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Isolation and culture of protoplasts from embryogenic cell suspension cultures of Lolium perenne L. and Festuca rubra L.

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ISOLATION AND CULTURE OF PROTOPLASTS FROM EMBRYOGENIC CELL SUSPENSION CULTURES OF *Lolium perenne* L. AND *Festuca rubra* L.

A Dissertation Presented

By

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DEDICATION

To my parents, brothers and sisters for their love and support throughout the course of my graduate education. These accomplishments would not have been possible without their support.

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I would like to thank the faculty and staff of the Department of Plant and Soil Sciences for their assistance in completing this research project, and especially Dr. William A. Torello for his support, guidance, friendship and honesty. I consider myself lucky to work with a person with those assets. I wish that every graduate student could have a similar advisor. In addition, I would like to thank Drs. David Mulcahy for his friendship, support and advise and Mike Marcotrigiano, Thomas Boyle and Allen Barker for their guidance. I would also like to acknowledge my friends Mr. Duane Chisholm and Dr. Floyd Woods for their friendship and special thanks for my friend, Marta Fernandez, for her unlimited support, friendship and determination to get me to that stage. Furthermore, I would like to thank Mrs. Debbie Clark for her understanding, helping in ordering chemicals and typing. I would like to thank my fellow graduate students in this Department for making my stay here enjoyable.

ABSTRACT

PROTOPLAST ISOLATION AND CULTURE OF COOL SEASON TURFGRASSES

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In monocots, regeneration of whole, viable plants from callus, suspension cultures and protoplasts is a prerequisite which must be met prior to the application of somatic hybridization or transformation techniques. The development of such systems for cool season turfgrasses such as red fescue (Festuca rubra L.) or perennial ryegrass (Lolium perenne L.) have not been reported.

Embryogenic callus and suspension cultures of red fescue and perennial ryegrass were initiated and maintained by physically reducing a large percentage of non-embryogenic cells by density separations or size filtration. Manipulation of inorganic and organic media constitutents as well as various growth regulator types and concentrations resulted in growth media which not only enhanced growth rates, but also the degree of somatic embryogenesis in both callus and suspension cultures. Plants were regenerated from callus and suspension cultures of both species. Rapidly growing embryogenic

suspension cultures were used as the primary source for isolating protoplasts for further culturing.

A highly efficient enzyme-isolation solution was developed in which great numbers of protoplasts could be isolated with minimal damage. Results indicate that this enzyme solution is effective in isolating protoplasts from suspension cultures without agitation and, therefore, greatly reduces the degree of damage normally associated with fragile protoplasts during isolation. Increasing the frequency of cell suspension subculture events prior to protoplast isolation also enhanced protoplast isolation.

Protoplasts were cultured in liquid media micro-droplets, plated directly in liquid media overlying solid media and cultured with or without conditioned media. Cell division and micro-colony formation occurred more frequently in conditioned media for both methods but remained very low. The use of liquid nurse culture media on protoplasts embedded in thin agarose layers or placed within feeder wells surrounded by nurse culture media significantly improved the degree of cell division. Microcalli up to 3.0 mm diameter developed rapidly and were transferred to liquid culture media. Currently, suspension cultures derived from protoplasts are being cultured.

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CHAPTER I

INTRODUCTION

Review of Somatic Embryogenesis and Plant Regeneration

Plant regeneration from cell cultures is an essential step prior to the application of many genetic manipulation techniques. Grasses and cereals are known to be comparatively recalcitrant with regard to regeneration of viable plants from tissue cultures.

Organogenesis is the direct formation of shoots and roots from cultures and is characterized by the development of adventious buds on segments of cultured shoots or by the direct organization of shoot meristems, in cell cultures (3,11,12,18,48,50,59,83). Organogenesis has also been reported to occur by derepression of existing shoot primordia in cultures (60,63-67). In monocots, regeneraticn by organogenesis has been reported to cease or become sporadic and transient after short culture periods (83).

Somatic embryogenesis is a mode of regeneration where complete plants are produced from embryos formed in culture. Within the past ten years, there has been much effort directed at establishing embryogenic cultures from numerous cereal crops and other grass species (67). Somatic embryogensis is comparatively stable and offers the advantage of regenerating plants during extended periods of culture. Furthermore, all evidence indicates that regeneration somatic embryos arise from single cells (64,65). As such, somatic

embryogenesis provides a high level of genetic stability, although exceptions do occur. For instance, embryos from sorghum (Sorghum bicolor L.) cultures have been reported to have a multicellular origin (81). The potentiality for plant regeneration through somatic embryogenesis is wide spread within the Gramineae and has suggested that embryogenesis might be the most preferable and common method for in vitro plant formation (1,2,3,5-10,13-22,65).

Embryogenesis and Plant Regeneration From Callus Cultures

Growth Regulators

The primary plant growth regulator used in cereal and grass cultures is 2,4-dichlorophenoxyacetic acid (2,4-D). It has been reported that 2,4-D is the most important external factor which universally controls the induction of somatic embryogenesis (64). At optimal levels, 2,4-D is believed to be required for the acquisition of embryogenic potential at the time when the explant proliferates into callus tissue. The presence of 2,4-D prevents development of embryos, and results in unorganized growth. Conversly, if 2,4-D is removed from the culture medium, embryogenesis has been shown to proceed further (63-65). However, in certain forage grasses, such as Dactylis glomerata L., 2,4-D does not prevent the initiation of embryogenesis in suspension cultures (19,22,43).

The optimal concentration depends upon the type of tissue explant and genotype. In most cases, supraoptimal 2,4-D levels (exceeding 10 mg/l) inhibit culture growth whereas, suboptimal concentrations favor the rapid growth of non-embryogenic (NE) cells over embryogenic (E) cells (31,32). Optimal 2,4-D levels must be determined for each species since NE cell types normally grow faster than E cells.

Somatic embryos have been shown to germinate on regeneration media lacking 2,4-D (63,64,65,67). In a few cases, the media were supplemented with either gibberellic acid (GA) or abscisic acid (ABA) to either promote embryo germination or to encourage the normal maturation of the embryos ,respectively (36,37,39,67). In other instances, 6-benzylaminopurine (BA), kinetin, or zeatin were incorporated into regeneration media to promote embryo germination (67). In general, a hormone-free medium stimulates plant regeneration in monocots and cytokinins, for the most part, are not required.

Nitrogen source

The form and concentration of nitrogen in growth media may significantly affect the extent of somatic embryogenesis. The addition of casein hydrolysate (CH) was found to be critical for the development of embryogenic cell suspensions in orchardgrass (Dactylis glomerata L.) (13). The embryogenic responses in these suspension cultures were reliably turned on or off by adding or deleting CH. Liquid suspension media lacking CH resulted in only root-bearing aggregates when transferred to regeneration medium. Similar results have been reported in cultures of other Gramineous species (67).

Type of explant

Embryogenic cell cultures have been obtained from explants of immature embryo (17,20,34,38,44,45,59,63), young inflorescences (3,6,59,79,81,82,85), mature embryos (2,5,61,62,67), young leaves (3,24,66,84), and roots (1,46) of many cereals and grasses. However, immature embryos and immature inflorescences have been reported to be the most reliable source of explants (7,11,40,56,67).

When immature embryos are cultured, callus is typically formed from the surrounding cells of the scutellum at its coleorhizal end (28,39,42,59,62,66,73,74,80). Normally, the immature embryos were cultured 10-14 days post-pollination (17,20,34,44,45,59,60). During that period, all the characteristic organs of the embryos have already been formed. Several researchers have found that it is necessary to place embryos in a face down position on the culture medium, leaving the scutellum exposed (2,5,14,20,28,32,38,44,45,59,75,76).

Immature inflorescences (IIF) are more important for clonal propagation, for maintenance of a desirable genotype and for embryo production than immature embryos. However, little attention has been focused on the IIF as opposed to immature embryo on embryoids production. The optimal time to use IIF in tissue culture is when individual floral primordia have started forming (7,8,13,15,18,19,44, 59,72,79,84). Normally, the inflorescences are sterilized when they are still enclosed by the surrounding leaves. Therefore, the concentration of commercial bleach and its duration of exposure might be reduced without risk of contamination. In contrast to immature embryos, the orientation of the IIF segments on the medium were not reported to be critical for the induction of embryogenic callus (9). When immature inflorescences are used, callus is proliferated from meristematic cells of the floral primordia and cells around the peripheral vascular bundles in the infloresences axis (59,72,79,84).

With leaf mesophyll explants, embryogenic cells usually arise from the lower epidermis between vascular bundles (24,25,36,76,86). Normally, the activity of intercalary meristems ceases early during

leaf development. As such, the older the leaf, the the callus that will be formed. Joarder et al. (30) studied the callusing frequency in relation to the leaf segment position and age and found that in successive one milliliter sections from an immature Lolium perrene L. leaf base, callus formed from only the first and second sections and not from the third or subsequent positions. The frequency of callusing from such explants decreased with distance from the base of the leaf and with the increased in the age of the leaf. They attributed such a relationship to the status of the mitotic index in the plants. Wernike and Brettell (82) noted that during differentiation the leaf tissue rapidly loses the ability to respond to convential tissue culture techniques. A similar response was observed in wheat by Wernicke and Milkoovtis (84) where cells the most basal leaf sections were highly meristematic and readily divided in culture. Vasil et al. (66,67) noted that little or no embryogenic tissue was produced if the explant contained fully differentiated vascular tissues.

Callus obtained from the above mentioned explants are reported to be compact, opaque, and nodular in appearance with a whitish pale or dark yellow in color typical of embryogenic cell types (E) callus. These calli are often surrounded by a translucent, friable, and soft tissue which is characteristically non-embryogenic cell types (NE) (1,67). NE tissue proliferate more rapidly in cereals and grasses (67). Nabors et al. (43) have reported that NE cells may be converted back to E cells, but such results are not repeatable for all species.

In all systems, E cells (from which embryoids are eventually produced) are small, rapidly dividing meristematic cells

characterized by a dense cytoplasm with large nuclei, prominent nucleoli and small vacuoles full of starch granules (67,73). The regenerative capacity of embryogenic callus often shows a progressive decline and sometimes a complete loss of embryogenic potential as cultures are maintained for long periods (43,67). Such decline in the ability to regenerate plants might be attributed to:

1. Genetic defects: Implying that decline may be due to nuclear changes (including polyploidy, aneuploidy, and chromosomal rearrangements) which normally takes place in long-term cultures (27,33,35,56). Such defects may be irreversible.
2. Physiological defects: The normal balance of metabolism within the cells or tissues may be altered and, may be become sensitive or insensitive to exogenous growth regulators (61,67). Embryogenic capacity may be restored by the addition of various media components (9,10,47,49,53).

According to Steward et al. (55), both processes described above may be involved in the decline and final loss of embryogenesis during prolonged serial subcultures. On the basis of the above information, if NE cell types are at a selective advantage for growth in the medium used, (as shown in repeated subcultures), the percentage of NE cells would increase while percentage of E cell types decline gradually (67). If a stage was attained in which callus or suspension cultures did not contain any embryogenic cells, the restoration of embryogenesis might be impossible (67). Conversely, if cultures contained E cells but were unable to express their E potential due to the presence of inhibitory substances, it may be possible to restore E

capacity by changing the composition of the growth medium in a way which would favor the growth of E over NE cells (53).

Embryogenic Cell Suspension Culture

Steward et al. (55) was the first to report plant regeneration through the process of embryogenesis in suspension cultures of carrot (Daucus carota L.). Thereafter, somatic embryogenesis was found to take place in many members within the Gramineae. In earlier reports, only NE suspension cells were observed in a number of crop species (67). These suspension cultures were composed mainly of proliferating root meristems containing large, vacuolated, and generally non-dividing cells originating from the surface of the explant calli (63,64,65,67). Plants could not be regenerated from these cell cultures. Gamborg et al. (21) were the first to report regeneration from suspension culture of a grass, Bromus inermis L. All regenerated plants were, however, albino. It was not until 1981 that embryogenic suspension cultures were induced and viable plants were regenerated by Vasil et al. in Panicum maximum L. (37,38), and Sacharrum officinarum L. (29). Embryogenic suspension cultures have been useful in propagation (37,39,46,47,71), isolation of genetic variants (58), or mutant cell lines, and as a source of protoplasts. Embryogenic suspension cultures are currently the only source of totipotent protoplasts in cereals and grasses (68,69). As such, it is essential that fast-growing embryogenic suspension cultures be initiated and maintained for future use in hybridization or transformation work.

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CHAPTER II

SOMATIC EMBRYOGENESIS AND PLANT REGENERATION FROM PERENNIAL RYEGRASS CALLUS CULTURES

Introduction

The application of tissue culture methods for crop improvement has been, for the most part, limited by the inability to regenerate complete and viable plants from cell cultures. There have been several studies concerning callus initiation and plant regeneration from grass species which have a dual role as a turf or forage grass (2,4,6,11,19,22). In 1956, Norstorg (15) reported the establishment of callus cultures of perennial ryegrass (Lolium perenne L.) from immature embryo explants, but plant regeneration was not accomplished. Ahloowalia (1) reported plant regeneration from callus cultures of a tripoloid hybrid non-viable triploid (2n=21) between perennial ryegrass and annual ryegrass (Lolium multiflorum L.); a hybrid which is normally lethal. Recently, Torello and Symington (18) reported callus induction and plant regeneration via organogenesis from 'Yorktown II' perennial ryegrass. As yet, the induction of embryogenic callus and plant regeneration through somatic embryogenesis in perennial ryegrasses have not been reported. Vasil et al. (20) have reported that plant regeneration through somatic embryogenesis is considered more reliable than organogenesis, producing a greater number of shoots over extended periods of culture. Regeneration through embryogenesis generally yields non-chimeric

genetically stable plants in contrast to organogenesis (10,19,21,22). There have been many reports implicating ethylene with decreased shoot formation (9,12,16). Grady and Brassahm (9) reported that shoot producing tobacco callus tissues have significantly reduced amounts of ethylene precursor ACC (1-aminocyclo propane-1-carboxylic acid) and typically produce very little ethylene compared with callus incapable of shoot formation. Similar results were obtained by Huxter et al. (12) in inhibiting the development of shoot primordia when ACC was applied exogenously to the callus cultures.

The objectives of this study were to develop a tissue culture system for the induction of embryogenic callus and to regenerate plants through somatic embryogenesis.

Materials and Methods

Callus Induction and Maintenance

Mature, dehusked caryopses or 1-1.25 cm crown sections of 'Diplomat' perennial ryegrass (*Lolium perenne* L.) were used as explant sources. Induction and maintenance medium was composed of half-strength Murashige and Skoog (MS) (14) basal salts supplemented with 6 mg/l of 2,4-dichlorophenoxyacetic acid (2,4-D), B-5 vitamins (8), 30 g/l sucrose and 8 g/l tissue culture agar (Carolina Biological Co.). The pH was adjusted to 5.7 prior to autoclaving at 121 ^{o}C for 20 min. Following autoclaving, 5 ml of induction medium was dispensed into 35x10 mm plastic petri plates. Approximately 10-15 dehusked caryopses were placed into each plate.

For callus induction from crown tissues, seeds were surface sterilized and germinated on hormone-free induction medium. Crown

segments were excised from 15-day-old seedlings and plated on callus induction media.

All cultures were incubated in the dark for 6 weeks at 26 ^{o}C $\pm$ 2.0. Calli were subcultured every 6 weeks by subdividing into small size with an average initial fresh weight of 1 g.

A second experiment was initiated to enhance the embryogenic capacity and plant regeneration of callus having very low regenerative ability. Cefotaxime, an antibiotic used to remove Agrobacterium tumefaciens contamination during co-cultivation experiments and implicated as a stimulant to embryo formation (13), was tested at 20, 40, 60, 80, 100, 200, 400, 600, or 800 mg/l callus maintenance medium as a pretreament prior to regeneration. Cultures were incubated under the same conditions as described above. Following a 6-week incubation period, visual assessment of the degree of embryogenesis was made and shoots arising from precocious germination of somatic embryos were counted.

Plant regeneration

Calli used in regeneration experiments had been subcultured periodically for the previous 18 months on callus maintenance medium. The following regeneration treatments all contained half-strength MS salts, B-5 vitamins, 45 g/l sucrose, and 8 g/l agar. Tewenty pieces of calli were transferred to different types of regeneration media:

1. Media concentrations of BA, kinetin, or zeatin at 0.0, 0.5, 1.0, 1.5, or 2.0 mg/l.

2. Abscisic acid (ABA) levels of 0.0, 0.8, 0.24, 0.32, 0.48, 0.66, 0.8, 2.65, 5.29, 7.9, 10.8, 15.9, 23.8, 47.6, or 71 mg/l. All media in this study contained 0.5 mg/l BA.
3. Media supplemented with either 10, 20, or 30 mg/l silver nitrate or 1, 2, or 3% activated charcoal (AC). All treatments were supplemented with 0.5 mg/l BA.
4. Media supplemented with 0.5 mg/l of filter-sterilized fluridone at 0.0, 0.5, 1.0, 1.5, or 2.0 mg/l BA for 2, 4, or 6 weeks prior to were transfer to fluridone free-regeneration with or without 2% AC.
5. Same as treament 4 except these compounds were incorporated directly to regeneration media.
6. Calli were pre-treated on callus maintenance medium supplemented with 60 mg/l cefotaxime for 6 weeks prior to transfer to regeneration media containing BA at either 0.0, 0.5, 1.0, 1.5, or 2.0 mg/l, with 0.5 mg/l fluridone, with or without 2% AC.

Every treatment was repeated twice with at minimum of 10 replications per treatment. Regeneration was compared by counting the number of shoots (albino and green) regenerated per g callus (initial fresh weight). All cultures were incubated at 26 ^{o}C $\pm$ 2.0 under a 16 hr light diurnal cycle (16.3 W.m^{-2} provided by fluorescent irradiation).

Results and Discussion

Calli were induced from both mature caryopses and crown tissues 5 weeks after placement on induction media. Two morphologically

distinct callus tissues were apparent in both sources. Embryogenic (E-type) calli were nodular, compact, white or opaque in appearance and surrounded by non-embryogenic (NE-type) calli which appeared yellowish to translucent, crystalline, and friable. Similar results have been reported for most cereal and grass cultures (20).

Crown explants produced more embryogenic callus tissue compared with mature caryopsis explants. The degree of embryogenesis for perennial ryegrass callus, however, was not as extensive as reported for red fescue Festuca rubra L. (19). Embryogenic callus tissue was also rapidly "diluted out" after each subculturing event, since NE callus tissue grew much faster than E-callus tissue.

Cefotaxime pretreatments to as high as 400 mg/l resulted in a gradual increase in E callus production. These results are in agreement with those reported by Mathias and Boyd (13) where cefotaxime was used as an alternative to carbeniciline in co-cultivation experiments with Agrobacterium tumeficiences due to its less toxic effect on cultured tissue. In this report, the addition of cefotaxime was found to have a stimulatory effect on the formation of embryogenic cultures and plant regeneration. Although calli were highly embryogenic, regeneration was extremely low.

Among the cytokinins, BA at 0.5 mg/l provided more shoot regeneration compared with either zeatin or kinetin (Table 1). All shoots regenerated were, however, albino.

Silver nitrate had no effect, at any concentration, on plant regeneration (Table 1). These results are in contrast to those reported for Triticum aestevum (16) and Nicotiana plumbaginifolia (16)

callus cultures, where silver nitrate effectively promoted shoot formation from calli with low regenerative capacities.

Fluridone, a carotene and ABA inhibitor, has been reported to induce preharvest sprouting or vivipary in Zea mays L. (7) and to enhance the growth of plantlets from callus-derived protoplasts of Saccharum officinarum L. (17). There was an increase in the number of plants produced from callus pretreated with fluridone for 6 weeks compared with calli pretreated for only 2 or 4 weeks of exposure (Table 2). The number of plants produced per g of callus was slightly greater when fluridone pretreated calli were placed on regeneration media with AC (Table 2). While most of the plants regenerated were albino, a green plants were obtained. Therefore, on the basis of the above results, the inclusion of fluridone enhanced the inducition of embryo germination (Fig. 1).

The addition of cefotaxime enhanced the number of embryogenic sectors in growing callus. However, it is interesting to note that callus grown on callus maintenance medium and supplemented with 6 mg/l 2,4-D and 60 mg/l cefotaxime induced embryo germination which normally requires the total removal of 2,4-D from the medium. A great numbers of plants were regenerated when callus was pretreated with 60 mg/l cefotaxime for 6 weeks prior to transfer to regeneration medium supplemented with 0.5 mg/l fluridone and 0.5 mg/l BA (Table 3). The type and not the concentration of cytokinin was found to be critical for regeneration of shoots. More green and albino plants were obtained when cultures were directly placed on regeneration media supplemented with 0.5 mg/l fluridone and 0.5 mg/l BA (Table 4).

Table 1. The effect of cytokinins, activated charcoal (AC) and silver nitrate ($AgNO_3$) on plant regeneration from perennial ryegrass after 6 weeks in culture. Standard regeneration medium (SRM) was of hormone-free medium, 1/2 strength MS, B-5 vitamins, 45 g/l sucrose, and 8 g/l agar. Every experiment was repeated at least twice.

Treatment (mg/l)	Mean albino shoot number[a]
SRM	0[b]
Zeatin	
0.5	2.1 ± 1.3[c]
1.0	2.2 ± 1.0
2.0	1.4 ± 1.0
BA	
0.5	6.9 ± 2.4
1.0	4.8 ± 1.0
2.0	2.8 ± 2.2
Kinetin	
0.5	2.1 ± 2.0
1.0	2.8 ± 2.1
2.0	1.5 ± 1.0
$AgNO_3$ +0.5 mg/l BA	
10	6.4 ± 3.0
20	7.3 ± 1.0
30	0
Activated charcoal (1%) + 0.5 mg/l BA	
1	1.1 ± 1.0
2	3.3 ± 1.3
3	3.4 ± 2.2

[a]All plants were albino.

[b]Shoot numbers are per g of callus (initial fresh weight).

[c]Mean ± SE.

Table 2. Extent of plant regeneration between 2, 4, and 6 weeks pretreatment with fluridone and BA prior to placement on standard regeneration medium and with or without 2% activated charcoal (AC). All regeneration media contained 0.5 mg/l fluridone. Numbers represent the mean shoot number per g callus (fresh weight).

Pretreatment media	Means of plants regenerated			
	+AC		-AC	
	Green	Albino	Green	Albino
2-week-pretreatment				
0.0 mg/l BA	0	0	0	0
0.5	0.8 ± 0.3[a]	3.9 ± 2.1	0	3.9 ± 1.0
1.0	0.4 ± 0.3	4.5 ± 2.5	0	2.9 ± 1.2
1.5	0	1.6 ± 1.2	0	2.6 ± 1.1
2.0	0	1.5 ± 1.1	0	2.6 ± 1.3
4-week-pretreatment				
0.0 BA mg/l	0	0	0	0
0.5	0	4.1 ± 2.2	0	5.6 ± 1.1
1.0	0	8.5 ± 1.1	0	7.3 ± 2.3
1.5	0	4.8 ± 2.1	0	4.4 ± 1.4
2.0	0	1.6 ± 1.7	0	2.6 ± 1.3
6-week-pretreatment				
0.0 BA mg/l	0	0	0	0
0.5	0	17.3 ± 3.2	0.4 ± 0.1	14.9 ± 3.4
1.0	0	24.7 ± 5.1	0	14.8 ± 2.8
1.5	0	14.5 ± 3.9	0	6.5 ± 2.5
2.0	0	11.0 ± 3.4	0	4.8 ± 1.8

[a]Mean ± SE.

Table 3. The effect of cytokinins and fluridone on plant regeneration from perennial ryegrass.

Treatment (mg/l)	Number of green plants		Number of albino plants	
	+[a]	-[b]	+	-
SRM (control)	0	0	0	0
0.5 BA +0.5 fluridone	1.1 ± 0.4[c]	-	15.3 ± 4.0	16.2 ± 3.2
0.5 kinetin +0.5 fluridone	0	5.2 ± 2.0	3.3 ± 1.9	11.1 ± 5.5
0.5 zeatin +0.5 fluridone	0	5.4 ± 3.3	5.6 ± 3.2	8.2 ± 4.8

[a]Cultures were maintained on a medium supplemented with 60 mg/l cefotaxime for 6 weeks prior to transfer to regeneration medium supplemented with cytokinins and 0.5 mg/l fluridone.

[b]Without cefotaxime.

[c]Mean ± SE.

Table 4. Effect of 6-benzylamino purine (BA) and activated charcoal (AC) concentration in standard regeneration medium (SRM) on shoot formation. Number represent the mean shoot number per g callus (fresh weight). All media contained 0.5 mg/l fluridone.

Regeneration treatment (BA mg/l)	Mean shoot formation[b]	
	+AC[a]	-AC
0.5	23.4 ± 4.1[c]	14.3 ± 3.9
1.0	24.6 ± 4.5	11.7 ± 4.4
1.5	11.0 ± 1.3	6.2 ± 2.1
2.0	7.1 ± 2.3	4.9 ± 1.5

[a]2% AC in SRM.

[b]All regenerated plants were albino.

[c]Mean ± SE.

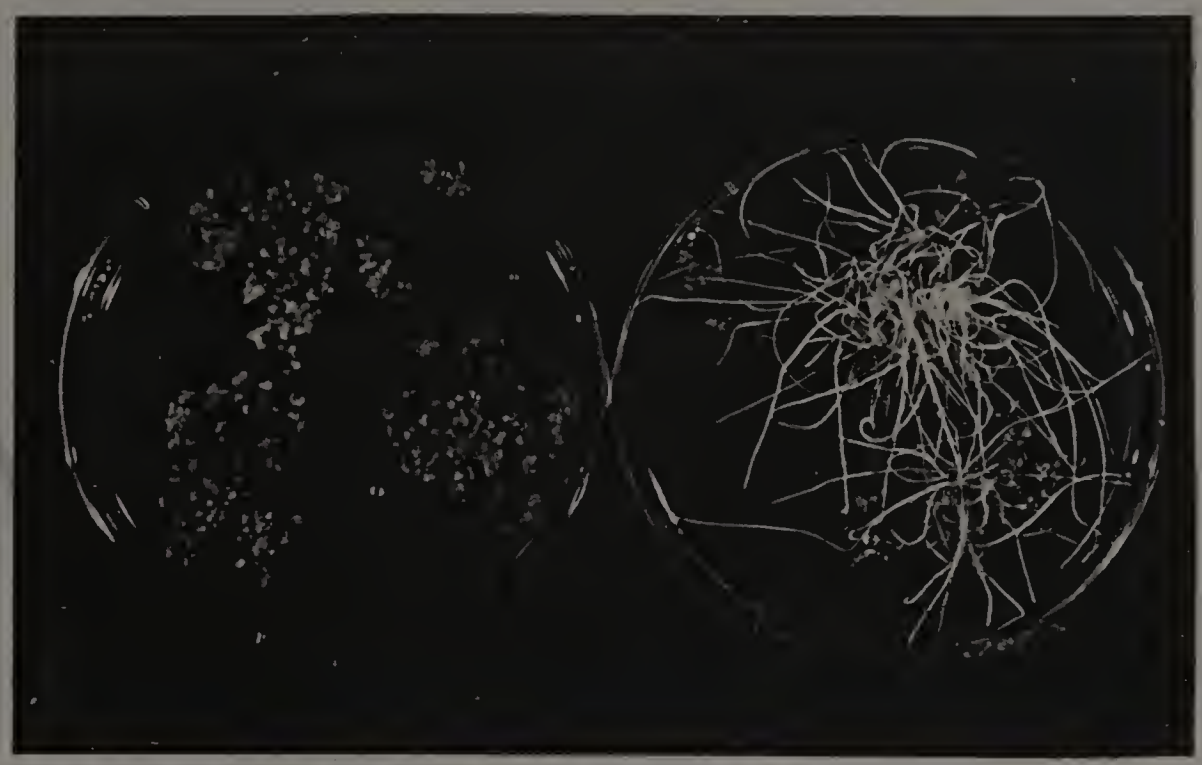

Figure 1. The effect of fluridone on plant regeneration from embryogenic callus of perennial ryegrass. (A) Without fluridone. (B) with 0.5 mg/liter fluridone.

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CHAPTER III

ENHANCED PLANT REGENERATION IN LONG-TERM CALLUS CULTURES OF RED FESCUE BY PRE-TREATMENT WITH ACTIVATED CHARCOAL

Introduction

Additions of activated charcoal (AC) to culture media have been shown to either enchance or inhibit growth and morphological development of different tissues in vitro. The beneficial effects of AC on embryogenesis and plant regeneration have been reported to be due to adsorption of inhibiting substances such as various phenolic compounds which accumulate during culture (3,6,11). Activated charcoal may also bind 1,5-hydroxy-methyl-furfural, a toxic compound derived from sucrose dehydration during autoclaving (10).

Inhibitory responses of AC have been reported to be due to the adsorption of various hormones, vitamins or other media constituents (1,10,11). As such, higher levels of these media components must be added to overcome inhibition by AC.

It is well established that 2,4-D is necessary for initiating and maintaining callus and suspension cultures for most cereal and grass species. However, 2,4-D and other growth regulators are normally deleted from regeneration media for optimum results (7,8). Therefore, additions of AC may quicken and/or enhance plant regeneration by adsorbing and reducing the residual activity of 2,4-D which may be retained in callus tissues after transfer to regeneration media. The effects of AC on 2,4-D activity have not, as yet, been reported.

The extent of somatic embryogenesis and the capacity for plant regeneration from our long-term callus cultures of red fescue (Festuca rubra L. 'Dawson') were greatly reduced after a 5 year period of maintenance. Therefore, the objectives of this research were to 1) evaluate the effects of AC pre-treatment on plant regeneration from long-term callus cultures of red fescue; and 2) determine if AC and reduces the activity of elevated and inhibitory levels of 2,4-D in culture media.

Materials and Methods

Callus Maintenance and Plant Regeneration

Embryogenic callus cultures of 'Dawson' red fescue used in this study were periodically subcultured (every 6-8 weeks) for over 5 years with the extent of embryogenesis and subsequent plant regeneration noticeably declining within the previous 6-month period. Cultures were maintained on a modified Murashige and Skoog (MS) (5) media supplemented with 5 mg/liter 2,4-D (7). The optimum regeneration media used periodically throughout the previous 5 year period was a hormone-free, 1/2 strength MS media supplemented with 10 g/liter sucrose and 8 g/liter of tissue culture grade agar (8).

Pre-treatment Effects of Activated Charcoal

Activated charcoal was added to callus maintenance media at 0.0, 10.0, 20.0 or 30.0 g/liter levels to evaluate the effects of AC pre-treatment on plant regeneration after transfer of calli to standard regeneration media. For each AC treatment, six calli weighing approximately 0.2 g (fresh weight) were placed on each of 20 petri-

plates of 100x15 mm for every AC treatment and were incubated in the dark at 26 ^{o}C $\pm$ 2.0 for 6 weeks before transfer to regeneration media. The extent of precocious germination of somatic embryos was visually assessed by comparison to controls at the end of the 6-week incubation period. Immediately following the AC pre-treatment period, calli from all treatments were transferred to regeneration media and incubated for 6-weeks under 16.3 W.m^{-2} provided by cool-white fluorescent irradiation. The effects of AC pre-treatment on regeneration were determined by comparing shoot number, root number, shoot length and root length on a per g callus (fresh weight) basis between all treatments.

Effect of Activated Charcoal on 2,4-D Activity

Embryogenic calli were cultured , as described previously but containing either 10 or 15 mg/liter 2,4-D with and without 20 g/liter AC. Exposure of red fescue callus to such elevated 2,4-D levels is inhibitory and results in a cessation of growth and discoloration of callus tissue (unpublished data). The effects of AC on elevated 2,4-D levels were assessed by comparison of overall regeneration capacity (previously described) between treatments with or without 20 g/liter AC.

Results and Discussion

A pre-treatment culture period on media containing AC significantly improved the extent of regeneration from long-term embryogenic red fescue callus cultures. Both shoot and root numbers per g of callus and shoot length were greater at all AC levels tested over controls (Table 5). Although callus growth was suppressed during

pre-treatment exposure to AC, a high degree of precocious germination of somatic embryos was apparent in AC treated calli. Such results were most likely due to the adsorption and/or reduction in activity of 2,4-D by AC. An increase in precocious germination and a reduction in callus growth are typical responses for red fescue cultured at sub-optimal 2,4-D levels (7). However, the extent of precocious germination and the capacity for plant regeneration had been greatly reduced in our 5-year old callus cultures. The increase in precocious germination was, therefore, due to AC addition to pre-treatment media which may also have adsorbed inhibiting compounds originating from callus tissue or the growth media. Several reports have implicated AC with adsorbtion of substances inhibitory to morphogenesis allowing for a greater capacity for plant regeneration (2,3,4,11).

The ability of AC to adsorb and reduce the activity of 2,4-D is shown on Table 6 where elevated and inhibitory levels of 2,4-D were used alone or with 20 g/liter AC. Significant and positive regeneration responses for shoot number, shoot length and root length resulted from the addition of AC to pre-treatment culture media. In treatments without AC, the overall regeneration capacity declined as 2,4-D concentrations were increased with shoot numbers being greatly decreased.

It is well established that 2,4-D is the preferred auxin source for cereal and grass cultures and that optimum plant regeneration occurs in the absence of 2,4-D. Recent evidence has shown that 2,4-D inhibits normal expression of cell differentiation keeping actively growing cultures in a relatively suppressed, non-morphogenic state which is reversed when cultures are transferred to 2,4-D-free media

(12,13). Although AC has been implicated in 2,4-D adsorption in this study, a reduction in 2,4-D activity cannot be fully responsible for the increase in the extent of regeneration. Control cultures (AC-free pre-treatment media) transferred to 2,4-D-free regeneration media exhibited a much reduced capacity for regeneration than has previously been obtained with this long-term culture. As such, the increased regeneration capacity induced by AC pre-treatment was most likely due to the adsorption of inhibiting substances characteristic of older cultures as well as the adsorbtion and/or reduction of 2,4-D activity.

Table 5. Effects of activated charcoal (AC) concentration on callus growth and plant regeneration in long-term callus cultures of red fescue.

AC level (g/liter)	Shoot number	Root number	Shoot length (cm)	Root length (cm)
0.0	6.7[a]	2.8	4.7	2.6
10.0	11.6	5.6	5.5	2.7
20.0	21.8	3.8	6.5	3.2
30.0	17.3	3.8	3.9	2.9
Significance				
Treatment	**	**	**	NS
Linear	**	NS	NS	NS
Quadratic	*	**	**	NS
Cubic	*	*	NS	NS

[a]Shoot (or root) numbers per g callus tissue (initial fresh wt.).

*,**,NSSignificant at 5% and 1% level and not significant respectively following an analysis of variance . Data represent the mean of 20 replications.

Table 6. The effects of elevated 2,4-D levels and activated charcoal (AC) pre-treatment on plant regeneration in red fescue.

Treatment		Shoot Number	Root Number	Shoot Length (cm)	Root Length (cm)
2,4-D (mg/liter)	AC (g/liter)				
5.0	0.0	6.7a	2.8	4.7	2.6
	20.0	21.8	3.8	6.5	3.2
10.0	0.0	4.6	5.0	5.3	3.5
	20.0	6.2	4.1	3.8	2.5
15.0	0.0	2.0	2.4	2.3	0.5
	20.0	4.6	4.9	4.2	2.1
Significance					
2,4-D		**	NS	NS	NS
AC		**	NS	*	**
2,4-D X AC		**	**	*	**

[a]Shoot (or root) numbers per g callus tissue (initial fresh wt.).

**,*,NS Significant at the 1% and 5% level and not significant respectively. Data represents the mean of 20 replications.

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C H A P T E R IV

ENHANCEMENT OF SOMATIC EMBRYOGENESIS AND PLANT REGENERATION WITH ELEVATED SUCROSE CONCENTRATIONS

Introduction

The most reliant mode of plant regeneration within the Gramineae has been somatic embryogenesis, in which embryogenic cultures maintain their regenerative capacity for extended periods (7,18,19). In many instances, however, the ability to regenerate plants may become sporadic and possibly lost over with extended subculturing (2,7,18,19). Reduction in regeneration capacity may be due to chromosomal abberations (15), the prolific growth of non-embryogenic over embryogenic cell types (19) or the accumulation of inhibitory substances. Barbara and Nickell (2) have reported a reduction in regeneration capacity of sugarcane callus after only several subcultures. Similar reductions in regenerative capacities have been reported for Triticum aestivum L. (4) and Hordeum vulgare L. (3).

Torello and Symington (18) reported extensive plant regeneration through somatic embryogenesis in red fescue (Festuca rubra L.) from long-term callus cultures. The extent of somatic embryogenesis and regenerative capacity from these cultures has been greatly reduced after a 5 year culture period.

Increased sucrose concentrations have been shown to enhance somatic embryogenesis in Daucus carota L. (20), Solanum melongena L. (6), Coix lacryma-jobi L. (17), Picea abies L. (1), Pinus taeda (21)

and Zea mays L. (10). In related studies, Rapela (14) found that sucrose enhanced organogenesis and somatic embryogenesis in inbreds of Zea mays L.. On the other hand, in Medicago sativa L., elevated levels of maltose was responsible for the enhancement of embryogenesis (16). It is not known, however, whether the stimulating effect of increased sucrose levels is due to nutritional or osmotic effects.

The objective of this study was to determine the interactive effects of high sucrose levels with various different basal media on embryogenesis and subsequent plant regeneration in long-term cultures of red fescue exhibiting a reduced regenerative capacity.

Materials and Methods

Culture Conditions

Initiation, maintenance and regeneration conditions for long term callus and suspension cultures of 'Dawson' red fescue have been previously reported (18). Embryogenic callus cultures were sub-cultured every 8 weeks for over 5 years.

In the first experiment, sucrose was added to either Murashige and Skoog basal salt (MS) (12), B-5 medium (5) or Gamborg, Miller and Ojima basal salt media (B5 basal salt) (5) media at 20, 30, 40, 50, or 60 g/l concentrations. Mannitol was added in other treatments at 5.5 and 21.9 g/l concentrations and supplemented with 20 g/l sucrose. All media used were supplemetned with 5 mg/l 2,4-dichlorophenxyacetic acid (2,4-D), B-5 vitamins (5) and 8 g/l agar. Two calli, each approximately 200 mg (fresh wt)were placed on each plate and were incubated in the dark at 26 ^{o}C $\pm$ 2.0 for 6 weeks prior to transfer to

regeneration medium. At least 10 replicates (plates) were used for each treatment.

In a second study, sucrose was added at 30, 45, or 60 g/l to MS liquid medium and embryogenic cell suspensions were used instead of callus. Initiation and maintenance of embryogenic suspension cultures of red fescue have been described in chapter 6. Suspension cultures were initiated from long -term callus cultures and were subcultured every 7 days for approximatly 1 year on 1/2 -strength MS medium supplemented with 4 mg/l 2,4-D and B-5 vitamins (5).

All suspension cultures were placed on a gyratory shaker (100 rpm) and incubated at 26 ^{o}C $\pm$ 2.0. The effect of pre-conditioning with sucrose on overall culture growth was determined by comparing the packed cell volume (PCV) of the control (30 g/l sucrose) with other treatments. Immediately following the pretreatment period, approximetly 2 g of cell aggregates with an average diameter of 2.5 mm, were transferred to regeneration medium containing the corresponding sucrose concentration (30, 45, or 60 g/l) and incubated as described below.

Plant Regeneration

The standard regeneration medium was hormone-free, half-strength MS basal medium supplemented with 10 g/l sucrose, and 8 g/liter agar (18). In experiment one, the degree of embryogenesis and precocious germination on callus cultures was assessed by visual comparison to control cultures 6 weeks after pretreatment with sucrose and prior to placement on regeneration medium. The extent of plant regeneration for both callus and suspension derived cultures was assessed after 6

weeks of incubation. Cultures were kept at 26 ^{o}C $\pm$ 2.0 with a 16 h light period (16.3 Wm^{-2}) by counting the number of shoots and roots and measuring shoot and root length. Regeneration conditions for suspension cultures were similar to those for callus cultures but contained higher sucrose concentrations as described above for pretreatment conditions.

Results and Discussion

The results of this study indicate that pretreatment with elevated levels of sucrose greatly enhanced the regeneration capacity of long-term embryogenic callus (Table 7) and suspension cultures (Table 8) of red fescue.

Figure 2 illustrates the degree of precocious germination from callus cultures 6 weeks after pretreatment with elevated concentrations of sucrose. The highest degree of embryogenesis and precocious germination occurred when MS basal medium was supplemented with 40 g/l sucrose.

Throughout this study, an M1 basal medium was shown to yield the best overall results with elevated sucrose concentrations. Increasing sucrose levels during pretreatment above the standard levels (30 g/l) significantly reduced the degree of albinism (Fig. 3) and enhanced the numbers and lengths of both shoots and roots regenerated on a gram callus fresh weight basis (Figs. 4-7). The optimal sucrose level for increased shoot numbers (which is considered more important than shoot length) was 40 g/l used in combination with the M1 basal media. Although shoot lengths were greatest at 60 g/l for B5 medium and B5 basal medium, shoot numbers at this relatively high level of sucrose

were fewer compared to MS basal media at 40 g/l sucrose. Similar results were obtained for root numbers in that 40 g/l sucrose was optimal for all 3 media tested but higher (50 g/l) sucrose levels yielded greater root lengths with MS basal media was the least effective. Root length, however, is also considered less important than the number of roots which are regenerated.

Results indicate that mannitol could not be substituted for the beneficial effects of sucrose (unpublished data). Supplemental addition of mannitol and concurrent reduction of sucrose resulted in discoloration and eventual death of callus tissues. Similar results obtained when mannitol was supplemented to the growth medium of sugarcane (11,12). In contrast to our results, Kavi Kishor and Keedy (8) found that callus derived from roots and embryos of rice exhibited better growth on Linsmaier and Skoog basal medium (9) if 2% sucrose was supplemented with 3% mannitol or 3% sorbitol.

The growth curve for suspension cultures at all sucrose levels is shown in Figure 8. The highest PCV was obtained if 45 g/l sucrose was added to the growth medium, followed by 30 and 60 g/l sucrose. However, the highest number of roots regenerated and the greatest shoot and root length occurred when 60 g/l sucrose was added to regenertion medium (Table 8). Plant regeneration derived from sucrose pre-treated callus tissue maintained after plating on standard regeneration medium with a normal level of sucrose. In contrast, no regeneration was observed for embryogenic suspension aggregates when they were placed on similar regeneration medium. Therefore, higher concentrations of sucrose were used in regeneration media for suspension cultures.

The results of this study indicate that it is possible to renew lost embryogenic and regeneration capacities in red fescue callus and suspension cultures if they are pretreated and regenerated on a medium supplemented with high levels of sucrose.

Table 7. Analysis of variance on the effects of sucrose concentration and three different basal media (MS basal salt, B5 medium, and B5 basal medium) on plant regeneration from long-term callus cultures of red fescue.

		Mean Squares				
Source	df	Albino shoot number	Shoot number	Root number	Shoot length	Root length
Media	2	96**	722*	72**	4NS	3**
Sucrose	4	183**	2038**	55**	9**	0.5*
Linear	1	464**	15167**	54**	29**	0.9*
Quadratic	1	86*	2036**	49**	0.1NS	0.6NS
Cubic	1	153**	714**	10NS	2NS	0.6NS
Sucrose x Media	7	45*	588**	9NS	3NS	2NS
Error	136	17	164	4.6	2	0.16

*,**,NS Significant at the 5% (*) or 1% (**) level or not significant (NS).

Table 8. The effect of elevated sucrose concentration pretreatments on subsequent shoot and root regeneration from embryogenic suspension cultures of red fescue[z].

Sucrose level (g/l)	Mean shoot number	Mean root number	Mean shoot length (cm)	Mean root length (cm)
30	4.7	7.5	6.9	1.1
45	23.3	13.0	14.0	2.3
60	34.8	17.0	17.5	3.8
LSD (0.05)	12.5	3.7	2.8	0.7

[z]Represents the mean of 10 replications.

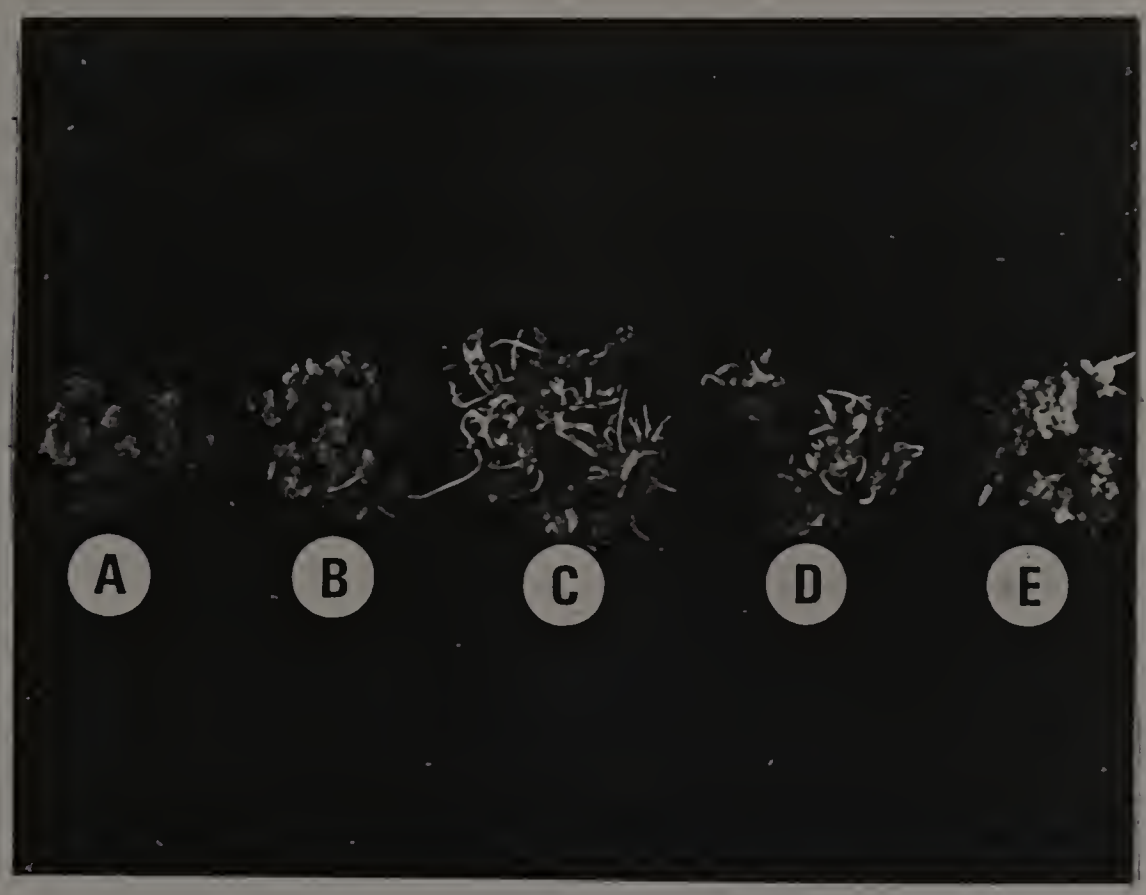

Figure 2. The effects of elevated sucrose on somatic embryogenesis and precocious germination in red fescue callus. (A) 20 g/liter sucrose. (B) 30 g/liter sucrose. (C) 40 g/liter sucrose. (D) 50 g/liter sucrose. (E) 60 g/liter sucrose.

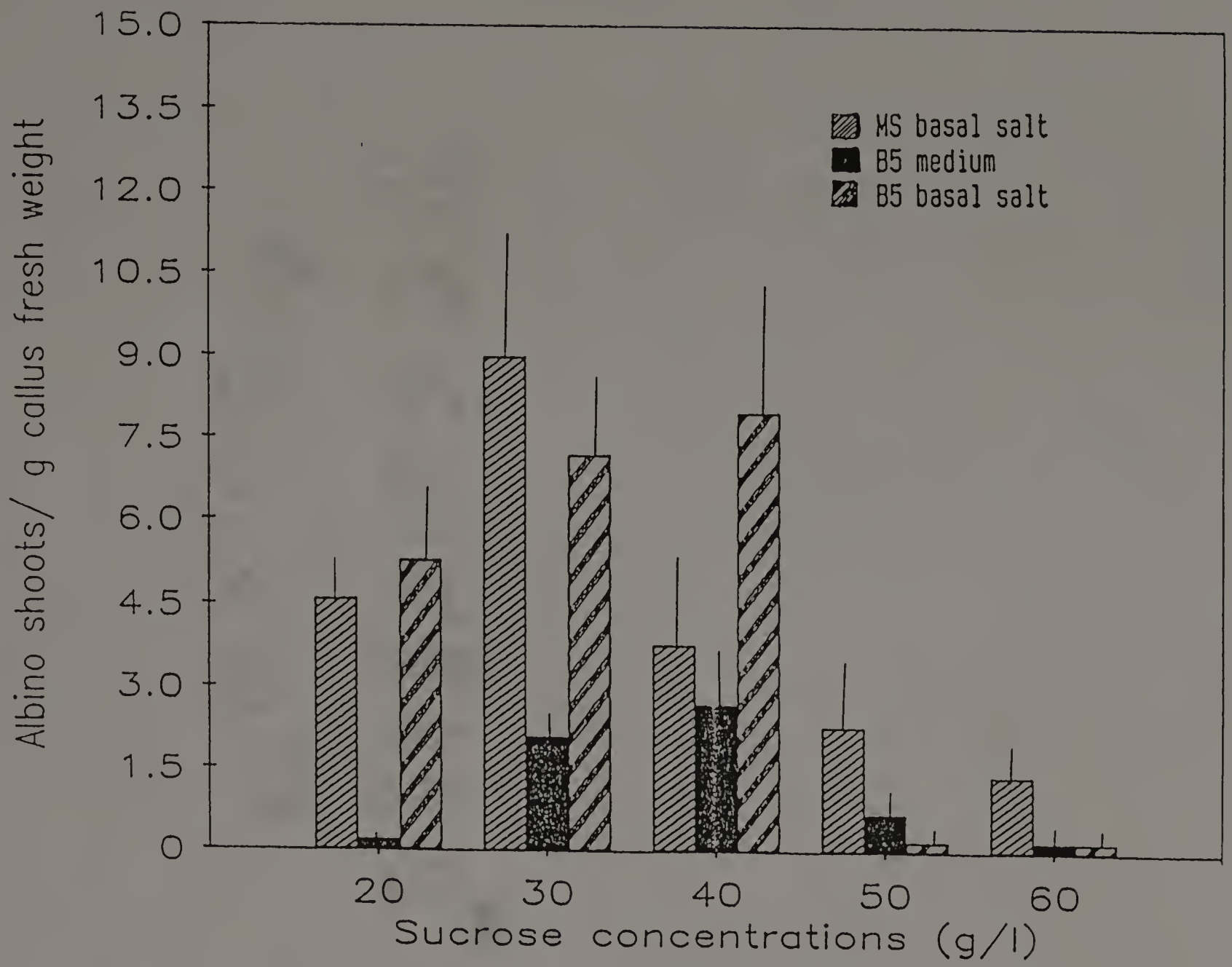

Figure 3. The effects of elevated sucrose levels and three different basal media (MS basal salt, B5 medium, and B5 basal salts) on albino shoots from long-term callus cultures (5 years old) of red fescue. Vertical line on each bar represents standard error of the mean.

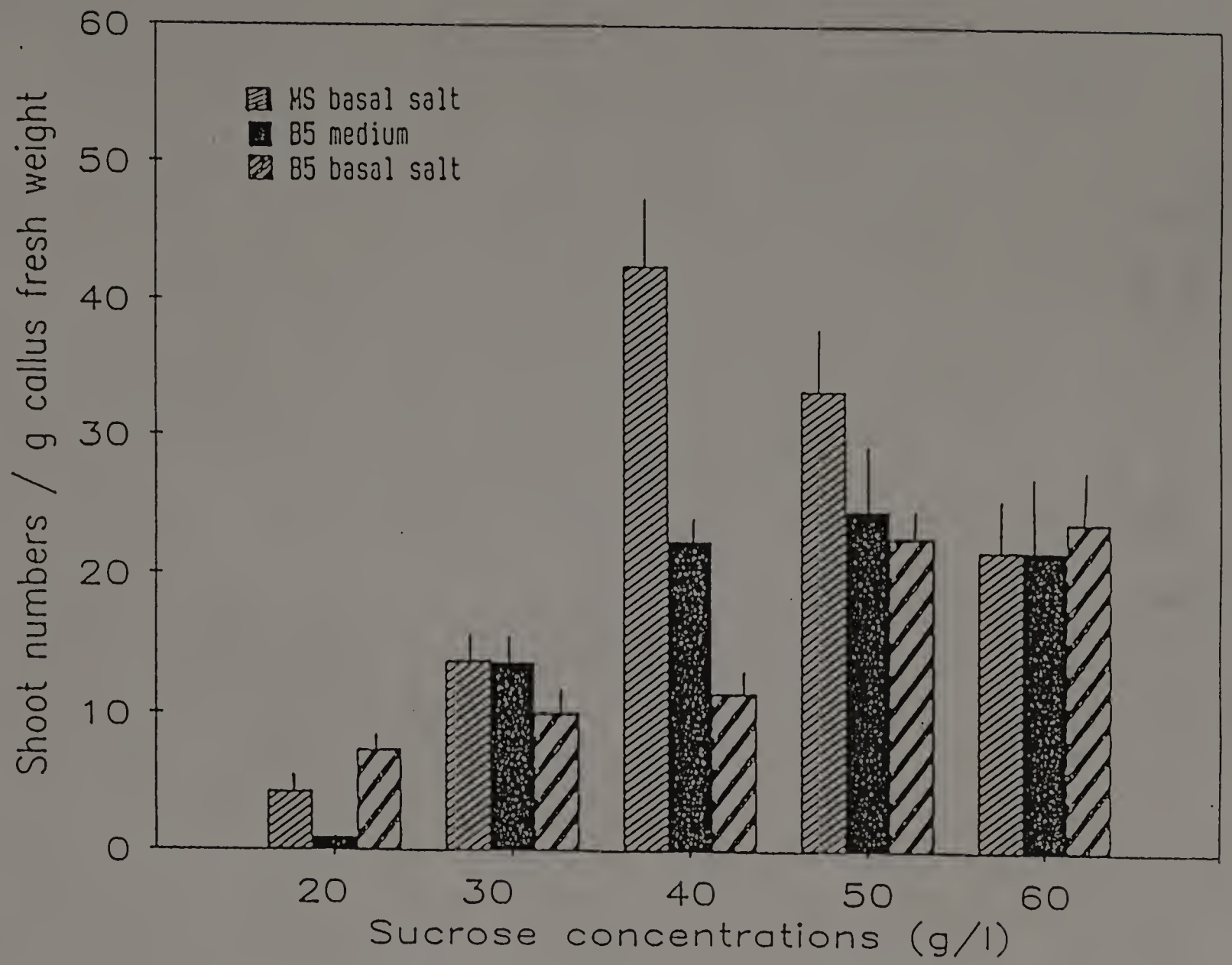

Figure 4. The effects of elevated sucrose levels and three different basal media (MS basal salt, B5 medium, and B% basal salts) on shoots number from long-term callus cultures (5 years old) of red fescue. Vertical line on each bar represents standard error of the mean.

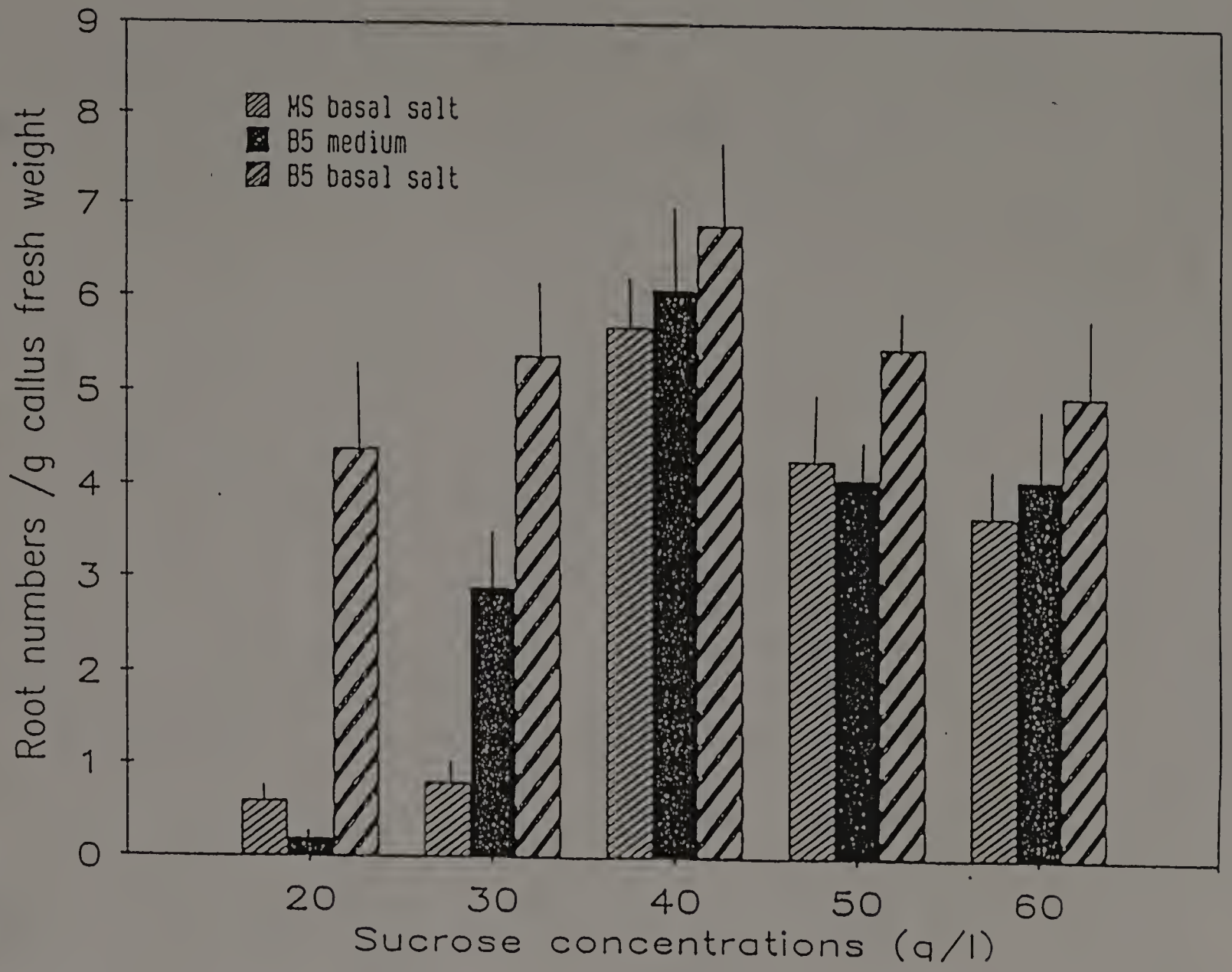

Figure 5. The effects of elevated sucrose levels and three different basal media (MS basal salt, B5 medium, and B5 basal salts) on root number from long-term callus cultures (5 years old) of red fescue. Vertical line on each bar represents standard error of the mean.

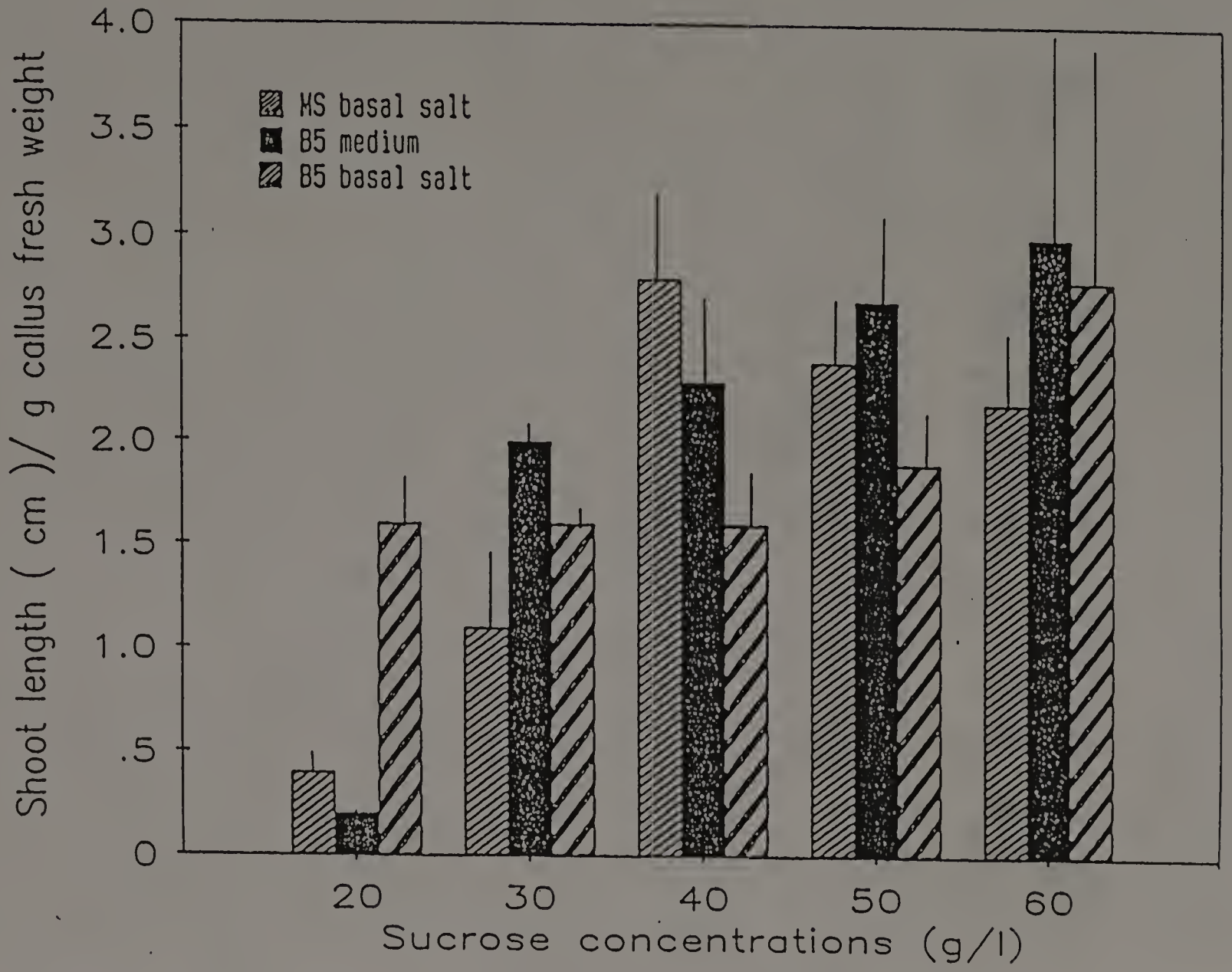

Figure 6. The effects of elevated sucrose levels and three different basal media (MS basal salt, B5 medium, and B5 basal salts) on shoot length from long-term callus cultures (5 years old) of red fescue. Vertical line on each bar represents standard error of the mean.

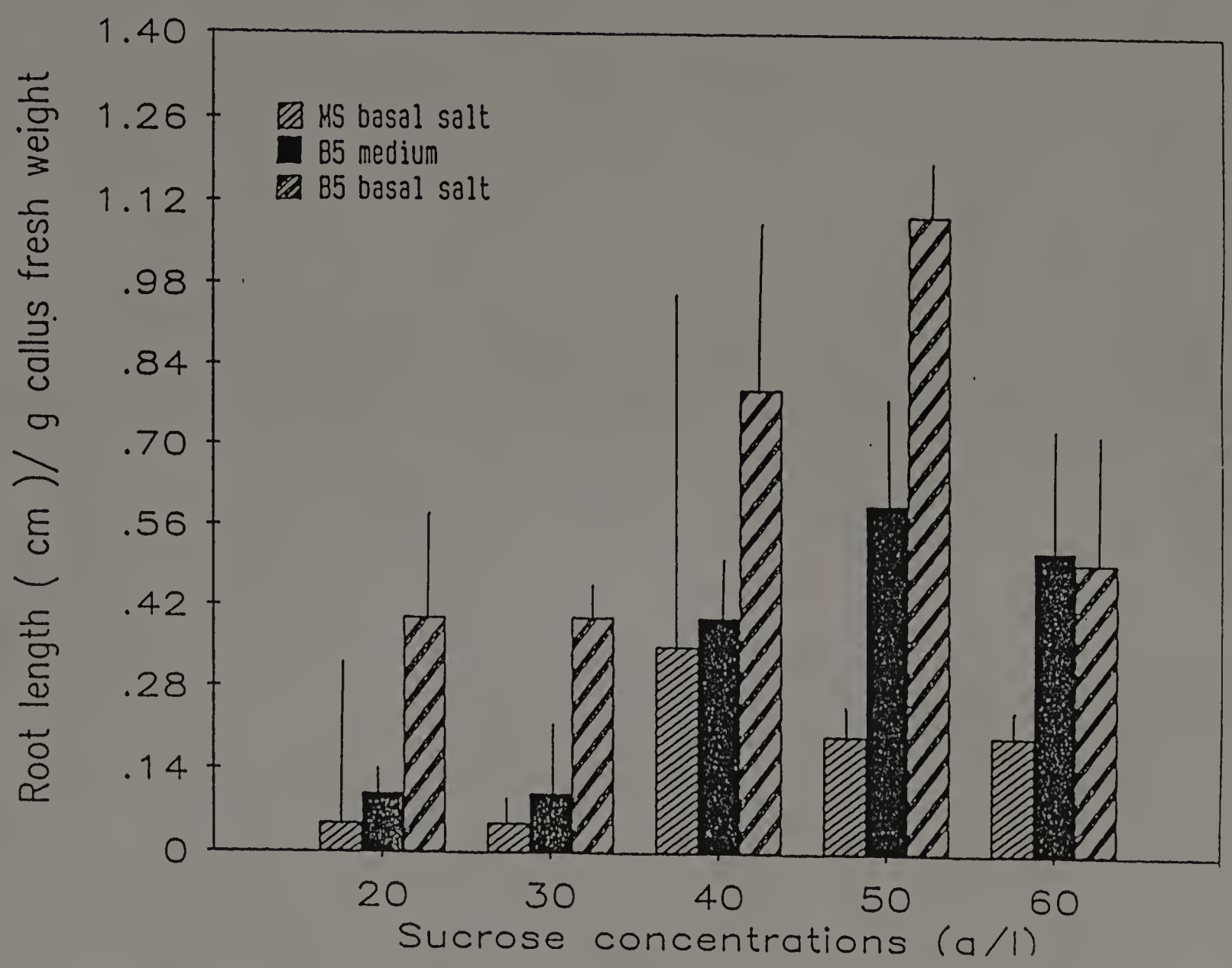

Figure 7. The effects of elevated sucrose levels and three different basal media (MS basal salt, B5 medium, and B5 basal salts) on rodt length from long-term callus cultures (5 years old) of red fescue. Vertical line on each bar represents standard error of the mean.

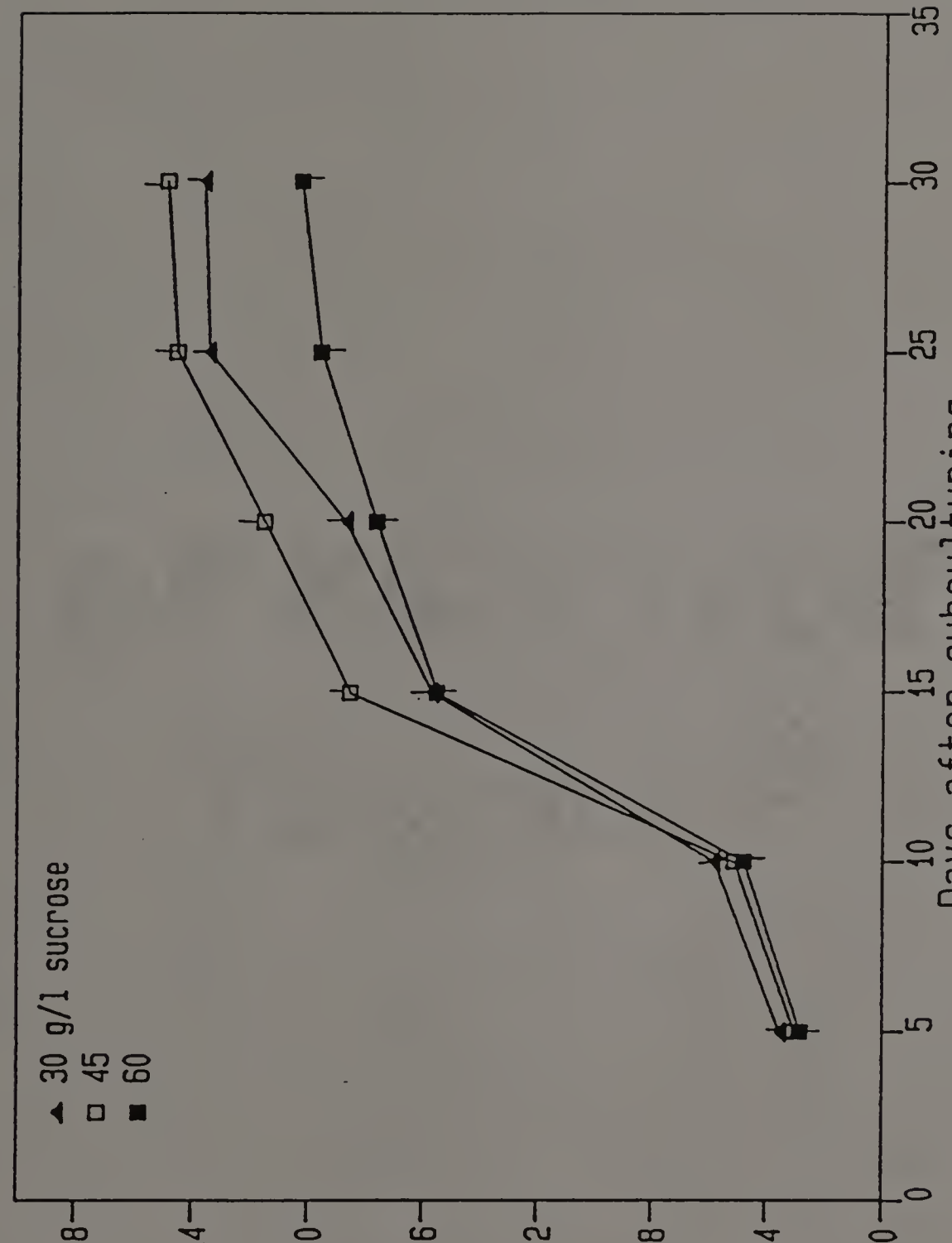

Figure 8: The effect of various concentrations of sucrose on the growth of suspension cultures of 'Dawson' red fescue. Vertical line on each bar represents standard error of the mean.

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CHAPTER V

SOMATIC EMBRYOGENESIS AND PLANT REGENERATION FROM SUSPENSION CULTURES OF PERENNIAL RYEGRASS

Introduction

Improvement of many crop species through somatic hybridization is limited by sporadic plant regeneration from cell cultures. With few exceptions, it has been especially difficult to regenerate plants from suspension cultures and protoplasts of cereals and grasses (8,11,12,13,20,29).

Plant regeneration through organogenesis has been reported to be multicellular in origin and, as such, plants produced may be chimeral (29). Furthermore, organogenesis is unstable and typically ceases or becomes sporadic after a few subculture periods in maize (2), wheat (2), oats (2), Lolium (4), Festuca (4), Phleum (4), Dactylis (4) and many others (29). In contrast, plant regeneration through somatic embryogenesis is of single cell origin (9) with a high degree of genetic stability (11,19). High regeneration rates over prolonged culture periods have also been reported for regeneration through somatic embryogenesis (24,25,26,28,30).

Plant regeneration via somatic embryogenesis has been reported for many turf, forage and cereal grasses such as Echinochloa muricata L. (1), Echinochloa crusgalli L. (1), Festuca rubra L. (23), Dactylis glomerata L. (8), Lolium multiflorum L. (3,5), Panicum maximum L. (11), Saccharum officinarum L. (12) and Triticum aestivum L. (19). Perennial ryegrasses (Lolium perenne L.) is a forage grass with a dual

role as a turf grass. Norstorg (18) has reported the establishment of tissue cultures of perennial ryegrass using immature embryo explants, but no regeneration was reported. Torello and Symington (22) reported plant regeneration from callus cultures through organogenesis. The induction of embryogenic callus and plant regeneration of perennial ryegrass through somatic embryogenesis has been described in Chapter II.

The limited successes in protoplast culture of grasses have depended primarily on the use of embryogenic suspension cells (20,24). Therefore, the objectives of this report were to induce the formation of a rapidly growing embryogenic suspension culture of perennial ryegrass and to evaluate the effect of various growth regulators and other media components on plant regeneration.

Materials and Methods

Callus Induction and Maintenance

Mature, dehusked caryopses of perennial ryegrass (Lolium perenne L. 'Diplomat') were cultured on callus maintenance medium which consisted of Murashige and Skoog basal medium (MS) (17) containing B-5 vitamins (7), 5 mg/l 2,4-D, 30 g/l sucrose, and 8 g/l tissue culture agar (Carolina Biological Co.). Embryogenic callus was induced 3-4 weeks after plating and was subcultured every 6-8 weeks for 18 months.

Induction and Maintenance of Embryogenic Suspension Cultures

Approximately 2 g of embryogenic callus were placed into 250-ml Erlenmyer flasks containing 50 ml of callus maintenance medium without agar. The medium pH was adjusted to 5.7 prior to autoclaving. Cultures were placed on a gyratory shaker at 100 rpm with a 16 h light

cycle at 26 ^{o}C ± 2.0. The resulting suspension culture was composed of mostly elongated, highly vacuolated and irregular shaped non-embryogenic cell types (NE) with very few embryogenic cells (E). Therefore, efforts were directed toward selectively increasing the percentage of E cells. The addition of numerous media components had no effect on selectively enhancing the growth of E cells in suspension. Therefore, separating cell types on the basis of physical characteristics became the only viable alternative for eliminating NE cell types. The first method tested was based upon differences in density between E and NE cell types and entailed the use of a discontinous Percoll gradient. Percoll gradients were sequentially layered from top to bottom at 5, 10, 15, 20, 25, 30, and 35% (v/v) concentrations in 50 ml centrifuge tubes. Five ml of suspension culture were placed onto Percoll gradients after filtering the suspension through a 75 um nylon mesh. Tubes were then centrifuged for 10 min at 80 x g to separate cells based on density. The highest proportion of single and embryogenic cells were sedimented cells at the 20-30% percoll boundries. These cells were removed with a sterile Pasteur pipette and then transferred to 50 ml centrifuge tubes.

The second method for separating cell types was based upon differences in size between E and NE cells. The average E cells of perennial ryegrass are small, ranging between 10-20 um in diameter, compared to non-embryogenic cells which are usually greater than 30 um. As such, this method entailed passing suspension through a nylon mesh with a 31 um pore size. Filtered cells were washed three times with a hormone-free medium by centrifuging for 80 x g for 10 min.

Sedimented cells were then resuspended in a hormone-free medium and used as stock culture.

The effect of cell density ranging approximately between 10^2-10^7 cells/ml of culture medium were studied to determine the optimal cell density for enhancing the formation of embryogenic cell clusters. Media used in this study was the same as in initial experiments except various concentrations of casein hydrolysate (CH) (1 or 3 g/l), cocount water (CW) (5 or 10%), or combinations of both were tested. Following the formation of embryogenic cell clusters, the effect of 2,4-D at concentrations ranging from 2 to 10 mg/l were evaluated to determine the optimal level for suspension growth. The effect of inoculum density at 5, 10, 15, 20, or 25 ml cell suspension to 45, 40, 35, 30, or 25 ml of fresh medium respectively was determined. The effect of elevated sucrose levels at 30, 45, 60 g/l were also evaluated. The medium used in the sucrose and dilution ratio studies was similar to that previously described except 3 g/l CH and 6 mg/l 2,4-D were used. Suspension growth was assessed by counting cells with a hemacytometer and/ or by packed cell volume (PCV). The method used for determing the doubling time is shown in Appendix B.

Every experiment was repeated at least 3 times and every treatment was replicated with 3 flasks. All cultures were placed on a gyratory shaker at 100 rpm and incubated at at 26 ^{o}C $\pm$ 2.0.

Plant Regeneration

To induce somatic embryo germination and plant regeneration, E suspension cell clusters and cell clumps (0.1-3.0 mm diameter) were plated on solidified half-strength MS basal medium supplemented with

45 g/l sucrose, B-5 vitamins, 8 g/l agar, 0.5 mg/l fluridone, and either 0.5 mg/l of benzylaminopurine (BA), 6-furfurylaminopurine (kinetin), or (6-[4-Hydroxy-3-methylbut-2-enylamino]aminopurine) (zeatin).

The effects of silver nitrate added to regeneration media at 10, 20, or 30 mg/l was tested for possible enhancement of shoot regeneration. In a seperate test, cefotaxime was added to suspension culture media at 60 ml/l medium 4 weeks prior to regeneration pretreament of cultures with cefotaxime was used to enhance embryo germination and plant regeneration. All cultures were incubated for 8 weeks under 16.3 Wm^{-2} of flourescent lighting with a 12-hr dark/12-hr light diurnal cycle at 26 $^{o}C \pm 2.0$.

Results and Discussion

Embryogenic suspension cells of Lolium perenne L. were comprised of 2 types of cells. Embryogenic cells (E) were small, rounded, with an average diameter of 10-20 um, and possessing a dense cytoplasm with a low level of vacuolation, whereas non-embrycgenic cells (NE) were highly vacuolated, comparatively large and elongated or ellipsoidal in shape. Embryogenic cells were also more dense than non-embryogenic cells. Newly initiated suspension cultures contained a high level of NE cells and relatively few E cells. Numerous media components were tested to increase the percentage of E cells. The addition of various compounds such as proline, sucrose, mannitol, abscisic acid (ABA), CH, and CW have been reported to enhance embryogenisis in other cultures within the Gramineae (27,28,29). Preliminary tests at wide concentration ranges for each of these compounds had no beneficial

effect and the percentage of NE cells remained high. Altering the dilution ratio of fresh medium to stock suspension has also been used to "dilute out" a high ratio of NE to E cells. Our results indicate that elimination of NE cells was successful only through the use of either a discontinuous pecoll gradient or filtering through a 31-um mesh screen which eliminated most of the large and highly vacuolated NE cells (unpublished data). Resultant suspensions from either method were composed predominantly of single cells with an average diameter of 10-20 um with less than 10 percent of the suspension being small cell clusters composed of 2-4 cells.

The effect of cell density at initiation was found to be critical in relation to the enhancement of cell division and embryogenic cell cluster formation. When the cell density was 10^6 cells/ml of media or greater, embryogenic clusters were formed within two weeks (unpublished data). The beneficial effects of high cell densities are similar to those obtained by Haplerin (10). The presence of a high cell density and the addition of CH were essential for stimulating cell division and shortening the time required for embryogenic cluster formation. The addition of CH was essential in maintaining fast-growing embryogenic suspension cultures (Table 9). Several unsuccessful attempts were made to replace this undefined medium component with combinations of glutamine, glycine, or aspargine. Gray et al. (8) reported that replacement of CH with glutamine, a major component of CH, resulted in the death of Dactylis glomerata L. embryogenic cell suspensions. In general, the omission of CH has yielded root bearing clumps without the production of somatic embryos for a number of grass species (2,8,16).

The effect of dilution ratio during subculture was assessed by using five different suspension/new media ratios. All five ratios tested resulted in rapid growth within the first 9 days after subculturing (Fig. 9). As expected, the maximum amount of PCV was higher when higher cell volumes (20 ml or 25 ml) were used since the initial density was higher. However, the results obtained in this experiment indicate that a low ratio of 10 ml:40 ml (stock culture:fresh medium) had a shorter doubling time along with a total PCV which was similar to higher ratios within 6 days of culture (Fig. 9). As such, this relatively dilute suspension/media ratio was used throughout the remainder of this study.

Higher suspension/media ratios were also shown to greatly increase the production of a mucilagenous substance. Mucilaginous materials were also produced if suspension cultures were left without subculturing for long periods (longer than 15 days). Similar results have been obtained for other grass cultures (8,22-28). Embryogenic capacity can also decrease due to the presence of mucilagenous materials in suspension (8,24,25,26).

The concentration of 2,4-D had a significant effect on the growth rate of suspension cultures (Fig. 10). The shortest doubling time and maximum PCV were observed with 6 mg/l 2,4-D. In a similar study, Gray et al. (8) reported that complete somatic embryo development of Dactylis glomerata L. was obtained even at relatively high 2,4-D concentrations in culture. In this study, somatic embryo develop was not inhibited by a relatively high 2,4-D concentration (Fig. 10).

There have been several reports concerning the beneficial effect of high sucrose levels on somatic embryogenesis (13,29,30,32). As such, the effect of sucrose on the degree of cell division and doubling time expressed as an increase in PCV was evaluated.

Figure 11 shows the comparison of PCV between cell suspensions subjected to 30, 45, or 60 g/l sucrose. Both 45 and 60 g/l levels resulted in more rapid growth rates than the typical 30 g/l level. Elevated sucrose levels have also been shown to enhance the degree of embryogenesis in callus cultures of red fescue (Festuca rubra L.) as shown in chapter 4.

The stages of somatic embryo development in perennial ryegrass suspension cultures were similar to those which have been reported to take place in zygotic embryo development (Figure 12). The doubling time for suspension cultures was greater when embryogenic clumps (0.1-0.3 mm diameter) developed in suspension. The development of embryogenic clumps in suspension was characteristic of fast growing cultures but numerous single cells were always present.

Although a large number of embryos at various stages of development were observed in embryogenic suspension cultures, very few embryos germinated to form plants. Abscisic acid and gibbrellic acid have been reported to promote embryo maturation and to enhance embryo germination (29). Neither growth regulator was found to be beneficial in this study when each was tested alone or in combination. The addition of various cytokinins to the standard regeneration medium (SRM) was beneficial but resulted in only albino plant formation (Table 10). A few green plants were produced when 0.5 mg/l fluridone was added to SRM. Fluridone is an inhibitor of carotene and ABA

synthesis (6). Srinivasan et al. (20) reported that fluridone increased the number of leaves in shoots produced from protoplast-derived callus of sugarcane. Addition of $AgNO_3$ only increased the number of albino plants regenerated. Some green plants were regenerated when suspensions were pretreated with 60 ml/l cefotaxime for 4 weeks prior to transfer to SRM supplemented with 0.5 mg/l BA and 0.5 mg/l fluridone (Table 10). The extent of embryogenesis was enhanced and the number of shoots regenerated increased with cefotaxime pretreament but, again, most shoots were albino. Mathias and Boyd (14) have reported similar results in that cefotaxime increased the number of regenerated plants significantly from wheat callus. It may be that the age of these suspension cultures (18 months) is responsible for the high degree of albinisim. Further work is underway in promoting green plant formation from perennial ryegrass suspension cultures.

Table 9. The effect of casein hydrolysate (CH) and coconut water (CW) on the formation of embryogenic clumps (expressed as PCV) from cell filtered through a 31-um nylon mesh. The PCV was determined 15 days after subculturing.

Addenda used	Mean packed cell volume (ml)
control(no addition)	0.1
CH(1%)	3.0
CH(3%)	5.0
CW(5%)	0.5
CW(10%)	0.5
CH(1%)+CW(5%)	3.0
CH(1%)+CW(10%)	3.0
CH(3%)+CW(5%)	5.2
CH(3%)+CW(10%)	5.5
LSD (0.05)	0.25

Table 10. The effect of cytokinins, fluridone and silver nitrate ($AgNO_3$) on plant regeneration from embryogenic suspension cultures of perennial ryegrass and the effect of a 4-week culture pretreament with 60 ml/l cefotaxime prior to transfer to regeneration media.

Treatment (mg/l)	Mean green shoot number	Mean albino shoot number
SRM (control)	0[a]	0
Zeatin		
0.5	0	3.0 ± 2.1
1.0	0	4.2 ± 2.2
2.0	0	3.2 ± 1.4
BA		
0.5	0	3.1 ± 0.0
1.0	0	3.6 ± 1.3
2.0	0	6.9 ± 1.1
Kinetin		
0.5	0	0
1.0	0	4.3 ± 2.0
2.0	0	0
Fluridone		
0.5 BA and 0.5 fluridone	3.1 ± 1.0	7.2 ± 1.0
0.5 kinetin and 0.5 fluridone	0	0
0.5 zeatin and 0.5 fluridone	0	0
$AgNO_3$ +0.5 mg/l BA		
10	0	10.2 ± 4.0
20	0	7.5 ± 4.0
30	0	0
Cefotaxime pretreament		
0.5 BA and 0.5 fluridone	2.1 ± 2.2	12.1 ± 2.2
0.5 kinetin and 0.5 fluridone	0	4.2 ± 1.4
0.5 zeatin and 0.5 fluridone	0	0

[a]Shoot numbers are per 1 ml of PCV.

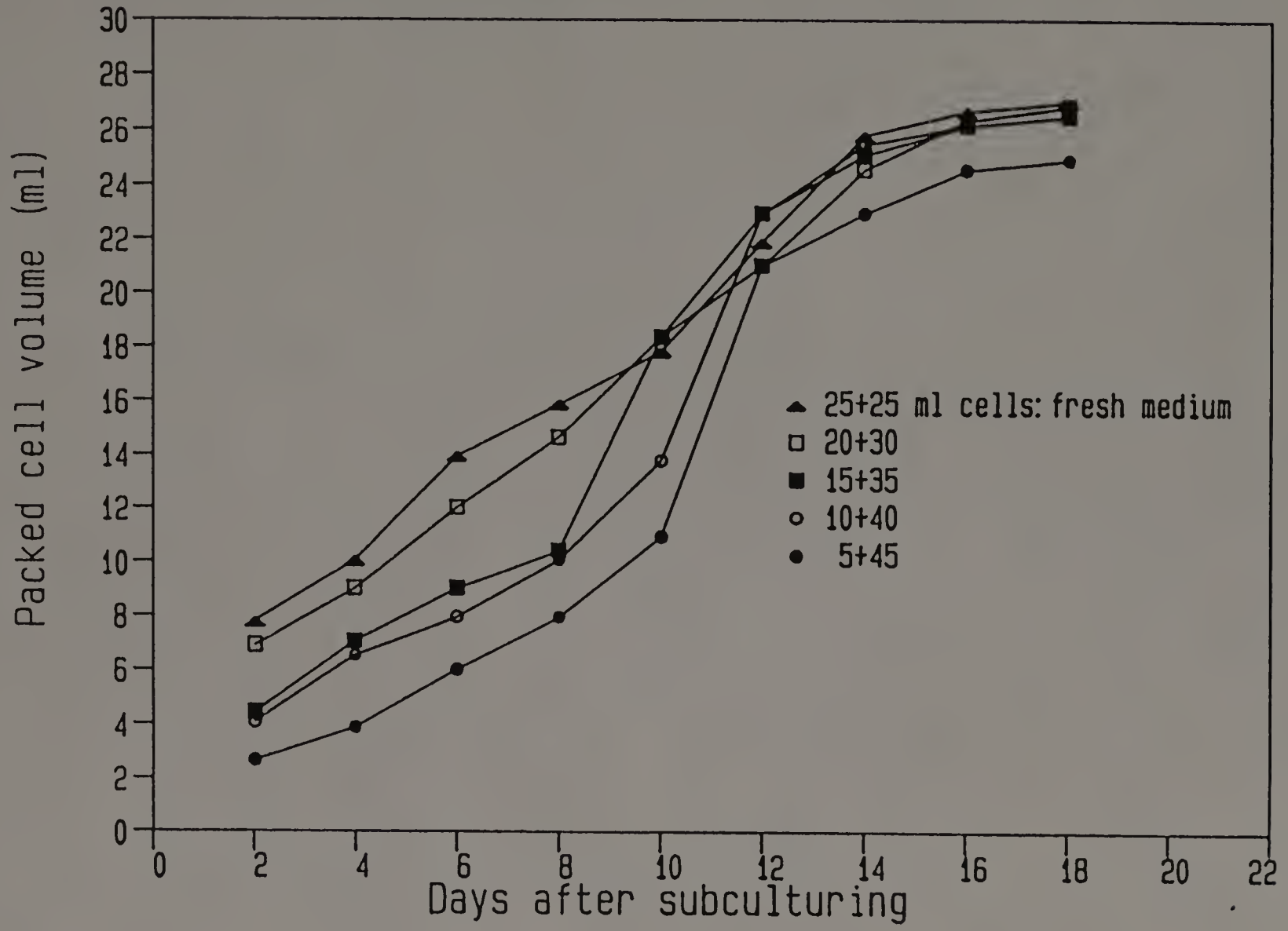

Figure 9. The effect of various dilution ratio on the growth of suspension cultures of 'Diplomat' perennial ryegrass.

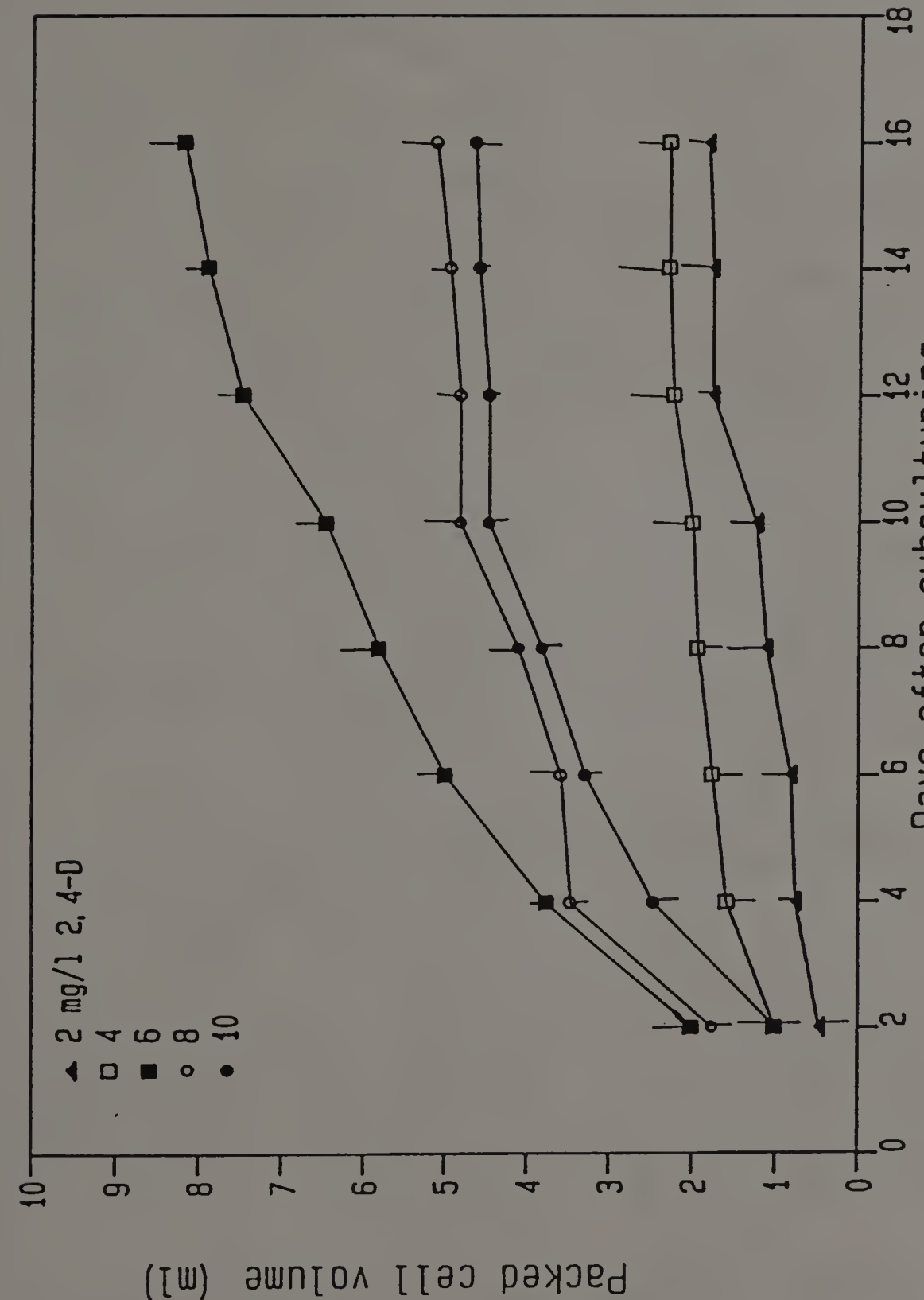

Figure 10. The effect of various 2,4-D levels on the growth of suspension cultures of 'Diplomat' perennial ryegrass. Vertical line on each bar represents standard error of the mean.

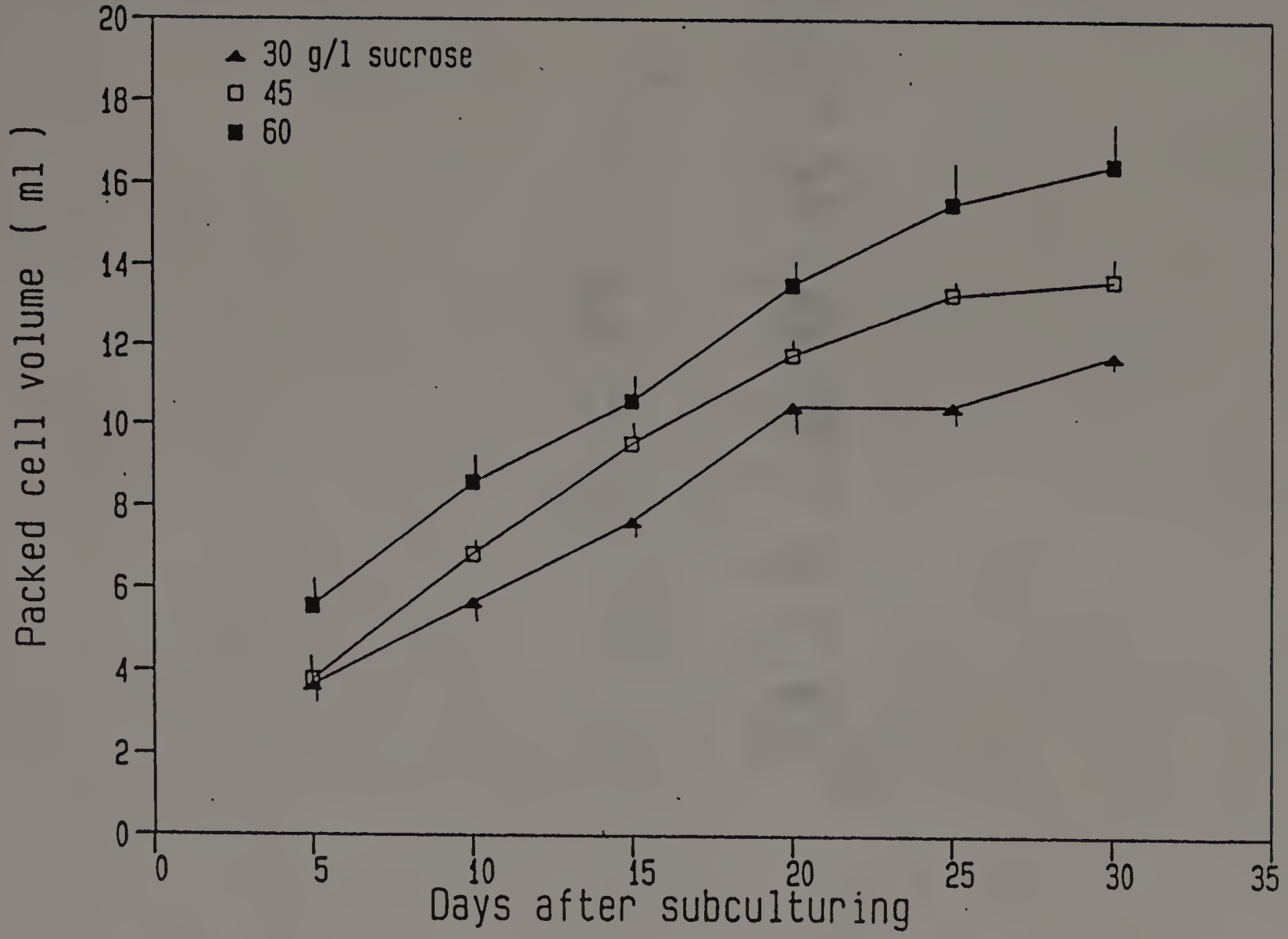

Figure 11. The effect of various sucrose levels on the growth of suspension cultures of 'Diplomat' perennial ryegrass. Vertical line on each bar represents standard error of the mean.

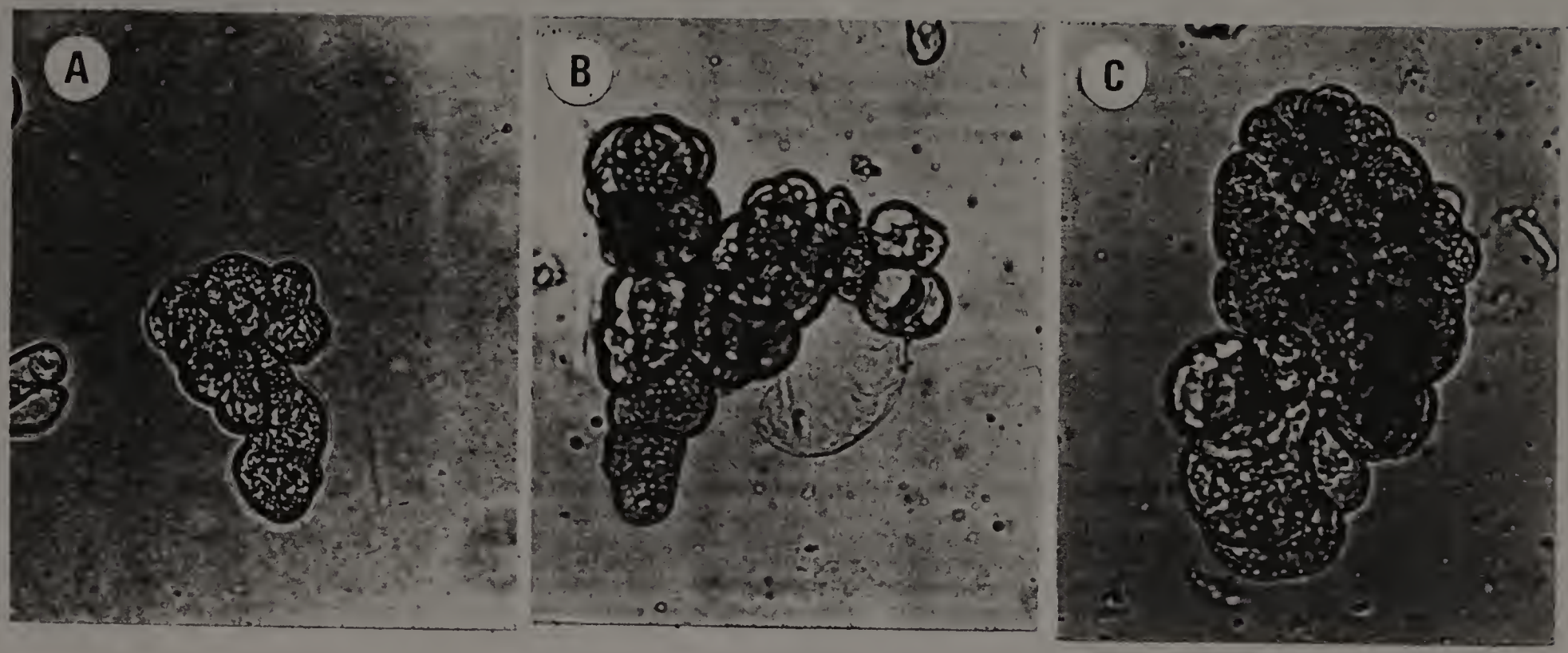

Figure 12. Somatic embryos at various stages of development in suspension cultures of perennial ryegrass. (A) Pro-embryoidal organization. X 200. (B) Initial formation of coleorhiza (Cr) and scutellum (Sc). X 100. (C) Fully developed somatic embryo. X 100.

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C H A P T E R VI

SOMATIC EMBRYOGENESIS AND PLANT REGENERATION FROM SUSPENSION CULTURES OF RED FESCUE

Introduction

The application of genetic transformation techniques for plant improvment of monocot species is limited by the difficulty in regenerating whole, viable plants from cell cultures. As such, it is essential to develop culture systems for plant regeneration from single cells and/or protoplasts (2,4,8,9,11-16).

Plant regeneration via somatic embryogenesis offers the advantages of long-term regeneration potential with a high regeneration rate (7,11,15,16). In monocots, plants regenerated through somatic embryogenesis are usually genetically stable, as opposed to regeneration through organogenesis where regeneration can become sporadic after 3-5 subcultures with a concomitant higher degree of genetic instability (5,7).

Plant regeneration from monocot species, especially grasses and cereals, has proven to be much more difficult compared to dicot species. Somatic embryogenesis has, however, recently been reported for numerous grasses and cereals (17) and is considered the most reliable mode for plant regeneration. Torello and Symington (11) have reported regeneration of whole viable plants through somatic embryogenesis from callus culture of red fescue (Festuca rubra L.).

The capacity for embryogenesis in red fescue was also shown to be stable following long periods of culture (11).

The objectives of this study were to establish a freely-suspended, fast-growing embryogenic suspension culture and to provide detailed observations of the various stages of embryo development leading to plant regeneration.

Materials and Methods

Callus Induction and Maintenance

Detailed procedures on the initiation and maintenance of red fescue callus cultures have previously been described (11). In general, embryogenic callus cultures were induced by placing mature, dehusked caryopses on a half-strength Murashige and Skoog basal medium (MS) (10) supplemented with 100 mg/liter inositol, 5 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D), and 8 g/liter tissue culture agar (Carolina Biological Co.). Embryogenic callus was subcultured every 6-8 weeks for the previous 5 years. Plant regeneration was periodically performed by placement of embryogenic callus on a hormone-free half-strength MS basal medium supplemented with 10 g/liter sucrose and 8 g/liter agar.

Induction and Maintenance of Suspension Cultures

Preliminary attempts to initiate suspension cultures were achieved by placing approximately 2 g of embryogenic callus into 50 ml of agar-free callus culture medium (as described above) The resultant suspension culture was prolific but highly heterogenous in regard to cell type and had less than 1% embryogenic (E) cells. In an effort to selectively enhance the percentage of E cells while gradually

decreasing the total number of nonembryogenic (NE) cells, the effects of various levels of, 1-naphthalene acetic acid (NAA), 6-benzylaminopurine (BA), 6-furfurylaminopurine (kinetin), 6[4-Hydroxy-3-methylbut-2-enylamino]purine (zeatin) and abscisic acid (ABA) were tested at various concentrations and found to be ineffective (Appendix A). In further preliminary studies, concentrations of selected amino acids (proline, serine, glutamine, glycine, arginine, alanine, lysine, tryptophane), sugars (sucrose, glucose, galactose, fructose, mannitol, or sorbitol), or undefined organic compounds such as casien hydrolysate (CH), coconut water (CW), or yeast extract (YE) were evaluated (Appendix A). Efforts were directed toward physically separating E from NE cells using either discontinuous percol gradients (5, 10, 15, 20, 25, 30, and 35 % (v/v) layers) or a filtering method using a 31 um screen mesh. Cells sedimenting at the 15-25% boundary layer of the percoll gradient were collected with a sterile pasteur pipette and transferred to 50-ml centrifuge tubes. Sedimented as well as filtered cells were centrifuged for 10 min at 80 x g, after which the supernatant was decanted off and the pellet resuspended in culture medium. The percentage of E to NE cells was estimated using a haemocytometer and found to be greater than 90% for both methods. Filtered suspensions were used as stock cultures in all further work.

The effect of cell densities (ranging between 10^3-10^7 cells/ml), culture media, and the media components (previously described) were tested using filtered, freely-suspended embryogenic cell cultures in an effort to enhance the formation and growth of embryogenic cell clumps (50-500 um diameter). The medium used during these tests was a

half-strength MS basal medium supplemented with B-5 vitamins (3) and 30 g/l sucrose. The effects of 2,4-D at concentrations ranging from 2 to 10 mg/l and the effects of CH at 1 or 3 g/l with or without CW at 5 or 10% were evaluated.

After suspensions had grown to produce cell clusters or clumps, the effect of dilution ratio was evaluated, using 5, 10, 15, 20, or 25 ml suspension medium to 45, 40, 35, 30, or 25 ml of fresh media, respectively. The method used fro determinig the doubling time is shown in Appendix B except 6 mg/l 2,4-D was replaced with 4 mg/l 2,4-D.

Every experiment was repeated at least 3 times and every treatment was replicated at 3 flasks. All cultures were placed on a gyratory shaker with 100 rpm and incubated at 26 $^{o}C \pm 2.0$.

Plant Regeneration

Plant regeneration was accomplished by transferring embryogenic cell clusters (averaging 1-5 mm in diameter) which developed over a 4-week period in suspension culture to the surface of a hormone-free 1/2 strength MS basal salt medium supplemented with B-5 vitamins, 30 g/l sucrose, and 8 g/liter tissue culture agar. Ten ml of that media were dispensed into 100x15 mm petri dish. Thereafter, cell aggregates were spread evenly on the surface of the above mentioned medium. All cultures were incubated under 16.3 $W.m^{-2}$ provided by fluorescent irradiation) with a 12 h diurnal cycle at $26^{o}C \pm -2.0$.

Results and Discussion

Among all treatments tested only CH has enhanced cell division in suspension culture. The percentage E to NE cells was, however, still

unacceptably low no matter what media components were used. In the numerous preliminary tests, suspension cultures retained a very low percentage of E cells. Embryogenic cells have been described as being spherical with an average diameter of between 10-25 um, with numerous starch granules and highly cytoplasmic with a negligible degree of vacuolation (Fig.15. Plate A). Non-embryogenic cells with an average diameter greater than 30-um were most prevalent, more vacuolated, and comparatively more elongated. Initial attempts to increase the percentage of E to NE cells by manipulating the cell density during subculture or addition of the many previously described compounds were not successful. Suspensions remained highly heterogenous in regard to cell type and consisted mainly of NE cells.

The use of both the percoll dicontinuous gradients and 31-um filtering method resulted in removing more than 90% of the NE cells. Separation by the percoll gradient method which is based upon cell density, was more tedious and time- consuming and cell division was slow and somewhat less synchronized.

Haplerin (6) has reported that the initial cell density per unit of fresh medium during subculturing was critical for inducing embryogenic clump formation in carrot. Similar results were obtained in this report, provided that the cell density during subculture was higher than 10^6 cells/ml media. Pre-filtered embryogenic cells had a tendency to agreggate and form clumps but many single cells were present during all phases of culture.

The addition of CH at either 1 or 3% increased culture growth and enhanced the proliferation of embryogenic clumps (Table 11). Similar

results were obtained for Dactylis glomerata L. by Gray et al. (4). Attempts to replace CH with other combinations of amino acids which have been reported to be present in CH were not successful (1). Coconut water has been reported to promote culture induction and enhance embryogenic cluster formation in grasses (4,8,9,12). Cocount water alone or in combination with CH had no beneficial effect on cell division or clump formation (Table 11).

The 2,4-D concentration had a significant effect on the growth of embryogenic cell suspensions. The fastest growth rate and maximum packed cell volumes (PCV) were attained at 4.0 mg/l 2,4-D (Figure 13).

The effect of dilution ratio during subculture (cell cluster volume/volume of subculture media) is shown in Figure 14. All five diltution ratios showed increases in PCV over time. Although the PCV for the dilute 5:45 and 10:40 ml ratios were initially low, they exhibited very fast rates of cell division and and comparatively shorter doubling times. All dilution treatments eventually had a similar PCV after 18 days in culture. The importance of dilution ratio was demonstrated by Ho and Vasil (8), Lu and Vasil (9) and Vasil and Vasil (12-16) where the addition of 8 ml stock culture into 40 ml of fresh media resulted in a suspension with a higher rate of division. The Figure 15 shows various stages of development of embryo formation in suspension culture. The result of various methods used for plating the freely-suspended cells is shown in Appendix C.

Plant Regeneration

Approximately 3 days after plating, green areas were noticed on the surface of most clumps from which shoots eventually emerged. All

regenerated plants were green and normal. Although prolific, the number of plants regenerated did not equal the number of embryos observed on the plated cell clumps which were, in essence, small calli. Non-synchronization of suspension growth might be responsible for incomplete regeneration, since the developmental stage of embryos in suspension was not the same.

After 18 months in suspension culture, all regenerated plants were appeared phenotypically stable (Fig. 16). Somatic embryogenesis in red fescue also provides the advantage of regenerating numerous plants after prolonged culture periods. Embryogenic suspension cultures of red fescue are currently being used for protoplasts isolation and culture, and should prove useful for the production of totipotent protoplasts from which a complete and successful plants regeneration can be produced.

Table 11. The effect of casein hydrolysate (CH) and coconut water (CW) on the formation of embryogenic clumps from cell suspensions initially filtered through 31 um nylon mesh. All media contained half-strength MS salts, B-5 vitamins, and 4.0 mg/l 2,4-D. Packed cell volumes were determined 30 days after subculturing.

Treatment	Packed cell volume (ml)
Control	0.2[z]
CH(0.1%)	2.8
CH(0.3%)	3.9
CW(5%)	0.35
CW(10%)	0.35
CH(0.1%)+CW(5%)	3.0
CH(0.1%)+CW(10%)	3.3
CH(0.3%)+CW(5%)	4.1
CH(0.3%)+CW(10%)	4.3
LSD (0.05)	0.13

[z]Represents the mean of 3 replicates.

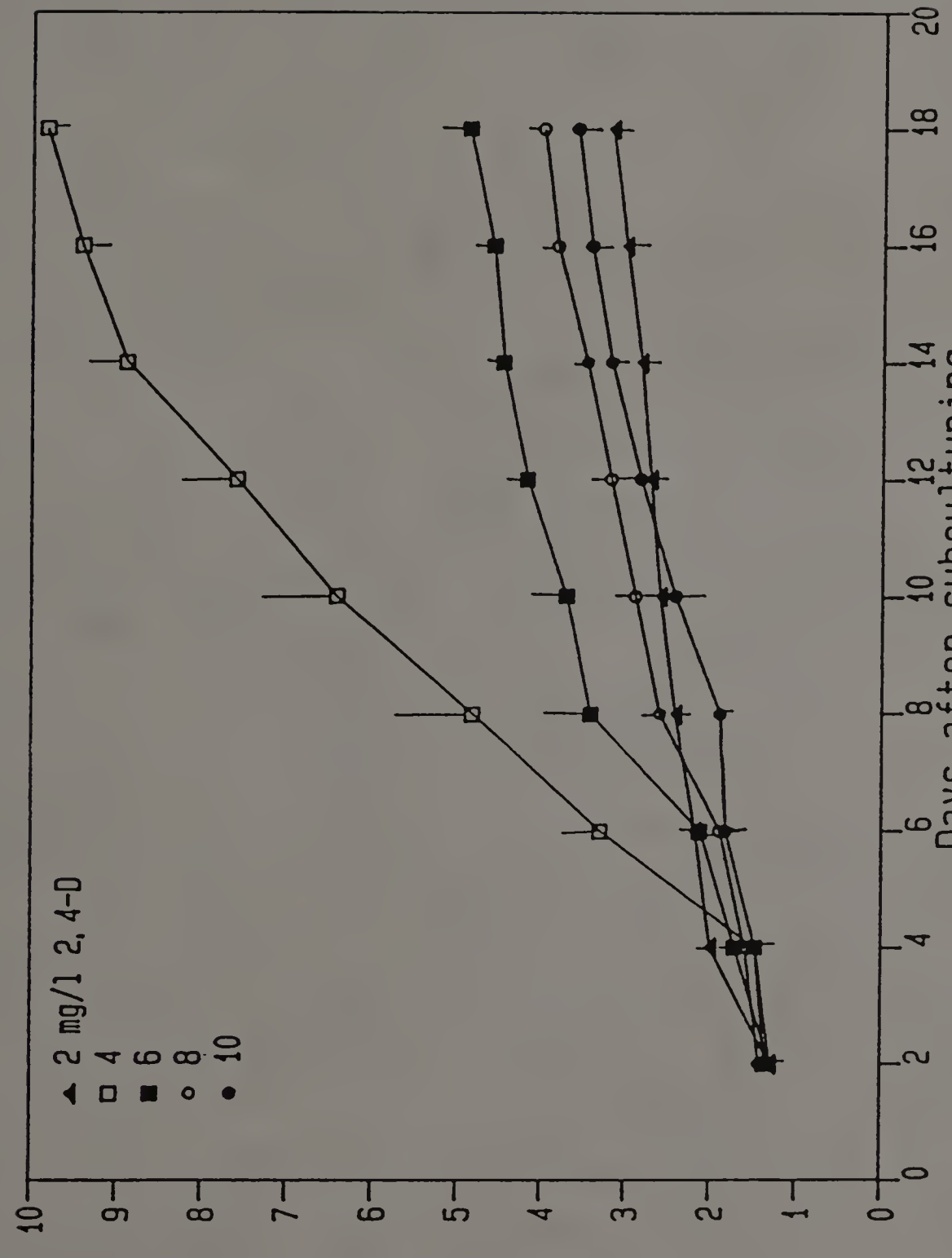

Figure 13. The effect of various 2,4-D levels on the growth of suspension cultures of 'Dawson' red fescue. Vertical line on each bar represents standard error of the mean.

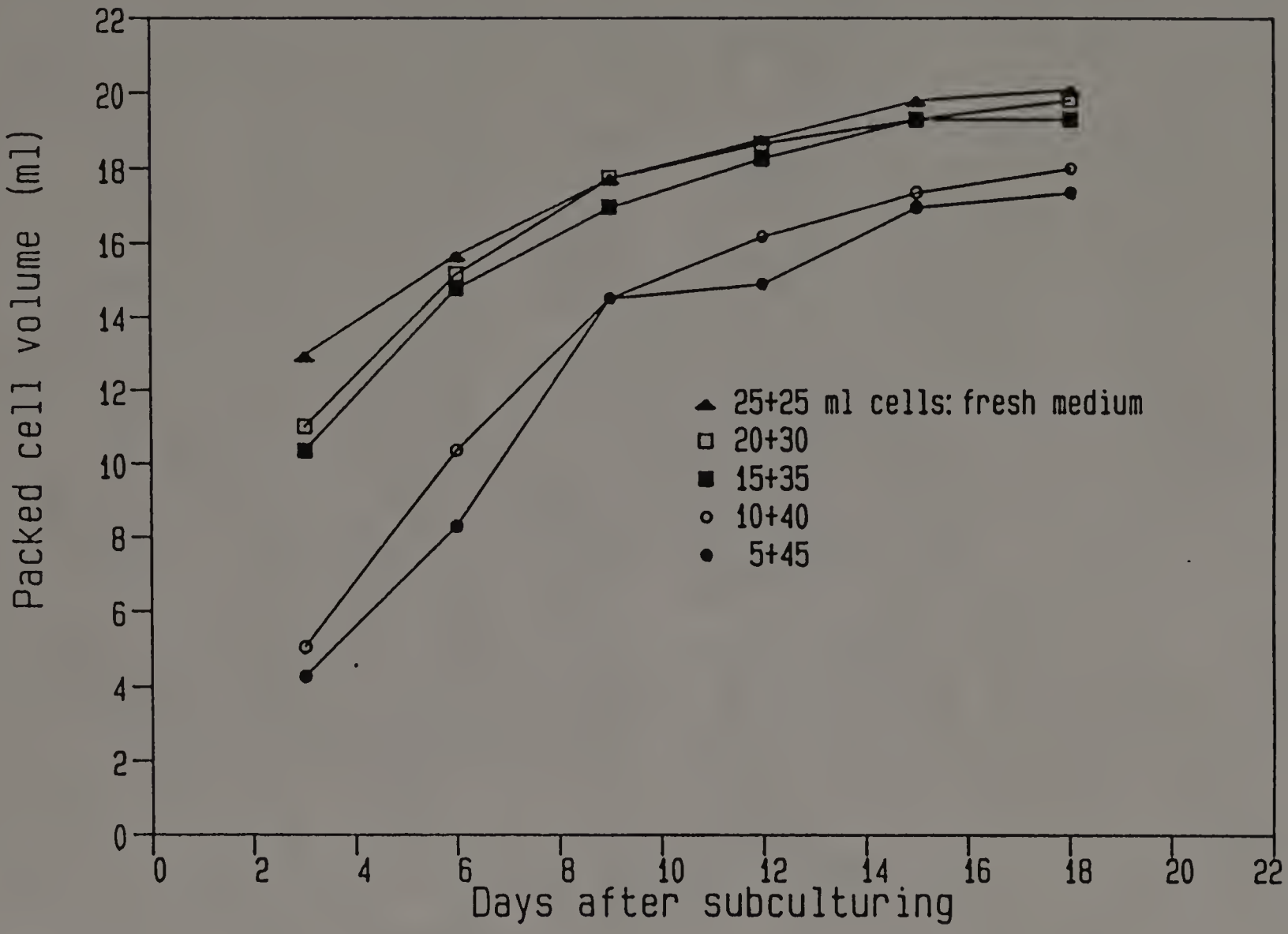

Figure 14. The effect of various dilution ration on the growth of suspension cultures of 'Dawson' red fescue.

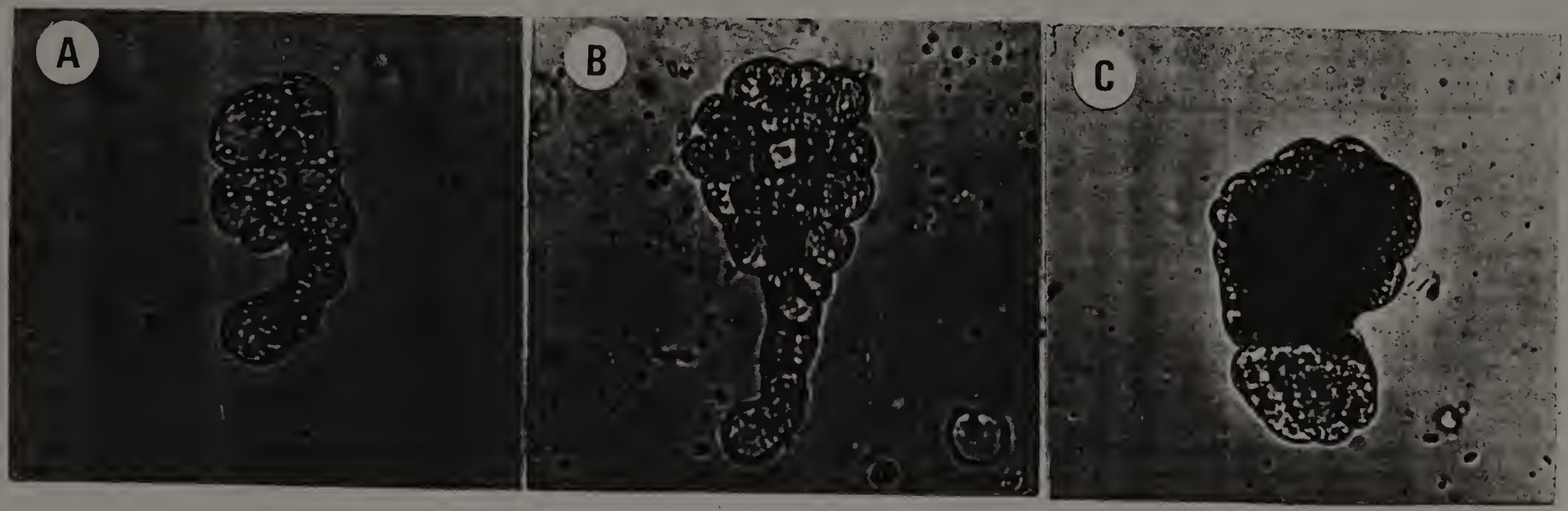

Figure 15. Somatic embryos at various stages of development in suspension cultures of 'Dawson' Red Fescue. (A) Pro-embryoidal organization. X 200. (B) Initial formation of coleorhiza (Cr) and scutellum (Sc). X 100. (C) Fully developed somatic embryo. X 100.

Figure 16. Plant regeneration from embryogenic cell suspension culture aggregates of red fescue.

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C H A P T E R VII

REVIEW OF PROTOPLAST ISOLATION AND CULTURE

Introduction

Numerous forage grass species have a dual role as turf-type grasses. Increased forage production and quality is essential to productivity within the meat, milk and wool industries. The multi-billion dollar turfgrass industry relies increasingly upon the introduction of new cultivars capable of performing under minimal maintenance and stressfull environmental conditions. A major advantage to cell culture procedures is found in providing new genetic variability which would be difficult or impossible to achieve by conventional methods of plant breeding. Therefore, there is no doubt of the potential rewards of protoplast technology are great.

There has been much interest in the application of protoplast technology for crop improvement by numerous methods, including direct genetic transformation. However, in monocots, most transformation or manipulative methodology must be preceded by the development of successful and repeatable protocols for protoplast isolation and culture followed by plant regeneration.

Since the initial successes of Takebe et al. (50) and Nagata and Takabe (40) in regenerating plants from mesophyll protoplasts of tobacco (Nicotiana tabaccum L.), numerous attempts have been made to obtain plants from protoplasts of other plant species.

Dicots have been found to be much more responsive to plant regeneration and somatic hybridization techniques than are monocots. Despite prolonged and extensive efforts, protoplasts of Graminaceous crops, including the cereals and grasses, continue to be relatively unresponsive, with rare exception, to treatment to induce plant regeneration (61).

The present position of protoplast culture in monocots from published reports can be summarized as follows:

A. There has been no notable progress toward the reproducable and sustained division of protoplasts isolated directly from differentiated plant tissue.

B. There have been a number of reports of protoplasts derived from cell cultures forming non-morphogenic cell cultures (5,8,32,60).

C. Several instances have been reported on plant regeneration from protoplasts isolated from embryogenic cell suspension cultures (2,60).

The regeneration of plants from protoplasts of Panicum maximum L. (38) and Pennisetum purpureum (59) has been very encouraging, athough regenerated plants did not progress beyond the small plantlet state. Recently, plant regeneration from protoplasts which developed into mature plants have been reported for Oryza sativa L. (14,37,60) and Saccharcum officinarum L. (49). The source of protoplasts in both cases was from embryogenic cell cultures. Procedures for maintaining morphogenic potential in suspension cultures and plant regeneration from protoplasts derived from them appears to be repeatable for these two species but varies considerably within other grass species.

Because of the general intractability of monocot protoplasts isolated directly from differentiated tissues, little information can be drawn from results of experiments concerning the effects of different treatments and procedures although numerous unsuccessful protocols have been reported. Information from model species (tobacco, petunia, potato, etc.) is undoubtedly valuable for application of protoplast techniques in difficult species. While the genetic principles seem to be similar for both, it is a mistake to assume that culture conditions and procedures are transferable. Such assumptions risk limiting our thinking and approaches to what are, in many respects, fundamentally different tissue culture systems.

A number of stages are required for protoplast culture and successful plant regeneration. Most of the variables (and they are numerous) have been approached empirically. There is rarely any clear idea of what is important and why certain treatments work and others do not.

1. Effects of Plant Genotype. There is considerable evidence for genetic control of cultured tissue and protoplast responses. It has been shown that certain families (e.g. Solanaceae), genera, species and even cultivars are more or less responsive than others to in-vitro treatment (30,48). Although there is much evidence of such variable responses, there are no reports on why some genotypes respond and others do not.

2. Environmental Effects. Plant growth and development is influenced by the surrounding environment and, therefore, it is not surprising that cells and protoplasts taken from plants exposed to

different conditions react differently. As such, previous growing conditions of donor plants will most likely have significant effects upon the potential for developing successful tissue culture protocols. Such effects have been reported for a wide range of pre-culture conditions (4).

3. Source of Protoplasts and Culture Conditions. Several thousands of culture conditions have been tested using differentiated mesophyll protoplasts isolated from numerous grasses and cereals (46). Only in one instance has cell division been sustained (47) leading to the formation of a callus culture which was non-morphogenic. There may be a novel growth regulator or a particular set or series of conditions which might induce mature mesophyll protoplasts to respond. For example, it has been noted that polyamines, which have been found to induce higher levels of nuclear division in isolated oat mesophyll protoplasts, may become recognized as important growth substances. However, isolated mesophyll protoplasts remain highly recalcitrant.

All evidence regarding the response of monocot explants in tissue culture suggests that meristematic plant tissues would most likely yield the most responsive protoplasts (60). A report of root forming cultures derived from protoplasts isolated from immature leaf sheaths of rice supports this concept (15). However, reproducible division of protoplasts from meristematic regions has not yet been achieved and may be a reflection of either difficulties in obtaining a sufficiently large yield of protoplasts or problems associated with morphogenetic competence (15).

4. Morphogenetic Competence of Cells. Numerous reports have shown that only a relatively small number of cells in a large culture

population are able to rapidly divide and differentiate after prolonged culture periods (57, 58). As such, only a small number of cells are "competent" enough to respond to imposed treatments for regeneration of whole plants. A similar phenomenon is observed in intact plants, where shoot and root apical meristems produce cells which eventually differentiate to form different functional and anatomical tissues. Differential gene expression may be largely responsible for the fact that only certain cells respond to external stimuli while others do not. Therefore, particular growth substances can have very different effects when acting upon cells and tissues of different origins within the plant (e.g. variation in hormone receptors).

Variation in the response of different explant tissues is well known in tissue culture. Within the Gramineae, a high degree of differentiation is usually associated with the loss of the ability to form callus. The response of immature leaf explants illustrate this point. At the base of leaves, callus is readily induced and is usually embryogenic and highly regenerable (63). In contrast, mature and older upper leaf segments usually loose the ability to form callus under similar conditions (63). Therefore, it must be assumed that as cells and tissues differentiate they become committed to particular functions and lose the ability to respond in culture (63). Vasil and Vasil (58) have obtained relatively large numbers of competent cells in embryogenic suspension cultures using an approach which could eventually be the best general method for a range of recalcitrant species, providing that genetic stability can be controlled. Such an

approach may be the only way to provide a large population of responsive protoplasts in species which lose their competence as tissues mature. The difficulty with their approach is in being able to recognize and define the cell types necessary to maintain morphogenetic competence in suspension cultures of different grass species. Recently, there has been a large number of reports on the induction of somatic embryogenesis from numerous grass species. This has been due, in small part, to the ability of workers to recognize early on the types of callus tissue capable of embryogenesis.

The work with protoplasts isolated from cereal and grass suspension cultures clearly demonstrates the mitotic integrity of the protoplasts and their morphogenetic competence. However, the problems currently faced in obtaining mature plants from protoplast-derived calli and somatic embryos must be resolved before a system can be effectively utilized in somatic hybridization or genetic transformation work. For the present and immediate future, embryogenic cell suspension cultures provide the most promising system for cultures of cereal and grass protoplasts. Such cell lines are currently available in Pennisetum americanum (57), P. purpureum (59), Panicum maximum (38), and Zea mays (4,47). Totipotent cell suspension cultures have also been reported in Lolium multiflorum (32) and Saccharum officinarum (49).

Protoplast Isolation, Culture and Plant Regeneration Within the Gramineae

Protoplast isolation

Although a great deal of literature has been published on the isolation, culture and fusion of protoplasts, comparatively little

work has been carried out with cereal and grass species. Early studies of plant protoplasts were hindered due to the lack of methods available in obtaining adequate quantities of protoplasts. Such problems have been overcome with the use of various fungal enzymes used to digest cell wall materials (17,18). After cell wall removal, protoplasts could be stimulated to regenerate cell wall material, form callus tissue, and then differentiate to form complete plants which in general exhibit the characteristics of the original plant (18,28). Many physiological, biochemical and genetic problems which had previously been unapproachable could, be studied using protoplast isolation and culture techniques (17).

The ability of the plasma membrane to undergo fusion has received considerable attention and this phenomenon has yielded the advantage of the formation of hybrid cells, which might ultimately lead to somatic hybrids (17,42). The amount of literature on this subject has become too vast to completely review, however, comprehensive reviews are available (2,11,23,24,26,60).

Procedures used to isolate protoplasts can greatly influence the properties of protoplasts affect their suitability for maintaining viability and subsequent cell division (18,60). Essential in the procedures for protoplast isolation is the removal of cell walls without causing irreversible damage to the protoplast (17). Prior to removal of cell walls, it is essential that cells be maintained in a solution of suitable osmotic potential (17). Mechanical procedures were initially used to isolate protoplasts, however, techniques have been developed for the removal of the cell wall by enzymatic digestion

which are now the predominant methods employed (2,17,18). Treatment of cells with cell wall degrading enzymes has resulted not only in the removal of cell walls but also other effects on protoplasts have been noted (e.g. increases in transaminase activity) (17). The most common enzymes used in the isolation of protoplasts appear to be "Macerozyme," a pectinase complex used to separate cells in combination with "Onozuka R-10," a cellulase complex for the degradation of cell wall materials (2,17,18,26,60). Protoplast isolation can be achieved between one and 18 hours depending upon the plant material, enzyme source and concentration, method of preperation, purification (16,18,26).

There are numerous cellulases, hemicellulases and pectinases from various biological sources now commercially available for protoplast research. Some enzymes are available as purified preparations such as Onozuka R-10. However, it has been shown that the effectiveness of crude and less costly preparations can be improved by gel filtration (11). Some enzyme preparations contain various contaminates, such as nucleases and/or proteases, which can adversely affect protoplast viability.

Protoplast isolation solutions consist of mixtures of enzymes dissolved in media containing osmotic stabilizers, the latter composed usually of various not metabolizable polyols, calcium and/or phosphate salts, and a buffer. The optimum conditions of osmolarity, pH, and enzymes required for protoplast isolation from a particular tissue are usually established empirically. The most common osmotic stabilizers were mannitol, sorbitol, glucose or mixtures of inorganic salts (60). Additions of potassium dextran sulfate have also shown to

be beneficial in stabilizing protoplasts (12,41). Solutions usually include a phosphate or MES (2-(N-morpholino)ethanesulfonic acid) buffer to minimize acidic shifts which may occur during digestion (11,23,28,60).

Various procedures have been employed for protoplast isolation. The tissue may first be treated with pectinase to loosen or release cells followed by incubation with one or more cellulases to digest cell walls (45). Explant tissues can also be subjected to a partial vacuum prior to incubation to facilitate infiltration of enzymes within tissues (51).

Organs or tissues may be placed in a plasmolyzing solution for a period of time, or transferred directly to the digestion mixture. Protoplasts were isolated enzymatically from seedling roots, hypocotyls and cotyledons of flax (Linum usitatissimum L.) (3). The cotyledons were cut into approximately 1 mm wide strips and hypocotyls and roots were cut longitudinally. The tissues for each explant were plasmolyzed in a salt solution containing 13% (w/v) mannitol for 2 hours then incubated in an enzyme solution of 2.0% (w/v) Rhozyme Hp-150, 4.0% (w/v) meicelase and 0.3% (w/v) macerozyme in CPW salt solution with 13% (w/v) mannitol. Enzyme incubation lasted 18 hours at 25 ^{o}C on a rotary shaker (30-40 rpm) (32). Vasil and Vasil (58) reported the isolation of protoplasts by mixing 10 ml of a 4-5 day-old embryogenic suspension culture (originated from immature embryos) of pearl millet (Pennisetum americanum L.) with 60 ml of a filter sterilized enzyme mixture (2.0% cellulysin, 1.0% macerozyme, 0.5% driselase, 0.5% Rhozyme, 0.25 M sorbitol, 0.25 M mannitol, 250 mg/l

glucose, 3 mM MES buffer prepared in a hormone-free medium at pH 5.6), incubated for 1 hr at room temperature followed by 19 hr at 14 ^{o}C in the dark (56).

After incubation, undigested tissue is removed by gravity filtering through a stainless steel (or nylon) filter and protoplasts are then collected and washed several times (in culture media) by centrifugation at ca 100 X g. The finer cell debris including fibers, tracheids etc. can be difficult to remove. Procedures designed to separate protoplasts from debris include floating on a 20% sucrose solution (15), aqueous dextran or a polyethylene glycol two-phase system (34), as well as numerous other methods (9,11,14,18,26, 29,37,60).

Lu et al. (39) have also reported the isolation of protoplasts from embryogenic suspension cultures derived from immature embryos and inflorescences of Guinea grass (Panicum maximum Jacq.) by mixing 0.75-1.5 mil of a 4-5 day-old suspension culture with 8-10 ml of a filter sterilized enzyme solution (2.0% Onozuka R-10, 0.15% pectolyase, 0.25% driselase, 0.5% Rhozyme, 0.15 M mannitol, 0.15 M sorbitol, 7.0 mM $CaCl_2$, 0.7 mM NaH_2PO_4 and 3.0 mM MES buffer at pH 5.6). Results have shown that protoplasts isolated from immature embryo and inflorescence-derived suspensions behaved similarly in culture (39). A large number of protoplasts divided and grew in a liquid culture medium to form hundreds of embryogenic cell aggregates and many globular proembryoids. Upon transfer to an agar medium, embryogenic callus was obtained which produced numerous embryoid-like structures. These embryoids germinated precociously to form green plants (39). The importance of using embryogenic cell suspensions as a source of

totipotent protoplasts from cereals and grasses have been noted in many reports (39,50,54-60). Jones and Dale (33) have also isolated viable protoplasts from embryogenic suspension cultures originating from embryogenic callus derived from immature embryos of Italian ryegrass (Lolium multiflorum L.). Protoplasts were isolated from suspension cultures during the log phase of growth (4 to 5 days after subculture). The isolation mixture was based on conditioned media and consisted of 10.0% mannitol, 1.0% meicelase, 1.0% cellulase R-10, 0.3% macerozyme and 0.1% pectolyase Y23. Chourey and Sharpe (7) described procedures for the establishment of cell suspension cultures from three cultivars of Sorghum bicolor. Protoplasts derived from these cultures showed sustained divisions leading to callus formation. The same authors reported that in several instances such calli have been returned to liquid medium giving rise to "protoclone" cell suspension cultures (7). Protoplasts from sorghum were isolated by the method described for maize [2.0% (w/v) cellulysin, 1.0% (w/v) driselase and 0.5% (w/v) pectinase dissolved in an osmoticum containing 0.2 M mannitol, 80.0 mM $CaCl_2$ and 0.5% w/v MES buffer at pH 5.8] (7). Vasil and Vasil (61) isolated protoplasts from an embryogenic cell suspension culture of Pennisetum purpureum which divided and produced globular embryoids and embryogenic masses. Cell masses continued growth on agar medium to form compact calli with embryoidal structures that eventually gave rise to green plants. The protoplasts were isolated from a 3-4 day-old suspension culture and the enzyme mixture consisted of cellulase R-10 (2.5%), mannitol (0.2 M), sorbitol (0.2 M), $CaCl_2$ (7.0 mM), NaH_2PO_4 (0.7 mM), and MES buffer (3.0 mM), at pH

5.7. Although somatic embryos and plantlets have now been obtained from protoplasts of P. americanum (55), P. maximum (39) and P. purpureum (59), it has not been possible in any case to grow the plantlets to maturity. Somatic embryos of Gramineae species formed in callus and cell suspension cultures germinate and grow into mature plants, but protoplast derived embryoids have thus far failed to do the same (55). Furthermore, somatic embryos formed in callus cultures are apparently easier to regenerate into plants than those which develop from plated suspension cultures. Increasing dissociation of cells from callus to cell suspensions to protoplasts appear to make it progressively difficult for the organization and maturation of somatic embryos and their subsequent development into mature plants (39,55,60). Heyser (30) described a system for the regeneration of callus and albino plantlets from protoplasts of proso millet (Panicum miliaceum L.). Protoplasts (2 x 10^6/g freshweight) were released from filtered cell clumps with 0.5-1.0% cellulysin and 0.1-0.2% Rhozyme in 600 mM KCl, 2.0 mM NH_4NO_3, 3.0 mM $CaCl_2$, 1.0 mM KH_2PO_4 at pH 5.5 diluted with conditioned LS medium.

Protoplast Culture

The recent upsurge in interest in plant cell and tissue culture and its applications toward plant improvement is a result of the development of numerous novel techniques associated with somatic genetic manipulation (60). Protoplasts can be cultured in liquid and occasionally on solidified media (32). When cultured in a liquid medium protoplasts may be incubated in 25-50 ml flasks (32). A convenient procedure consists of culturing suspensions of protoplasts

in liquid droplets (11,35). The protoplasts are suspended in culture medium at ca 10^4/ml which is usually distributed in 50-100 ul drops in petri dishes sealed with Parafilm and incubated in humidified plastic boxes at 25-28 ^oC under low light intensity or darkness (11). After cell wall regeneration and division has been initiated, fresh media with a lower osmolarity is added. Eventually a cell suspension culture may be formed. Alternatively, cells grown in liquid can be transferred to agar media by mixing with an equal volume of agar nutrient medium cooled to about 40 ^oC (11,42). Aliquots are then poured into petri dishes which are sealed with parafilm and incubated (59). The advantage of this procedure is the fixed or embedded position of cells which facilitates further observation of individual cells or cell clusters.

Protoplasts have nutritional requirements similar to those of cultured plant cells. The mineral salt levels established for plant cell cultures have been modified to meet the particular requirements of protoplasts (24,25). A typical medium consists of:

1. Osmotic stabilizers: mannitol and sorbitol have been the compounds most frequently used to maintain osmolarity and are used separately or in combination. Very little is known about the rate of absorption and metabolism of these compounds by plant cells, but sorbitol can be utilized by cell cultures as a carbon source (10). Glucose could also be used as an osmotic stabilizer as well as various combinations of mineral salts.
2. Inorganic nutrients: mineral salts supply the inorganic nutrients required by plant cells. Protoplasts have been

cultured on a range of mineral salt media varying widely in total salt concentration (25). It has proven beneficial to increase the calcium concentration of protoplast media by 2-3 times over that normally used for plant cells (4-6 mM) (7,24,60). After cell regeneration and division have been re-established, regular plant cell culture media are satisfactory.

3. Carbon sources: Glucose is perhaps the preferred and most reliable carbon source. Plant cells grow about equally well on glucose and sucrose, however, sucrose alone may not always be satisfactory for plant protoplasts (35). Some media contain 1-3 mM ribose or (some other pentose) as supplementary carbon sources (35,36,45).

4. Vitamins: The vitamins used for protoplast culture generally include only those present in standard plant tissue culture media. If protoplasts are to be cultured at very low densities in defined media, there may be a requirement for additional vitamins (36).

5. Organic nitrogen supplements: Protoplast media frequently contain one or more amino acids. A convenient approach is to add casein hydrolysate (36,43,44) which appears to meet the needs of protoplasts if inorganic nitrogen is inadequate. Additions of 1-5 mM L-glutamine can improve growth (6,22) and coconut milk at 1-5% (v/v) can have beneficial effects on the survival and growth of protoplasts (16,36).

6. Growth hormones: Auxins and cytokinins are required to induce cell division and plant regeneration from protoplasts. Auxins which have proven effective include 2,4-dichlorophenoxyacetic acid (2,4-D) and napthaleneacetic acid (NAA). Indole-3-acetic acid (IAA) has been used less frequently which may relate to its instability and possible adverse effect on protoplasts . Cytokinins have been shown to be required for protoplast division (22,60). The effective cytokinins include kinetin (35), benzyladenine (43,60), zeatin (16) and isopentenyl adenosine (1).

Mesophyll Protoplast Culture

There have been many attempts to culture protoplasts of grasses from mesophyll tissue. Early successes in protoplast culture were achieved with relative ease with mesophyll protoplasts of Solanaceous species. Unfortunately, the success with model genera such as Nicotiana and Petunia proved to be of little value for the culture of mesophyll protoplasts within the Gramineae (48,60). The most exhaustive and persistant efforts were made by Potrykus et al. (48) who used tens of thousands of variations in culture media but met with little success. A similar and equally disappointing experimental method was used by Galston et al. (21) for the culture of protoplasts of Avena sativa L.. Those experiences, unfortunate and disappointing though they were, highlighted the problems faced in the culture of grass mesophyll protoplasts in which variations in culture media do not seem to overcome the extreme recalcitrance of mesophyll protoplasts within the Gramineae.

Occasionally, reports of mesophyll protoplasts undergoing 1 or 2 cell divisions have appeared in the literature. By all accounts, these were isolated and unpredictable events. The sustained cell divisions which have been reported from protoplasts isolated from young stem tissues of Zea mays (48) were later found not to be reproducible. A similar claim of callus formation from shoot apex derived protoplasts of Saccharum spp. (20) was also unconvincing and required further documentation. To date, there is not a single unequivocal demonstration of sustained divisions induced in mesophyll protoplasts of any species with the Gramineae (60).

Protoplasts From Cell Cultures

In contrast to problems faced in the culture of mesophyll protoplasts within the Gramineae, protoplasts isolated from cell cultures have been successfully cultured in many grass species.

Non-Morphogenic Cell Cultures. Protoplasts isolated from callus tissues of Oryza sativa (14) and from suspension cultures of Sorghum bicolor (5) and Lolium multiflorum (33) have been cultured to obtain callus tissues. In most cases, consistently high plating efficiencies for grass protoplasts (15-25%) have been obtained. Since the original donor cell cultures were not morphogenetically competent, protoplasts derived from those calli have also failed to undergo any organ formation.

Morphogenic Cell Cultures. Vasil and Vasil (55,60) have reported success in culturing protoplasts derived from morphogenic cell suspension cultures of Pennisetum americanum. Similar reports with other species within the Gramineae (58-61) led Vasil and his group to

believe that protoplasts isolated from morphogenically competent cell cultures may be capable of forming callus tissue from which plants could be regenerated (55). Since the isolation of protoplasts from compact morphogenic callus cultures proved to be difficult and inefficient. Vasil and Vasil (56) isolated protoplasts from cell suspension cultures of P. americanum which were shown to be embryogenic. Subsequent culture of these protoplasts gave rise to plants by somatic embryogenesis. Embryogenic calli, somatic embryos and plantlets have been obtained from protoplasts isolated from other embryogenic cell suspension cultures within the Gramineae (60). These results have been repeated several times during subsequent years (60). Vasil and Vasil (55) reported that in initial experiments, the plating efficiency of protoplasts was only 1-2% and were not suitable for genetic manipulation experiments. However, they were able to increase these low percentages considerably. Nevertheless, plantlets obtained from germinating embryoids have failed to develop into mature plants and there was no single instance where the plantlets could be transferred to soil. In general, plantlets became brown and necrotic after forming several leaves and roots (55).

The early success in plant regeneration from protoplasts of Panicum americanum have been repeated in P. miliacum (30), P. maximum (39), P. purpureum (59), Oryza sativa (13,38,51) and Saccharum officinarum (49) with significant improvement in plating efficiency. Such advances were made possible by improving the nature of the cell suspension cultures which were more finely dispersed and homogenous in nature, and were neither heterogenous nor contained any meristemoids. Embryogenic calli, somatic embryos and green plantlets were obtained

from protoplasts derived from 2 species which were isolated from embryogenic cell suspension cultures (39,59,60). As in P. americanum, the protoplast-derived plantlets did not continue growth beyond the formation of several green leaves and roots, and could not be transferred to soil and grown to maturity (58).

The inability of protoplast-derived plantlets to continue growth appears to be related to the physiological and developmental maturity of the precociously germinating embryoids (39,60). It should be noted that the embryoids from callus and plated cell suspension cultures gave rise to plantlets which were grown to maturity. Furthermore, it has been shown to be much easier to regenerate plants from embryogenic calli compared to those formed in plated cell suspension cultures (55). Therefore, there seems to be less potential for regeneration as cultures become more dissociated. Walker et al. (61) reported that a diameter of 105 um represents the lower limit for callus aggregates that are morphogenetically competent in alfalfa (Medicago sativa L.). Single cells and smaller aggregates may become competent with further division.

A significant factor in increasing the plating efficiency of cultured protoplasts in the above mentioned grasses was the use of embryogenic cell suspension cultures which consisted predominantly of small, dense embryogenic cells (55). Such cell lines were obtained by the manipulation of dilution ratios and the duration of each sub-culture (7,30,39,40,58,59).

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CHAPTER VIII

PROTOPLAST ISOLATION AND CULTURE FROM EMBRYOGENIC SUSPENSION CULTURES OF PERENNIAL RYEGRASS

Introduction

Crop improvement via somatic hybridization techniques will depend on development of efficient and reliable procedures for plant regeneration from protoplasts or single cells. In cereals and grasses, embryogenic cell suspension cultures have been the only proven source of totipotent protoplasts (9,13,14). Plant regeneration from protoplasts of embryogenic cultures of Pennisetum americanum (L). K. Schum (12), Panicum maximum Jacq. (6), Pennisetum purpureum Schum (14), and Saccharum officinarum L. (9) have been reported. Regenerants from these species could not be grown to full maturity. Attempts to regenerate plants from protoplasts from Panicum melianum Schum (2) and Lolium multiflorium L. (3) have been limited to the regeneration of albino plants or callus respectively. Recently, Yamada et al. (15) and Kyozuka (5) successfully regenerated mature plants from protoplasts of various cultivars of rice. Srinivasan and Vasil (9) also regenerated viable plants from protoplasts of Sacharum officinarum L.. Vasil and Vasil (14) have been successful in producing somatic embryos from protoplasts of Zea mays L.. To date, rice (5,8,11,15) and sugarcane (9) are the only plants within the Gramineae which have been regenerated from protoplasts and grown to full maturity.

Protoplast isolation and culture are influenced by a large array of factors including the particular growth phase of calli or suspension cultures, quality and age of cultures, type and concentration of isolating enzymes and osmotica, pH, and numerous other media amendments. The culture conditions as well as the methods used in protoplast isolation can greatly affect the viability of protoplasts, the degree of cell division, colony formation, and regeneration.

At present, no information has been reported on protoplast isolation or culture from perennial ryegrass (Lolium prenne L.). Isolation, maintenance and plant regeneration from embryogenic callus and suspension cultures of perennial ryegrass has been described in Chapter II and III.

The objectives of this study were to develop a protocol for rapid protoplast isolation from embryogenic tissues and secondly, to develop a culture media which would support cell division.

Materials and Methods

Callus Cultures

Detailed procedures for the formation and culture of embryogenic callus of 'Diplomat' perennial ryegrass have previously been described in Chapter II. Embryogenic callus cultures used in this study were periodically subcultured (every 4-6 weeks) for over 12 months. The ability for plant regeneration from such cultures has been reported in Chapter II. Cultures have been maintained on a modified Murashige and Skoog basal medium (7) supplemented with 4 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D), 30 g/l sucrose, and B-5 vitamins (1) and 8 g/l

agar (Carolina Biological Co.). The optimum regeneration medium consists of a hormone-free, half strength MS medium supplemented with 0.5 mg/l fluridone, 0.5 mg/l benzylamino purine (BA), B-5 vitamins, 30 g/l sucrose and 8 g/l of tissue culture grade agar.

Cell Suspension Culture

Approximately 2 g of embryogenic callus tissue were placed in 250 ml flasks containing 50 ml of a half strength MS basal medium supplemented with 6 g/l 2,4-D, B-5 vitamins, and 45 g/l sucrose. Media pH was adjusted to 5.7 prior to autoclaving. Cultures were placed on a gyratory shaker at 100 rpm and incubated at 26 ^{o}C $\pm$ 2.0. Suspensions were routinely subcultured every 7 days by dispensing 5 ml of cell suspension to 45 ml of fresh medium.

Protoplast Isolation

Protoplasts were isolated from embryogenic cell suspension cultures. Two weeks prior to protoplast isolation, cell suspensions were subcultured every 48 h in order to maintain a high degree of cell division. After centrifugation, 5 ml of packed cells were placed in 60x15 mm petri dishes containing 5 ml of protoplast isolation medium (Table 12). The enzyme mixture was dissolved in distilled water and the pH was adjusted to 5.7 (unless otherwise mentioned). The isolation mixture was incubated for 5-6 h without agitation. Protoplasts were separated from undigested cells and cell clumps by passing them through a 31-um nylon mesh with 5 volumes of protoplast culture medium (Table 13, medium A). After centrifugation (80 x g), pelleted protoplast were washed 3 times in protoplast culture medium by centrifuging at 80 x g for 3 min at each washing. Kao and Michaluk

vitamins (KM) (4), dimethyl sulfoxide (DMSO), and the various phytohormones used were filtered sterilized (Nalgene, 0.2 um pore size) prior to addition to culture media. This procedure was found to be the most effective method of isolation and was routinely used unless otherwise mentioned. The number of protoplasts released was estimated using a haemocytometer (American Optical, U.S.A.), and the number of protoplasts released per ml of packed cells was calculated.

Protoplast viability was estimated quantitively after 5-6 h of incubation by either exclusion of Evans blue or by staining with phenosafranin at a 0.01% final concentration to assess the percentage of dead protoplasts. At least 1000 protoplasts were scored in every viability determination.

Calcofluor White stain (Sigma Chemical Co.) (0.1%) dissolved in 0.3 M mannitol was mixed with an equal volume of protoplast suspension to confirm the absence of undigested cell wall material after washing or to detect the reformation of cell walls after plating.

Conditioned Media

Conditioned media was obtained by centrifuging 50 ml of freely suspended embryogenic suspension cells taken at either 2, 4, 6, 8 or 10 days after subculturing. The supernatant (conditioned media) was then filtered through a 0.2 um sterile disposable syringe. Conditioned media taken between 3 and 5 days after subculture (exponential growth phase) supported the highest degree of protoplast division and, therefore, was used throughout the study.

Protoplast Culture

Protoplasts were cultured by 4 methods. The first was a single droplet method with conditioned or nonconditioned media. The protoplasts were suspended in 200 ul droplets ($5x10^5$-10^6 protoplast/ml) and were dispensed onto petri dishes (60x15 mm) and sealed with parafilm strips.

In the second method, 400 ul of protoplast suspensions were plated onto a shallow layer of solidified conditioned or nonconditioned media in petri dishes (35x10 mm) and then sealed with parafilm.

The third method was a nurse culture technique utilized the "Ultraclone growth chambers" (UGC) (Earl-Clay Labrotories, Inc. 890 Lamont Avenue, Novato, CA 94947) which are semi-permeable, and flexible semi-solid culture wells. Protoplasts in UGC wells can be concentrated into a specific growth area and separated from surrounding nurse cells by a semi-permeable barrier. Droplets of protoplast suspension (100 ul) were placed inside Ultraclone growth chambers which were then placed in petri dishes. The components of the suspension culture medium where the nurse cells grew was similar to the protoplasts culture medium. Suspension culture media was similar to protoplast culture media. Two UGC units were placed in the center of each petri plate then parafilm sealed and incubated.

The fourth method was the Mixed Nurse Method developed by Kyozuka et al. (5) where 0.5 ml of protoplast suspension was mixed with an equal volume of molten low melting agarose culture medium (2% agarose, Bethesda Research Lab) in 35x10 mm plastic petri dishes. Protoplasts were embedded at the bottom of the agarose media after solidification

to provide a close solid-liquid interface and to facilitate observation with an inverted microscope. Agarose containing embedded protoplasts was solidified for at least 1 hour before being transferred to nurse culture media to ensure that no suspension cells would become accidentially embedded in the agarose. Solidified agarose cultures were then cut into squares (5 mm x 5 mm) and transferred to 60 x 15 mm Petri dishes containing cell suspensions.

In the third and the fourth culturing methods, Nurse cells (approximately 50 mg/plate) were taken from suspension cultures growing at an exponential growth phase and were washed at least three times with a hormone-free protoplast washing/culture medium (Table 13, medium A) before corresponding concentrations of hormones were added. Ten ml of fresh Nurse culture media were dispensed into Petri plating surrounding the UGC wells or the agarose squares. Liquid medium containing nurse cells was changed every 8-10 days. Plates were incubated on a shaker at very low speed (ca. 10 rpm) in the dark at 26 $^{o}C \pm 2.0$. After two weeks of culture, the osmotic stabilizers in the nurse liquid medium (Table 13, medium A) were removed and replaced with the final culture media (Table 13, medium B).

Various concentrations of 2,4-D, BA, and kinetin were evaluated for their effect on cell division and colony formation.

Results and Discussion

Protoplast Isolation

High protoplast yield was observed after 5-6 h using of incubation in the isolation solution. After filtering and washing, clean protoplast suspensions (10^6-$5x10^6$/ml) devoid of contaminating

cells were obtained. Isolated protoplasts were, for the most part, densely cytoplasmic, with very small vacuoles, and a high starch content and varied in size between 10-30 um (Fig. 17. plate A).

The yield of protoplasts was influenced by the growth conditions of embryogenic suspension cells and period of subculturing. The amount of protoplasts isolated from cell suspensions subcultured every 48 hr was greater with a higher percentage of viability when compared with suspensions which were subcultured every 7 days. The age of suspension cultures had a great effect on the size of the protoplasts with these cells yielding large protoplasts (20-40 um) which were more vacuolated and contained fewer starch granules. In general, larger protoplasts took a longer time, to divide or were unable to enter division. In contrast, protoplasts isolated from cells subcultured every 48 h were highly cytoplasmic, less vacuolated, smaller (12-25 um) and divided more readily. The addition of pectolyase Y-23 to the isolation mixture was essential for obtaining a great number of viable protoplasts and could not be replaced by macerase, pectinase, Rhozyme-HP-150, or their various combinations. There was also a higher protoplast yields from embryogenic suspension cells compared to embryogenic callus.

Agitation during incubation was necessary to obtain high protoplast yields from callus cultures but was not required when embryogenic suspensions were used. Isolation without agitation resulted in less protoplast damage and greatly reduced the extent of undigsted cells, partially digested, or cellular debris, thus making the washing sequence much more efficient (Fig. 18). The viability and

yield of protoplasts was greatly affected by the type and concentrations of enzymes, osmotica, and inorganic salts. Among the numerous cellulases tested, cellulysin at 1.25% (w/v) in combination with driselase 1.25% (w/v), and (w/v) cellulase type VII (Penicillium funiculosum) 3.5% (w/v) incubation yielded the best results (Fig. 12).

The age of the suspension culture and growth phase had a substantial effect on the yield, viability, and degree of damage during the washing of protoplasts. There was a rapid increase in the number of suspension cells in culture every 48 h as expressed by packed cell volume (Fig. 14). Cell walls in these cultures were comparatively thin, and the isolation of protoplasts from such cells was more efficient, showing a higher frequency of protoplasts with resumed division (Fig. 17, 19).

The ratio of packed suspension cell volume and enzyme solution volume also affected protoplast yield and their ability to withstand changes in osmotic pressure. The greatest protoplast populations were obtained when 0.2 ml of packed cells were used with 1.0 ml of isolation mixture (Table 14). The rest of the parameters which have been evaluated in the course of optimizing the isolation solution for embryogenic cell suspension and embryogenic callus tissues are shown in appendices D and G respectively.

Protoplast Culture

The use of the mixed nurse method (5) resulted in the increasing the percentage of protoplasts reforming cell walls, budding, and resuming cell division. Such method has provided a higher percentage

of protoplasts with resumed cell division than the other methods tested (data not presented). The presence of nurse cells in the bathing medium around agarose squares was more effective than the UGC-well method. The UGC method was, however, more effective compared to the liquid droplet method or plating protoplasts on thin layers of conditioned or non conditioned media. UGC wells have an important advantage over the mixed nurse method, however, in that nurse cells can be changed without disturbing protoplast cultures.

Cell division was noticed as early as 24 hr after protoplasts were embedded in 2% agarose using the mixed nurse cell method compared to 2-3 days for other methods tested. After 2-3 weeks of culture, several visually apparent colonies were formed in mixed nurse cultures compared to 3-4 weeks for the UGC method was used. Nurse cells were kept in contact with protoplasts for 14 days with the mixed nurse method and about 20 days with the UGC method. An experiment is currently in a progress to test the relationship between the concentration and time exposure of nurse cells and the efficiency of cell division.

The effects of various concentrations of 2,4-D alone and in combination with 0.5 mg/l BA, zeatin and kinetin were investigated. The effect of BA in combination with 2,4-D was more effective in increasing the percentage of protoplasts forming cell walls, budding, and dividing (Table 16). In contrast, 2,4-D alone had no effect at all concentrations tested and zeatin were kinetin were less effective in inducing cell division than BA. The percentage of protoplasts reforming cell walls and dividing decreased steadily with levels of 2,4-D greater than 0.25 mg/l. The resynthesis of cell walls and

division were evident among a large percentage of protoplasts 24-48 h after plating (Table 15). The pecentage of protoplasts that eventually regenerated into visually apparent colonies has, however, remained low (around 3%).

Efforts continue to center upon the enhancement of colony formation and growth, with the ultimate goal of plant regeneration.

Table 12. Protoplast Isolation Mixture

1.25 % (w/v) cellulysine (Sigma Chemical Co.)
1.25 % (w/v) Driselase (Kyowa Harko Hogyo Co., Tokyo, Japan)
0.025 % (w/v) Pectolyase (Sigma Chemical Co.)
3.5 % Cellulase type VII (Sigma Chemical Co.)
0.3 M Mannitol
2.0 % (w/v) Glycine
0.3 mM MES(2[N-Morpholino)ethane sulfonic acid)
3.0% (w/v) Sodium Thiosulfate

Table 13. Composition of washing and culture medium required for the culture of perennial ryegrass protoplasts.

Ingredient	First 2 weeks Medium A	Final medium Medium B
MS basal salt	1/2 strength	1/2 strength
Vitamins	KM (4)	B-5 (1)
Sugars and sugar alcohols (g/l):		
Mannose	0.25	
Fructose	0.25	
Ribose	0.25	
Xylose	0.25	
Rhamnose	0.25	
Cellibiose	0.25	
Glucose	36.024	
Sucrose	20.0	30
Mannitol	36.445	
Stabilizers (g/l):		
KCl	18.64	
$MgSO_2$	9.19	
$CaCl_2$	15.41	
Sodium thiosulfate	15.0	
MES(buffer) (2[N-Morpholino) ethanesulfonic acid)	2.79	
Organic nitrogen (g/l):		
Casein hydrolysate	0.2	3
Glycine	10.0	
Growth regulator (mg/l):		
2,4-D	0.25	6
BA	0.5	
Agarose	20.0 g/l	
DMSO (Dimethyl sulfoxide)	2%	
Coconut water	5%	

[a]Various levels of 2,4-D, NAA, BA, zeatin, and kinetin were incorporated to this medium, pH 5.7 (KOH). All vitamins, DMSO and growth regulators were filtered sterilized.

Table 14. The effect of packed cell volume/enzyme mixture ratio on the efficiency of protoplasts released and degree of damage from perennial ryegrass embryogenic suspension cells after 6 h of incubation without agitation.

Packed cell volume (PCV/ ml)	Protoplast yield per ml[a]
0.2	$1x7x10^5$
0.4	$1.4x10^6$
0.6	$1.7x10^6$
0.8	$2.2x10^6$
1.0	$2.2x10^6$
1.2	$1.4x10^6$

[a]Degree of protoplast damage estimated visually at the end of incubation period was negligible.

Table 15. The combined effect of 2,4-D with kinetin, zeatin, or BA on cell wall formation, budding, and division from perennial ryegrass cv. Diplomat protoplasts using the mixed nurse method. Data were collected 21 days after plating and represent the mean of 3 replications.

2,4-D (mg/l)	Cytokinins (0.5 mg/l)	% Cell wall formation	% budding	% division
0.1	Kinetin	59 ± 10[a]	44 ± 4	10 ± 2
	Zeatin	55 ± 5	47 ± 9	10 ± 4
	BA	80 ± 17	57 ± 15	25 ± 6
0.25	Kinetin	72 ± 4	49 ± 2	12 ± 2
	Zeatin	61 ± 6	52 ± 6	10 ± 2
	BA	93 ± 16	64 ± 4	29 ± 5
0.5	Kinetin	63 ± 9	46 ± 7	8 ± 2
	Zeatin	55 ± 5	39 ± 10	9 ± 3
	BA	82 ± 11	52 ± 9	19 ± 4
1.0	Kinetin	51 ± 6	31 ± 4	4 ± 1
	Zeatin	39 ± 4	18 ± 2	5 ± 1
	BA	67 ± 10	27 ± 6	10 ± 2
2.0	Kinetin	44 ± 6	16 ± 3	4 ± 1
	Zeatin	22 ± 2	11 ± 2	3 ± 1
	BA	52 ± 12	20 ± 4	5 ± 2
3.0	Kinetin	32 ± 3	11 ± 2	4 ± 1
	Zeatin	20 ± 2	6 ± 1	3 ± 1
	BA	45 ± 3	16 ± 3	3 ± 1
4.0	Kinetin	21 ± 3	8 ± 4	3 ± 1
	Zeatin	17 ± 4	13 ± 2	3 ± 1
	BA	41 ± 6	14 ± 3	3 ± 1
5.0	Kinetin	17 ± 3	6 ± 1	2 ± 0
	Zeatin	17 ± 3	13 ± 2	2 ± 0
	BA	29 ± 7	14 ± 4	2 ± 0

[a]Mean ± standard error.

Table 16. The effect of protoplast culture methods on the percent of protoplasts with cell division and colony formation. Data represents the average of 3 replications. Data were collected 21 days after plating. Microcalli ranged in size from 0.1 to 0.5 mm in diameter.

Culture method	% Protoplasts dividing (Visual estimate)	% Colony formation (Visual estimate)
Mixed nurse method	16-18	2-3
Ultraclone growth chamber	2-5	0.5
Liquid droplet:		
In conditioned medium	1	-[z]
In nonconditioned medium	< 0.5	-
Liquid thin layer:		-
In conditioned medium	1	-
In nonconditioned medium	< 0.5	-

[z]The degree of colony formation was negligible.

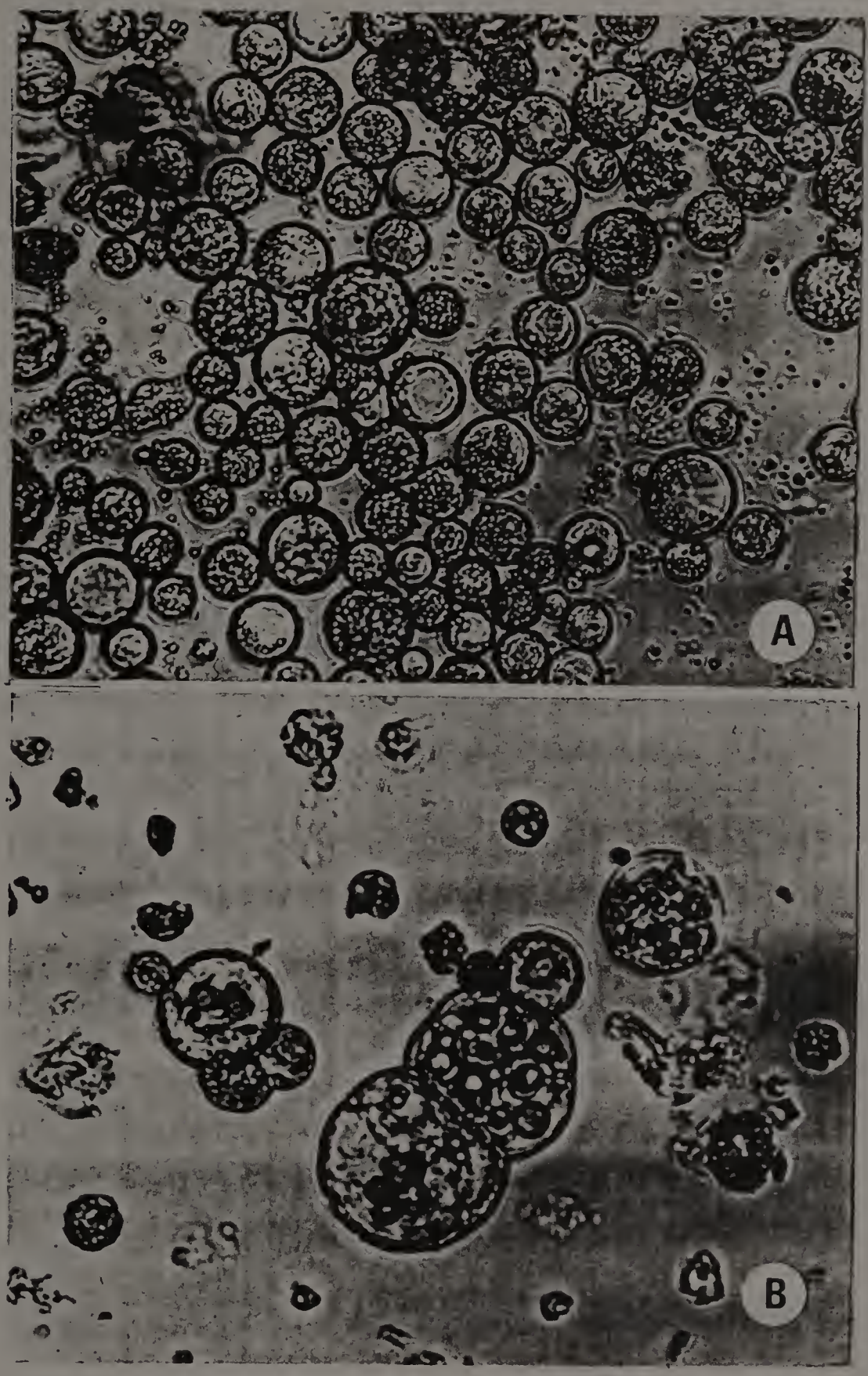

Figure 17. (A) Protoplasts isolated from embryogenic suspension cultures of perennial ryegrass. (B) Cultural protoplasts undergoing first divisions.

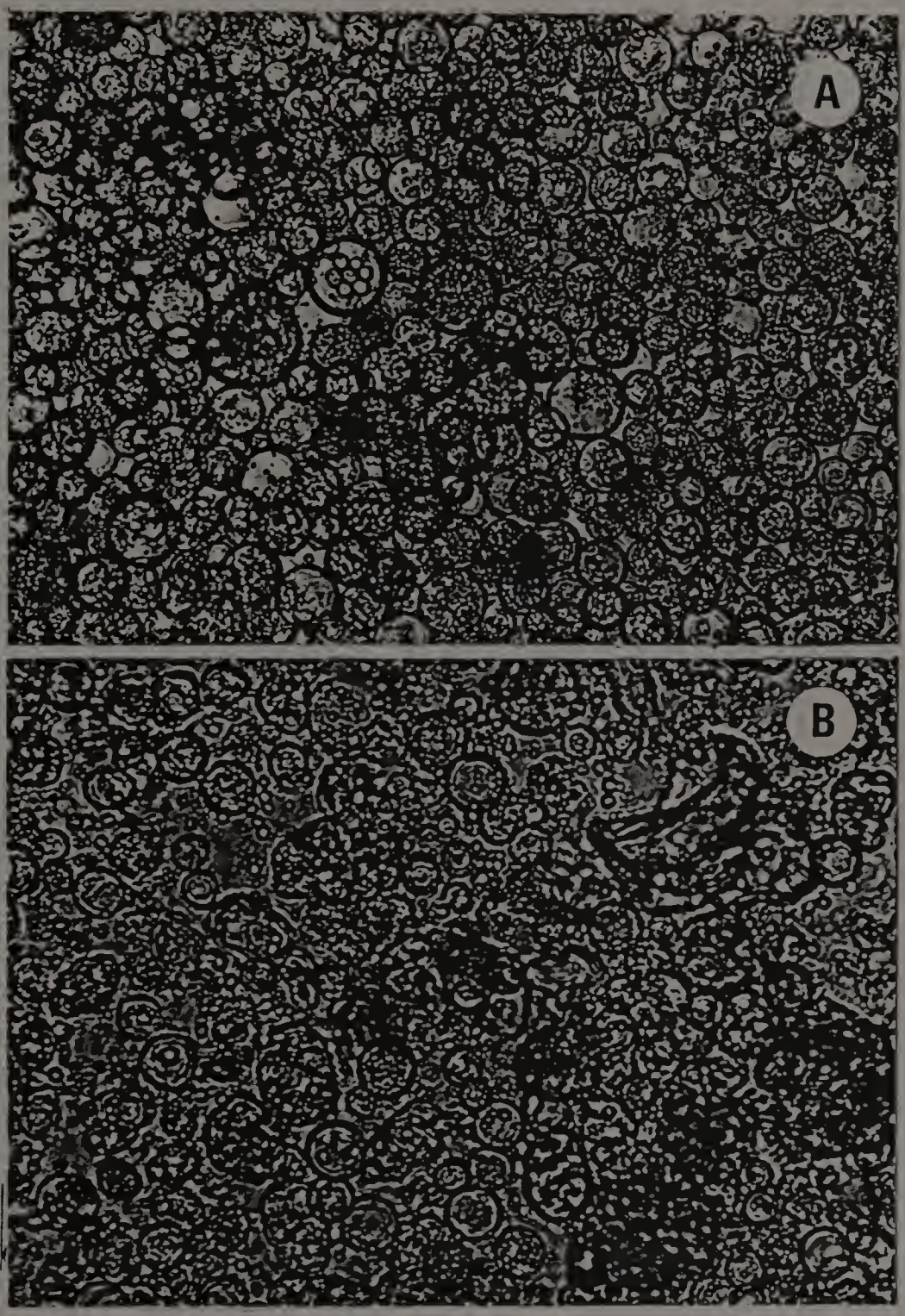

Figure 18. The effect of agitation during isolation on protoplast integrity. (A) No agitation, numerous intact protoplasts. (B) With agitation. a high degree of damage.

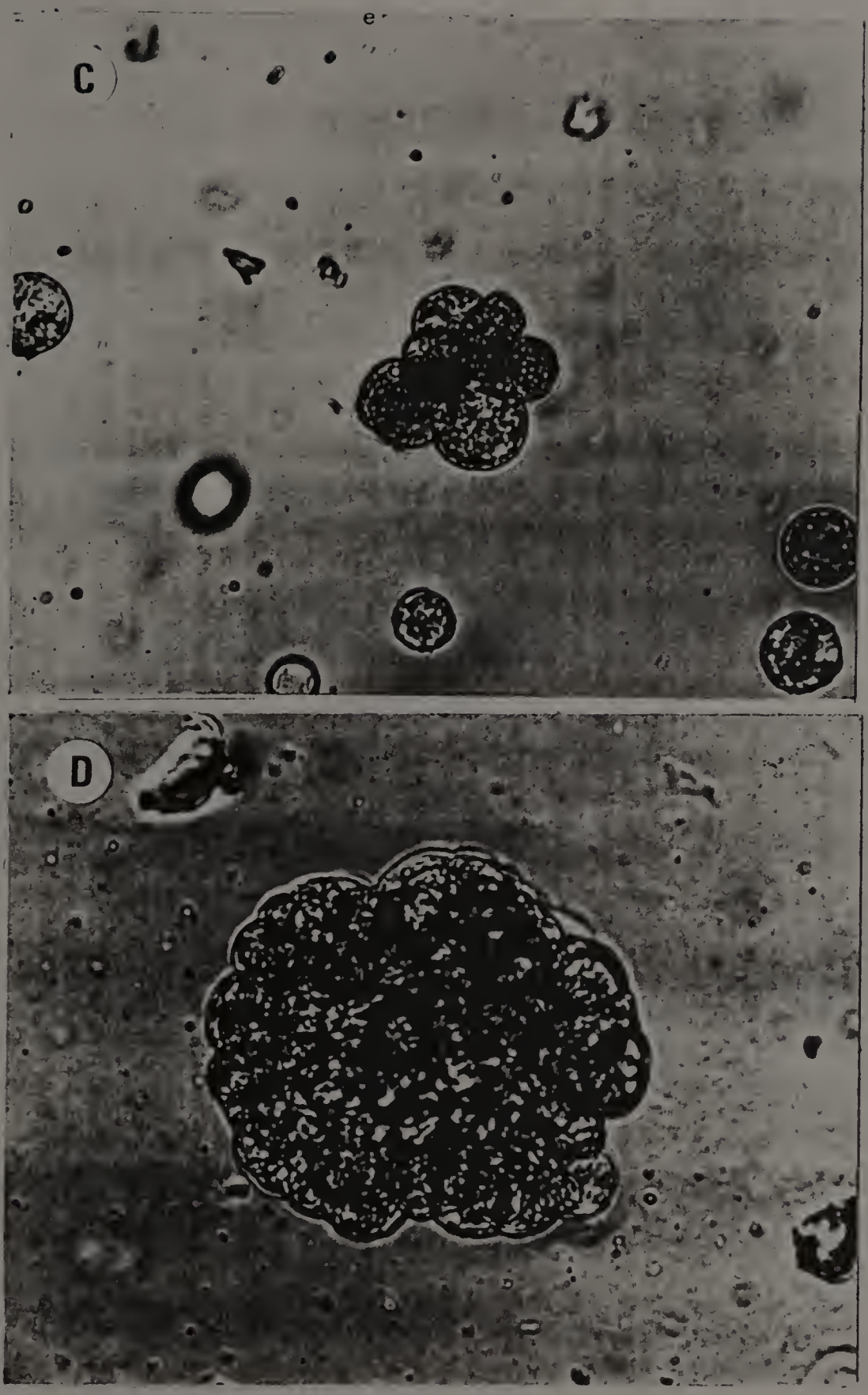

Figure 19. (C) Protoplast division and development of cell clusters during protoplast culture. X 200. (D) Formation of micro-calli from protoplasts of perennial ryegrass. X 100.

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C H A P T E R IX

PROTOPLAST ISOLATION AND CULTURE FROM EMBRYOGENIC SUSPENSION CULTURES OF RED FESCUE

Introduction

Crop improvement certain various somatic hybridization and genetic transformation techniques cannot be realized until single cells or protoplasts can be successfully cultured and regenerated into viable plants. Many important advances have been reported for protoplast isolation and culture within various families, particularly within the Solanaceae. For many dicot species, leaf mesophyll tissue is the primary source for protoplast. In contrast, protoplasts isolated from mesophyll tissue of monocots species cannot, as yet, be successfully cultured (11). The use of non-embryogenic grass and cereal cell cultures as sources for protoplasts have resulted only in the formation of non-morphogenic callus tissue which has been incapable of plant regeneration (1,10,11). Embryogenic cell suspension cultures have been the only proven source of totipotent protoplasts resulting in plant regeneration (8,12,13,15,16,17).

At present, no information has been reported on protoplast isolation and culture from red fescue (Festuca rubra L.). Induction, maintenance and plant regeneration from embryogenic callus and suspension cultures of red fescue has been described by Torello et al. (13) and in Chapter VI respectively. The extent and duration of embryogenesis and plant regeneration from this turfgrass species is

also known to be prolific for long culture periods (14). Therefore, the objectives of this study were to develop protocols for the rapid isolation of viable protoplasts from embryogenic suspension cultures and to develop protoplast culture conditions which would support cell divison.

Materials and Methods

Callus Induction and Maintenence

Embryogenic callus cultures were initiated from mature caryopses of "Dawson" red fescue and maintained on a medium consisting of a 1/2 strength Murashige and Skoog basal media and vitamins (MS) (9) supplemented with 4 mg/l 2,4-D, 30 g/l sucrose and 8 g/l tissue culture agar (Carolina Biological Co.). Callus has been subcultured every 6-8 weeks for the previous 5 years.

Induction and Maintenance of Suspension Cultures

Embryogenic suspension cultures of red fescue were obtained by placing 2 g of callus tissue into a liquid medium consisting of 1/2 MS salts supplemented with 4 mg/l 2,4-D, B-5 vitamins (2), 3 g/l casein hydrolysate, and 45 g/l sucrose. Suspension cultures were placed on a gyratory shaker at 100 rpm and incubated at 26 ^{o}C $\pm$ 2.0. All cultures were subcultured every 7 days by adding 10 ml of suspension culture into 40 ml of fresh medium in 250 ml Erlenmyer flasks. Plant regeneration from embryogenic suspension cultures of red fescue has been reported in Chapter VI.

Protoplast Isolation

Subculture frequency for suspension cultures was increased to 2-day intervals to enhance the rate of cell division prior to protoplast isolation. Cells and cell clusters were harvested by centrifuging 50 ml of the suspension culture at 80 x g, removing the supernatant and immersing the pellet in 5 ml of the enzyme isolation mixture (Table 12). Protoplast isolation mixtures were incubated at 26 ^{o}C $\pm$ 2.0 without agitation for 4 h. Released protoplasts were separated from undigested cells and debris by filtration through a 31-um pore size nylon mesh followed by centrifugation for 3 min at 80 x g. The enzyme solution was decanted off and the pellet was washed and resuspended with 5 ml of solution (A) (Table 17) and centrifuged again as described above. The procedures were repeated with washing solution B and solution C. Protoplast numbers were estimated with a hemocytometer. Protoplast viability was determined with Evans Blue as well as phenosafranin staining (18). Calcofluor white was used to determine if all cell wall material had been removed and to assess the progress of cell wall formation during culture.

Protoplast Culture

Protoplast plating (culture) techniques evaluated were the mixed nurse cell method (7), Ultraclone Growth Chambers (UCG), liquid media droplet and liquid media thin layer with or without conditioned media. Agarose embedded protoplasts as well as UGC wells were immersed in 60x15 mm petri dishes containing 10 ml of suspension cultures supplemented with media components described for the protoplast culture medium. The effects of various concentrations of 2,4-D alone

and in combination with 0.5 mg/l BA, zeatin or kinetin were also investigated. Detailed descriptions of these culture methods have been described in previous chapter. The period between plating of protoplasts and the gradual reduction of osmotic stabilizers was approximately 3 weeks. Efforts to decrease the levels of these compounds earlier than 3 weeks have failed due to the great sensivity of protoplasts to change in osmotic pressure. Therefore, protoplasts were initially cultured on medium A (Table 18) for 3 weeks prior to being transferred to the final culture medium (Table 18, medium B). All media components were filter sterilized (0.2 um pore size) and adjusted to a pH of 5.7.

Conditioned media was taken between 2-3 days after subculture of suspension (exponential growth phase). Nurse cells were taken from the same growth phase. The concentration of osmotic stabilizers were similar to protoplast culture media for conditioned media and nurse cells cultures.

Results and Discussion

Protoplast Isolation

Large numbers of protoplasts (exceeding 5×10^6 protoplasts/ml) were isolated from embryogenic suspension cultures. The viability of isolated protoplasts always exceeded 95%. These results were obtained without agitation during isolation which led to considerably less protoplast damage and debris accumulation (Fig 20, plate A).

The growth phase of embryogenic suspension cultures used in isolation affected protoplast yield (Table 19). Protoplasts were more easily isolated from cultures subcultured every 2 days compared to

those subcultured at 7-day intervals. In general, 2-day intervals yielded smaller cells having thinner cell walls and a higher rate of division. Furthermore, protoplasts isolated from cultures having 7-day subculture intervals showed a greater percentage of lysis during protoplast washing.

The ratio of harvested suspension cells [packed cell volume (PCV)] to enzyme solution volume also affected protoplast yield. The maximum number of viable protoplasts were obtained when a 1/1 ratio (V/V) was used. The enzyme isolation solution was, therefore, considered highly efficient since ratios of 1:10 or greater are usually necessary for most grass/cereal cultures (4,5,15,16,17). Higher volumes of this isolation mixture proved to be detrimental in regard to protoplast yield, viability and increased quantities of cellular debris which was difficult to remove.

Agitation during isolation had a negative effect on the overall number and viability of protoplasts as well as increasing the extent of hard to remove cellular debris. Longer isolation periods and higher speeds of agitation resulted in higher levels of damage (Table 19). The rest of the parameters which have evaluated in the course of optimizing the isolation mixture from embryogenic cell suspension and embryogenic callus tissues are shown in appendices E and F respectively.

Protoplast Culture

Protoplasts were initially cultured in a medium where the osmotic pressure was maintained relatively high with inorganic salts (solution A in Table 17) plus other compounds (Table (18) with the osmoticum and

stabilizers omitted). In this media, protoplasts remained intact without any apparent changes for about 3-4 days before they began to degenerate. Therefore, to induce cell wall formation and cell division, protoplasts were transferred gradually, in a series of washing steps, through a reduction of salt concentration while increasing the concentration of glucose and sucrose (Table 17). After washing through these three media, protoplasts were cultured for 3 weeks in medium A transferred to medium B (Table 18).

In preliminary attempts, protoplasts were cultured in liquid micro-droplets or liquid thin layers. After a 4 week incubation period, a few small micro-colonies (100-300 um diameter) of callus were formed which did not proceed beyond that stage of growth for both culture methods. Unsuccessful attempts were made to enhance the percentage of protoplasts with resumed cell division and colony formation by evaluating various concentrations of amino acids, growth regulators, carbohydrates and numerous other compounds. In general, culture of protoplasts in a single droplet resulted in a higher frequency of cell division compared to plating protoplasts in a higher volume of liquid media plated on thin layer solid medium. These results may be attributed to more rapid media conditioning or less growth factor dispersal and a greater capacity for oxygen replenishment in the micro-drop method.

Increased cell wall formation and division frequency were observed for both the liquid drop and thin layer method when condition media was utilized (Table 20). The particular growth phase of the suspensions culture from which conditioned medium was taken had a significant effect on the frequency of colony formation. The best

results were obtained when conditioned medium was taken from cell suspension 2-3 days after subculturing. Cell division and micro-colony formation was further increased by embedding protoplasts in an agarose medium or with the use of UGC wells. The mixed nurse method included the use of nurse cells (suspension media) and have been previously described. Protoplasts of red fescue were very sensitive to the reduction in the levels of various osmotica and stabilizers present in the protoplast culture medium and the surrounding suspension. After 3 weeks of culture, protoplasts were moved to standard suspension culture media. The percentage of protoplasts with resumed cell divsion and colony formation was greater with protoplast embedded in agarose compared to UGC inserts (Table 20). These differences may be attributed to the inability of certain metabolites from the nurse culture to cross through the UGC wall and/or to the presence of unknown or toxic substances associated with these culture wells. Increased plating efficiencies for rice were also reported by Kyozuka et al. (7) using the agarose-mixed nurse culture method.

The addition of 0.5 mg/l BA was found to be essential for colony formation (Table 21). Figure 20, plate B shows protoplasts with resumed cell division 48 hr after plating. The growth of protoplasts were negligible when 2,4-D was used alone or in combination with zeatin or kinetin. The percentage of protoplasts reforming cell walls and dividing decreased steadily with increasing levels of 2,4-D greater than 0.1 mg/l.

The addition of DMSO to protoplast culture media for all plating methods, resulted in in shortening of the time interval between

plating and cell divsion and increased the percentage of protoplasts with resumed cell division (Table 20). The effects of DMSO were also reported to enhance the frequency of cell divison for Saccharum officinarum L. (13) and Hibiscus rosa-sinensis L. (3) protoplasts. Hahne and Hoffmann (4) attributed the enhancement of cell division of protoplasts of Hibiscus rosa-sinensis to the effects of DMSO which supported the reinstallation of a net of cortical microtubules in the protoplast which was considered critical for inducing consecutive divisions. Similar results were obtained in this research where the addition of DMSO was very essential for resuming cell division and obtaining colony formation. However, it is important to note that DMSO had no effect if applied to protoplasts isolated from suspension cultures at the end of the exponential phase growth (7 days after subculture) (Unpublished data).

The quality and particular growth phase of suspension cultures from which protoplasts were isolated had a great effect on the number, viability and culturing success indicated in this study. It has been apparent that young, fast growing embryogenic suspension cultures are necessary for a reasonably high degree of cell division and microcalli production from red fescue protoplasts. Work is currently underway to culture microcalli as suspension cultures for ultimate goals of embryogenesis and plant regeneration.

Table 17. The sequences of washing steps used to transfer Festuca rubra L. protoplasts from high inorganic solutions to the appropriate culture medium.

Ingredients	Washing solutions		
	A	B	C
Mannitol	–	50 mM	0.2 M
Sorbitol	–	50 mM	–
Sucrose	–	15 mM	58 mM
Glucose	–	–	0.2 M
KCl	0.3 M	0.2 M	0.1 M
$CaCl_2$	30 mM	30 mM	30 mM
$MgSO_4$	30 mM	30 mM	30 mM
Sodium thiosulfate	120 mM	120 mM	120 mM
MES (buffer)	3 mM	3 mM	3 mM
pH	5.7	5.7	5.7

Table 18. Composition of washing and culture medium required for the culture of red fescue protoplasts.

Ingredient	First 3 weeks (medium A)	Final medium (medium B)
MS basal salt	1/2 strength	1/2 strength
Vitamins	KM (6)	B-5 (2)
Sugars and sugar alcohols (g/l)		
Mannose	0.25	
Fructose	0.25	
Ribose	0.25	
Xylose	0.25	
Rhamnose	0.25	
Cellibiose	0.25	
Glucose	36.024	
Sucrose	20.0	30
Mannitol	36.445	
Stabilizers (g/l)		
KCl	18.64	
$MgSo_4$	9.19	
$CaCl_2$	15.41	
Sodium thiosulfate	15.0	
MES(buffer) (2[N-Morpholino) ethanesulfonic acid)	2.79	
Organic nitrogen (g/l)		
Casein hydrolysate	0.2	3
Glycine	10.0	
Growth regulator (mg/l)		
2,4-D	0.1	4
BA	0.5	
Agarose	10.0 g/l	
DMSO (Dimethyl sulfoxide)	2%	
Coconut water	5%	

[a]Various levels of 2,4-D, NAA, BA, zeatin, and kinetin were incorporated to this medium, pH 5.7(KOH). All vitamins, DMSO and growth regulators were filtered sterilized.

Table 19. The effect of subculturing period on the efficiency of protoplasts released and degree of damage from red fescue embryogenic suspension cells after 6 h of incubation with no agitation 26 $^{o}C \pm 2.0$.

Subculturing period (days)	Incubation period (h)	Protoplasts yield per ml	Degree of damage
Shaking (100rpm)			
2	1	$6x10^6$	+++[a]
	3	$4x10^6$	++++
	6	$5x10^5$	++++
	9	$3x10^5$	++++
	12	$8x10^3$	++++
7	1	$3x10^6$	+++
	3	$1x10^6$	++++
	6	$3x10^5$	++++
	9	$3x10^3$	++++
	12	$1x10^3$	++++
No shaking			
2	1	$6x10^5$	-
	3	$1x10^6$	-
	6	$3x10^6$	-
	9	$5x10^6$	-
	12	$8x10^6$	-
7	1	$2x10^5$	-
	3	$6x10^5$	-
	6	$1x10^6$	-
	9	$3x10^6$	-
	12	$4x10^6$	-

[a]Degree of protoplast damage estimated visually at the end of incubation period, ++++ maximum damage; +++ 30% damage, ++ 20% damage; +, 10% damage; - no damage.

Table 20. The effect of protoplast culture methods on percent division and colony. Data represents the average of 3 replications. Data were collected 21 days after plating. Microcalli ranged in size from 0.1 to 0.5 mm in diameter.

Culture method	% Protoplasts dividing (Visual estimate)		% Colony formation (Visual estimate)	
	+DMSO	-DMSO[a]	+DMSO	-DMSO
Mixed nurse method	10	3	1	< 0.25
Ultraclone growth chamber	2	0.5	-[b]	-
Liquid droplet:				
In conditioned medium	2	-	-	-
In nonconditioned medium	-	-	-	-
Liquid thin layer:				
In conditioned medium	>0.25	-	-	-
In nonconditioned medium	-	-	-	-

[a]The same protoplast culture medium shown in Table 18 except DMSO was omitted.

[b]The degree of cell division colony formation was negligible.

Table 21. The combined effect of 2,4-D with kinetin, zeatin, or BA on cell wall formation, budding, and division from red fescue cv. Dawson protoplasts using the mixed nurse method. Data represent the average of three replications. Data were taken 21 days after plating.

2,4-D (mg/l)	Cytokinins (0.5 mg/l)	% Cell wall formation	% budding	% division
0.1	Kinetin	10 ± 2a	2 ± 0.0	-
	Zeatin	6 ± 1	2 ± 0.0	-
	BA	75 ± 9	49 ± 6	10 ± 1.0
0.25	Kinetin	13 ± 2	4 ± 1	1 ± 1.0
	Zeatin	8 ± 2	3 ± 2	-
	BA	63 ± 8	33 ± 3	5 ± 2
0.5	Kinetin	4 ± 1	1 ± 0.0	-
	Zeatin	4 ± 0.0	1 ± 0.0	-
	BA	55 ± 9	29 ± 5	5 ± 3
1.0	Kinetin	4 ± 1	-	-
	Zeatin	3 ± 1	-	-
	BA	44 ± 4	27 ± 3	3 ± 1
2.0	Kinetin	-	-	-
	Zeatin	2 ± 0.0	-	-
	BA	30 ± 3	8 ± 2	1 ± 0.0
3.0	Kinetin	-	-	-
	Zeatin	2 ± 0.5	-	-
	BA	27 ± 3	3 ± 0.0	-
4.0	Kinetin	-	-	-
	Zeatin	1 ± 0.0	-	-
	BA	20 ± 3	1 ± 0.0	-
5.0	Kinetin	-	-	-
	Zeatin	1 ± 0.0	-	-
	BA	20 ± .3	1 ± 0.0	-

[a]Mean ± standard error.

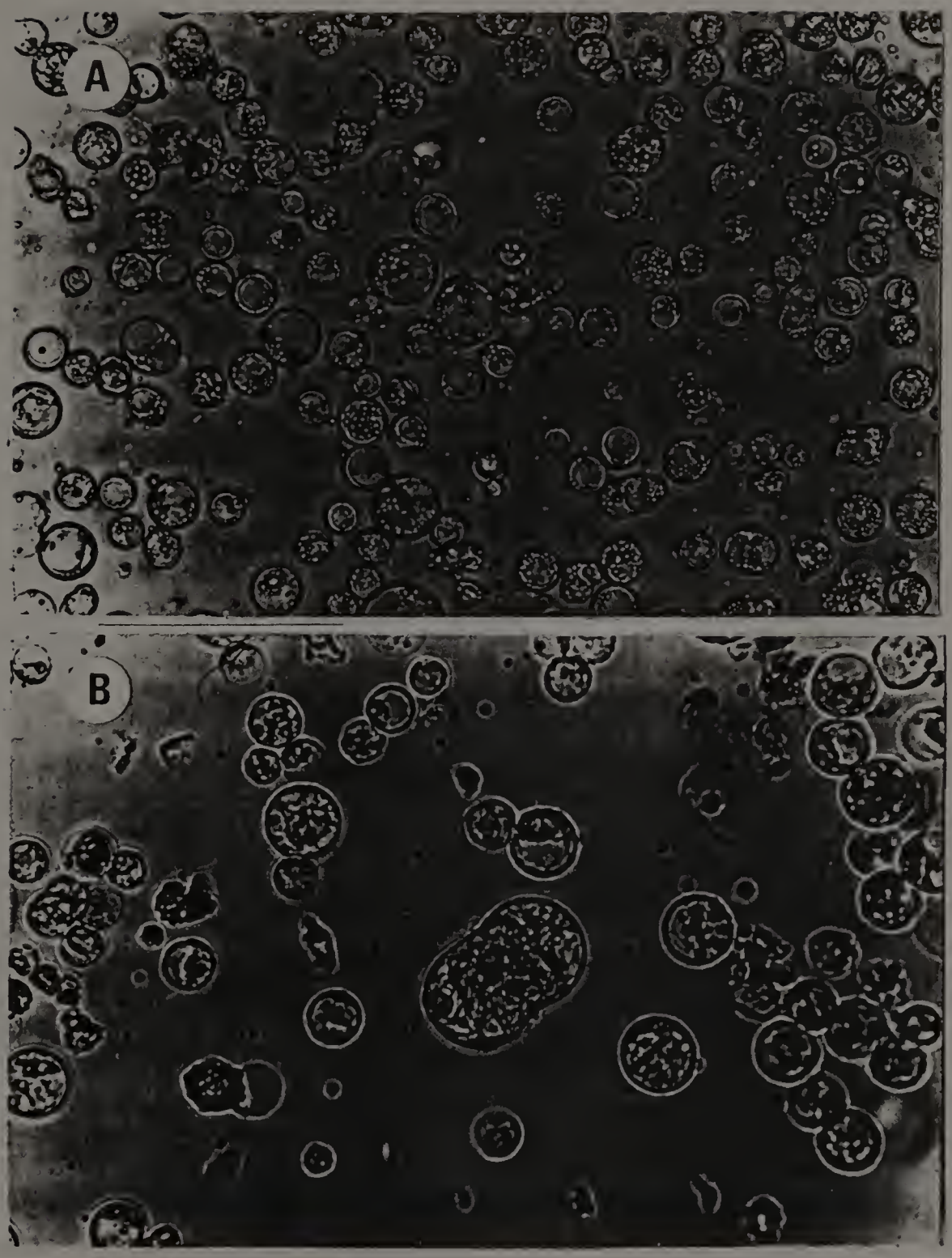

Figure 20. (A) Protoplasts isolated from embryogenic suspension cultures of red fescue. (B) Cultural protoplasts undergoing first divisions.

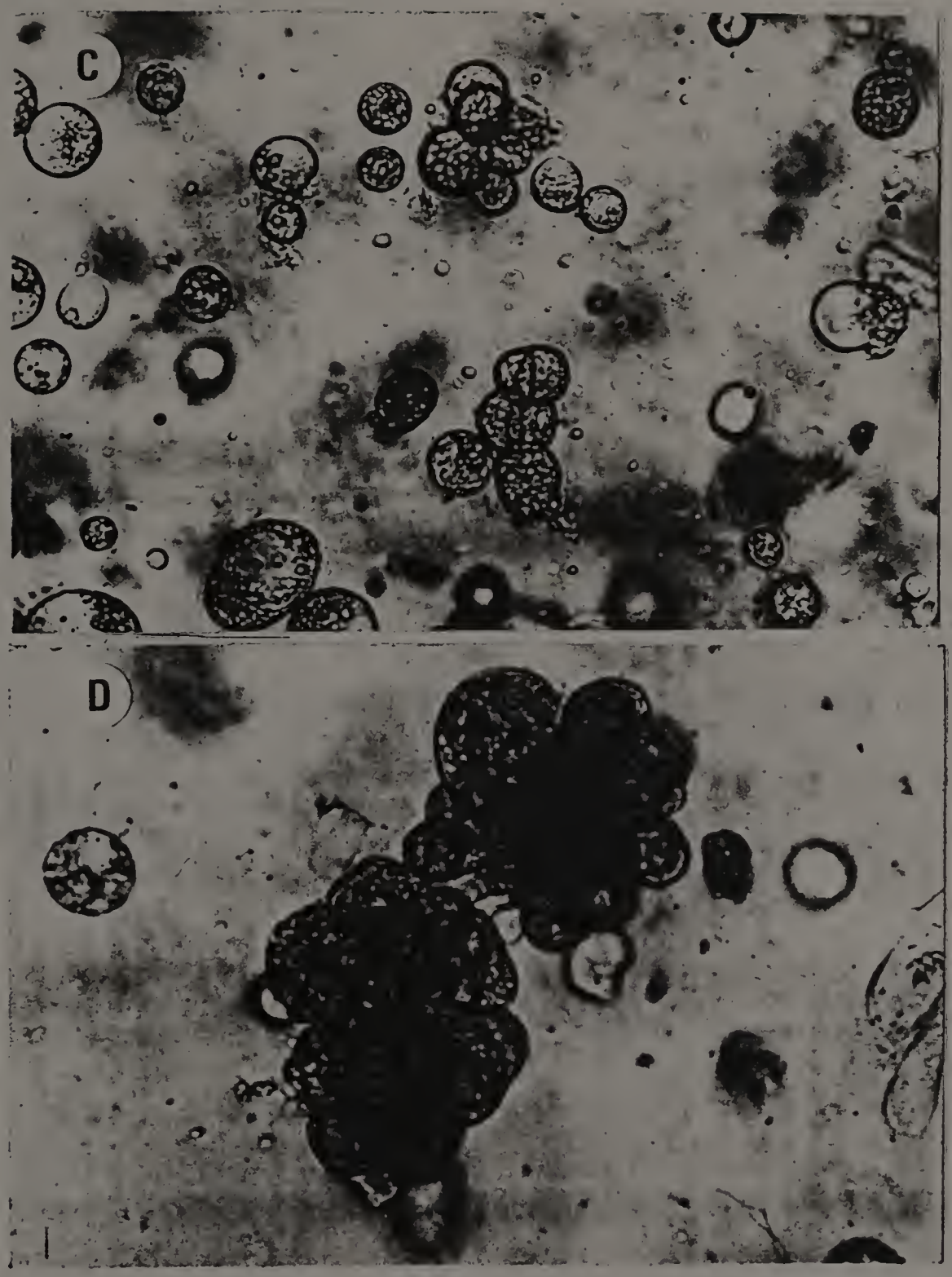

Figure 21. (A) Protoplast division and development of cell clusters during protoplast culture. X 200. (B) Formation of micro-calli from protoplasts of red fescue. X 100.

CHAPTER X

CONCLUSION

Stable embryogenic cell suspension cultures are difficult to establish. Availability of highly embryogenic callus is critical for the establishment of suspension cultures. Embryogenic callus of both red fescue and perennial ryegrass were obtained and it was observed that calli derived from crown tissue were more embryogenic than when mature caryopses were used as explants. Plant regeneration was easily achieved in red fescue by transferring to a hormone free medium. Although the embryogenic potential in perennial ryegrass was high, plant regeneration was hard to achieve. These results indicate that there is most likely a difference between embryo induction and embryo germination mechanisms and is probably genotype dependent. The failure of perennial ryegrass somatic embryos to germinate may also be attributed to the presence of some inhibitors which have to be reduced or eliminated prior to successful regeneration. The addition of some other substances used to enhance plant regeneration, such as activated charcoal, casien hydrolysate, sucrose, proline and others, have not improved embryo germination in perennial ryegrass. Only the addition of either cefotaxime or fluridone in combination with BA in the regeneration media have enhanced plant regeneration. The mode of action of these substances is not yet known, but it may be related to negating the effect of inhibitors.

Somatic embryogenesis has been shown to be a reliable and efficient method for the regeneration of plants from forage grasses. Therefore, protoplasts isolated from embryogenic tissue would be more likely to retain a greater morphogenetic capacity. The embryogenic capacity of the callus used in this study showed a progressive decline in its morphogenic capacity through repeated subcultures. Addition of activated charcoal (AC) resulted in an increase in the percentage of plants regenerated per callus. The favorable effect of AC was attributed to its ability to adsorb various inhibitors such as ABA, phenolics, or other compounds which might be present in culture, especially if cultures were left for long periods of time without transferring to fresh medium. The pretreatment of cultures with elevated sucrose levels dramatically re-instated high levels of embryogenesis and plant regeneration in long-term cultures of red fescue. These effects have been attributed to nutritional rather than osmotic factors as evidenced by the decrease in growth and appearance of necrotic tissue when mannitol was used at similar levels.

This research has also provided alternative methodology for separating embryogenic cell (E) types from non-embryogenic cell (NE) types in suspension cultures. The first method was a percoll discontinuous gradient which was based on the difference in density between E and NE cell types. The second method was based on the difference in size between E and NE cell types and entailed filtration through 31-um nylon mesh. Cells collected from both of these methods have resulted in an almost total elimination of NE cells. Both separation techniques have proven essential in the production of E

cell clusters and colonies. The addition of a wide range of compounds which have been reported to enhance E cell development have resulted in either enhancing or suppressing both E and NE cells.

The isolation and culture of protoplasts from embryogenic suspension cultures of both species has not been reported. Normally, an isolation mixture is very specific and limited to individual species. Results of this work provides the first isolation mixture which has proven effective for protoplast isolation from cell cultures of different species (red fescue and perennial ryegrass). Furthermore, agitation of isolation mixtures is a common practice to enhance the release of protoplasts. With the isolation mixture developed, agitation was not required for the isolation of high populations of viable protoplasts. During agitation, large amounts of protoplasts are released but a high percentage retain cell wall fragments. Moreover, agitation always results in high levels of cell debris which is very hard to eliminate during the protoplast washing sequence and, as such, overall numbers and viability are decreased.

Although a callus or suspension culture can undergo rapid cell division, the same cells usually do not divide after their cell walls are removed. It seems that cells lose their ability to undergo mitosis when their protoplasts are isolated. As yet, no explanation for this phenomenon is known, but some possibilities may include the following:

1. The enzyme preparations used to remove the cell walls may contain impurities and enzymatic activities that may damage cells.

2. Changes in the cell shape are known to disorganize arrays of cortical microtubles and the cells are then unable to repair the disrupted microtubles.
3. Enzymatic digestion of the cell walls may liberate molecules of low molecular weight that are take up and trigger the reaction in protoplasts such as depolymerization of microtubles.

The above-mentioned factors might be dependent on the genotype of the explant. The best example is the requirement for the inclusion of dimethyl sulfoxide (DMSO) to stimulate cell division of protoplasts of red fescue while DMSO was not as essential for perennial ryegrasses. The effect of DMSO might be attributed to its direct effect on the properties of plasma membranes including lipid composition, fluidity and permeability of ions.

The results presented in this dissertation provide the first steps toward the improvement of these species through the use of somatic hybridization, microinjection, electroporation, and various DNA uptake techniques. More work must, however, be directed towards the regeneration of viable plants from protoplasts prior to the use of these methods.

APPENDIX A

SOURCE AND CONCENTRATION OF COMPOUNDS TESTED TO ENHANCE PERCENTAGE OF EMBROYGENIC CELL TYPE OVER NONEMBRYOGENIC CELL TYPE

Table 22. The source and the concentration of the compounds which have been tested to enhance the percentage of embryogenic cell (E) type over the nonembryogenic cell (NE) type. All of these compounds have not resulted in selective enhancement of the E cell types over N cell types when tested alone or in various combination.

Compound	Level tested
Phytohormones:	
NAA	0.1,0.5,1.0,2.0,3.0,4.0,5.0 mg/l
2,4-D	1,2,3,4,5,6,7,8,9,10 mg/l
BA	0.1,0.3,0.5 mg/l
Kinetin	0.1,0.3,0.5 mg/l
Zeatin	0.1,0.3,0.5 mg/l
ABA	10,20,30,40,60,80,100,200,300,400-800 ug/l
Amino acids:	
Alanin	25,50,75,100 mM
Arginine	25,50,75,100 mM
Glycine	25,50,75,100 mM
Glutamine	25,50,75,100 mM
Lysine	25,50,75,100 mM
Proline	25,50,75,100 mM
Serine	25,50,75,100 mM
Tryptophane	25,50,75,100 mM
Sugars:	
Sucrose	10,20,30,40,50,60,70 g/l
Glucose	10,20,30,40,50,60,70 g/l
Galactose	10,20,30,40,50,60,70 g/l
Fructose	10,20,30,40,50,60,70 g/l

Table 22. (Continued)

Sugar alcohol:	
Sorbitol	25,50,75,100,200,300-1000 mM
Mannitol	25,50,75,100,200,300-1000 mM
Organic compounds:	
CH	0.5,1.0,1.0,2.0,3.0,4.0,5.0,6.0,7.0 g/l
YE	0.5,1.0,1.0,2.0,3.0,4.0,5.0,6.0,7.0
CW	5,10,15,20,25 ml/l

A P P E N D I X B

DETERMINATION OF DOUBLING TIME FOR EMBRYOGENIC CELL SUSPENSION CULTURE

Determination of The Doubling Time For Embryogenic Suspension Cultures

Ten ml of suspension cells were dispensed into each of the 21 Erlenmyer flasks (250 ml) containing 40 ml of fresh medium. The PCV was determined every 3 days. Fast-growing freely-suspended cells used in the above mentioned studies, were taken at the exponential phase and were used in all experiments. All media used in the above studies contained 6 mg/l 2,4-D. The pH of all media was adjusted to 5.7 prior to autoclaving. Every experiment was repeated at least three times and with three flasks for every treatment. Every culture was placed on a gyratory shaker at 100 rpm and 26 $^{o}C \pm 2.0$.

A P P E N D I X C

THE EFFECT OF PLATING METHODS IN PLATING EFFICIENCY

Plating Techinques

Embryogenic suspension cells used in the following studies were taken from culture growing at the exponential phase. Culture were filtered through a 105-um nylon mesh and cells were concentrated to the desired density by centrifuging for 10 min at 80 x g before plating. Every treatment was represented with 3 plates and every plating method was repeated at least three times. Petri dishes were sealed with a parafilm strips and were placed in the dark at 26-28 ^{o}C $\pm$ 2.0.

Muliple Drop Culture Techniques

Five drops (100-ul) of each treatment under investigation were placed in the middle of 100x15 mm plastic petri dish with Eppendroff digital micropipet. Similar volumes of embryogenic cells (ca. 10^6) were added to the drop of the corresponding treatment in the Petri dish.

Cell Culture in Liquid Medium

The relationship between cell density and the frequency of cell division and clump formation was evaluated by the addition of 5 to 25 ml of suspension cells and bringing the combination up to 50 ml. Cultures were placed on a gyratory shakers at 100 rpm and at 26 ^{o}C $\pm$ 2.0. Packed cell volume was measured at 3-day intervals after subculturing.

Cell Plating in a Stationary Liquid Medium

Five ml of solidified medium with 8 g/l agar was poured into 60x15-mm Falcon Petri dish, and 1 ml of suspension cells (10^6) were placed on the top of the agar.

Cell Plating in Agar

200 ul of unsolidified medium of each treatment was poured in 60x15-mm Falcon Petri dish. Another 200 ml of freely-suspended cells (10^6/ml) was mixed with the warm agar.

Table 23. Effect of plating methods on cell division in suspension cultures, expressed as an increase in packed cell volume determined 15 days after plating. All media contained 2 mg/l 2,4-D.

Plating method:	Packed cell volume (PCV)
Multiple drop culture technique	0.25
Cell culture in liquid medium	5.5
Cell plating in thin stationary liquid medium	z
Cell plating in agar	z

[z]No colony formation was noticed.

A P P E N D I X D

DETERMINATION OF THE OPTIMAL ENZYME MEDIUM FOR PROTOPLAST ISOLATION OF PERENNIAL RYEGRASS EMBRYOGENIC CELL SUSPENSION CULTURE

Table 24. The effect of mellibiose on the efficiency of protoplast released and degree of damage from perennial ryegrass embryogenic suspension cells after 6 h of incubation with no agitation at 26 ^{o}C ± 2.0.

Mellibiose concentration (%)	Protoplast yield per ml	Degree of damage[a]
0.3	$2.2x10^6$	+
0.5	$2.9x10^6$	+

[a]Degree of protoplast damage estimated visually at the end of incubation period, ++++ maximum damage; +++ 30% damage, ++ 20% damage; +, 10% damage; - no damage.

Table 25. The effect of calcium chloride on the efficiency of protoplast released and degree of damage from perennial ryegrass embryogenic suspension cells after 6 h of incubation with no agitation at 26 ^{o}C ± 2.0.

$CaCl_2$ (M)	Protoplast yield per ml	Degree of damage[a]
0.2	$3.0x10^6$	+
0.3	$8.0x10^6$	-
0.4	$1.0x10^7$	-
0.5	$7.0x10^6$	-
0.6	$5.0x10^6$	-

[a]Degree of protoplast damage estimated visually at the end of incubation period, ++++ maximum damage; +++ 30% damage, ++ 20% damage; +, 10% damage; - no damage.

Table 26. The effect of macerase on the efficiency of protoplast released and degree of damage from perennial ryegrass embryogenic suspension cells after 6 h of incubation with no agitation at 26 ^{o}C ± 2.0.

Macerase concentration (%)	Protoplast yield per ml	Degree of damage[a]
0.2	$1.2x10^6$	-
0.4	$1.9x10^6$	+
0.6	$2.7x10^6$	-
0.8	$3.9x10^6$	-
1.0	$2.7x10^6$	++

[a]Degree of protoplast damage estimated visually at the end of each incubation period, ++++ maximum damage; +++ 30% damage, ++ 20% damage; +, 10% damage; - no damage.

Table 27. The effect of mannitol on the efficiency of protoplast released and degree of damage from perennial ryegrass embryogenic suspension cells after 6 h of incubation with no agitation at 26 ^{o}C ± 2.0.

Mannitol (M)	Protoplast yield per ml	Degree of damage[a]
0.2	$1.5x10^6$	+
0.3	$4.2x10^6$	-
0.4	$3.6x10^6$	-
0.5	$3.1x10^6$	+
0.6	$2.0x10^6$	+
0.7	$9.0x10^5$	+
0.8	$5.0x10^5$	+

[a]Degree of protoplast damage estimated visually at the end of incubation period, ++++ maximum damage; +++ 30% damage, ++ 20% damage; +, 10% damage; - no damage.

Table 28. The effect of subculturing period on the efficiency of protoplast released and degree of damage from perennial ryegrass embryogenic suspension cells after various periods of incubation with no agitation at 26 $^{o}C \pm 2.0$.

Subculturing period (days)	Incubation period (h)	Protoplast yield per ml	Degree of damage[a]
Shaking (100 rpm)			
2	1	$5.6x10^6$	+++
	3	$4.0x10^6$	++++
	6	$9.0x10^5$	++++
	9	$8.0x10^5$	++++
	12	$5.0x10^5$	++++
7 days	1	$1.1x10^6$	++
	3	$3.0x10^5$	++++
	6	$1.5x10^5$	++++
	9	$1.1x10^5$	++++
	12	$1.0x10^5$	++++
No Shaking			
2	1	$2.4x10^5$	-
	3	$6.1x10^5$	-
	6	$2.5x10^6$	-
	9	$4.7x10^6$	-
	12	$6.7x10^6$	-
7	1	$6.2x10^5$	-
	3	$2.3x10^5$	-
	6	$2.8x10^5$	-
	9	$1.5x10^6$	-
	12	$2.9x10^6$	-

[a]Degree of protoplast damage estimated visually at the end of incubation period, ++++ maximum damage; +++ 30% damage, ++ 20% damage; +, 10% damage; - no damage.

Table 29. The effect of sodium thiosulfate on the efficiency of protoplasts released and degree of damage from perennial ryegrass embryogenic suspension cells after various periods of incubation with the enzyme mixture at 26 °C ± 2.0 on a gyratory shaker at 100 rpm.

Sodium thiosulfate concentration (1%)	Incubation period (min)	Protoplast yield per ml	Degree of damage[a]
0.0	15	$4.0x10^5$	+
	30	$1.1x10^6$	++
	60	$7.0x10^5$	++++
3	15	$6.0x10^5$	-
	30	$3.0x10^6$	-
	60	$5.6x10^6$	+
4	15	$9.0x10^6$	-
	30	$3.1x10^6$	-
	60	$5.4x10^6$	+
5	15	$7.0x10^5$	-
	30	$3.4x10^6$	-
	60	$5.1x10^6$	+
6	15	$4.0x10^5$	-
	30	$3.0x10^6$	-
	60	$4.9x10^6$	+

[a]Degree of protoplast damage estimated visually at the end of incubation period. ++++, maximum damage; 30% maximum damage; ++, 20% damage; +, 10% damage; - no damage.

Table 30. The effect of sodium thiosulfate on the efficiency of the protoplast released and degree of damage from perennial ryegrass embryogenic suspension cells after 6 h of incubation without agitation at 26 ^{o}C ± 2.0.

Sodium thiosulfate (%)	Protoplast yield per ml	Degree of damage[a]
0.0	$7.0x10^5$	+
3.0	$5.1x10^6$	-
4.0	$4.0x10^6$	-
5.0	$3.5x10^6$	-
6.0	$2.4x10^6$	-

[a]Degree of protoplast damage estimated visually at the end of incubation period. ++++, maximum damage; 30% maximum damage; ++, 20% damage; +, 10% damage; - no damage.

Table 31. The effect of cellulysine on the efficiency of the protoplast released and degree of damage from perennial ryegrass embryogenic suspension cells after 6 h of incubation without agitation at 26 ^{o}C ± 2.0.

Cellulysine concentration (%)	Protoplast yield per ml	Degree of damage[a]
1.0	$2.0x10^6$	-
1.25	$4.5x10^6$	-
1.5	$3.5x10^6$	+
1.75	$2.8x10^6$	+
2.0	$2.2x10^6$	+

[a]Degree of protoplast damage estimated visually at the end of incubation period. ++++, maximum damage; 30% maximum damage; ++, 20% damage; +, 10% damage; - no damage.

Table 32. The effect of pH on the efficiency of the protoplast released and degree of damage from perennial ryegrass embryogenic suspension cells after 6 h of incubation without agitation at 26 ^{o}C ± 2.0.

pH of the enzyme mixture	Protoplasts yield per ml	Degree of damage[a]
4.0	$3.6x10^6$	-
4.5	$2.3x10^6$	-
5.0	$4.0x10^6$	-
5.5	$4.3x10^6$	-
6.0	$2.4x10^6$	-
6.5	$1.3x10^6$	-
7.0	$3.0x10^5$	-
7.5	$6.0x10^5$	-
8.0	$3.5x10^6$	-

[a]Degree of protoplast damage estimated visually at the end of incubation period. ++++, maximum damage; 30% maximum damage; ++, 20% damage; +, 10% damage; - no damage.

A P P E N D I X E

DETERMINATION OF THE OPTIMAL ENZYME MEDIUM FOR PROTOPLAST ISOLATION OF RED FESCUE EMBRYOGENIC CELL SUSPENSION CULTURE

Table 33. The effect of mellibiose on the efficiency of protoplasts released and degree of damage from red fescue embryogenic suspension cells after 6 h of incubation with no agitation 26 ^{0}C ± 2.0.

Mellibiose concentration (%)	Protoplast yield per ml	Degree of damage[a]
0.3	$5.0x10^{6}$	+
0.5	$6.5x10^{6}$	++

[a]Degree of protoplast damage estimated visually at the end of incubation period, ++++ maximum damage; +++ 30% damage, ++ 20% damage; +, 10% damage; - no damage.

Table 34. The effect of mannitol on the efficiency of protoplasts released and degree of damage from red fescue embryogenic suspension cells after 6 h of incubation with no agitation 26 ^{o}C ± 2.0.

Mannitol (M)	Protoplast yield per ml	Degree of damage[a]
0.2	4.0×10^6	+
0.3	9.0×10^6	-
0.4	7×10^6	-
0.5	4×10^6	-
0.6	2×10^6	-
0.7	8×10^5	+
0.8	5×10^5	+

[a]Degree of protoplast damage estimated visually at the end of incubation period, ++++ maximum damage; +++ 30% damage, ++ 20% damage; +, 10% damage; - no damage.

Table 35. The effect of calcium chloride on the efficiency of protoplasts released and degree of damage from red fescue embryogenic suspension cells after 6 h of incubation with no agitation 26 $^{o}C \pm 2.0$.

$CaCl_2$ (M)	Protoplast yield per ml	Degree of damage[a]
0.2	$3.0x10^6$	-
0.3	$7.0x10^6$	-
0.4	$1.0x10^7$	-
0.5	$7.0x10^6$	-
0.6	$5.0x10^6$	-

[a]Degree of protoplast damage estimated visually at the end of incubation period, ++++ maximum damage; +++ 30% damage, ++ 20% damage; +, 10% damage; - no damage.

Table 36. The effect of macerase on the efficiency of protoplasts released and degree of damage from red fescue embryogenic suspension cells after 6 h of incubation with no agitation 26 ^{0}C ± 2.0.

Macerase concentration (%)	Protoplast yield per ml	Degree of damage[a]
0.2	$5.0x10^6$	+
0.4	$5.0x10^6$	+
0.6	$6.0x10^6$	+
0.8	$3.0x10^6$	+
1.0	$3.0x10^6$	+

[a]Degree of protoplast damage estimated visually at the end of incubation period, ++++ maximum damage; +++ 30% damage, ++ 20% damage; +, 10% damage; - no damage.

Table 37. The effect of sodium thiosulfate on the efficiency of protoplasts released and degree of damage from red fescue embryogenic suspension cells after various periods of incubation with the enzyme mixture at 26 ^{o}C ± 2.0 on a gyratory shaker at 100 rpm .

Sodium thiosulfate concentration (%)	Incubation period	Protoplast yield per ml	Degree of damage[a]
0.0	15	$5.0x10^5$	+
	30	$2.0x10^6$	++
	60	$6.0x10^5$	++++
1.0	15	$1.1x10^6$	-
	30	$4.0x10^6$	-
	60	$2.0x10^6$	+
2	15	$3.0x10^6$	-
	30	$4.0x10^6$	-
	60	$8.0x10^6$	+
3	15	$6.0x10^6$	-
	30	$9.0x10^6$	-
	60	$9.0x10^6$	+
4	15	$6.0x10^6$	+
	30	$7.0x10^6$	+
	60	$5.0x10^6$	+
5	15	$3.0x10^6$	+
	30	$7.0x10^6$	+
	60	$5.0x10^6$	+
6	15	$3.0x10^6$	+
	30	$7.0x10^6$	+
	60	$2.0x10^6$	+

[a]Degree of protoplast damage estimated visually at the end of incubation period. ++++, maximum damage; 30% maximum damage; ++, 20% damage; +, 10% damage; - no damage.

Table 38. The effect of sodium thiosulfate on the efficiency of the protoplast released and degree of damage from red fescue embryogenic suspension cells after 6 h of incubation without agitation at 26 ^{o}C ± 2.0.

Sodium thiosulfate (%)	Protoplast yield per ml	Degree of damage[a]
0.0	$7.0x10^5$	+[a]
1.0	$1.0x10^6$	-
2.0	$2.8x10^6$	-
3.0	$8.0x10^6$	-
4.0	$9.0x10^6$	-
5.0	$7.0x10^6$	-
6.0	$4.0x10^6$	-

[a]Degree of protoplast damage estimated visually at the end of incubation period. ++++, maximum damage; 30% maximum damage; ++, 20% damage; +, 10% damage; - no damage.

Table 39. The effect of cellulysine on the efficiency of the protoplast released and degree of damage from red fescue embryogenic suspension cells after 6 h of incubation without agitation at 26 ^{o}C ± 2.0.

Cellulysine concentration (%)	Protoplasts yield per ml	Degree of damage[a]
1.0	$4.0x10^6$	-
1.25	$9.0x10^6$	-
1.5	$7.0x10^6$	-
1.75	$7.0x10^6$	-
2.0	$5.0x10^6$	+

[a]Degree of protoplast damage estimated visually at the end of incubation period. ++++, maximum damage; 30% maximum damage; ++, 20% damage; +, 10% damage; - no damage.

Table 40. The effect of pH on the efficiency of the protoplast released and degree of damage from red fescue embryogenic suspension cells after 6 h of incubation without agitation at 26 $^{o}C \pm 2.0$.

pH	Protoplast yield per ml	Degree of damage[a]
4.0	$5.0x10^6$	-
4.5	$4.0x10^6$	-
5.0	$4.3x10^6$	-
5.5	$8.0x10^6$	-
6.0	$5.0x10^6$	-
6.5	$4.4x10^6$	-
7.0	$2.0x10^5$	-
7.5	$8.0x10^5$	-
8.0	$4.0x10^5$	-

[a]Degree of protoplast damage estimated visually at the end of incubation period. ++++, maximum damage; 30% maximum damage; ++, 20% damage; +, 10% damage; - no damage.

Table 41. The effect of packed cell volume/enzyme mixture ratio on the efficiency of the protoplast released and degree of damage from red fescue embryogenic suspension cells after 6 h of incubation without agitation at 26 ^{o}C ± 2.0.

Packed cell volume (PCV/ml)	Protoplast yield per ml	Degree of damage[a]
0.2	$1.0x10^6$	-[a]
0.4	$2.0x10^6$	-
0.6	$6.0x10^6$	-
0.8	$7.0x10^6$	-
1.0	$9.0x10^6$	-
1.2	$7.0x10^6$	-

[a]Degree of protoplast damage estimated visually at the end of incubation period. ++++, maximum damage; 30% maximum damage; ++, 20% damage; +, 10% damage; - no damage.

A P P E N D I X F

DETERMINATION OF THE OPTIMAL ENZYME MEDIUM FOR PROTOPLAST ISOLATION OF RED FESCUE EMBRYOGENIC CALLUS

Table 42. The effect of mellibiose on the efficiency of protoplasts released and degree of damage from red fescue embryogenic callus culture after 4 h of incubation at 100 rpm at 26 ^{o}C ± 2.0.

Mellibiose concentration (%)	Protoplast yield per ml	Degree of damage[a]
0.3	$4.0x10^5$	++
0.5	$6.0x10^5$	+

[a]Degree of protoplast damage estimated visually at the end of incubation period, ++++ maximum damage; +++ 30% damage, ++ 20% damage; +, 10% damage; - no damage.

Table 43. The effect of calcium chloride on the efficiency of protoplasts released and degree of damage from red fescue embryogenic callus culture after 4 h of incubation at 100 rpm at 26 $^{o}C \pm 2.0$.

$CaCl_2$ (M)	Protoplast yield per ml	Degree of damage[a]
0.2	$2.0x10^5$	+
0.3	$6.0x10^5$	-
0.4	$9.0x10^5$	-
0.5	$4.0x10^5$	-
0.6	$3.0x10^3$	-

[a]Degree of protoplast damage estimated visually at the end of incubation period, ++++ maximum damage; +++ 30% damage, ++ 20% damage; +, 10% damage; - no damage.

Table 44. The effect of macerase on the efficiency of protoplasts released and degree of damage from red fescue embryogenic callus culture after 4 h of incubation at 100 rpm at 26 ^{o}C ± 2.0.

Macerase concentration (%)	Protoplast yield per ml	Degree of damage[a]
0.2	$3.0x10^5$	-[a]
0.4	$6.0x10^5$	-
0.6	$6.0x10^5$	-
0.8	$6.0x10^5$	-
1.0	$5.0x10^5$	-

[a]Degree of protoplast damage estimated visually at the end of incubation period, ++++ maximum damage; +++ 30% damage, ++ 20% damage; +, 10% damage; - no damage.

Table 45. The effect of mannitol on the efficiency of protoplasts released and degree of damage from red fescue embryogenic callus culture after 4 h of incubation at 100 rpm at 26 ^{o}C ± 2.0.

Mannitol (M)	Protoplast yield per ml	Degree of damage[a]
0.2	$2.0x10^5$	-
0.3	$5.0x10^5$	-
0.4	$7.0x10^5$	-
0.5	$3.0x10^5$	+
0.6	$1.0x10^5$	+
0.7	$5.0x10^4$	+
0.8	$2.0x10^4$	+

[a]Degree of protoplast damage estimated visually at the end of incubation period, ++++ maximum damage; +++ 30% damage, ++ 20% damage; +, 10% damage; - no damage.

Table 46. The effect of subculturing period on the efficiency of protoplasts released and degree of damage from red fescue embryogenic callus culture after 4 h of incubation at 100 rpm at 26 $^{o}C \pm 2.0$.

Subculturing period (days)	Protoplast yield per ml	Degree of damage[a]
2	$7.5x10^2$	+
4	$8.0x10^2$	+
6	$1.0x10^3$	+
8	$1.0x10^3$	-
10	$3.0x10^3$	-
12	$6.0x10^3$	-
14	$8.0x10^4$	-
16	$1.0x10^5$	-
18	$6.0x10^5$	+
20	$4.0x10^5$	++

[a]Degree of protoplast damage estimated visually at the end of incubation period, ++++ maximum damage; +++ 30% damage, ++ 20% damage; +, 10% damage; - no damage.

Table 47. The effect of callus ratio/enzyme mixture ratio on the efficiency of protoplast released and degree of damage from red fescue embryogenic callus after 4 hrs of incubation with the enzyme mixture at 26 ^{o}C ± 2.0 with agitation at 100 rpm.

Callus weight (g/ml)	Protoplast yield per ml	Degree of damage[a]
0.2	$7.0x10^5$	-
0.4	$1.1x10^6$	-
0.6	$1.3x10^6$	+
0.8	$8.0x10^5$	+
1.0	$4.0x10^5$	+
1.2	$1.4x10^5$	++

[a]Degree of protoplast damage estimated visually at the end of incubation period. ++++, maximum damage; 30% maximum damage; ++, 20% damage; +, 10% damage; - no damage.

Table 48. The effect of cellulysine on the efficiency of protoplast released and degree of damage from red fescue embryogenic callus after 4 hrs of incubation with the enzyme mixture at 26 $^{o}C \pm 2.0$ with agitation at 100 rpm.

Cellulysine concentration (%)	Protoplast yield per ml	Degree of damage[a]
1.0	$3.0x10^5$	-
1.25	$8.0x10^5$	-
1.5	$8.0x10^5$	-
1.75	$5.0x10^5$	+
2.0	$1.0x10^5$	+

[a]Degree of protoplast damage estimated visually at the end of incubation period. ++++, maximum damage; 30% maximum damage; ++, 20% damage; +, 10% damage; - no damage.

Table 49. The effect of pH on the efficiency of protoplast released and degree of damage from red fescue embryogenic callus after 4 hrs of incubation with the enzyme mixture at 26 $^{o}C \pm$ 2.0 with agitation at 100 rpm.

pH of the enzyme mixture	Protoplasts yield per ml	Degree of damage[a]
4.0	$2.0x10^5$	+
4.5	$4.0x10^5$	+
5.0	$5.0x10^5$	+
5.5	$5.0x10^5$	-
6.0	$5.0x10^5$	-
6.5	$3.0x10^5$	-
7.0	$8.0x10^4$	-
7.5	$6.0x10^4$	-
8,0	$5.0x10^4$	-

[a]Degree of protoplast damage estimated visually at the end of incubation period. ++++, maximum damage; 30% maximum damage; ++, 20% damage; +, 10% damage; - no damage.

A P P E N D I X G

DETERMINATION OF THE OPTIMAL ENZYME MEDIUM FOR PROTOPLAST ISOLATION OF PERENNIAL RYEGRASS EMBRYOGENIC CALLUS

Table 50. The effect of mellibiose on the efficiency of protoplasts released and degree of damage from perennial ryegrass embryogenic callus culture after 6 h of incubation without agitation at 26 $^{o}C \pm 2.0$.

Mellibiose concentration (%)	Protoplast yield per ml	Degree of damage[a]
0.3	$9.0x10^5$	+
0.5	$1.1x10^6$	-

[a]Degree of protoplast damage estimated visually at the end of incubation period, ++++ maximum damage; +++ 30% damage, ++ 20% damage; +, 10% damage; - no damage.

Table 51. The effect of calcium chloride on the efficiency of protoplasts released and degree of damage from perennial ryegrass embryogenic callus culture after 6 h of incubation without agitation at 26 $^{o}C \pm 2.0$.

$CaCl_2$ (M)	Protoplast yield per ml	Degree of damage[a]
0.2	$5.0x10^5$	-
0.3	$2.0x10^6$	-
0.4	$1.0x10^6$	-
0.5	$8.0x10^5$	+
0.6	$4.0x10^5$	++

[a]Degree of protoplast damage estimated visually at the end of incubation period, ++++ maximum damage; +++ 30% damage, ++ 20% damage; +, 10% damage; - no damage.

Table 52. The effect of macerase on the efficiency of protoplasts released and degree of damage from perennial ryegrass embryogenic callus culture after 6 h of incubation without agitation at 26 $^{o}C \pm 2.0$.

Macerase concentration (%)	Protoplast yield per ml	Degree of damage[a]
0.2	$1.0x10^5$	-
0.4	$9.0x10^5$	-
0.6	$7.0x10^5$	-
0.8	$3.0x10^5$	+
1.0	$1.0x10^3$	++++

[a]Degree of protoplast damage estimated visually at the end of incubation period, ++++ maximum damage; +++ 30% damage, ++ 20% damage; +, 10% damage; - no damage.

Table 53. The effect of mannitol on the efficiency of protoplasts released and degree of damage from perennial ryegrass embryogenic callus culture after 6 h of incubation without agitation at 26 $^{o}C \pm 2.0$.

Mannitol (M)	Protoplast yield per ml	Degree of damage[a]
0.2	$5.0x10^5$	-
0.3	$2.0x10^6$	-
0.4	$1.0x10^6$	-
0.5	$7.0x10^5$	-
0.6	$5.0x10^5$	+
0.7	$1.0x10^5$	+
0.8	$1.0x10^5$	+

[a]Degree of protoplast damage estimated visually at the end of incubation period, ++++ maximum damage; +++ 30% damage, ++ 20% damage; +, 10% damage; - no damage.

Table 54. The effect of subculturing periods on the efficiency of protoplasts released and degree of damage from perennial ryegrass embryogenic callus culture after 6 h of incubation without agitation at 26 $^{o}C \pm 2.0$.

Subculture period (days)	Protoplast yield per ml	Degree of damage[a]
2	$3.0x10^3$	-
4	$4.0x10^4$	-
6	$6.0x10^4$	-
8	$1.0x10^5$	-
10	$7.0x10^7$	-
12	$9.0x10^5$	-
14	$5.0x10^6$	-
16	$3.0x10^6$	+
18	$9.0x10^4$	+
20	$6.0x10^4$	+

[a]Degree of protoplast damage estimated visually at the end of incubation period, ++++ maximum damage; +++ 30% damage, ++ 20% damage; +, 10% damage; - no damage.

Table 55. The effect of sodium thiosufate on the efficiency of protoplast released and degree of damage from perennial ryegrass embryogenic callus after various periods of incubation at 26 ^{o}C ± 2.0 with agitation at 100 rpm.

Sodium thiosulfate concentration (%)	Incubation period (min)	Protoplast yield per ml	Degree of damage[a]
0.0	15	$1.0x10^5$	+
	30	$7.0x10^5$	++
	60	$4.0x10^4$	+++
1.0	15	$5.0x10^5$	-
	30	$1.0x10^6$	-
	60	$8.0x10^5$	++
2.0	15	$1.0x10^6$	-
	30	$3.0x10^6$	-
	60	$8.0x10^6$	+
3.0	15	$2.0x10^6$	-
	30	$3.0x10^6$	-
	60	$3.0x10^6$	+
4.0	15	$5.0x10^6$	-
	30	$8.0x10^6$	-
	60	$6.0x10^6$	+
5.0	15	$9.0x10^5$	-
	30	$3.0x10^6$	-
	60	$1.0x10^6$	+
6.0	15	$5.0x10^5$	-
	30	$1.0x10^6$	-
	60	$9.0x10^5$	+

[a]Degree of protoplast damage estimated visually at the end of incubation period. ++++, maximum damage; 30% maximum damage; ++, 20% damage; +, 10% damage; - no damage.

Table 56. The effect of sodium thiosulfate on the efficiency of the protoplasts released and degree of damage from perennial ryegrass embryogenic callus after 6 h of incubation in enzyme mixture without agitation at 26 ^{o}C ± 2.0.

Sodium thiosulfate (%)	Protoplast yield per ml	Degree of damage[a]
0.0	$3.0x10^5$	+
1.0	$6.0x10^5$	-
2.0	$8.0x10^5$	-
3.0	$1.0x10^6$	-
4.0	$8.0x10^5$	-
5.0	$4.0x10^5$	-
6.0	$1.0x10^5$	-

[a]Degree of protoplast damage estimated visually at the end of incubation period. ++++, maximum damage; 30% maximum damage; ++, 20% damage; +, 10% damage; - no damage.

Table 57. The effect of cellulysine on the efficiency of the protoplasts released and degree of damage from perennial ryegrass embryogenic callus after 6 h of incubation in enzyme mixture without agitation at 26 ^{o}C ± 2.0.

Cellulysine concentration (%)	Protoplast yield per ml	Degree of damage[a]
1.0	8.0×10^5	+
1.25	2.0×10^6	-
1.5	1.0×10^6	+
1.75	8.0×10^5	+
2.0	4.0×10^5	+

[a]Degree of protoplast damage estimated visually at the end of incubation period. ++++, maximum damage; 30% maximum damage; ++, 20% damage; +, 10% damage; - no damage.

Table 58. The effect of pH on the efficiency of the protoplasts released and degree of damage from perennial ryegrass embryogenic callus after 6 h of incubation in enzyme mixture without agitation at 26 $^{o}C \pm 2.0$.

pH of the enzyme mixture	Protoplast yield per ml	Degree of damage[a]
4.0	$4.0x10^5$	-
4.5	$7.0x10^5$	-
5.0	$7.0x10^5$	-
5.5	$7.0x10^5$	-
6.0	$7.0x10^5$	-
6.5	$2.0x10^5$	-
7.0	$1.0x10^5$	-
7.5	$4.0x10^4$	-
8.0	$2.0x10^4$	-

[a]Degree of protoplast damage estimated visually at the end of incubation period. ++++, maximum damage; 30% maximum damage; ++, 20% damage; +, 10% damage; - no damage.

Table 59. The effect of callus enzyme mixture ratio on the efficiency of the protoplasts released and degree of damage from perennial ryegrass embryogenic callus after 6 h of incubation in enzyme mixture without agitation at 26 ^{o}C ± 2.0.

Callus weight (g)	Protoplast yield per ml	Degree of damage[a]
0.2	$5.0x10^5$	-
0.4	$8.0x10^5$	-
0.6	$6.0x10^5$	-
0.8	$3.0x10^5$	-
1.0	$1.0x10^5$	-
1.2	$6.0x10^4$	-

[a]Degree of protoplast damage estimated visually at the end of incubation period. ++++, maximum damage; 30% maximum damage; ++, 20% damage; +, 10% damage; - no damage.

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