THE EFFECT OF GROWTH REGULATORS ON *IN VITRO* CALLUS INITIATION IN EXCISED WHEAT ROOTS

by

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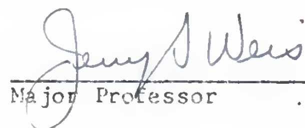

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INTRODUCTION

The field of plant tissue culture had its origin in the ideas and early experiments of Haberlandt (1902) but it was thirty years later before successful *in vitro* cultures were obtained. White (1931) surveyed the early history of the field and by a series of experiments with excised root tips, emphasized the suitability of the Kott-Robbins root tip method for study plant nutrition (White 1932a, b; 1933a, b). White's work culminated in 1934 when he demonstrated potential unlimited growth of excised tomato roots *in vitro* (White 1934). About the same time Gautheret (1934, 1935, 1938) was making an intensive study of the culture of cambial tissues, and in 1939, Gautheret, Nobecourt, and White published independently the successful cultivation of tobacco and carrot tissues. These tissues are still in culture today and they represent the first true plant tissue culture in the form of unorganized mass of cells (White 1963).

Plant tissue culture could be divided into four major classes according to the organ or tissues involved: a) root cultures, b) embryo culture, c) stem culture, and d) culture of undifferentiated masses or callus culture. Roots have perhaps been the most intensively studied because their growth is primarily by an increase in length with little or no increase in diameter and they will float on the surface of liquid media which makes aeration no problem in their culture (White 1953).

Research has shown that with the proper medium nearly all parts of dicotyledonous plants can be cultured. However, this is not the case

with tissues and organs excised from monocotyledonous plants. Loo (1945) succeeded in culturing the apical meristem of Asparagus officinalis. Morel and Wetmore (1951) found that it was possible to obtain the in vitro proliferation of Amorphophallus rivieri tissues for an indefinite time providing coconut milk was present in the medium. Zeibur and Brink (1951) noted a stimulation of in vitro Hordeum embryo growth as well as embryos from other genera by Hordeum endosperm. Straus and LaRue (1954) reported the successful culturing of maize endosperm tissue. Tamaoki and Ullstrup (1958) achieved growth of excised corn endosperm and meristem tissue on a modified Nitsch's medium supplemented with Difco yeast extract or coconut milk. They found that the actively growing cultures were very friable while the slower growing tissues were more compact.

Carew and Schwarting (1958) successfully obtained callus tissue from excised rye embryos on a modified Heller's medium containing sucrose, yeast extract, and 2,4-D.[a] Casein hydrolysate added to the medium gave maximum growth for sub-culturing the tissues. Norstog (1961) reported in vitro growth of excised Hordeum vulgare L. embryos on various media provided when coconut milk was added. However, the addition of various amino acids to the medium didn't promote growth and differentiation in Hordeum tissues in the absence of coconut milk. Webster (1966) obtained callus from the oat seedling in the presence of IAA and NAA within six weeks after treatment, and this callus was maintained three years through several sub-cultures. Callus was also obtained from onion with best growth occuring on media containing 2,4-D, ethylenediaminotetraacetic

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[a]Abbreviations used = 1. 2,4-D (2,4-dichlorophenoxyacetic acid); 2. IAA (Indole - 3 - acetic acid); 3. NAA (naphthaleneacetic acid); 4. GA (gibberellic acid); 5. K (Kinetin); 6. CM (coconut milk; 7. BM (basal medium).

acid (EDTA) and sucrose, although it grew slowly and survived only one year. He also reported that callus formed more readily in the light than in the dark.

Yamada et al. (1967) reported that 2,4-D was necessary for callus induction from Oryza sativa on a completely synthetic medium. Kinetin was not essential for callus induction and indoleacetic acid was effective only at higher concentration. Yamada et al. (1964) made a cytological study of cultured cells of Tradescantia paludosa. They obtained calli from young flower buds, roots and stems, and found the calli contained diploid meristematic colonies where cell division took place and giant cells with polyploid nuclei as a result of endomitosis. Carter et al. (1967) reported successful callus formation from emerging roots and shoots of Avena sativa seeds when they were placed directly on media containing IAA. Yatazawa et al. (1967a) obtained callus tissues on the roots of rice seedling cultured on a modified Heller medium. They found that 2,4-D and yeast extract were necessary for growth, and that although no organ formation was apparent, some tissue differentiation was observed. Mohan Ram and Steward (1964) obtained proliferated growth of several cultivated varieties of banana fruit tissue cultured at different stages of development. Smith (1965) noted that excised embryo shoot apices of wheat were, like most other monocotyledons, difficult to grow. Warick and Fuchs (1968) obtained callus formation of wheat root from seeds directly germinated on agar medium of Knopp's solution containing 2,4-D. Klein and Edsall (1968) reported that callus tissues were derived from the primary root tips of rice, corn, wheat, oat in media containing mineral salts, sucrose, 2,4-D, coconut milk, and sources of amino acids.

Examination of information from above and data from other sources

reveal several major differences between culture techniques for dicotyledonous and monocotyledonous tissues. The latter almost universally has a relatively slower growth rate and somewhat limited life span compared to the former. Also there is an almost universal use of complex additives i.e. yeast extract, casein hydrolysate, coconut milk, etc. in the medium for culturing monocot tissues. In many cases the absolute need for these components or the active fraction of the complex additive has not been ascertained. Lastly, the use of various growth regulators for the initiation and maintenance of monocot cultures is far from being understood.

Thus, with these questions in mind, a study was made of the role of various growth regulators on the initiation and development of callus from wheat roots. Data are also present on the effect of presence or absence of the root tip on callus development and of histological studies of developed callus tissues.

MATERIALS AND METHODS

Culturing of Tissues

Seeds of *Triticum vulgare* L. var Bison were sterilized 10 minutes in 0.2% mercurous chloride ($HgCl_2$) containing 0.001% Tween 20, were washed several times with sterile distilled water and were placed in petri dishes on sterile 1% agar. Seeds were germinated in the dark for 48-72 hours at 30°. A 30 mm section was excised from primary roots in the following manner: a) section with the root tip intact, and b) section with the apical 3 mm removed. Two to three root sections were transferred aseptically to 125 ml Erlenmeyer flasks containing 50 ml of culture medium and plugged with cotton.

The culture medium (Table I) was that used by Murashige and Skoog (1962) and was supplemented by the addition of IAA, 2,4-D, NAA, kinetin, gibberellic acid, coconut milk, alone and in combination. The pH was adjusted to 5.7-6.0 with 0.1 NKOH, the medium solidified with 1% agar and sterilized 20 minutes at 16 p.s.i.

The tissues were grown in the dark at 27 ± 2 for five, seven, or ten weeks.

Histological Study

Callus tissue was fixed in a mixture of glacial acetic acid: formalin (85: 15 v/v) for three days and then dehydrated through a graded series of t-butyl alcohol. The tissues were embedded in paraffin and 10 μ sections were mounted on glass slides and stained with safranin in

absolute ethanol with fast green as the counterstain and tannic acid: ferric chloride as a mordant (Sass, 1958).

Protein Determination

Determination of the soluble protein in tissues grown on various media was done according to the Folin method of Lowry *et. al.* (1951). Optical density was read at 660 mu in a B and L Spectronic 20 equipped with a wide-range photo tube.

Table I. Composition of Murashige's revised medium.

A. Mineral Salts

Major Elements			Minor Elements		
Salts	mg/l	mM	Salts	mg/l	μM
NH_4NO_3	1650	N41.2	H_3BO_3	6.2	100
KNO_3	1900	18.8	$MnSO_4 . H_2O$	22.3	100
$CaCl_2 . 2H_2O$	440	3.0	$ZnSO_4 .4 H_2O$	8.6	30
$MgSO_4 .7 H_2O$	370	1.5	KI	.83	5.0
$KH_2 PO_4$	170	1.25	$Na_2MoO_4 .2 H_2O$	0.25	1.0
Na_2 - EDTA	37.31	Na0.20	$CuSO_4 .5 H_2O$	0.025	0.1
Fe $SO_4 .7 H_2O$	27.81	Fe0.10	$CaCl_2 .6 H_2O$	0.025	0.1

5 ml/l of a stock solution containing 5.57 g Fe $SO_4 .7 H_2O$ and 7.45 g Na_2 - EDTA per liter of H_2O.

B. Organic Constituents

Sucrose	30 g/l
Myo - inositol	100 mg/l
Nicotinic acid	0.5 mg/l
Pyridoxin - HCl	0.5 mg/l
Thiamin - HCl	0.1 mg/l

Table II. The effect of growth regulators on callus development, elongation, and branching of intact wheat root sections.[1]

Medium	Age (weeks)	Callus development[3]	Increase in length, mm	Branching[4]
BM	6	0	15	1
BM + NAA[2]	6	1	3	1
BM + K + GA	6	0	22	0
BM + GA + IAA	6	0	2	3
BM + K + 2,4-D + CM	6	0	0	0
BM + K	7	0	15	0
BM + IAA	7	0	5	3
BM + K + IAA	7	0	0	1
BM + K + GA + IAA	7	0	3	1
BM + 2,4-D	8	3	0	2
BM + GA	8	0	15	3
BM + K + 2,4-D	8	2	0	1
BM + K + NAA	8	0	1	1
BM + GA + 2,4-D	8	3	0	2
BM + GA + NAA	8	0	0	3

1. All data average of 12 sections
2. Concentrations of additives in mg/l: K = 0.5; NAA = 0.2; 2,4-D = 2; GA = 0.3; IAA = 0.2
3. 0 = no callus; 1 = slight callus on 50% or less of sections; 2 = slight callus on nearly all root sections; 3 = good callus development on all root sections.

Table II. Cont.

4. 0 = no branch roots; 1 = branch roots on 25% or less of sections; 2 = branch roots on 25% to 50% of sections; 3 = branch roots on more than 50% of sections

Table III. The effect of growth regulators on callus development, elongation, and branching of wheat root sections with tip removed.[1]

Medium	Age (weeks)	Callus development[3]	Increase in length, mm	Branching[4]
BM + K + 2,4D[2]	5	3	0	2
BM + K + NAA	5	0	0	1
BM	6	0	0	3
BM + 2,4-D	6	3	0	2
BM + GA	6	0	0	3
BM + GA + NAA	6	1	0	3
BM + K + GA	7	0	0	1
BM + K + GA + IAA	7	0	0	1
BM + K + IAA	7	0	0	1
BM + K	8	0	0	1
BM + NAA	8	1	0	2
BM + IAA	8	0	0	3
BM + GA + IAA	8	0	0	3
BM + GA + 2,4-D	8	3	0	2
BM + K + 2,4-D + CM	8	0	0	1

1. All data the average of 12 sections

2. Concentrations of additives in mg/l: K = 0.5; NAA = 0.2; 2,4-D = 2; GA = 0.3; IAA = 0.2

3. 0 = no callus; 1 = slight callus on 50% or less of sections; 2 = slight callus on nearly all root sections; 3 = good callus on all root sections

Table III. Cont.

4. 0 = no branch roots; 1 = branch roots on 25% or less of sections; 2 = branch roots on 25% to 50% of sections; 3 = branch roots on more than 50% of sections.

Table IV. Protein Content of root callus tissues at varying time intervals.

Medium	µg Protein/ Mg Fresh weight		
	8 weeks	10 weeks	11 weeks
BM + 2,4-D	2.8 ± 0.3	2.9 ± 0.3	3.9 ± 0.4
BM + 2,4-D + GA	2.3 ± 0.4	2.5 ± 0.3	2.9 ± 0.3
BM + 2,4-D + K	2.9 ± 0.2	———	———

RESULTS

The data in Table II show of the growth regulators tested, callus initiation and developmented occurred most readily on intact root sections in the presence of 2,4-D. The addition of other additives, i.e., kinetin or GA, did not obviate callus development. Interestingly, callus development in the presence of 2,4-D was totally suppressed when coconut milk was present in the medium. The same responses were noted when root sections with the tips removed were used (Table III).

Root sections with the tip intact, which developed little to no callus, underwent varying degrees of elongation dependent upon additives in the medium (Table II). Greatest elongation occurred when either kinetin or GA was used alone or in combination with no auxin in the medium. No elongation occurred in root sections with the tips removed (Table III).

Many of the root sections developed lateral branches regardless of whether callus initiation and development occurred. Branching was more pronounced in decapitated sections than with intact sections. The growth regulator content of the medium had little influence on the pattern of branching except that it was greatly reduced in all cases where kinetin was present.

Experimentation was conducted to see if any correlation could be made between callus growth and the protein content of the tissues. Data in Table IV indicate that although there is a general increase in protein content as the tissue increase in size and age there is no significant difference as a result of different growth regulators. There is some indication

that added GA tends to reduce the protein content of the developing tissues without appreciably affecting tissue weight.

Histological examination of the callus developing from root sections is shown in Figure 1, A through F. Callus formation typically developed from cortical cells of the root sections (Fig. 1-A and 1-E) and totally encased the root (Fig. 1-B). Closer examination of the callus cells is shown in Figures 1-C and 1-D. Considerable variation was noted in the cells although nearly all were large thin-walled parenchymatous cells with large vacuoles and nuclei. In the sections studied no cells were found undergoing division.

Although no external differentiation was noted in any of the developing calli, internal differentiation was evident. Scattered vascular elements, particularly vessel-like cells with spiral thickenings were found throughout the sections and occasionally organized masses of endodermal cells surrounding a partially organized vascular stele in which abnormally shaped trachery elements were found (Fig. 1-F).

Fig. 1-A. L. S. of Wheat root showing callus development from cortical cells. x40.

Fig. 1-B. X. S. of wheat root with surrounding callus cells. Darker compact cells are those of root itself with loosely arranged thin-walled cells making up callus. x100.

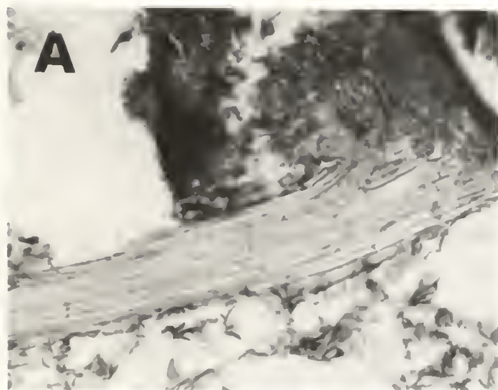
Fig. 1-C. X. S. of callus showing thin-walled porenchyma cells displaying a wide variation in shape. x450.

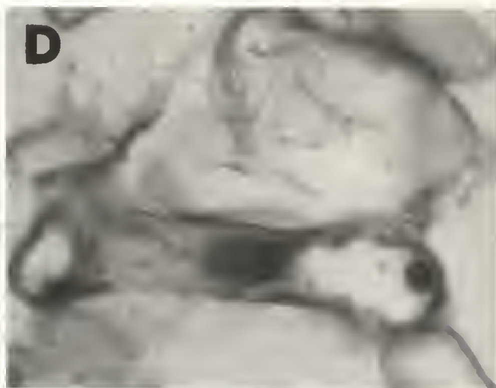
Fig. 1-D. X. S. of a parenchyma cell in the callus tissue showing large vacuoles with a central nucleus. x950.

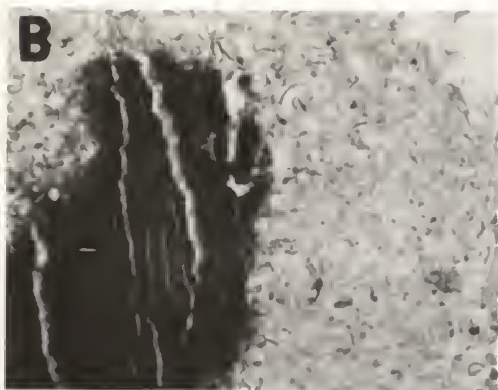
Fig. 1-E. L. S. of root callus with showing callus origin from cortical cells of the root. x450.

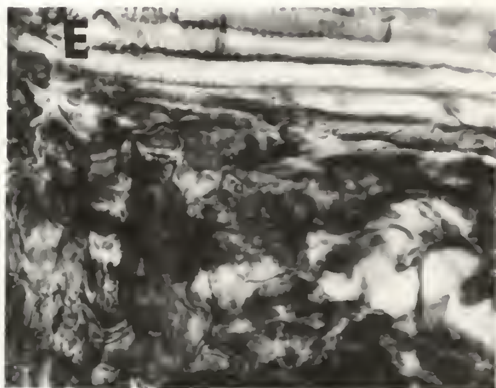
Fig. 1-F. X. S. of sclerenchymatous cells in the wheat callus organized into a stele showing an endodermis (arrow). x450.

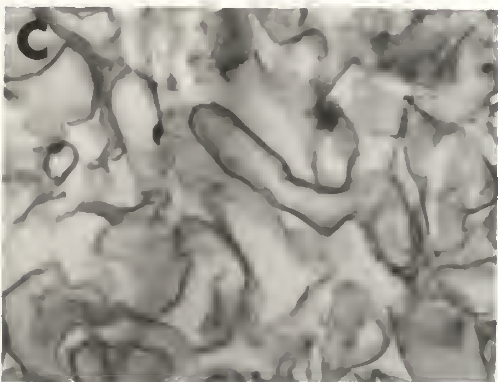
FIGURE 1

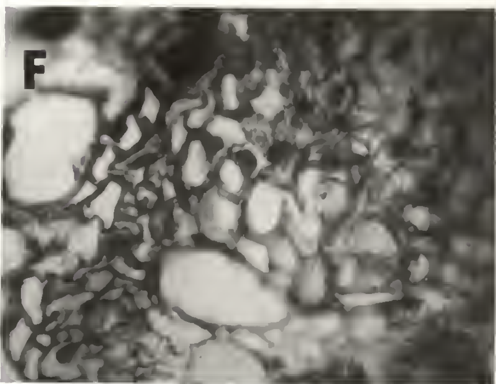












DISCUSSION

Callus Initiation

The fact the basal medium itself failed to support callus growth on wheat root segments suggests specific growth regulators are required for initiation and proliferation of callus. 2,4-D was shown in all cases to be superior to the other growth regulators used for callus formation (Tables II and III). This is similar to that reported in the culture of rice seedlings by Yamada *et al.* (1967) and Yatazawa *et al.* (1967a, b), the culture of roots from several monocotyledonous plants (Warwick and Fuchs, 1968), the *in vitro* culture of corn endospern (Tamaoki and Ullstrup, 1958) and banana fruit tissue (Mohan-Ram and Steward, 1964).

The addition of other auxins to the medium promoted little or no callus formation. IAA was totally inactive while callus initiation was stimulated by NAA with little subsequent development occurring. Reasons for the inactivity of these compounds in this response are not known although concentration may be a factor. Yatazawa *et al.* (1967b) reported that callus initiation and development by IAA or NAA on rice stems and roots cultured *in vitro* occurred only when its concentration exceeded that of 2,4-D by 50-100 times. Such concentration differences were not used in this study.

Kinetin has also been shown to vary in its ability to stimulate callus development in the presence of 2,4-D. Carter *et al.* (1967)

showed that kinetin enhanced 2,4-D initiation of callus formation from oat tissues only if kinetin exceeded the concentration of 2,4-D by a large factor. Yamada et al. (1967) reported a similar finding with callus development from rice seedlings cultured in vitro. In this study kinetin showed no particular enhancement of callus development although an optimum concentration between K and 2,4-D was not studied.

Results of experiments listed in Tables II and III show that the addition of GA to the medium had no effect on callus induction. This confirms the work of Carew and Schwarting (1958) who showed GA concentration of 10-30 gm/l failed to support callus initiation from rye embryos, that of Mohan-Ram and Steward (1964) on the failure of GA to produce significant increase in callus from banana fruit.

Perhaps the most contradictive results obtained in this study were those found when coconut milk was added to the medium. Carew and Schwarting (1958) reported that coconut milk obviated callus development from several monocotyledonous roots. Data presented here show that when 15% coconut milk was present in the medium, the callus initiating properties of 2,4-D were negated. An early report by Zeibur and Brink (1951) noted that although coconut milk promoted callus development from several dicotyledonous embryos, it had no effect on embryos from Hordeum. Also Mohan-Ram and Steward (1964) noted a decrease of the in vitro growth of excised banana fruit tissue when coconut milk was present with 2,4-D. The reasons for these discrepancies are unclear though it may well be related to the age of the coconut from which the milk was extracted (Hildebrandt, 1962).

Segments with and without the root tip were used in this study and it was found that the presence or absence of the tip had no bearing on

callus induction and growth. This suggests that not only cells from the meristematic region of the root but also these in the region of elongation are capable of being stimulated to divide and produce a callus growth. This is particularly evident in Fig. 1-A where callus development is noted to occur from root cells of the cortex.

Branching of Root Segments

The stimulating effect of the basal medium on the formation of lateral branches from intact root segments is similar to that found in onion root tips by Krikorian and Katz (1968). This indicates increased pericycle activity, an event that normally does not take place in the intact root.

The addition of growth regulators to the medium alters the branching response to the basal medium. Kinetin, alone or in combination with other additives, shows a significant inhibition of branching while IAA and GA both tend to stimulate branching. Data from Tables II and III show that this stimulation is greater when the root tip is absent. This suggests the root tip imposes certain inhibitors to branching, much like the apical dominance phenomenon of many stems, probably through the synthesis of active substances which, in the presence of exogenously applied growth regulators, provide a supra-optimal concentration.

Elongation of Root Segments

Data in Tables II and III show that the presence of the root tip is requisite for elongation of the root segments. This elongation occurs on the basal medium without any added growth regulators. This suggests substances necessary for elongation are synthesized in the meristematic

area of the tip, a fact long since recognized. In the presence of exogenous auxin, elongation is greatly reduced indicating an optimal level of auxin has been surpassed.

The addition of K and GA alone to the medium had little effect on elongation of sections. However, when added simultaneously, a synergistic effect is noted and elongation is greatly enhanced. Yamada et al. (1967) noted a similar response with rice seedlings when 0.001 to 1.0 p.p.m. kinetin was used in the presence of GA. Smith (1965) however noted that kinetin had little affect on the in vitro elongation of wheat shoots at concentration below 0.05 mg/l while higher concentrations were inhibitory.

Thus it is apparent that to maintain root cultures as intact entities, the basal medium should contain little to no exogenous auxin, the tip must remain undamaged, and development of callus must be prevented. This elongation can be enhanced by the addition of both kinetin and gibberellic acid although the optimal concentration of the latter factors remain to be determined.

Histological Observation

Microscopic examinations of 10 week old wheat calli indicated there was little, if any, external tissue organization. The callus consisted basically of large parenchymatous cells similar to the ground tissue found in mature wheat roots although more diversity in cell size was noted in callus cells. This is probably due to the position phenomenon of cells found in the intact root but which is not associated with cell organization in the callus. Cells from calli also show a diversity in cell shape (Fig. 1-C and 1-D), an obvious nucleus and a rather highly

vacuolated cytoplasm. No dividing cells were found in any of the sections studied.

Yatazawa et al. (1967a) noted similar findings in a study of callus tissue from rice root tips. He observed no organization such as root or shoot primordia in his cultures. Gamborg and Eveleigh (1968) reported that suspension cultures of Thatcher wheat root callus tissue often formed roots, particularly in early sub-cultures. This ability to form additional roots diminished as the number of sub-cultures increased.

Although the tissue used in this study was cultured on a solid medium, growth on a liquid medium can be accomplished. When calli are grown in shake-culture on the basal medium minus agar, considerable root proliferation is noted from calli in the presence of 2,4-D. This proliferation of secondary roots appears to be obviated by the addition of vitamin free casein hydrolysate or yeast extract (unpublished data from J.S. Weis).

Internal differentiation is apparent in most of the calli examined. Vascular elements, especially vessels with spiral wall thickenings, whose origin is uncertain, are noted. Fiber-like cells with sclerenchymatous characteristics were also found. These were organized into a stele-like structure that shows a definite endodermis (Fig. 1-F). In most cases the origin of these vascular areas is considered to be the callus based on cells surrounding the "bundle."

Yamada et al. (1964) noted scattered meristematic colonies in Calli of *Tradescantia paludosa* where cell division was observed. They also reported the finding of giant cells with polyploid nuclei. But in general their findings were similar to those reported in this histological study. Internal differentiation was also reported in rice root

calli by Yatazawa et al. (1967a). They noted an outer stratified layer of one to three cells with numerous mitotic figures while inside the tissue was composed of larger isodiametric parenchymatous cells containing scattered vascular elements. Also noted were several lysigenous lacunae. Photomicrographs of suspension cultures of wheat and barley root callus cells by Gamborg and Eveleigh (1968) showed numerous cell aggregates quite similar to the callus observed in this study (Figs. 1-C and 1-D).

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THE EFFECT OF GROWTH REGULATORS ON *IN VITRO* CALLUS INITIATION IN EXCISED WHEAT ROOTS

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The *in vitro* culture of various cells, tissues, and organs has become an important tool in the understanding of plant growth and development. Past experimentation has shown that with the proper medium nearly all tissues derived from dicotyledonous plants may be cultured. Such is not the case with tissues obtained from monocotyledonous plants. This study was undertaken to investigate the affects of various growth regulators on callus initiation and development from roots of *Triticum vulgare* L. var. Bison.

Experimentation showed that of the growth regulators tested only 2,4-dichlorophenoxyacetic acid at 2 mg/l promoted the development of callus from excised wheat root segments. Other auxins such as idole-3-acetic acid or naphthalene acetic acid alone or in combination, effected little to no callus development. The addition of gibberellic acid or kinetin to the basal medium in the presence of 2,4-dichlorophenoxyacetic acid did not increase callus development. Adding 15% coconut milk to the basal medium was ineffective alone in the initiation of callus and totally negated the affect of 2,4-dichlorophenoxyacetic acid on callus development. Callus development occurred as readily in root sections with the tip excised as those sections with the tip intact providing 2,4-dichlorophenoxyacetic acid was present in the medium.

No significant change in protein content of calli was found as the tissue aged although the presence of gibberellic acid in the medium slightly reduced protein content while having little affect on callus weight.

Histological examination showed that although no external differentiation of the developing callus tissue was discernible, internal

differentiation occurred. Scattered vessel-like cells were noted in the callus masses as well as organized groups of sclerenchymatous cells closely resembling an endodermal structure of a vascular stele.