gaba		
tyr		
aaba		
met		
val		
phe		
ile		
leu	0.5	0.067
lys		

Appendix III. Continued.

Genotype K4
 Medium B5mod
 Subculture 3

	Picomoles mg ⁻¹ fresh wt	Proportion of total amino acids
asp	11.8	0.087
glu	27.9	0.205
asn	6.7	0.049
ser	15.4	0.113
gln	0.7	0.005
his	13.4	0.099
hser		
gly	6.1	0.045
thr	3.9	0.029
arg	0.7	0.005
ala	30.2	0.222
gaba		
tyr	6.0	0.044
aaba	2.1	0.016
met		
val		
phe	5.3	0.039
ile	1.0	0.008
leu	2.7	0.020
lys	2.3	0.017

Genotype K4
 Medium B5PAA
 Subculture 3

asp		
glu		
asn		
ser		
gln		
his	8.6	0.955
hser		
gly	0.4	0.045
thr		
arg		
ala		
gaba		
tyr		
aaba		
met		
val		
phe		
ile		
leu		
lys		

Appendix IV. Petiole-derived calli of alfalfa, cultured as per Chapter 2, Sections 2.2 and 2.3

a. Day 2
b. Day 9
c. Day 14

d. Day 18
e. Day 23
f. Day 30

Appendix V. Development of alfalfa somatic embryos via suspension culture, as per Chapter 3, Section 3.1

- a. Sterile *in vitro*-propagated shoots
- b. Petiole segments on Day 0
- c. Calli after 21 d culture on B5h

- d. Embryogenic suspension
- e. Embryogenic suspension
- f. Embryo and callus formation following about 10 d culture on BOi2Y

Appendix VI. GDH activity-stained gel

Lanes represent (from left to right):

Genotype	Medium
K4	B5mod
K4	B5PAA
K10	B5mod
K10	B5PAA
K21	B5mod
K21	B5PAA
K28	B5mod
K28	B5PAA
A70.34	B5mod

END

26 05 93

FIN