ESTABLISHMENT AND CHARACTERIZATION OF EMBRYOGENIC SUSPENSION CULTURES OF WHEAT (*Triticum aestivum* L.)

By

JOHN PAUL FELLERS

Bachelor of Science

Oklahoma State University

Stillwater, Oklahoma

1989

Submitted to the Faculty of the Graduate College of the
Oklahoma State University in partial fulfillment
of the requirements for the
Degree of
MASTER OF SCIENCE
May, 1992

ESTABLISHMENT AND CHARACTERIZATION OF

EMBRYOGENIC SUSPENSION CULTURES

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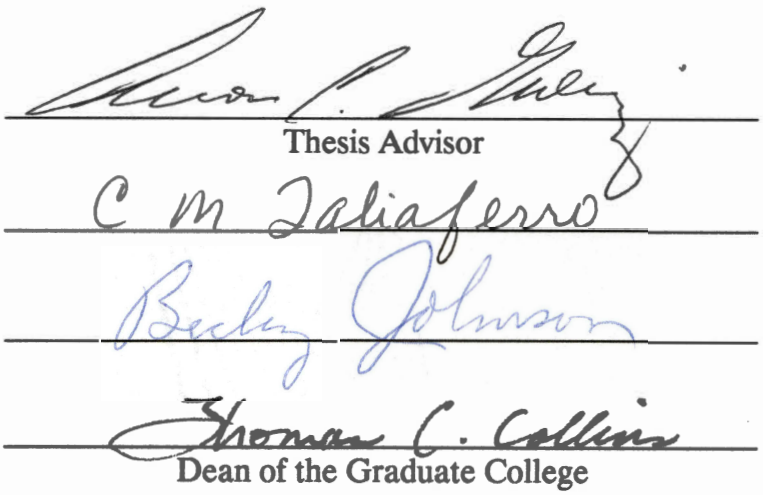
Thesis Approved:



Thesis Advisor

Dean of the Graduate College

ACKNOWLEDGMENTS

I would first like to thank all the people that have contributed their time, effort, and encouragement during my time at Oklahoma State University. I cannot thank enough Dr. Arron Guenzi for the numerous opportunities he has given me throughout the years. Thank you for your leadership, inspiration, and valuable help that you have given me as a major professor, and as a friend. I would also like to thank the other members of my committee, Dr. Charles M. Taliaferro and Dr. Becky Johnson for their insight and suggestions. A special thanks goes to Dr. David R. Porter for his expertise and guidance.

I would like to dedicate this thesis to my parents, Kenneth and Sue Fellers, to my lovely wife, Courtney, and to my sister Karen. Their constant support, love, encouragement, and patience have made this all worthwhile. Thanks for letting me follow my dreams.

Finally I would like to thank my friends and fellow graduate students that have made this time enjoyable and memorable. They are Troy W., Dave H., Jackie D, George S., Roger W., Greg J., Mihdzar K., and Mark G.

TABLE OF CONTENTS

Chapter Page

INTRODUCTION 1

I. FACTORS AFFECTING SOMATIC EMBRYOGENESIS IN CEREALS: LITERATURE REVIEW 2

Introduction 3
Literature Review 4
Explant 4
2,4-D Concentrations 5
Basal Salts 6
Sucrose 6
L-Proline 7
Suspension Cultures 7
References 10
Tables 14

II. ESTABLISHMENT AND MAINTENANCE OF EMBRYOGENIC SUSPENSION CULTURES OF WHEAT (*Triticum aestivum* L. em. Thell.) 16

Abstract 18
Introduction 20
Materials and Methods 21
Plant Material 21
Callus Initiation and Maintenance 21
Experiment I 21
Experiment II 22
Experiment III 22
Light and Scanning Electron Microscopy 22
Suspension Cultures 23
Initiation and Maintenance 23

Chapter Page

Characterization of Cell Suspension Growth 24
Regeneration of Plants from Callus Derived from Suspension Cultures 24
Results 25
Callus Initiation and Maintenance 25
Experiment I 25
Experiment II 25
Experiment III 26
Light and Scanning Electron Microscopy 26
Suspension Culture Characterization 26
Regeneration of Plants and Suspension 26
Discussion 28
References 32
Tables 36
Figures 41

III. PROTEINS ASSOCIATED WITH SOMATIC EMBRYOGENESIS OF WHEAT CALLUS 48

Abstract 50
Introduction 51
Materials and Methods 53
Callus Culture 53
In Vivo Labeling of Proteins 53
Protein Isolation, Electrophoresis, and Analysis 53
Results 55
Discussion 57
References 59
Table 62
Figures 63

LIST OF TABLES

Table Page

CHAPTER I

1. Comparison of Media Components Used for Wheat (*Triticum aestivum* L.) Suspension Cultures 14

2. Comparison of Suspension Culture Media Components Used for Monocotyledon Plants Similar to Wheat 15

CHAPTER II

1. Treatment Combinations for the Evaluation of 2,4-D, Proline, and Sucrose on Three Cultivars of Wheat (Experiment I) 36

2. Treatment Combinations for the Evaluation of Basal Salts, Sucrose, and Proline on Callus Growth of Bobwhite (Experiment II) 36

3. Treatment Combinations of Sucrose and 2,4-D on the establishment of Embryogenic Bobwhite Calli (Experiment III) 37

4. Media Used to Establish Embryogenic Suspension Cultures 37

5. Plating Schemes for Regeneration of Plants from Suspension Cells 38

6. Significance Levels from Analysis of Variance for Main Effects of Cultivar, 2,4-D Concentration, Proline Concentration and Their Interactions on Callus Relative Growth Rates and Percent Embryogenic Calli 39

7. Significance Levels from Analysis of Variance for Main Effects of Basal Salts, Sucrose Concentration, L-Proline Concentration and Their Interactions on Relative Growth Rates of Bobwhite Calli 39

Table Page

8. Significance Levels from Analysis of Variance for Main Effects of Sucrose, 2,4-D Concentrations, and Their Interactions on Relative Growth Rates of Bobwhite Callus..... 40

9. Significance Levels from Analysis of Variance for Main Effects of Sucrose, 2,4-D Concentrations, and Their Interactions on Percent Embryogenic Calli of Bobwhite..... 40

CHAPTER III

1. Unique Proteins Synthesized in E-callus from Two or More Treatments.. 62

LIST OF FIGURES

Figure Page

CHAPTER II

1. Calli Relative Growth Rates as Influenced by Cultivar, Proline Concentration, and 2,4-D Concentration ($LSD_{(0.05)}$ = .004) 41

2. Percent Embryogenic Calli as Influenced by Cultivar, Proline Concentration, and 2,4-D Concentration ($LSD_{(0.05)}$ = 6.1) 41

3. Influence of Basal Salts, Sucrose, and Proline on Relative Growth of Bobwhite Calli ($LSD_{(0.05)}$ = .012) 42

4. Influence of Basal Salts, Sucrose, and Proline on Shoot Regeneration of Bobwhite Calli 42

5. Sucrose and 2,4-D Effects on Relative Growth of Bobwhite Callus ($LSD_{(0.05)}$ = .009) 43

6. Sucrose and 2,4-D Effects on Percent Embryogenic Calli of Bobwhite ($LSD_{(0.05)}$ = 13.3) 43

7. Thin Section of Embryogenic Bobwhite Callus (Bar = 50 um) 44

8. Scanning Electron Micrographs of Bobwhite Embryogenic Tissue Magnified 33X and 30X, Respectively 45

Figure Page

9. Relative Fresh Weight of Embryogenic Suspension Cultures. (A - 9.0MS58P (Treatment 1 and 2); B - 9.0MS58 (Treatment 3 and 4); C - 9.0MS87 (Treatment 5 and 6); D - 5.6MS58 (Treatment 7) and ND7532 (Treatment 8)) 46

10. A) Embryogenic Callus Derived from Suspension Cultures Magnified 10 Times B) Somatic Embryogenesis and Organogenesis of Callus Derive from Suspension Culture. C) Albino and D) Green Plant Regenerated from Callus Derived from Suspension Cultures. 47

CHAPTER III

1. Fluorograph of ^{35}[S]-labeled Proteins of 105 d-old E-callus Growing on 9 uM 2,4-D. Arrows ( ) Indicated E-proteins that were Unique Across Two or More Treatments 63

2. Aligned and Overlaid Protein Spot Maps of *in vivo*-labeled Proteins of 105 d old Callus Growing on 9.0 uM 2,4-D. Dark Spots (71 total) are Proteins Present in E-callus and not in NE-callus. Numbered E-proteins are Present in Two or More Treatments 64

3. Aligned and Overlaid Protein Spot Maps of *in vivo*-labeled Proteins of 105 d old Callus Growing on 5.6 uM 2,4-D. Dark Spots (33 total) are Proteins Present in E-callus and not in NE-callus. Numbered E-proteins are Present in Two or More Treatments 65

4. Aligned and Overlaid Protein Spot Maps of *in vivo*-labeled Proteins of 271 d old Callus Growing on 9.0 uM 2,4-D. Dark Spots (43 total) are Proteins Present in E-callus and not in NE-callus. Numbered E-proteins are Present in Two or More Treatments 66

5. Aligned and Overlaid Protein Spot Maps of *in vivo*-labeled Proteins of 271 d old Callus Growing on 5.6 uM 2,4-D. Dark Spots (39 total) are Proteins Present in E-callus and not in NE-callus. Numbered E-proteins are Present in Two or More Treatments 67

INTRODUCTION

Each chapter in this thesis conforms to the Publications Handbook and Style Manual of the American Society of Agronomy. Chapters will be submitted for publication in Crop Science, a Crop Science Society of America publication.

CHAPTER I

FACTORS AFFECTING SOMATIC EMBRYOGENESIS IN CEREALS: A LITERATURE REVIEW

INTRODUCTION

Winter wheat is Oklahoma's most important crop commodity. It ranks second in value only to the cattle industry, which in itself relies heavily on wheat for winter pasture. With exports of wheat accounting for more than one-half of the state's cash receipts, the importance of wheat and its improvement becomes obvious. Plant tissue culture offers a novel approach for the application of biotechnology to wheat improvement.

Plant transformation, caused by the insertion of recombinant DNA into the plant genome, is one of the primary goals of plant biotechnology. Tissue culture is an important component of the application of this technique. Regeneration of plants from cell and tissue culture is essential for the recovery of transgenic plants. Since wheat is resistant to infection by *Agrobacterium tumefaciens*, direct DNA uptake by protoplasts or direct DNA uptake via microprojectiles by intact cells are the systems currently being explored to introduce recombinant DNA into the wheat genome.

Suspension cultures are ideal sources of protoplasts (Vasil and Vasil, 1982; Vasil et al., 1990). The cells grow in small clusters, and thus provide optimal release of protoplasts upon enzyme digestion. They are also ideal cell types for microprojectile-based transformation systems. The limitation with current suspension culture and protoplast methodologies is unreliable plant regeneration. Regenerable suspension culture systems have been established for monocotyledon species such as *Zea maize* (Kamo et al., 1987; Rhodes et al., 1988), *Oryza sativa* L. (Yamada et al., 1986; Ghosh-Biswas and Zapata, 1990) and *Pennisetum americanum* (Vasil and Vasil, 1982). Regeneration of wheat has been reported (Harris et al., 1988; Maddock, 1987; Redway et al., 1990b; Vasil et al., 1990, Wang and Nguyen, 1990; Yang et al., 1991; Chang et al., 1991), however, these techniques were either non-reproducible or the regenerated plants were sterile.

LITERATURE REVIEW

Suspension cultures have been established in several monocot species; rice (Yamada et al., 1986; Ghosh-Biswas and Zapata, 1990; Abe and Futsuhara, 1991), maize (Kamo et al., 1987; Felker and Goodwin, 1988; Kamo and Hodges, 1986), pearl millet *(Pennisetum americanum)* (Vasil and Vasil, 1982), *Panicum maximum* Jacq. and *Pennisetum purpureum* (Karlson and Vasil, 1986), barley (*Hordeum vulgare* L.) (Lührs and Lörz, 1986; Jähne et al., 1991), red fescue (*Fescuta rubra*) (Zaghmout and Torello, 1989), and wheat (Harris et al., 1988; Inagaki et al., 1988, Vasil et al., 1990; Redway et al., 1990b; Wang and Nguyen, 1990; Chang et al., 1991; Yang et al., 1991). There are several steps that must be accomplished before regenerable suspensions can be achieved. First, a highly regenerable source of callus tissue is required to initiate the suspensions. Second, suspensions must be maintained for extended periods of time and still preserve their plant regeneration capacity.

Vasil and Vasil (1986) stated that several factors play a critical role in the control of regeneration in tissue cultures of cereal and other grass species. They are the physiological state and developmental stage of the explant, concentration of plant growth regulators, growing conditions of the donor plants, nutrient medium, subculture interval, and genotype. They also stated that the most important factors are the explant source and the concentration of 2,4-D. Tables 1 and 2 show various nutrient mediums, 2,4-D concentrations and explants that have been used in suspension culture systems.

Explant

Tissue cultures start with an explant source. The immature embryo is the most successful one for wheat. In 1982, Ozias-Akins and Vasil found evidence of somatic embryogenesis in callus derived from immature embryos. They reported that callus arose from the scutellum of immature embryos on MS medium with 2,4-D concentrations of 4.5 to 36 uM and that 9.0 uM of 2,4-D proved to be the

optimum level. Sears and Deckard (1982) concluded that plant regeneration from tissue cultures derived from immature embryos was predictable and stable when a responsive genotype is used. Redway et al. (1990a) found that immature embryos 1.0 to 1.5 mm long were the most suitable for embryogenic callus formation while anthers responded poorly and inflorescences gave intermediate values.

2,4-D Concentration

The growth regulator, 2,4-dichlorophenoxyacetic acid (2,4-D), is the most variable media component in tissue culture of cereals. 2,4-D is a synthetic auxin that is used in dicotyledon weed control. Monocotyledon plants have the ability to withstand high concentrations. When used in tissue culture of cereals, it is used to control and manipulate differentiation of callus tissue. Sears and Deckard (1982) found that transfer of totipotent calli to media void of 2,4-D prior to adequate shoot initiation often resulted in rapid initiation of root development. Ozias-Akins and Vasil (1982) found that 2,4-D must be in high concentrations to prevent germination of the immature embryo. Zamora and Scott (1983) found 90 to 100% of the leaf segments used as explants produced calli on medium that contained 10 to 40 uM 2,4-D in the culture medium. Galiba and Erdai (1986) found that calli growth was more rapid on a medium containing 2.25 uM 2,4-D than on one with 9.0 uM. However, it was noted that there was a higher frequency of differentiation on the 2.25 uMM medium.

Basal Salts

Basal salts have been found to be an important factor in tissue culture. Some of the formulations developed for plant tissue culture are B5 (Gamborg et al, 1968), MS (Murashige and Skoog, 1962), LS (Linsmair and Skoog, 1965) and N6 (Chu et al., 1975). In the early research of wheat it was found that MS-based medium, that was worked out for tobacco (*Nicotiana tobacum)*, proved to be the most successful

in producing callus. It has been used in many callus and suspension culture systems (Harris et al, 1988; Morris et al., 1986; Vasil et al., 1990). Kamo and Hodges (1986) found that growth of callus was better on N6 rather than MS for maize. They also found that suspension cells would initially grow better in a N6 medium, but after several months there was no significant difference between the two. Rhodes et al. (1988) found that N6 basal medium, supplemented with asparagine and proline, was more suitable for the growth of maize protoplasts than the MS medium. Redway et al. (1990a) found that wheat callus formation on MS medium was significantly higher than on other media tested.

Sucrose

Sucrose is the most commonly used sugar in cereal tissue culture media. Sucrose concentrations range from 58 mM to 87 mM (Tables 1 and 2). Bregitzer et al. (1989) found that immature oat embryos plated on a 175 mM MS media, with no hormones, produced somatic embryos that germinated to form plantlets when transferred to a 58 mM sucrose MS-based medium. They also found that non-friable callus, when transferred to a 175 mM sucrose medium would induce the somatic embryos to mature, and these embryos would later germinate when transferred to the 58 mM sucrose medium. Galiba and Erdai (1986) reported that the maximum number of wheat calli developing shoot primordia were attained at 58 mM sucrose. They concluded that this concentration was the optimum for growth and shoot formation. Brown et al. (1989) concluded that additional sucrose, added to the basal medium at low concentrations, stimulated shoot regeneration at low concentrations. They also reported that higher concentrations were inhibitory to growth and regeneration.

Not only can the sugar in the medium be used as a carbon source, but also as an osmoticum. Zhou et al. (1991) found that increased medium osmolarities

significantly increased the percentage of green plants regenerated from anther-derived callus.

L-Proline

L-proline is another common additive to basal medium. In the plant, L-proline is a storage compound for reduced nitrogen and is important in osmotic adjustment. In 1985, Armstrong and Green reported the importance of L-proline in tissue culture of maize. They compared MS and N6 basal media, and the addition of L-proline to them. They found the formation of somatic embryoids and friable callus initiation increased linearly with the addition of up to 25 mM L-proline to N6 medium. Proline additions of up to 9 mM to MS medium did not stimulate embryoid formation. They concluded that this difference could be attributed to the nitrogen source difference in the basal medium. Büter et al. (1991) found that the addition of L-proline in combination to cold treatments, increased embryogenic callus formation 6 to 10 times in maize anther culture.

Suspension Cultures

Suspension culture is another method of growing plant cells in a defined medium. Reliable systems have been established for tobacco and carrot. However, cereals have been very recalcitrant. Embryogenic callus tissue is difficult to recover from suspension cultures. Vasil and Vasil (1986) defined true embryogenic suspension cultures of grasses as being finely dispersed, free of any callus pieces, organized tissues, or meristems and meristemoids, and being comprised mostly of groups of small, richly cytoplasmic and starch-containing meristematic cells. Tables 1 and 2 show the different media, explants, and species that have been used in suspension culture of grasses. Vasil and Vasil (1982) reported that an embryogenic suspension culture was established from cultured inflorescence segments of pearl millet. These cultures were grown for 2 to 3 wk, at 27^{o} C, rotated at 150 rpm in a shaker, and refreshed at 3 to 4 d intervals with 10 to 20 ml of fresh media.

Zaghmout and Torello (1989) reported that reliable regeneration of whole plants had been established in red fescue. The cultures were initiated from embryogenic callus, placed in a modified MS medium, grown in a 250 ml flask, and rotated at 100 rpm at approximately 26^{o} C.

In wheat, there have been several reports of callus formation and plant regeneration from protoplasts (Harris et al., 1988; Vasil et al., 1990;). These protoplasts were derived from suspension cultures which were established from embryogenic callus cultures derived from immature embryos and anthers. In these suspension cultures, the 2,4-D concentration ranged from 4.5 to 22.5 uM, and sucrose ranged from 58 to 87 mM. There are few reports of plants directly regenerated from suspension culture cells. Redway et al. (1990a) reported the establishment of stable, embryogenic wheat suspension cultures. Compact, nodular and embryogenic callus, 5 to 8 months after callus initiation, was used to establish suspensions. One gram fresh weight of callus was placed in 125 ml flasks containing 15 ml of liquid MS medium with 9 uM 2,4-D. Nine to eleven day old cultures of six month old suspensions were plated and plants regenerated. The calli formed were much like the calli used to established the cultures, and were embryogenic. Redway et al. concluded that a MS medium with 87 mM sucrose and 9.0 uM 2,4-D was most suitable and the addition of IAA and zeatin to the regeneration medium proved plant regeneration.

Wang and Nguyen (1990) were able to regenerate plants from long-term cell cultures of wheat. Suspensions cells were grown in low concentrations of 2,4-D, thus root competent cells could be isolated away from those with shoot regeneration potential. Suspensions were maintained for 2.5 years and still maintained regeneration potential. By using this type of selection strategy, they were able to obtain regeneration frequencies of 80%. Yang et al. (1991) used a similar strategy. However, they initiated their suspensions with primary embryogenic scutellum callus

not from aged callus. By using low concentrations of 2,4-D (1 uM), embryogenic clusters, that did not differentiate, were selected and used to continue the suspension. Cell clusters were continuously selected and placed on differentiation medium. They concluded the optimum 2,4-D concentration for long term retention of plant regeneration was 5 uM.

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Table 1. Comparison of media components used for wheat (*Triticum aestivum* L.) suspension cultures.

Citation	Basal Medium	2,4-D	Callus Explant
		-uM-	
Chen et al., 1985	B5[1]	4.5	mesocotyl
Yamada et al., 1986	LS[2]	10	embryos
Morris et al., 1986	MS[3]	11.5	embryos
Kumpaisal et al., 1987	LS	10	embryos
Maddock, 1987	MS	11.5 and 22.6	embryos and inflorescence
Harris et al., 1988	MS	5	anthers
Inagaki et al., 1988	B5	9.0	haploid embryos
Wang and Nguyen, 1989	MS	4.5	embryos
Redway et al., 1990b	MS	9.0	embryos
Wang and Nguyen, 1990	MS	0.45 and 2.3	embryos
Chang et al., 1991	MS	0.45	embryos
Yang, et al., 1991	MS	1 and 5	embryos

1 - B5 (Gamborg et al., 1968)
2 - LS (Linsmair and Skoog, 1965)
3 - MS (Murashige and Skoog, 1962)

Table 2. Comparison of suspension culture media components used for monocotyledon plants similar to wheat.

Author	Basal Medium	2,4-D	Species
		-uM-	
Vasil and Vasil, 1982	MS[1] and N6[2]	11.3	pearl millet
Kamo and Hodges, 1986	MS and N6	4.5 - 9	maize
Wang et al., 1986	MS	3.4	maize
Yamada et al., 1986	LS[3]	20	rice
Kamo et al., 1987	N6	4.5 - 18	maize
Zaghmout et al., 1989	1/2 MS	22.6	red fescue
Abe and Futsuhara, 1991	MS and N6	6.8 - 11.3	rice

1 - MS (Murashige and Skoog, 1962)
2 - N6 (Chu et al., 1975)
3 - LS (Linsmair and Skoog, 1965)

CHAPTER II

ESTABLISHMENT AND CHARACTERIZATION OF EMBRYOGENIC SUSPENSION CULTURES OF WHEAT (*Triticum aestivum* L.)

Establishment and Characterization of Embryogenic Suspension Cultures of Wheat (*Triticum aestivum* L)

John P. Fellers, Arron C. Guenzi*, and Charles M. Taliaferro

J.P. Fellers, A.C. Guenzi*, and C.M. Taliaferro, Dep. of Agronomy, Oklahoma State Univ., Stillwater, OK 74078.
Contribution of the Oklahoma Agric. Exp. Stn., Journal No._____.
Received _____, 1992. * Corresponding author

ABSTRACT

Improved suspension cell culture systems are needed to facilitate the application of recombinant DNA technology for wheat germplasm enhancement. Suspension cells grow as small clusters and are ideal targets for transformation by microprojectiles. The limitation with current suspension culture methods is the lack of reliable plant regeneration. The objective of this research was to develop embryogenic suspension cultures of wheat with plant regeneration capability. This study evaluated three wheat cultivars, 'Bobwhite', 'Mit', and 'Pavon 66', and the effects of medium basal salts, 2,4-D, sucrose, and L-proline concentrations on establishment of rapidly growing and highly embryogenic callus and suspension cultures. In the first experiment, the three cultivars were evaluated on a Murashige and Skoog (1962) (MS) basal medium. The medium components evaluated were 5.6 or 9.0 uM 2,4-D and 0 or 12 mM L-proline. In the second experiment, Bobwhite was evaluated on different basal media, MS and N6 (Chu et al., 1975), 58 or 87 mM sucrose, and 0 or 12 mM L-proline. The third experiment evaluated the response of Bobwhite to 58 or 87 mM sucrose and 5.6 or 9.0 uM 2,4-D. Percent embryogenic calli was visually estimated and verified with light and scanning electron microscopy. The most highly embryogenic callus was produced by Bobwhite on medium with MS basal salts, 58 mM sucrose, 5.6 uM 2,4-D and 0 proline. The suspension culture methodology producing the most regenerated plants utilized callus tissue produced on solid medium with MS basal salts, 87 mM sucrose, 9.0 uM 2,4-D, and 0 proline. Suspension cells were grown in similar medium without the gelling agent and subsequently plated on solid medium with MS basal salts, 58 mM sucrose, and 2.3 uM 2,4-D. Four green and 13 albino plantlets were regenerated from suspension derived calli. The suspension culture system requires further optimization, however,

the highly embryogenic callus tissue produced by the protocol may be utilized as target tissue for transformation with microprojectiles.

Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; IAA- Indole-3-acetic acid; NAA - α-Napthaleneacetic acid. RG - Relative growth; %EC - Percent embryogenic calli.

INTRODUCTION

Suspension cultures, because of their small cell cluster size provide ideal cell types for protoplast and microprojectile-based transformation systems. The limitation with suspension culture and protoplast methods is the lack of reliable plant regeneration. Regenerable protoplast and suspension systems have been reported in *Zea maize* L., (Rhodes et al., 1988; Kamo and Hodges, 1986; Kamo et al., 1987; Mórocz et al., 1990), *Hordeum vulgare* L. (Jähne et al., 1991, Lührs and Lörz 1986), *Oryza sativa* L. (Yamada, et al., 1986; Ghosh-Biswas and Zapata, 1990; Abdulla et al., 1986) and *Triticum aestivum* L. (Maddock, 1987; Harris et al., 1988; Inagaki et al., 1988; Chang et al., 1991; Vasil et al., 1990; Redway, et al., 1990b; Yang et al., 1991). All have had varying degrees of success. In wheat, however, none of the regenerated plants have been fertile.

There are two steps that must be accomplished before regeneration from cell suspensions can be achieved. First, a highly regenerable source of callus tissue is required to initiate the suspension. Second, suspensions must preserve their plant regeneration capacity for extended periods of time. Vasil and Vasil (1986) stated that several factors play a critical role in the control of regeneration in tissue cultures of cereal and other grass species. They are the physiological state and developmental stage of the explant, concentration of 2,4-D, growing conditions of the donor plants, nutrient medium including amount of sucrose, subculture interval, and genotype. They also stated the most important factors are the explant source and the 2,4-D concentration.

The objectives of this research were to establish highly embryogenic and rapidly growing callus and suspension cultures. This involved the evaluation of cultivars, basal salts, sucrose, L-proline, and 2,4-D concentrations.

MATERIALS AND METHODS

Plant Material

Three genotypes of hard red wheat, *Triticum aestivum* L. cv. Bobwhite, Mit, and Pavon 66, were used, depending upon the experiment. Bobwhite (Heering et al., 1991), Pavon 66 (Zhou et al., 1991), and Mit (Wang and Nguyen, 1990) have all shown good response in tissue culture. Caryopses were removed from wheat spikes 10 to 14 days post anthesis. Surface sterilization was performed by sequentially washing the seed in: 1) 70% (v/v) ethanol for 5 min, 2) 20% (v/v) clorox with 60 uL of Triton X-100 for 15 minutes, and 3) 2 rinses in sterile RO-deionized water. Immature embryos were asepticly removed and plated with the shoot-root axis in contact with the solid medium (Sears and Deckard, 1982).

Callus Initiation and Maintenance

Experiment I. The effects of 2,4-D and L-proline were compared in a 2 x 2 factorial arrangement with five replicates on the three cultivars mentioned above. The 2,4-D concentrations were 5.6 uM and 9.0 uM. L-proline concentrations were 0 and 12 mM (Table 1). Basal salts were a modified Murashige and Skoog (1962) (MS) (Sears and Deckard, 1982). Other ingredients that were held constant were 2.9 uM thiamine-HCl, 1.7 mM L-asparagine, 0.55 mM myo-inositol, 4.1 uM of nicotinic acid, and 2.25 g L^{-1} Phytagel (Sigma Chemical Co., St. Louis, MO). Media were autoclaved at 121° C, 18 psi, for 15 min. Approximately 50 ml of medium was poured into 100 x 25 mm sterile petri plates. Twenty-five embryos (5 per plate) from each cultivar were plated for each treatment. A medium, described by Redway et al. (1990a) (RV) that contained 9.0 uM of 2,4-D and 87 mM sucrose, was used as a comparison. Calli were grown in the dark at 21° C and transferred every 3 to 4 weeks to fresh medium. Relative growth rates (RG) were determined by the following formula as described by Singer and McDaniel (1986).

$$\text{Relative Growth (RG)} = (\ln FW_2 - \ln FW_1)/(t_2 - t_1);$$

where FW_1 = Initial fresh weight; FW_2 = Final fresh weight; t_1 = day initial weight taken; t_2 = day final weight taken. Visual estimations of percent embryogenic calli (%EC) were made at the end of transfer period five.

Experiment II. A 2^3 factorial experimental design was used to compare the effects of basal salts, sucrose, and L-proline on the establishment of rapidly growing and highly embryogenic calli. All treatments were replicated 10 times. The basal salts were an MS, as described above, and N6 (Chu et al., 1974). Two concentrations of sucrose were evaluated, 58 and 87 mM, and also 0 and 12 mM L-proline (Table 2). Additional ingredients which were constant were listed previously. Concentrations of 2,4-D and asparagine were 5.6 uM and 5.3 mM, respectively. Media was raised to pH 5.85 and autoclaved for 15 min, at 18 psi and 121° C. One hundred 'Bobwhite' embryos, approximately 1.0 mm in length were plated (15 per plate) on each treatment and grown in the light at 21° C. Calli were transferred to fresh medium every 3 to 4 wk. At the end of transfer period six, 10 of the best calli from each treatment were transferred to 1.1 uM 2,4-D for shoot regeneration.

Experiment III. 'Bobwhite' was used to test the effects of 2,4-D and sucrose on RG and %EC. The experimental design was a 2 x 2 factorial with 10 replicates. Concentrations of 2,4-D were 5.6 and 9.0 uM and concentrations of sucrose were 58 and 87 mM (Table 3). Constants were 2.9 uM thiamine-HCl, 1.7 mM L-asparagine and L-glutamine, 0.55 mM myo-inositol , 4.1 uM of nicotinic acid and 100 mg L^{-1} casein hydrolysate. Forty embryos (4 per plate) were plated per treatment and placed in the dark at 21° C. Fresh weights were determined and %EC estimated at the end of each transfer period. Relative growth rates were calculated as described previously.

Light and Scanning Electron Microscopy

Tissues visually classified as embryogenic were fixed in formaldehyde-acetic acid-alcohol (FAA), dehydrated in a tertiary-butyl alcohol series, and embedded in

paraplast. Sections 10-12 um thick were stained with the fast green and safranin series.

Tissue for scanning electron microscopy was fixed for 2 h in 1.6% (v/v) gluteraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. After two, 20 minute buffer washes, the tissue was left in a third buffer wash overnight. The next day the tissue was fixed in 1% (v/v) OsO_4 in 0.1 M sodium cacodylate buffer for 2 h and then dehydrated in a graded ethanol series to 100% ethanol. The tissue was then critical point dried, mounted on aluminum stubs, and coated with 200 Å Au/Pd.

Suspension Cultures

Initiation and Maintenance. Embryogenic callus from experiment I was used to establish suspension cultures. Two different media were used for the suspensions. Both contained 9.0 uM of 2,4-D, 1.7 mM of L-asparagine and L-glutamine, 0.55 mM myo-inositol, 100 mg L^{-1} of casein hydrolysate, and 0.9 uM NAA (Redway et al., 1990b). Treatments consisted of 58 mM and 87 mM sucrose. One gram of Bobwhite callus, from different solid media (Table 4) were placed in 30 ml of liquid contained in a 125 ml DeLong flask. Cultures were grown in a rotary incubator at 150 rpm, in the dark, and at 21° C. Suspensions were refreshed every 5 to 7 days by removing 10 ml of old media and replacing it with a like amount.

Two months after initiation, flask contents were poured onto a 1 mm mesh sieve. Cell clusters were forced through the sieve, washed with 20 ml of fresh media, and collected in a sterile 50 ml centrifuge tube. The tube was then centrifuged at 500 *x* g, 30 ml of the supernatant was placed in a new 125 ml flask, and the remainder discarded. One gram of the cell pellet was placed in the new flask containing the conditioned medium. Suspensions were then subcultured every two weeks by removing 10 ml of suspended cells and placing it in 20 ml of fresh media. Cultures were refreshed as described above every other week.

Characterization of Cell Suspension Growth. Two 1.5 ml samples of suspended cells were pipetted into pre-weighed 1.5 ml microcentrifuge tubes. The tubes were centrifuged in a swinging bucket rotor at 1000 *x* g. The supernatant was removed and fresh weights determined. Tubes were then dried for 24 h at 80^{o} C. Tubes were allowed to cool to room temperature and dry weights determined. A rapidly growing, non-embryogenic wheat cell line, ND7532 (Gatschet et al. in preparation), was used as a comparison in the growth determinations.

Regeneration of Plants from Callus Derived from Suspension Cells

Once a month, 1 ml of suspended cells were plated on solid media for regeneration. In all cases, the medium contained MS basal salts, 58 mM sucrose, 0.55 mM thiamine-HCl, 1.7 uM L-asparagine, 1.7 uM L-glutamine, 0.55 mM myo-inositol, 100 mg L^{-1} casein hydrolysate, and 4.1 uM of nicotinic acid. Regeneration methods are described in Table 5. Cultures were evaluated for embryogenic and organogenic potential.

Regenerated shoots were moved to medium with no 2,4-D, 4.6 uM zeatin, and 5.7 uM IAA for 2 to 3 wk for further development. Plantlets were then transferred to a rooting medium with half the concentration of MS-basal salts and without plant growth regulators.

RESULTS

Callus Initiation and Maintenance

Experiment I. Cultivar and proline main effects and their interaction were highly significant ($p<0.01$) for %EC (Table 6). When comparing relative growth rates, Mit had similar response over all treatments to that of Bobwhite (Fig. 1). However, Bobwhite had the highest %EC for all treatments combinations (Fig. 2). Adding proline in the medium decreased the %EC in Mit and Pavon 66 but did not significantly affect Bobwhite calli.

For Pavon 66, as 2,4-D concentration increased and when proline was added, the amount of %EC decreased significantly. When proline was added to the lower concentration of 2,4-D, the percent embryogenic calli would decrease without affecting relative growth (Fig. 2). The addition of proline increased the percentage of embryogenic tissue in Bobwhite cultures with 9 uM 2,4-D.

The optimum medium for Bobwhite was 5.6 uM 2,4-D with no proline. For Mit, the optimum growth was attained with 9.0 uM 2,4-D and no proline. Although Pavon 66 had a high %EC at 5.6 uM 2,4-D and 0 proline, it did not have growth rates as great as Mit or Bobwhite.

Experiment II. Immature embryos initiated callus at a frequency ≥ 95% for all treatments. MS basal salts produced an overall greater RG rate than the N6 (Fig. 3). The 58 mM concentration sucrose was optimum for calli growth in the presence of either basal media. However, the addition of proline to MS basal salts and 87 mM sucrose seemed to enhance growth later in the experiment. As the 2,4-D concentration was reduced to induce plant regeneration, MS basal salts with 58 mM sucrose proved to be optimum for plantlet regeneration (Fig. 4). The addition of proline inhibited shoot regeneration.

Experiment III. There was a highly significant main effect of 2,4-D on %EC and RG (Tables 7 and 8). There was also a significant interaction of sucrose and auxin level despite no significant main effect of sucrose. This experiment showed that the optimum medium for obtaining rapidly growing calli and calli with the highest %EC was 5.6 uM 2,4-D and 58 mM sucrose (Fig. 5 and 6)

Light and Scanning Electron Microscopy

Embryogenic structures in thin sections stained dark red indicating dense cells and rapid cell division (Fig. 7). The structures were identified as somatic embryos in various stages of development. Structures similar to those found by Ozias-Akins and Vasil (1982, 1983) were found in the tissue selected as embryogenic and scanned by the electron microscope (Fig. 8).

Suspension Culture Characterization

Seven months after initiation suspensions were doubling their relative fresh and dry weights within 4 to 6 days (Fig. 9). Treatment 1 doubled in relative fresh weight in 8 days but did not reach the density of the other treatments (Figure 9A). Treatment 4 had erratic growth and did not reach a peak cell density during the experiment (Figure 9B). Most of the remaining treatments grew linearly between day 3 and 8, and reached a peak cell density 18 d after initiation. All treatment combination responses, except that of number 1 were equivalent to that of the control.

Regeneration of Plants from Suspension Cells

Four green and 13 albino plantlets (Fig. 10) were regenerated from suspension derived calli. The green plantlets were derived from cells that were initiated on MS based 87 mM sucrose medium, transferred to liquid MS based 87 mM sucrose medium, and subsequently plated on solid medium containing 58 mM sucrose. One green plant was regenerated from method 1,3,4, and 5 (Table 5). Five of the albino plantlets were regenerated from method 1, three from method 2, and

the remainder from method 3 through 5. Ten, whether albino or green, were derived from the scheme of initiating the calli on 9.0 uM 2,4-D and 87 mM sucrose and placed in liquid media containing the same sucrose and 2,4-D concentrations. Seven albino plants were from calli initiated on 9.0 uM 2,4-D and 58 mM sucrose and also transferred to liquid medium containing the same sucrose and 2,4-D concentrations. Green plants formed roots and were transferred to soil. These plants are currently growing in the greenhouse.

DISCUSSION

Response in tissue culture is highly genotype dependent. Sears and Deckard (1982) reported that the wheat cultivars 'ND7532' and 'Roughrider', were the only genotypes out of 39 evaluated that maintained tissue culture response after 420 d. Bapat et al. (1988) found 20 of 33 genotypes cultured showed response in tissue culture. Of these, only five had %EC greater that 70%. Of the three cultivars we evaluated, Bobwhite clearly performed the best regardless of media composition (Fig. 1; Fig. 2). Although medium could be optimized for Mit and Pavon, even the optimized medium for these cultivars resulted in significantly lower growth rates and percentages of embryogenic tissue when compared to Bobwhite. Bobwhite had excellent relative growth rates and production of embryogenic calli across all treatments. Because of Bobwhite's superior performance in Experiment I, subsequent experiments were designed to further optimize callus and suspension culture media for this genotype.

Proline is produced in response to stress and is a storage compound for reduced nitrogen (Büter et al., 1991). The addition of L-proline did not enhance the production of embryogenic calli. Our results indicate that proline reduced the formation of embryogenic tissue. These results are opposite of those observed by Armstrong and Green (1985) in maize. They found that the addition of up to 25 mM L-proline increased the formation of somatic embryos in maize. The value of proline to enhance somatic embryogenesis may be species and genotype dependent. For maximum relative growth rates and percentage of embryogenic callus of Bobwhite, proline was not required. However, the addition of proline did significantly inhibit of shoot development from Bobwhite callus (Fig. 4).

N6 basal salts significantly inhibited both relative growth rates and shoot regeneration of Bobwhite callus (Fig. 4; Fig. 5). Our results are in contrast to those

obtained for maize callus (Kamo and Hodges, 1986; Armstrong and Green, 1985) and wheat protoplasts (Gou et al., 1991) where N6 enhances the recovery of embryogenic callus.

The influence of sucrose as an independent effect was not significant (Tables 6, 7 and 8). However, there were significant interactions in relative growth and embryogenic potential with both proline (Table 7) and 2,4-D (Table 8). Relative growth rates were greatest when sucrose was at 58 mM and 2,4-D was at 5.6 uM. This is in agreement with Galiba and Erdei (1986). Bobwhite callus grew well when sucrose was at 87 mM and proline was added (Fig. 3) indicating that the increased osmotic stress by higher sucrose concentrations (Brown et al., 1989) can be nullified by the addition of proline. However, this did not increase the regeneration of shoots.

Our results indicate that the optimum 2,4-D concentration for production of embryogenic calli that was rapidly growing, was 5.6 uM (Fig. 1, 2, 5, and 6). Results of Redway et al. (1990) are in contrast to ours, however. They found that 9 uM 2,4-D had the highest formation of embryogenic calli. They also reported that calli growing on 87 mM sucrose and 9 uM 2,4-D was optimum for establishing suspension cultures with regeneration potential.

Accumulation of fresh weight by cell suspensions was influenced by the interaction of sucrose concentrations of the callus and suspension media. Rapidly growing suspension cultures were established when callus was transferred to suspension culture medium with a sucrose concentration the same as the callus medium (Fig. 9). Sucrose concentration of the callus medium also significantly affected the regeneration of green plantlets from suspension cell. This is consistent with results of Zhou et al. (1991).

Sucrose concentration of the cell suspension medium influenced plant regeneration. Ninety-five percent of the plants regenerated from suspension

culture-derived callus grown in 58 mM sucrose were albino. Forty-five percent of the albino plants were regenerated from callus derived from suspension cells grown in 87 mM sucrose. Yang et al. (1991) and Redway et al. (1990) reported successful plant regeneration from suspension cells. Their sucrose concentration of 87 mM produced results that are in agreement with ours.

Plants were regenerated from plating methods 1 through 5, all of which contained 58 mM sucrose (Redway et al., 1990b). The greatest number of plants were recovered from methods 3,4, and 5. The gradual reduction of 2,4-D is similar to the method described by Sears and Deckard (1982). However, the amount of time involved in callus induction and proliferation, suspension initiation, and then plant regeneration from suspension cells, greatly reduces the potential of plant regeneration. Reducing the 2,4-D level from suspension cultures to plating medium allows for rapid organogenesis of the suspension derived cells (Wang and Nguyen, 1990). Redway et al. (1990b) plated suspension cultures on various 2,4-D levels (0.2 to 9.0 uM) but did not state which was optimum. Our results indicate the rapid decrease of 2,4-D to a concentration of 2.3 uM 2,4-D, then to 1.1 uM after 3 to 4 wk with supplemental IAA and zeatin optimized green plant regeneration.

The addition of IAA and zeatin to the regeneration medium simulates events which occur during embryo development in the seed. As post anthesis zygotic embryos develop, IAA and zeatin concentrations increase. Our results agree with those of Vasil et al. (1990) which indicate that the addition of these hormones will enhance somatic embryo development and germination. We found no interaction between 2,4-D, zeatin and IAA concentrations in the production of embryogenic calli from suspension cultures (unpublished). However, some calli plated on zeatin and IAA medium in methods 3 to 5 ceased growth and turned brown. These results are consistent with observations made by Chang et al. (1991).

In summary, the best medium for developing rapidly growing, highly embryogenic callus cultures of Bobwhite contained MS basal salts, 5.6 uM 2,4-D, 58 mM sucrose without additional L-proline. The method for optimal plantlet regeneration from suspensions was to establish calli on an MS based medium with 9.0 uM 2,4-D and 87 mM sucrose. After 6 months, transfer highly embryogenic calli to a MS based liquid medium containing 9.0 uM 2,4-D and 87 mM sucrose. Embryogenic callus was recovered by plating suspension cells on a MS based medium with 2.3 uM 2,4-D with 58 mM sucrose. As callus was subcultured, the 2,4-D concentration was reduced to 1.1 uM with the addition of 4.6 uM zeatin and 5.7 uM IAA until shoots were produced.

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Table 1. Treatment combinations for the evaluation of 2,4-D, proline, and sucrose on three cultivars of wheat (Experiment I).

Treatment Code	Basal Salts	2,4-D	Sucrose	L-proline
		-uM-	-mM-	-mM-
5.6MS58	MS	5.6	58	0
5.6MS58P	MS	5.6	58	12
9.0MS58	MS	9.0	58	0
9.0MS58P	MS	9.0	58	12
9.0MS87(RV)	MS	9.0	87	0

Table 2. Treatment combinations for the evaluation of basal salts, sucrose, and proline on callus growth of Bobwhite (Experiment II).

Treatment Code	Basal Salts	2,4-D	Sucrose	Proline
		-uM-	-mM-	-mM-
5.6MS58	MS	5.6	58	0
5.6MS58P	MS	5.6	58	12
5.6MS87	MS	5.6	87	0
5.6MS87P	MS	5.6	87	12
5.6N658	N6	5.6	58	0
5.6N658P	N6	5.6	58	12
5.6N687	N6	5.6	87	0
5.6N687P	N6	5.6	87	12

Table 3. Treatment combinations of sucrose and 2,4-D on the establishment of embryogenic Bobwhite calli (Experiment III).

Treatment Code	2,4-D	Sucrose	L-proline
	-uM-	-mM-	
5.6MS58	5.6	58	0
9.0MS58	9.0	58	0
5.6MS87	5.6	87	0
9.0MS87	9.0	87	0

Table 4. Media used to establish embryogenic suspension cultures

	Callus Medium			Suspension Medium	
Treatment	2,4-D	Sucrose	Proline	2,4-D	Sucrose
	-uM-	-mM-	-mM-	-uM-	-mM-
1	9.0	58	12	9.0	58
2	9.0	58	12	9.0	87
3	9.0	58	0	9.0	58
4	9.0	58	0	9.0	87
5	9.0	87	0	9.0	58
6	9.0	87	0	9.0	87
7	5.6	58	0	9.0	58
8*	9.0	87	0	9.0	87

* - ND7532 non-embryogenic cell suspension that was used as a growth comparison.

Table 5. Plating schemes for regeneration of plants from suspension cells.

Transfer Period§	Plant Growth Regulators	Method 1	2	3	4	5	6	7
		uM						
0§	2,4-D	9	5.6	3.4	2.3	1.1	0.5	-
	Zeatin	-	-	4.6	4.6	4.6	4.6	4.6
	IAA	-	-	5.7	5.7	5.7	5.7	5.7
1	2,4-D	5.6	3.4	0.5	0.5	0.5	-	-
	Zeatin	-	-	4.6	4.6	4.6	4.6	4.6
	IAA	-	-	5.7	5.7	5.7	5.7	5.7
2	2,4-D	3.4	2.3	0.5	0.5	0.5	-	-
	Zeatin	-	-	4.6	4.6	4.6	4.6	4.6
	IAA	-	-	5.7	5.7	5.7	5.7	5.7
3	2,4-D	2.3	1.1	0.5	0.5	0.5	-	-
	Zeatin	-	4.6	4.6	4.6	4.6	4.6	4.6
	IAA	-	5.7	5.7	5.7	5.7	5.7	5.7
4	2,4-D	1.1	0.5	0.5	0.5	0.5	-	-
	Zeatin	4.6	4.6	4.6	4.6	4.6	4.6	4.6
	IAA	5.7	5.7	5.7	5.7	5.7	5.7	5.7
5	2,4-D	0.5	0.5	0.5	0.5	0.5	-	-
	Zeatin	4.6	4.6	4.6	4.6	4.6	4.6	4.6
	IAA	5.7	5.7	5.7	5.7	5.7	5.7	5.7

§ - Transfer period is 3 to 4 wk.

Table 6. Significance levels from analysis of variance for main effects of cultivar, 2,4-D concentration, proline concentration and their interactions on callus relative growth rates and percent embryogenic calli.

		Transfer Period[1]				
Source	df	2	3	4	5	5 %EC
Cultivar[§]	2	**	**	**	*	**
Proline	1	**	**	*	+	**
Cult *x* Pro	2	**	+	**	ns	**
2,4-D	1	ns	ns	ns	ns	ns
Cult *x* 2,4-D	2	ns	*	ns	ns	ns
Pro *x* 2,4-D	1	ns	ns	ns	ns	*
Cult *x* Pro *x* 2,4-D	2	ns	ns	ns	ns	ns
Error	279					

1 - Transfer period is 3 to 4 wk.

§ - +, *, ** F-test significant at P=0.1, 0.05, and 0.01, respectively. ns = non-significant (P>0.1)

Table 7. Significance levels from analysis of variance for main effects of basal salts, sucrose concentration, L-proline concentration and their interactions on relative growth rates of Bobwhite callus.

		Transfer Period[1]			
Source	df	3	4	5	6
Basal[§]	1	**	**	ns	ns
Sucrose	1	ns	**	*	ns
Suc *x* Basal	1	**	**	ns	ns
Proline	1	+	**	ns	ns
Basal *x* Pro	1	*	**	**	*
Suc *x* Pro	1	*	ns	**	**
Basal *x* Suc *x* Pro	1	ns	**	+	**
Error	71				

1 - Transfer period is 3 to 4 wk.

§ - +, *, ** F-test significant at P=0.1, 0.05, and 0.01, respectively. ns = non-significant (P>0.1)

Table 8. Significance levels from analysis of variance for main effects of sucrose, 2,4-D concentrations, and their interactions on relative growth rates of Bobwhite callus.

		Transfer Period[1]					
Source	df	1	2	3	4	5	6
Sucrose[§]	1	ns	ns	ns	ns	*	ns
2,4-D	1	**	**	**	**	*	*
Suc *x* 2,4-D	1	ns	+	+	*	**	ns
Error	153						

1 - Transfer period is 3 to 4 wk.

§ - +, *, ** F-test significant at P=0.1, 0.05, and 0.01, respectively. ns = non-significant (P>0.1)

Table 9. Significance levels from analysis of variance for main effects of sucrose, 2,4-D concentrations and their interactions on percent embryogenic calli of Bobwhite.

		Transfer Period[1]					
Source	df	1	2	3	5	5	6
Sucrose[§]	1	+	ns	+	ns	ns	ns
2,4-D	1	**	ns	**	**	+	ns
Suc *x* 2,4-D	1	ns	**	*	**	**	**
Error	156						

1 - Transfer period is 3 to 4 wk.

§ - +, *, ** F-test significant at P=0.1, 0.05, and 0.01, respectively. ns = non-significant (P>0.1)

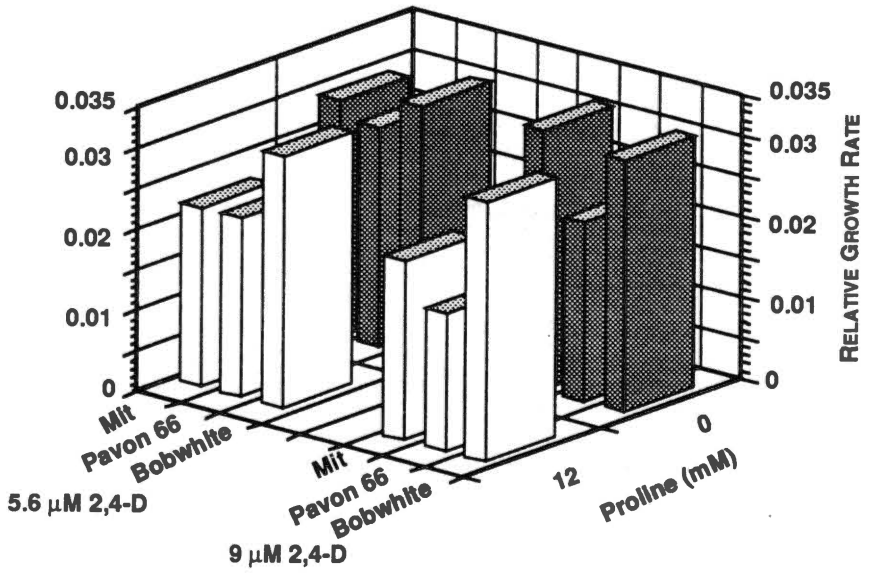

Figure 1. Calli relative growth rates as influenced by cultivar, proline concentration, and 2,4-D concentration ($LSD_{(0.05)}$=.004).

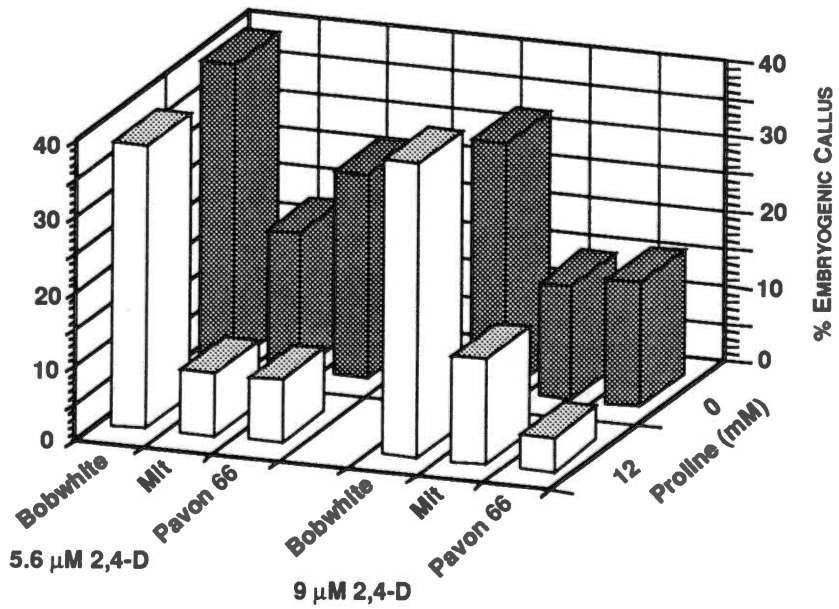

Figure 2. Percent embryogenic calli as influenced by cultivar, proline concentration, and 2,4-D concentration ($LSD_{(0.05)}$= 6.1).

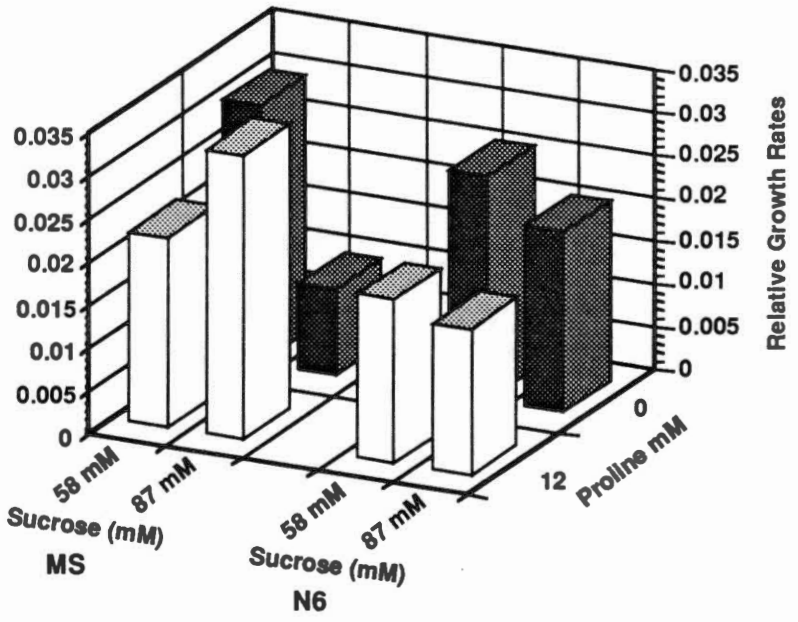

Figure 3. Influence of basal salts, sucrose, and proline on relative growth of Bobwhite calli ($LSD_{(0.05)} = 0.012$).

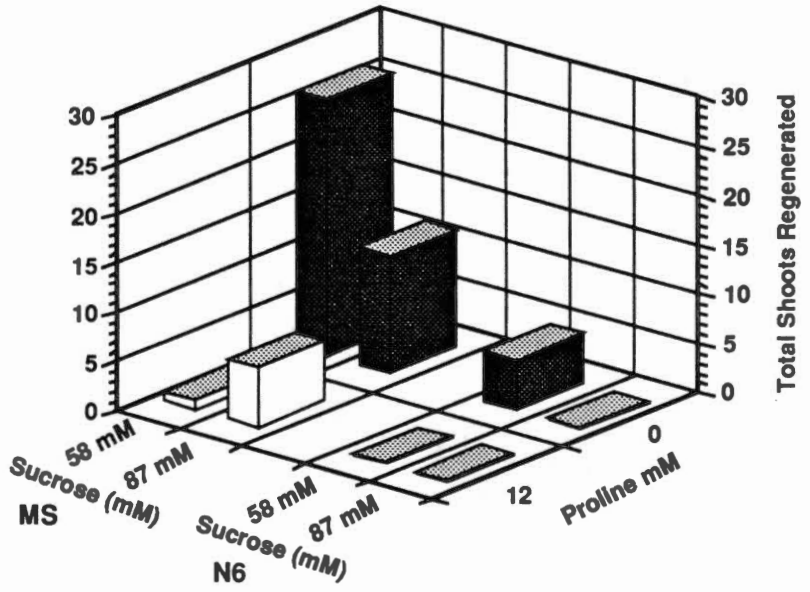

Figure 4. Influence of basal salts, sucrose, and proline on shoot regeneration of Bobwhite calli.

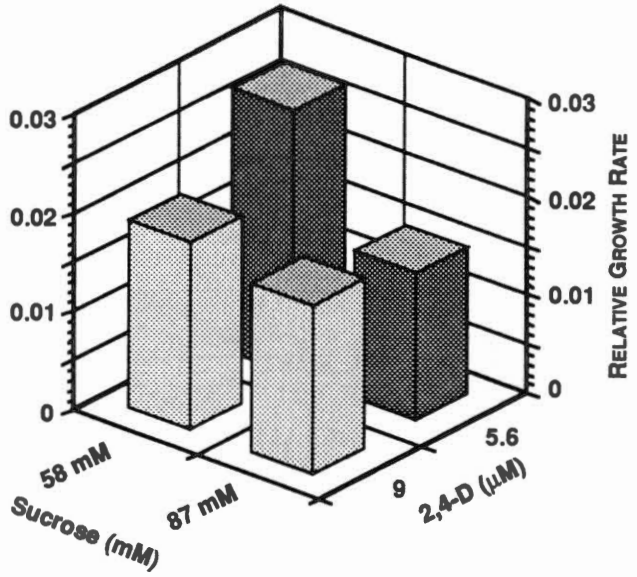

Figure 5. Sucrose and 2,4-D effects on relative growth of Bobwhite callus ($LSD_{(0.05)} = 0.009$)

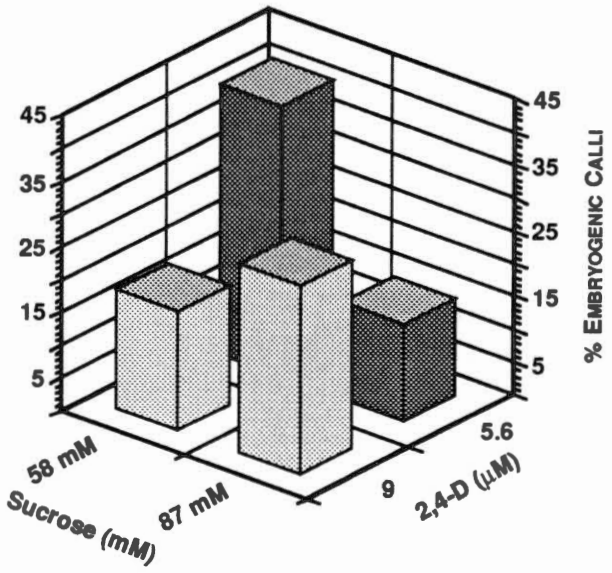

Figure 6. Sucrose and 2,4-D effects on percent embryogenic calli of Bobwhite ($LSD_{(0.05)} = 13.3$).

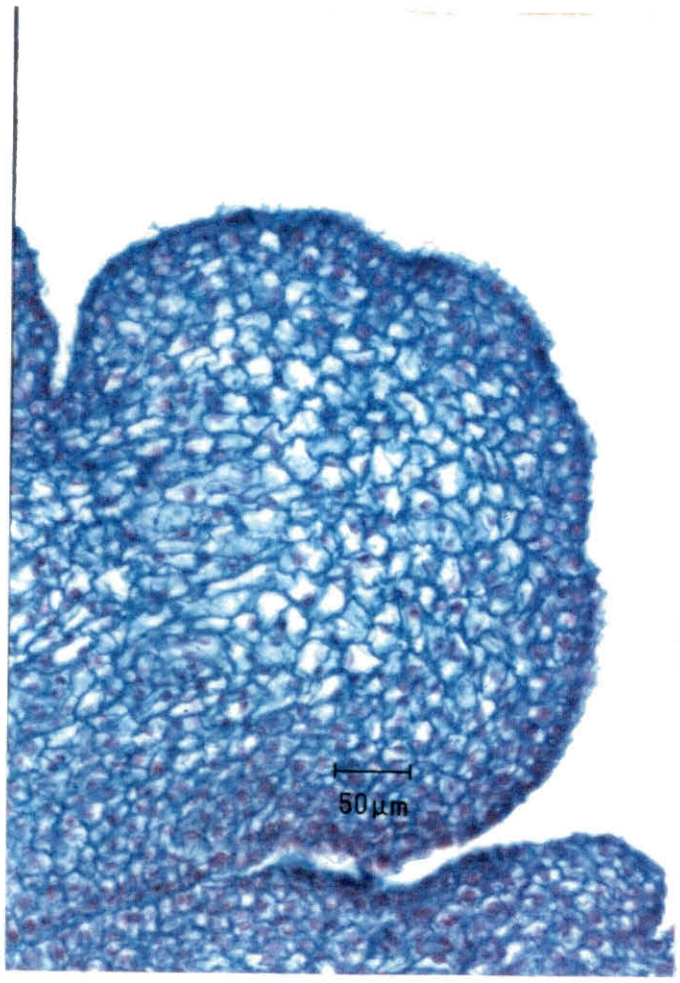

Figure 7. Thin section of embryogenic Bobwhite callus (bar = 50 um).

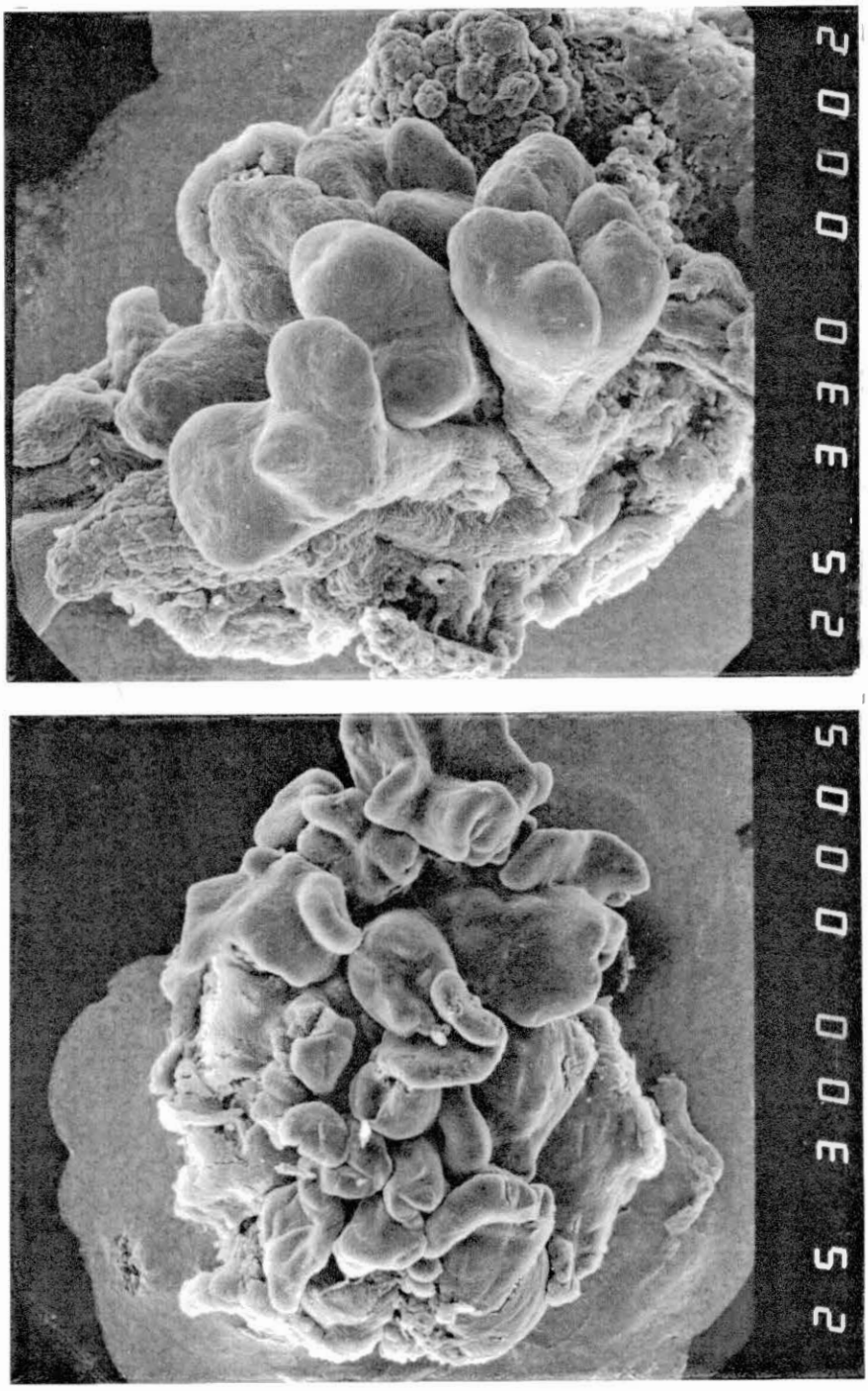

Figure 8. Scanning electron micrographs of Bobwhite embryogenic tissue magnified 33X and 30X, respectively

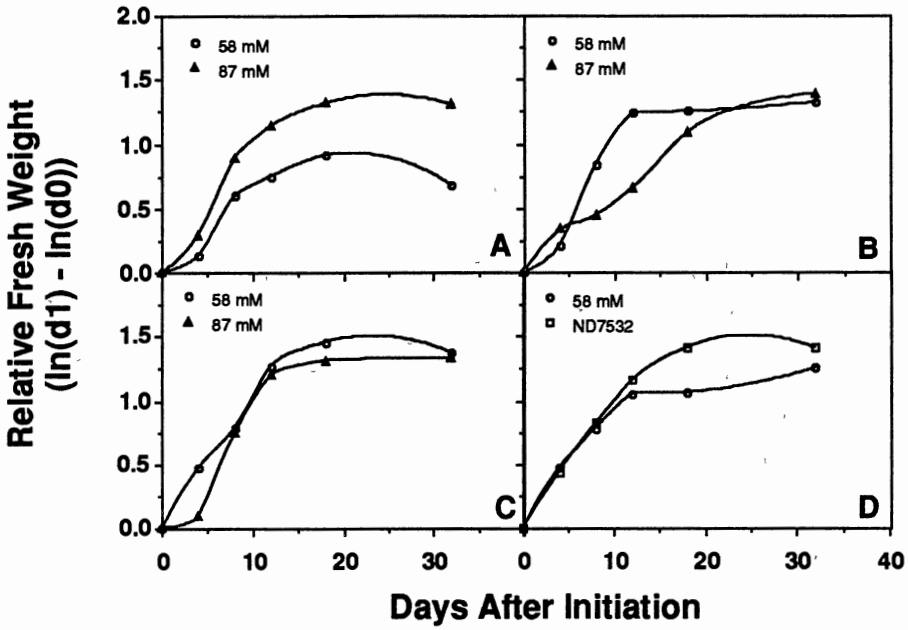

Figure 9. Relative fresh weight of embryogenic suspension cultures. (A - 9.0MS58P (Treatments 1 and 2); B - 9.0MS58 (Treatments 3 and 4); C - 9.0MS87 (Treatments 5 and 6); D - 5.6MS58 (Treatment 7) and ND7532(Treatment 8)).

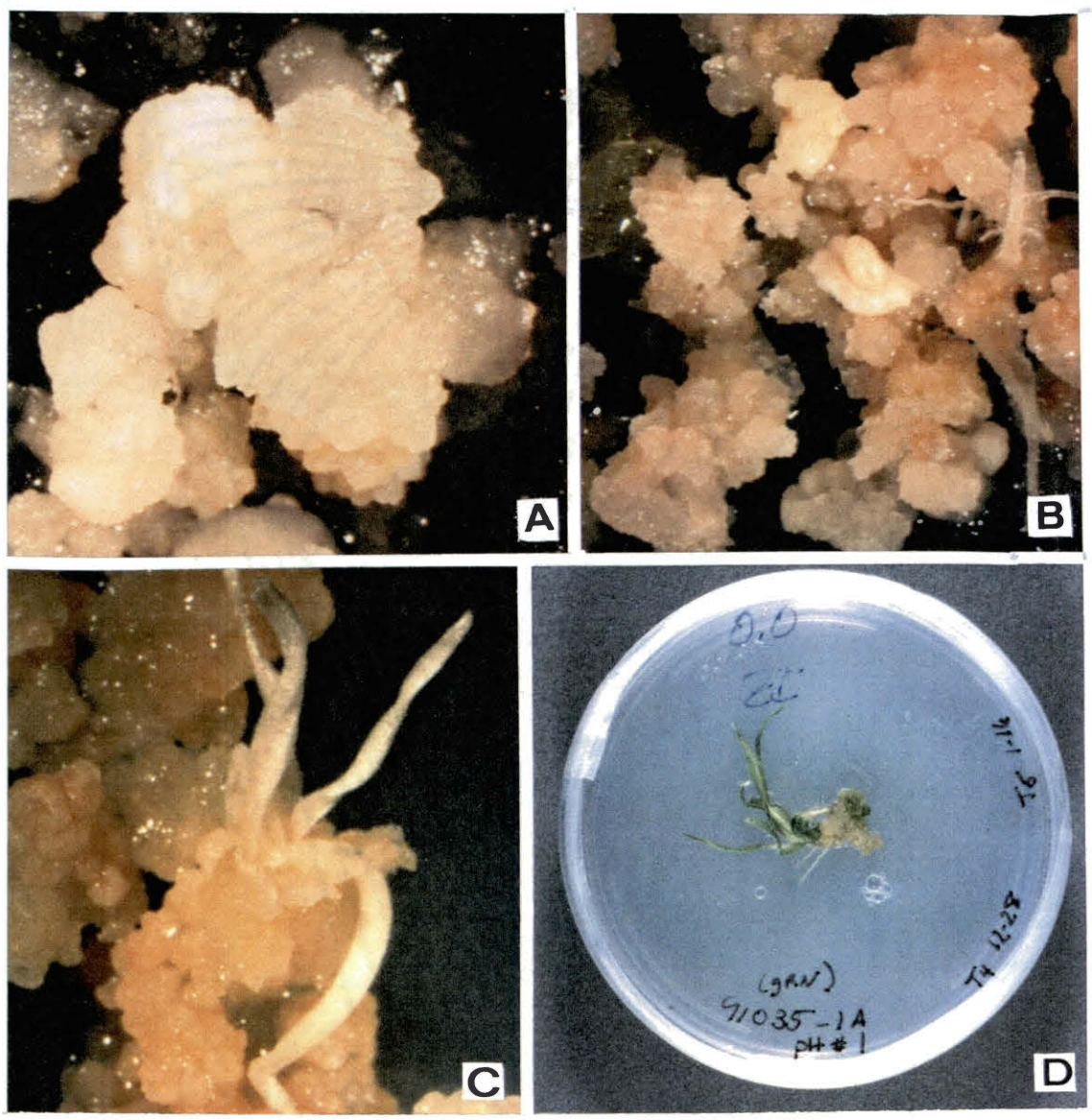

Figure 10. A) Embryogenic callus derived from suspension cultures magnified 10 times B) Somatic embryogenesis and organogenesis of callus derived from suspension cultures. C) Albino and D) green plant regenerated from callus derived from suspension cultures.

CHAPTER III

PROTEINS ASSOCIATED WITH SOMATIC EMBRYOGENESIS OF WHEAT CALLUS

Proteins Associated with Somatic Embryogenesis of Wheat

John P. Fellers, Arron C. Guenzi*, and David R. Porter

J.P. Fellers, A.C. Guenzi*, Dep. of Agronomy, Oklahoma State Univ., Stillwater, OK 74078; and D.R. Porter, USDA-ARS, Plant Sci. Res. Lab., Stillwater, OK 74075.
Contribution of the Oklahoma Agric. Exp. Stn., Journal No._____, Received _____, 1991. * Corresponding author

ABSTRACT

A marker protein for embryogenic potential could be useful in determining if target tissue for microprojectile bombardment has the ability to regenerate plants. The identification of such a protein in wheat callus cultures was approached by using isoelectric focusing and SDS-polyacrylamide gel electrophoresis of proteins *in vivo* labeled with ^{35}[S]-methionine and cysteine. Protein profile differences were examined in embryogenic (E-callus) and non-embryogenic (NE-callus) wheat callus 105 and 271 d after callus initiation growing on 5.6 and 9 uM 2,4-dichlorophenoxyacetic acid (2,4-D). Proteins unique to E-callus were identified by computer assisted analysis of scanned images of fluorographs of *in vivo* labeled proteins of E- and NE-callus. Thirty-three embryogenic proteins (E-proteins) were identified in 105 d old E-callus growing on 5.6 uM 2,4-D, 71 E-proteins in 105 d old callus on 9 uM 2,4-D, 43 E-proteins in 271 d old callus on 5.6 uM 2,4-D, and 39 E-proteins in callus 271 d old callus growing on 9 uM 2,4-D. Of these E-proteins, 10 were in callus 105 d callus old regardless of 2,4-D concentrations. One E-protein was present in both 2,4-D concentrations of 271 d old callus. Two E-proteins with relative molecular weights/pIs of 43.0/7.6 and 27.0/8.0 were present in E-callus from three of the four treatments. These proteins could be used as markers for determining if tissue has embryogenic potential.

INTRODUCTION

Somatic embryogenesis in cereals is dependent on numerous factors such as growing conditions of the donor plant, physiological state and developmental stage of the explant, genotype, 2,4-dichlorophenoxyacetic acid (2,4-D) concentration, and nutrient medium (Vasil and Vasil, 1986). Understanding the biochemical and molecular basis of somatic embryogenesis would help to improve plant regeneration potential from somatic tissues.

Protein changes associated with embryogenesis have been studied in *Daucus carota* L. (Sung and Okimoto, 1983; Hatzopoulos et al., 1990; Kiyosue, et al., 1991;), *Dactylis glomerata* L. (Hahne, et al., 1988), *Pisium sativum* L. (Stirn and Jacobsen, 1987), *Brassica napus* L., (Pechan, et al., 1991), and *Picea abies* L. (Wann et al., 1987). Proteins specific for stages of embryogenesis in carrot were identified for both somatic and zygotic embryos (Sung and Okimoto, 1983). Similar proteins have been found in pea tissue cultures that differed only in their regeneration potential (Stirn and Jacobsen, 1987). Genes from cDNA libraries have also been linked to embryogenic potential in carrot (de Vires, et al., 1988; Wilde et al., 1988).

Even though 2,4-D does not directly regulate genes controlling somatic embryogenesis in carrot (Borkird, 1988), it has a strong effect in the development of somatic embryos in cereal tissue culture (Wernickle and Milkovits, 1986; Bhaskaran and Smith 1990; Scott et al., 1990; Carmen, 1990). Despite reports of regeneration from cereals after extended periods of culture (Wang and Nguyen, 1990), the window for regeneration of fertile plants in cereals is limited. The identification of a marker protein for embryogenic potential would allow for rapid identification of embryogenic tissue for use in initiating embryogenic suspension cultures and as an indication of loss of regeneration potential with culture age.

The objective of this research was to evaluate protein synthesis in embryogenic (E-callus) and non-embryogenic (NE-callus) callus tissue as influenced by 2,4-D concentration and tissue age.

MATERIALS AND METHODS

Callus Culture

Immature embryos, excised 10 to 14 d after pollination, from the hard red spring wheat cultivar 'Bobwhite' (*Triticum aestivum* L.) were plated on a solidified MS (Murashige and Skoog, 1962) basal medium as modified by Sears and Deckard (1982). Calli were transferred every 3 to 4 weeks to fresh medium and grown at 20° C in the dark. Calli from two age groups, 105 and 271 d after initiation, were visually classified and separated into embryogenic and non-embryogenic, and grown on either 5.6 and 9 uM for a total of eight treatment combinations. These calli were cultured for two months prior to *in vivo* labeling to insure accurate classification.

In vivo Labeling of Proteins

Nine hundred milligrams of tissue from each treatment were placed on 25 ml of medium, as described above, contained in a 60 x 20 mm sterile petri plate. Trans-^{35}[S]-label™ (c. 70% L-methionine, 15 % L-cysteine, 1123 Ci/mmole, ICN Biomedicals, Inc., Irvine, CA) was applied to medium around each callus, but not on it. Five hundred uCi of label was applied to each plate. Tissue was cultured for 21 d in the dark at 20° C.

Protein Isolation, Electrophoresis, and Analysis

Proteins were isolated and denatured as described by Ramagopal (1987). Proteins were then solubolized in O'Farrells buffer (O'Farrell, 1975). Measurement of uptake and incorporation of labeled amino acids into proteins was determined by trichloracetic acid (TCA) precipitation as described by Mans and Novelli (1961). Isoelectric focusing polyacrylamide gel electrophoresis (IEF-PAGE) was performed using procedures described by Damerval et al. (1986), with some modification. IEF gels contained 3.4 % Servalyt 3 - 10 and 0.8 % Servalyt 5 - 7 ampholytes. Gels were 1-mm in diameter and 13.5-mm long. Equal counts (1,000,000 cpm) of TCA-precipitatable ^{35}[S]-labeled proteins were loaded onto each gel. IEF was

performed at 16,200 volts x hours in electrode buffers 0.1 N NaOH catholyte and 0.06 % H_3PO_4 anolyte. The gels were extracted from the tubes as described by Porter et al. (1992) and equilibrated for 1 h. Tube gels were then placed on 16 x 20 cm x 1 mm slab gels made of 10.7% acrylamide for separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Damerval et al., 1986). Slab gels were electrophoresed at 25 mA gel^{-1} in a migration buffer described by Laemmli (1970). Gels were fixed and fluorographed (Skinner and Griswald, 1983), dried (Porter et al., 1989) and exposed to Kodak X-Omat x-ray film contained in an X-Omatic cassette with intensifying screens. Exposure time was adjusted to a standard of 1 x 10^6 cpm day^{-1} gel^{-1} at -70° C. Fluorographs were analyzed using a two-dimensional scanner (Protein and DNA Imageware Systems, Hunington Station, NY). Molecular weight standards were co-electrophoresed in the second dimension for determination of relative molecular weights (M_r). Relative pI (pI_r) values were assigned by estimating the pH gradient of the IEF gel based on ampholyte composition. Relative density values (DV_r) were estimated from spot intensity during image analysis. Ninety-five proteins were identified as being consistent between all treatments and classifications. These were used as 'landmark' proteins to allow for alignment of 2D-gels from the various treatments. Between 200 and 275 proteins were identified by image analysis for each gel. Identification of E-proteins was made by comparing images of E-callus with that of NE-callus at the same 2,4-D concentration and age. Comparison were then made across 2,4-D concentrations and tissue age to identify E-proteins which were consistently synthesized.

RESULTS

Image analysis of the fluorographs (Fig. 1) indicated a two-fold increase in the number of E-proteins associated with 105 d-old E-callus growing on 9 uM 2,4-D in comparison with E-callus of the same age but growing on 5.6 uM 2,4-D. Seventy-one E-proteins were identified in E-callus growing on 9 uM 2,4-D for 105 d (Fig. 2) and 33 in E-callus growing on 5.6 uM 2,4-D for 105 d (Fig. 3). Eleven of these were found to be unique to the 105 d callus age on 9 uM 2,4-D (Table 1). E-proteins EP20.8/5.6, EP25.9/4.5, EP38.4/5.4, EP43.0/7.6, and EP53.3/5.7 were detected in 9 uM 2,4-D E-callus at greater intensities than in 5.6 uM 2,4-D E-callus at 105 d. In contrast, the intensities of E-proteins EP36.2/5.1 and EP39.1/4.9 were approximately doubled in 5.6 uM 2,4-D at 105 d. Three proteins, EP25.8/6.5, EP43.8/7.6, and EP53.4/4.9 had relatively the same intensities in both 2,4-D concentrations at 105 d. Even though total number of E-proteins were approximately the same for 271 d old callus growing on 5.6 and 9 uM 2,4-D [43 (Fig. 4) verses 39 (Fig. 5), respectfully], only EP27.0/8.0 was found to be unique when comparisons were made across the 2,4-D concentrations. It was detected in greater amounts in 9 uM 2,4-D calli.

One E-protein was found at both 2,4-D concentrations and was influenced by age. EP43.0/7.6 was present in E-callus growing on 5.6 and 9 uM 2,4-D and was more prevalent in younger tissue (105 d). EP27.0/8.0 was present in callus growing on 9 uM 2,4-D at 105 and 271 d, but was more prevalent in older tissue (271 d) (Table 1).

To determine which E-proteins were consistent in all the treatments, comparisons were made of fluorographs of tissue from different 2,4-D concentrations and age. Although no single protein was found in all four treatments, two proteins were detected in each of three treatments. EP27.0/8.0 was found in all E-callus except 105 d old E-callus growing on 5.6 uM 2,4-D (Table 1).

The intensity of EP27.0/8.0 decreased as the 2,4-D concentration was lowered. The other E-protein, EP43.0/7.6, also decreased in intensity as 2,4-D was reduced. The EP43.0/7.6 protein was present, however, in both E-callus and NE-callus in the 271 d-old treatment on 9 uM 2,4-D medium.

DISCUSSION

The biochemical and molecular basis of somatic embryogenesis has been extensively studied in carrot suspension cultures. Protein patterns of E-callus and NE-callus of carrot are relatively similar, however a few specific proteins have been identified to be involved with embryogenesis (Kiyosue et al., 1991, Kiyosue et al., 1992; de Virese et al., 1988). Grass species, in contrast, have a more complex pattern of proteins related to somatic embryogenesis (Hahne et al., 1988; Chen and Luthe, 1987). The synthetic auxin, 2,4-D, has no direct effect on embryogenesis in carrot (Borkird, et al., 1988), but has a direct effect on somatic embryo development in cereals (Bhaskaran and Smith, 1990). It has been suggested that somatic embryogenesis in wheat is controlled by the synthetic auxin 2,4-D (Sears and Deckard, 1982) and that prolonged maintenance at high 2,4-D levels can delay development of the embryos (Wernicke and Milkovits, 1986). However, after extended periods of time, the cells can loose the potential to regenerate altogether. In suspension cultures, the 2,4-D is maintained at a high level to cause rapid proliferation of the cells, but embryogenic potential is often lost.

Proteins specific to embryogenic growth have been reported in other species. Sung and Okimoto (1981) identified a 43 kD protein, specific to embryogenic potential in carrot. Proteins in the 40 to 44 kD range were also associated with somatic embryogenesis in rice (Chen and Luthe, 1987). EP43.0/7.6, which we identified, appears to be similar to these. The other protein that we were able to identify, EP27.0/8.0, was similar to ECP31 in carrot, (Kiyosue, 1991), and the E-proteins identified in *Brassica napus* L. (Pechan, et al., 1991), and rice (Chen and Luthe, 1987). If these proteins are in fact related, this would indicate that some embryogenic proteins have been strongly conserved during evolution.

The intensity of EP43.0/7.6 decreased as the 2,4-D concentration increased and also as the callus got older when grown on 5.6 uM 2,4-D (Table 1). As

previously mentioned, high concentrations of 2,4-D and prolonged culture times reduce somatic embryogenic development. The effect of 2,4-D concentration and culture age on the expression of EP43.0/7.6 reflects the effects of 2,4-D and age on embryogenic potential. This close association holds promise in the use of EP43.0/7.6 as a maker for somatic embryogenesis. Further studies are needed to determine if the same proteins are synthesized by using *in vitro* translation of mRNA from E-callus and NE-callus of the same treatments. Further research is also needed to extend these observations to E and NE- suspension cultures.

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Table 1. Unique proteins synthesized in E-callus from two or more treatments.

E-Protein	M_r -kD-	pI_r	105 d 5.6 uM[1]	105 d 9 uM	271 d 5.6 uM	271 d 9 uM
EP 20.8/5.6	20.8	5.6	4101* (24)§	3003 (56)	-	-
EP 24.9/7.2	24.9	7.2	5101 (105)	-	-	6001 (118)
EP 25.8/6.5	25.8	6.5	101 (217)	1 (224)	-	-
EP 25.9/4.5	25.9	4.5	1101 (99)	3 (333)	-	-
EP 27.0/8.0	27.0	8.0	-	7007 (68)	7003 (12)	8101 (192)
EP 29.3/4.5	29.3	4.5	-	107 (68)	104 (12)	-
EP 36.2/5.1	36.2	5.1	1407 (518)	1210 (237)	-	-
EP 38.4/5.4	38.4	5.4	1401 (30)	203 (369)	-	-
EP 39.1/4.9	39.1	4.9	7401 (121)	7306 (63)	-	-
EP 40.4/8.4	40.4	8.4	-	8302 (602)	8201 (73)	-
EP 43.0/7.6	43.0	7.6	6503(72)	7402 (147)	6306 (44)	-
EP 43.8/7.6	43.8	7.6	2505 (58)	2403 (58)	-	-
EP 44.0/5.4	44.0	5.4	-	1414 (127)	1308 (41)	-
EP 44.8/7.4	44.8	7.4	-	6404 (49)	6301 (201)	-
EP 44.8/7.2	44.8	7.2	-	6401 (108)	5304 (84)	-
EP 48.5/7.8	48.5	7.8	1405 (388)	-	-	1503 (89)
EP 53.3/5.7	53.3	5.7	1706 (15)	603 (268)	-	-
EP 53.4/4.9	53.4	4.9	6705 (135)	7605 (133)	-	-
EP 56.5/7.4	56.5	7.4	-	6610 (175)	6607 (330)	-
EP 57.6/8.4	57.6	8.4	-	8606 (134)	8608 (63)	-
EP 78.5/7.4	78.5	7.4	-	6805 (105)	6801 (80)	-
EP 82.9/7.2	82.9	7.2	-	6807 (15)	5901 (22)	-
EP 82.9/7.3	82.9	7.3	-	6806 (66)	5903 (21)	-

[1] - Concentrations of 2,4-D

* - Spot numbers assigned by image analysis according to protein position on the gel.

§ - Relative density value

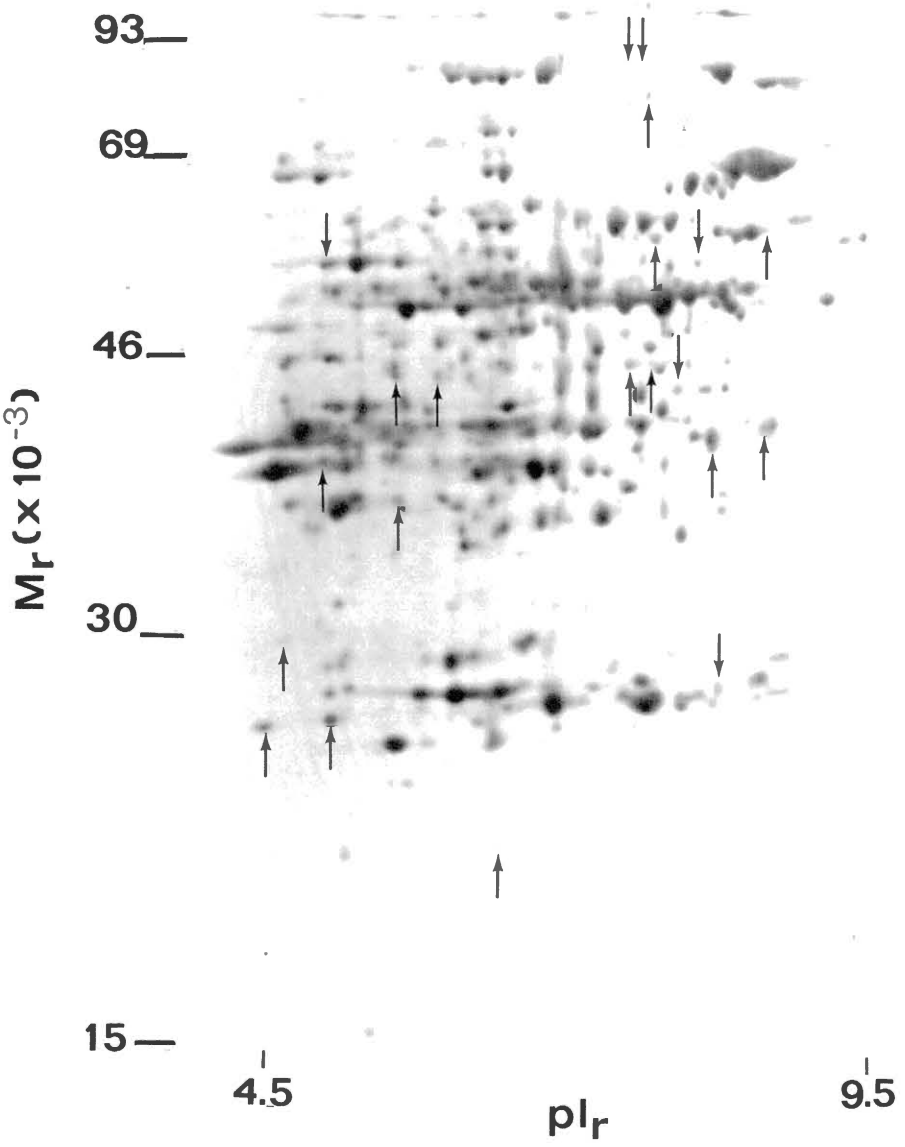

Figure 1. Fluorograph of ^{35}[S]-labeled proteins of 105 d-old E-callus growing on 9 uM 2,4-D resolved by 2D-PAGE. Arrows (↓↑) indicate E-proteins that were unique across two or more treatments.

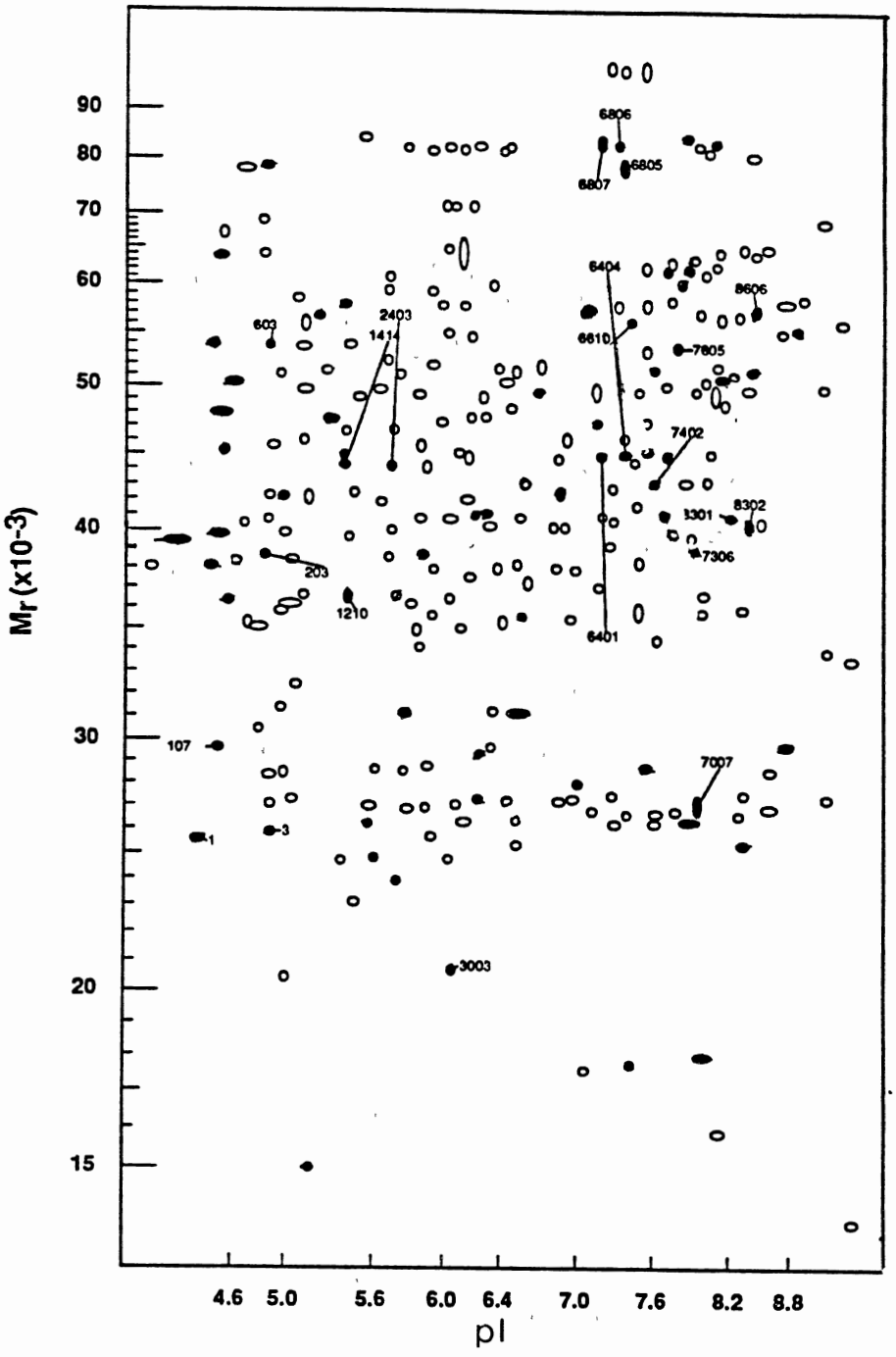

Figure 2. Aligned and overlaid protein spot maps of *in vivo*-labeled proteins of 105 d old callus growing on 9.0 uM 2,4-D. Dark spots (71 total) are proteins present in E-callus and not in NE-callus. Numbered E-proteins are present in two or more treatments.

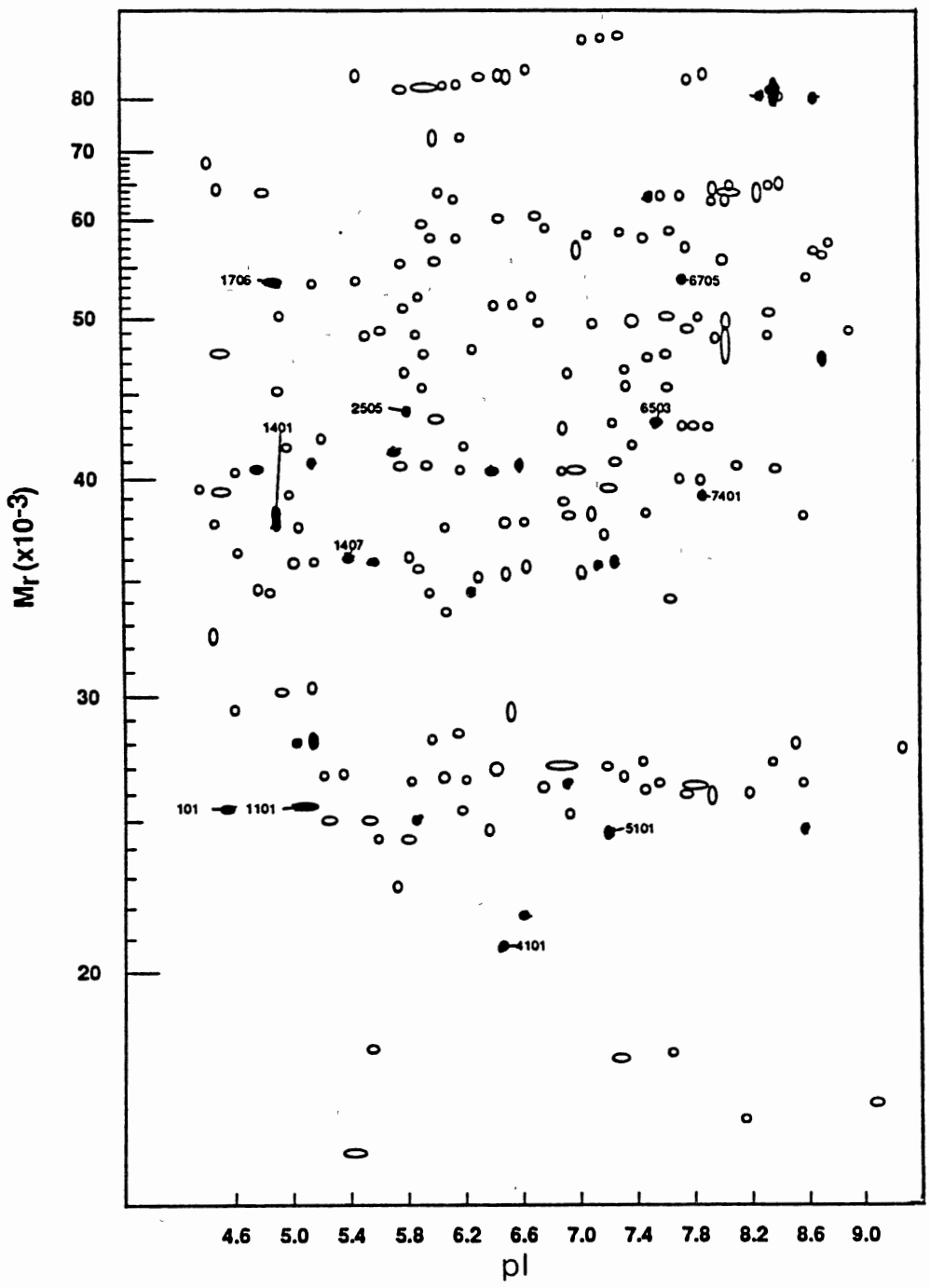

Figure 3. Aligned and overlaid protein spot maps of *in vivo*-labeled proteins of 105 d old callus growing on 5.6 uM 2,4-D. Dark spots (33 total) are proteins present in E-callus and not in NE-callus. Numbered E-proteins are present in two or more treatments.

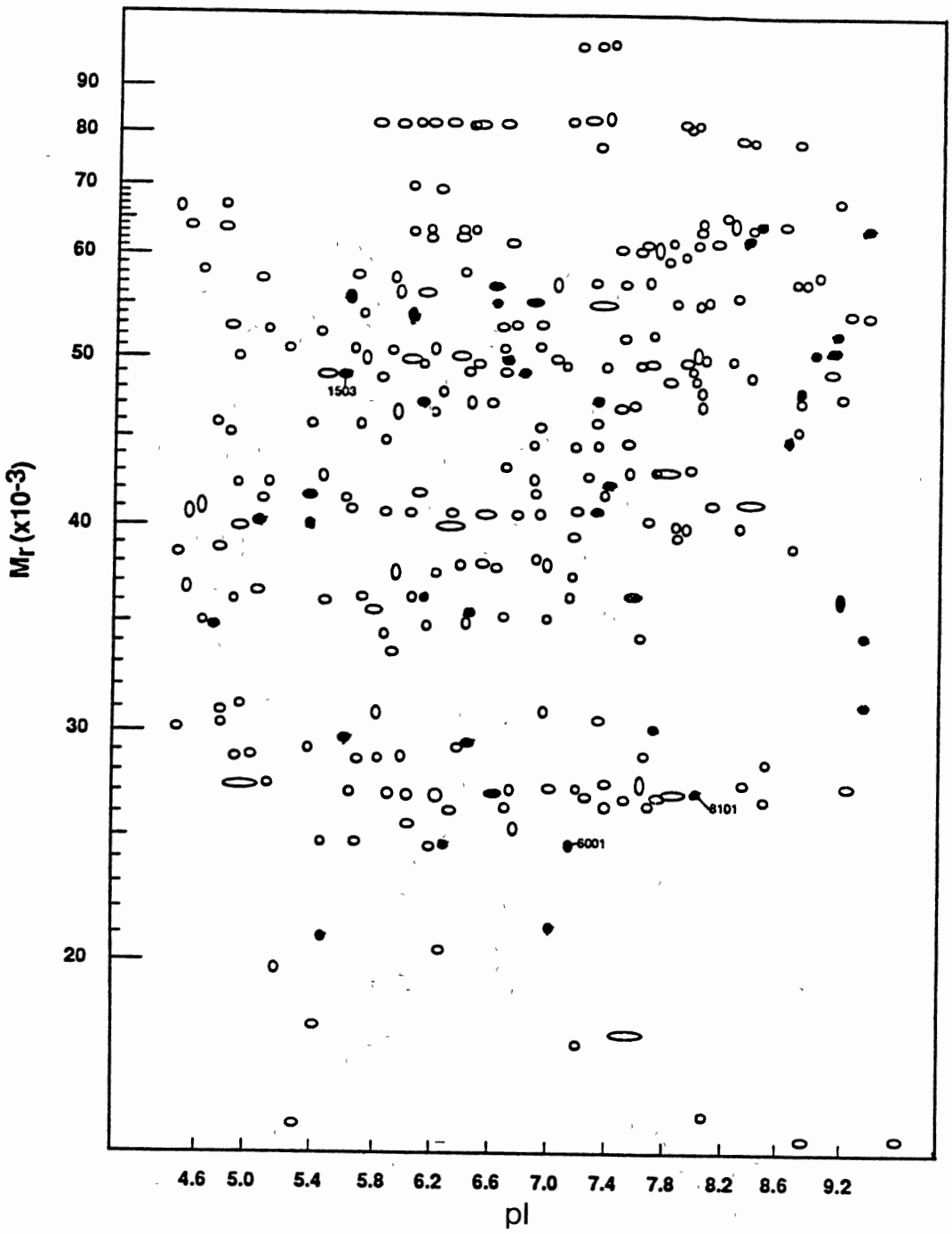

Figure 4. Aligned and overlaid protein spot maps of *in vivo*-labeled proteins of 271 d old callus growing on 9.0 uM 2,4-D. Dark spots (43 total) are proteins present in E-callus and not in NE-callus. Numbered E-proteins are present in two or more treatments.

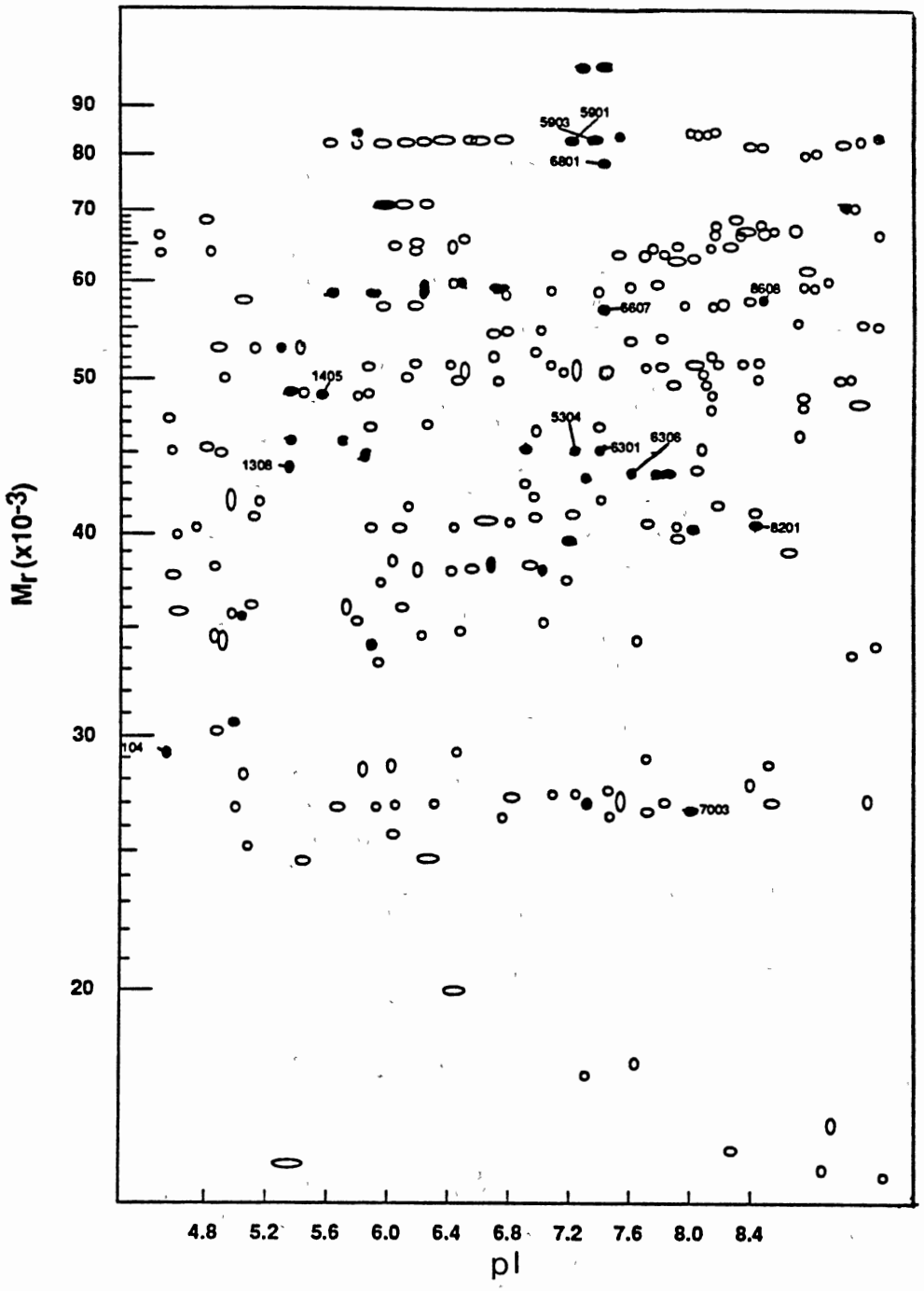

Figure 5. Aligned and overlaid protein spot maps of *in vivo*-labeled proteins of 271 d old callus growing on 5.6 uM 2,4-D. Dark spots (39 total) are proteins present in E-callus and not in NE-callus. Numbered E-proteins are present in two or more treatments.

VITA 2

John P. Fellers

Candidate for the Degree of

Master of Science

Thesis: ESTABLISHMENT AND CHARACTERIZATION OF EMBRYOGENIC SUSPENSION CULTURES OF WHEAT (*Triticum aestivum* L.)

Major Field: Agronomy

Biographical:

Personal Data: Born in Kiowa, Kansas, July 23, 1967, the son of Kenneth and Sue Fellers.

Education: Graduated from Burlington High School, Burlington, Oklahoma, in May 1985; received Bachelor of Science in Agronomy from Oklahoma State University in December, 1989; completed the requirements for the Master of Science degree at Oklahoma State University in May, 1992.

Professional Experience: Graduated Research Assistant, Department of Agronomy, Oklahoma State University, January 1990 to May 1992. Graduate Teaching Assistant, Department of Agronomy, Oklahoma State University, January 1991 to May 1991. Research Assistant, Department of Agronomy, Oklahoma State University, January 1987 to May 1987; May 1988 to December 1989.

Professional Memberships: American Society of Agronomy, Crop Science Society of America, Alpha Lambda Delta.