STUDIES ON THE DEVELOPMENT OF BARLEY EMBRYOS IN CULTURE

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Alan W. Cockerline

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THESIS

This is to certify that the

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ABSTRACT

STUDIES ON THE DEVELOPMENT OF

BARLEY EMBRYOS IN CULTURE

By Alan W. Cockerline

The purpose of this investigation was to make a study *in vitro* of the morphological development of the embryo and young seedling of barley, *Hordeum distichon* L. (Hannchen, C.I. 531, a two-rowed variety). The embryos selected for study were excised from caryopses in various levels of development. The culture media were entirely synthetic, with special emphasis being placed on the carbohydrate source, and in particular the sugars.

The cultured samples ranged in level of development from the relatively undifferentiated late proembryos on up through the level of late differentiation to "mature" embryos. For purposes of the investigation the cultured embryos were grouped into four levels of morphological differentiation. These were: level I - late proembryo; level II - early differentiation; level III - middle differentiation; and level IV - late differentiation.

Under the experimental conditions of this investigation the morphological responses of the cultured embryos appeared to be directly related to the level of embryonic differentiation at the time of inoculation into culture. The cultured

mbryos did not follow the normal sequence of _in situ_ and _in ivo_ development. Though no responses were observed for ultured late proembryos, embryos excised and placed in ulture at early and middle differentiation germinated recociously to form aberrant plantlets, while embryos ultured at late differentiation germinated readily to roduce "normal" seedlings.

It would seem that the development of callus, under the xperimental conditions, is characteristic for embryos ultured at early and middle differentiation. In the cul- ures of early differentiating embryos the callus formation s extensive and apparently a forerunner of subsequent evelopment.

Thirteen sugars and seven intermediate (glycolytic) espiratory products were used as primary organic carbon ources in the media. In general, the pentoses and the ntermediate respiratory products were poor carbon sources. ucrose was the most satisfactory sugar for the culture of arley embryos under the experimental conditions. The esponses exhibited by the cultured embryos to the various arbon sources appear to be characteristic of the level of ifferentiation at the time of inoculation and the variation between carbon sources is a matter of degree rather than type of response. Level IV responds most favorably to a sugar concentration of 3%; level III to 2%; and, level II to 4%.

It would seem that the responses of the various levels of development are somewhat related to the concentration of agar in the media. Over a range of from 0.65 to 0.8% agar, the most satisfactory concentration for the culturable levels, was 0.8%.

STUDIES ON THE DEVELOPMENT OF BARLEY EMBRYOS IN CULTURE

By

Alan W. Cockerline

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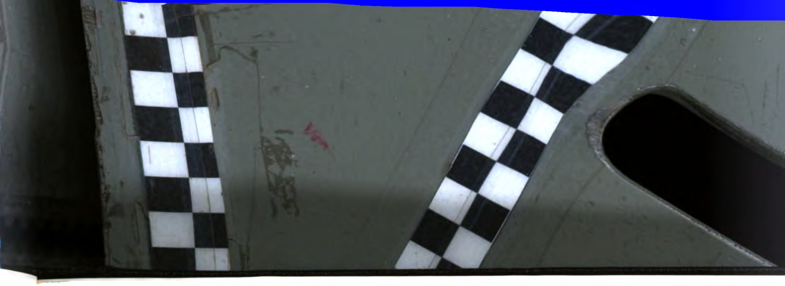

PLATE I. Seedlings germinated from caryopses rolled in a moist paper towel; two days after germination, and four days of incubation.

PLATE I

INTRODUCTION

The purpose of this investigation was to make a study *in vitro* of the morphological development of the embryo and young seedling of barley, *Hordeum distichon* L. (Hannchen, C.I. 531, a two-rowed variety). The embryos selected for study were excised from caryopses in various levels of development. The culture media were entirely synthetic, with special emphasis being placed on the carbohydrate source, and in particular the sugars.

The research problem was planned to give answers to the following questions:

1. Can barley embryos be cultured *in vitro*, when using well-defined media devoid of biological extracts such as coconut milk, etc.?

2. When cultured *in vitro*, does the embryo follow its "normal" *in situ*, *in vivo* sequence of embryological development?

3. Is precocious germination characteristic of the embryo in purely synthetic media?

4. How does root and shoot development *in vitro* compare with "normal" germination?

5. Eight arbitrary stages of embryological development can be readily recognized and excised for culture. Do these levels of differentiation exhibit characteristic responses *in vitro*?

6. Do qualitative and quantitative variations in the primary organic carbon source have a pronounced effect on the development of the embryo, and seedling <u>in vitro</u>?

7. Will variations in the concentrationof agar have any effect on growth responses?

8. Are there quantitative and qualitative differences in sugars extractable by means of 80% ethanol reflux which might be useful in attempting to further characterize the various stages of embryological development?

GENERAL CONCEPTS

When reduced to its simplest form, the technique broadly classified as "plant tissue culture," may be defined as follows: "the cultivation of isolated plant tissues and organs *in vitro*" (White, 1951). In addition to the study of "normal" and tumor tissues, the *in vitro* studies of plants have included among others, considerations of the following: root culture; endosperm culture; shoot apex culture; and, the culture of flower buds.

Gustaf Haberlandt (1902) was the first to formulate specifically the concept of plant tissue culture, *per se* (White, 1943). His work is generally considered as being basic to the establishment of this field. In addition, he was the first investigator to express clearly the idea of cultivating isolated plant cells *in vitro*, as well as being the first to make well-organized studies of the cultivation of cells.

The crux of Haberlandt's classic paper, "Kulturversuche mit isolierten Pflanzenzellen," as translated by White (1954), is contained in the following excerpt:

> There has been, so far as I know, up to the present, no planned attempt to cultivate vegetative cells of higher plants in suitable nutrients. Yet the results of such attempts should cast many interesting sidelights on the peculiarities and capacities which the cell, as an elementary organism, possesses: they should make possible

conclusions as to the interrelations and reciprocal influences to which the cell is subjected within the multicellular organism.

The term "embryo culture" has frequently been used in a rather vague sense. It is most commonly employed to describe the growth and development of plant embryos (as well as ensuant seedlings) in various culture media; with however, a seeming indifference towards age, size, and level of differentiation of the embryos at the time of excision and inoculation.

The *in vitro* study of plant embryos has been approached in three general ways: (a) seedling culture; (b) culture of parts of embryos and seedlings; and (c) embryo culture *in toto*.

Two fundamental problems in the development of successful techniques for the cultivation of excised plant tissues and organs have been: the choice of tissues which were responsive to such an approach; and, the development of satisfactory media. The technique of tissue culture, as such, has now advanced well beyond the point wherein it is an end in itself.

Early investigators in the field of plant embryo culture were primarily concerned with such objectives as: overcoming certain doemancy phenomena in some seeds; and, obtaining hybrids which were previously considered to be unobtainable. Currently interest is centered on such aspects as: processes involved

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involved in the course of germination; morphogenesis *in vitro*; and, specific nutritive requirements.

A full appreciation of the subject of plant embryo culture necessitates familiarity with, and comprehension of the following concepts: precocious germination; pregerminal cultures; and, postgerminal cultures.

Dieterich (1924) in his work with relatively undifferentiated embryos of the Cruciferae, and Gramineae, frequently observed that the embryos instead of continuing their embryonic development, produced a premature germination. This he referred to as "künstliche Frühgeburt," or artificial early germination. Such a form of development is often described in the literature as precocious germination.

Rijven (1952) pointed out, that one should distinguish between "pregerminal" and "postgerminal" cultures. Germination according to Rijven, begins "at the moment the embryonic tissues (in a state of cell division and plasmotic growth) become separated by an intercalated section of incipient cell elongation. The appearance of the cell elongation marks off a new phase of the life cycle, as it affects the nature of growth and structure." He thus divides embryo culture into two distinct phases: (a) pregerminal embryo culture, dealing with embryos excised from the ovule during embryogenesis, and with the attempt to prolong the embryonic growth

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growth until a fully differentiated embryo results; and (b) postgerminal embryo culture, which includes embryos excised at similar or later stages, and dealing with processes involved in the embryo's germination, and its subsequent seedling development.

HISTORICAL REVIEW

An historical review of plant embryo culture must of necessity include numerous citations from the broader field of plant tissue culture. As one might expect, many of the fundamental techniques and basic media utilized for the cultivation of plant embryos have evolved naturally from the extensive investigations of isolated plant tissues and organs *in vitro*.

The history and much of the literature pertaining to plant tissue culture has been described and discussed in various reviews and manuals, such as those of: Gautheret (1942, 1945, 1947, 1955); Guilliermond (1942); Nobecourt (1943); Rappaport (1954); Riker and Hildebrandt (1958); Street (1957); and, White (1931, 1936, 1941, 1943, 1946, 1951, 1954).

In addition, the work with plant tissue cultures has in itself derived many ideas and techniques from that done with animal tissue cultures. The amount of research carried on with animal tissue cultures has been far more extensive than that with plants (Riker and Hildebrandt, 1958). Reviews on this subject, amongst others, have been published by Parker (1950) and Murray (1953).

Following Haberlandt's paper, "Kulturversuche mit isolierten Pflanzenzellen" (1902), many believed that each

individual cell had the capacity to divide and proliferate indefinitely. Early attempts to maintain cells in a proliferating state were unsuccessful. Positive results in the area of cultures produced from single plant cells were not published until 52 years later. Muir _et al_. (1954) reported on cultures produced from single cells of marigold (crown gall origin) and tobacco (from "normal" stem). Cultures of animal tissues grown from single isolated cells were reported as early as 1948 by Sandford, Earle, and Likely. Subsequent papers relative to the cultivation of plant tissues from single cells, were published by: Nickell (1956); Muir _et al_. (1958); Steward _et al_. (1958a, b); and, Jones _et al_. (1960).

Two early works might well be ranked as classics in the field of plant embryo culture. They are those of Brown and Morris (1890) and Hannig (1904). Brown and Morris's paper, "Research on the germination of some Gramineae. I," published in the Journal of the Chemical Society (London), is one of the earliest publications on the subject of plant embryo culture. Brown and Morris were primarily concerned with the post-germinal culture of mature grass embryos. Hannig, who made studies on various representatives of the Cruciferae and in particular of _Raphanus sativus_, should be given credit as the first investigator to report on the pregerminal culture of relatively immature plant embryos _in vitro_.

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The works of Hannig, Brown and Morris, together with the subsequent investigations of Stingl (1907), Buckner and Kastle (1917), Andronescu (1919), Molliard (1921) and Dieterich (1924), established a foundation for future *in vitro* investigations of plant embryos.

Throughout the literature are to be found numerous papers describing the effects of various biological additives, such as coconut milk and tomato extract, on pre- and postgerminal embryonic development. Stingl's paper, "Experimentelle Studie über die Ernährung von pflanzlichen Embryonen" (1907) must be ranked as one of the forerunners in this field of endeavor. His media *per se* consisted of the endosperm of closely related grass species, into which mature, excised cereal embryos were implanted.

Andronescu's contributions to plant embryo culture were two simple but very fundamental observations: seedlings resulting from cultured *Zea Mays* embryos were frequently either dwarfed, or weak and etiolated. Earlier investigators undoubtedly had made similar observations. It remained, however, for Andronescu to publish these findings thereby making it possible for others to realize that when viewed in the light of their own research, such was not unusual but rather a common occurrence. Numerous reports on a wide variety of materials have since substantiated his results.

After removing the cotyledons, Buckner and Kastle cultured embryos of lima bean on nutrient agar and obtained relatively normal seedlings. Molliard's paper, "Sur le development des plantules fragmentees" (1921) was one of the earliest indications that there are some regions of the plant embryo, particularly the cotyledons, which have a greater proliferation capacity than others. Many papers dealing with the cultivation of embryos which have had organs removed and/or the culture of isolated organs and organ-fragments have since been published. The principal contribution of the forementioned authors was, I believe, to be found in their then unique approach to the subject of plant embryo culture. In almost any _in vitro_ study of growth and development, a familiarity with some of the abnormal and unusual manifestations which may be attributed to technique (such as wounding, etc.), rather than to the treatment _per se_, permits a more meaningful interpretation of the results.

Dieterich's pregerminal cultures of relatively undifferentiated embryos of the Cruciferae and the Gramineae were in essence an extension of Hannig's investigations. The basic idea of precocious germination, as described in detail under the section entitled GENERAL CONCEPTS, can be traced directly to his publication, "Über Kultur von Embryonen ausserhalb des Samens" (1924), in which he described the condition known

"kunstliche Fruhgeburt." Unfortunately to this date, the roblem of precocious germination is the rule, rather than he exception, in pregerminal embryonic studies.

Extensive use has been made of postgerminal or seedling culture as a means of obtaining viable plants from hybrid crosses. This technique has frequently permitted the development of a young plant, which was otherwise impractical or impossible when a fully ripened seed was used. Excision and culture of the embryo has been one method of circumventing the problem of abortion <u>in situ</u>, and <u>in vivo</u>. The initial impetus for this line of research was provided by Laibach's work with flax in 1925. Amongst others, subsequent reports with somewhat similar objectives, were those of: Jorgensen (1928); Laibach (1929); Werckmeister (1934); Starstead (1935); Skirm (1936); Lammerts (1942); Smith (1944); Blakeslee and Satina (1944); Cummings (1945); MacLean (1946); Sanders (1950); Konzak <u>et al</u>. (1951); and, Weaver (1957, 1958).

Media

Media used for the culture of plant embryos are either liquid or partially solidified with agar. Liquid media are reported only rarely. In addition to distilled water the various media contain: mineral salts; carbohydrates; nitrogenous compounds; vitamins; auxins; and frequently biological extracts such as coconut milk and tomato extract.

Media used for the culture of plant embryos are almost as numerous as the papers on this subject. Understandably the requirements of the material to be cultured will vary, and so accordingly will that portion of the environment formed by the culture base. Once a given medium has been published, its subsequent citations usually appear in a modified form. Media frequently used for the culture of plant embryos, are those of: Tukey (1934); Randolf and Cox (1943); White (1943); and Rijven (1952). An additional medium satisfactory for this type of investigation is that of LaRue (1955, unpublished). The composition of the aforementioned media will be found in Tables 1 and 2 of the APPENDIX.

Mineral Nutrition

Certain solutions appear with a high degree of regularity. These are as follows: Berthelot's Trace Elements (1934); Randolf and Cox's Mineral Solution (1943); White's Mineral Solution (1943); and, Nitch's Trace Elements (1951). The composition of these various solutions is to be found in Appendix Tables 1 and 2.

The early investigators made use of very simple media. Over the years the direction has been towards that of increasing complexity. The more recent solutions have attempted to take into consideration the nutritional requirements of

the material to be cultured, in so far as they are known.

Iron salts used in the various media have had a tendency to precipitate. This accordingly renders the medium iron deficient. Street *et al.* (1952) believe that the precipitation of the iron salts may result in an increased pH of the medium. A number of investigators have replaced the relatively unstable iron sulfate by complexes such as ferric citrate. It would seem that the use of chelates permits one to maintain embryos in culture over a much longer period of time without having to renew the media or stock solutions.

Unfortunately very little is known of the role of the micro-elements *per se*, in the nutrition of plant embryos *in vitro*. Investigations on this subject have been primarily limited to the role of certain trace elements in plant tissue cultures.

According to White (1951), excised tomato roots require iron, copper and molybdenum. White also believes that zinc, manganese, boron, and iron are probably necessary. Nobecourt (1938) and Gautheret (1939) introduced into their media a complex of accessory salts as suggested by Berthelot (1934) including beryllium, titanium, cobalt, nickel, and boron. An absolute necessity for such elements in tissue culture, has yet to be demonstrated. Various investigators have made use of Berthelot's complex additives, and were unable to detect any significant influence.

Boll and Street (1951) growing tomato roots in White's medium, using highly purified chemicals, found that additions of copper (0.01 ppm) and molybdenum (0.0001 ppm) were necessary for successful growth. It should be noted however, that in normal reagent grade salts, these chemicals seem to be generally present as impurities in concentrations closely paralleling those added by Boll and Street.

Possibly the failure to grow very young stages of some embryos in culture may in part be due to the lack of some micro element or group of trace elements in the medium. I doubt this. Rather I believe that the situation is a great deal more complex, and is related to the environmental organization as a whole.

Carbon Nutrition - Carbohydrates

Carbohydrates in the medium supply a source of energy for the maintenance of the various processes in the cultured embryo. Some investigators place great credence in their value as osmotic agents.

Early workers such as Kotte (1922) and Robbins (1918, 1922) assumed that monosaccharides, especially glucose, would be a most suitable carbon source. Tukey (1933) grew peach embryos on media containing 0.5 to 2% glucose. In 1934 he reported that although 2% glucose was beneficial for embryos removed from the seed at an early stage of development, it was inhibitory for later stages.

White (1934, 1940b), Thielman (1938) and LaRue (1936a), showed that in the culture of tissue fragments, and embryos, sucrose was a more satisfactory energy source. Van Overbeek *et al*. (1944) noted that in the culture of the "heart shaped" relatively immature *Datura* embryos, sucrose was a much better carbon source than glucose.

Doerpinghaus (1952) cultured "heart shaped" *Datura* embryos (10 species) in media containing sucrose, glucose, fructose, mannose, and glycerol. He found sucrose to be a superior carbon source for all species tested. The other sugars showed a species dependence. Three species of the "stramonium" type grew well on 4% sucrose. *D. meteloides* grew well on all sugars except fructose. *D. discolor* grew only on sucrose and glucose.

LaRue (1936b) succeeded in culturing smaller embryos than had previously been reported. He observed that younger embryos require a higher sugar concentration than do the older ones. Tukey (1938), reported that the concentration of sugars for excised peach embryos, varied with the time lapse between fertilization and excision. In 1942, Lammerts found that for peach, tangerine, and apricot embryos, 2% sucrose is better for cultures of relatively undifferentiated embryos, while a concentration of 0.5% is more satisfactory for the mature ones. Sanders (1950) found that growth in Datura stramonium embryos, with 4% sucrose was 42 times that obtained with 0.5%. Over the same range, comparable embryos of three other Datura species increased only 1.2 to 2.7 times. Sanders' cultures were restricted to relatively well differentiated embryos. In 1953 Rietsma et al. published a report on the culture of Datura stramonium embryos over an incubation period of 8 days. They observed that while the "pre-heart" stages grew well on 8-12% sucrose, the "late heart," and "torpedo" stages (mature embryos) developed more satisfactorily at 4%. The authors suggest that "culturing of plant embryos in very early stages can be made possible by studying the relations between the phase of development and nutritive requirements."

Ziebur et al. (1950) and Ziebur and Brink (1951) reported limited success in the culture of young Hordeum embryos on a medium containing 12.5% sucrose. They noted that a concentration of 2% was quite satisfactory for the well differentiated embryos.

Purvis (1944) in excised and vernalized rye embryos, found flowering is increased with rising sucrose concentrations, reaching a maximum at 2%.

In 1947 Steinberg reported that sucrose was superior to

ll other sugars tested for the culture of tobacco seedlings. He noted, however, that substantial growth was obtained with both glucose and fructose.

Knudson (1916) and Knudson and Lindstrom (1919) cultured intact albino corn seedlings in both light and dark. They found little difference between sucrose, glucose, and fructose as carbon sources. The sugars failed to sustain growth, although those plants cultured with sugar in the media lived longer.

In 1923 Brannon reported that fructose was superior to glucose in intact seedling cultures of timothy. However it was not found to be superior for pea, alfalfa, radish or Bryophyllum. Juhren and Went (1949) cultured squash seedlings in the dark. They reported that fructose was a more effective carbon source than was sucrose. They believed that "fructose may be more effectively utilized than sucrose in the synthesis of certain growth promoting substances."

Burstrom (1941, 1948) cultured excised wheat roots in media containing sucrose, glucose, and fructose. The most satisfactory growth responses were obtained from glucose. In additional cultures of excised wheat, flax, and sunflower roots using galactose in addition to the above mentioned sugars; galactose was found to be "toxic" to wheat roots, but not to flax or sunflower. Burstrom concluded that "galactose

was used for respiration instead of for synthetic purposes."

Rijven (1952) found that 8% sucrose was the most suitable concentration, and carbon source, for Capsella bursa-pastoris. A mixture of equal parts of isotonic solutions of fructose and glucose did not yield as good or better results.

In White's (1940a) cultures of excised tomato roots sucrose was found to be superior to glucose, as well as to equi-molecular concentrations of maltose, raffinose, and arabinose. Dormer and Street (1949) reported that excised tomato roots utilized sucrose at a greater rate than glucose or fructose, or equal molecular quantities of the two. They concluded that the absorption of sugar was closely related to phosphorylation.

Smith (1932) and Hill and Patton (1947) reported that reducing sugars undergo partial hydrolysis with autoclaving. In their cultures of excised tomato roots, Street and Lowe (1950) assumed that there was no significant hydrolysis when sucrose was autoclaved. Rijven (1952) reported that he did not find any difference in the activity of un-autoclaved and autoclaved monosaccharides and disaccharides in his Capsella cultures. After autoclaving sucrose, Ball (1953) found from 0.7-0.8% glucose or fructose present. He notes that callus cultures of Sequoia sempervirens when grown on two differently sterilized media (heat sterilized and filter sterilized),

exhibit quite different growth patterns. Undoubtedly there are differences in media which have been either heat or filter sterilized. Ball made analyses of his media relative to hydrolytic products before and after sterilization. Those investigators who did not find any difference between the two based their conclusions solely on the manner in which the cultured tissues or embryos reacted to this kind of treatment.

Nickell and Burkholder (1950) reported that in their studies of Rumex tumors in vitro, sucrose, glucose, and fructose were the best carbon sources. Their's was one of the first publications listing soluble starch as a good organic carbon supplement. All organic acids were, with the exception of aspartic, rated as being poor carbon sources. Pyruvic acid at all levels (0.001-0.032 M or 88 ppm to 3817 ppm) proved to be inhibitory. The carbon sources and their ratings are summarized as follows: (1) Excellent - sucrose, glucose, fructose, and soluble starch; (2) Good - raffinose, melobiose, cellobiose, glycerol, and aspartic acid; (3) Poor - galactose, xylose, arabinose, lactose, sorbose, rhamnose, sorbitol, inositol, mannitol, sodium acetate, oxalic acid, maleic acid, and tartaric acid.

Lee (1950a, 1950b) cultured seedlings of Lycopersicum esculentum Mill. He substituted equimolecular concentrations

of maltose, glucose, fructose, raffinose, and arabinose for 2% sucrose. The seedlings exhibited a greater growth response with sucrose than with any of the other sugars. In general no significant responses were observed with either raffinose or arabinose.

One of the most exhaustive series of publications dealing with the effects of various carbon compounds in tissue culture, are those of Hildebrandt and Riker (1946, 1947, 1948, 1949, and 1953). These investigators studied the effects of various sugars, polysaccharides, organic acids, and alcohols on callus cultures of marigold, paris-daisy, periwinkle, sunflower, and tobacco tissues. Glucose, fructose, and sucrose were reported as excellent carbon sources for all five species. However, as would be expected, with certain species specific effects were observed. Significant increases in weight were noted for marigold on mannose, maltose, and cellobiose. Significant increases were also reported for periwinkle on galactose, maltose, lactose, and cellobiose. Only slight increases were listed for: marigold on starch, dextrin, pectin; paris-daisy on maltose, lactose, cellobiose, raffinose; starch, inulin, dextrin, pectin; sunflower on maltose, lactose, raffinose, starch, inulin, dextrin, pectin; and tobacco on xylose, mannose, lactose, raffinose, starch, dextrin, and pectin.

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All organic acids as a substitute for sucrose were unfavorable for growth although slight increases were observed. The organic acids were incorporated into the media at a concentration of 0.5% or 5000 ppm. They included such acids as: succinic, stearic, fumaric, glutaric, acetic, malic, formic, tartaric, and glycolic.

Alcohols at a concentration of 0.5% as the sole carbon source proved to be unfavorable for all tissues. The study included such alcohols as : methanol, ethanol, butanol, mannitol, dulcitol, and erythritol.

The sugar concentrations ranged from 0.015 to 8.0%. Glucose, sucrose, and fructose were best in a range of from 0.5 to 2.0%, with an optimum of 1.0 to 2.0%.

Almost as good for sunflower, as glucose, fructose, and sucrose, were: cellobiose and maltose. For periwinkle, raffinose, galactose, and lactose were almost equal in response to glucose, sucrose, and fructose. Concentrations of sugars below 0.5% and above 4.0% gave little or no growth.

Van Overbeek _et al_. (1944) based their explanation for the superiority of sucrose upon the findings of Duodoroff _et al_. (1943), who reported that dry preparations of _Pseudomas saccharophila_ will readily form glucose-1-phosphate with sucrose, but not with glucose or fructose, or a mixture of both. Van Overbeek _et al_. reason there should be in these

substances a difference in availability for phosphorylation.

A similar type of explanation was that of White (1951) who concluded that "sucrose utilization probably involves an enzymatic phosphorylation at the surface of the absorbing cells so that the material actually absorbed is freshly formed (nascent) hexose-phosphate which cannot be replaced by hexose and phosphate in uncombined form nor by the addition of preformed hexose-phosphates to the medium."

Plantefol (1938) and Plantefol and Gautheret (1939) found that carrot tissues can be adapted to glycerol as a carbon source, if first grown in a mixture of glucose and glycerol.

Embryos and tissues of various plant species react in different ways to the various sugars, both quantitatively and qualitatively. In general sucrose would seem to be the most satisfactory organic carbon source for plant embryos. However, as is quite apparent from the variety of citations on this subject, one must be very careful when making a broad generalization from very specific data collected under equally restrictive conditions. Consideration should be given to the following factors when evaluating the various sugars: availability of the different sugars; paths of translocation; changes during translocation; specific roles of these sugars in growth and development; differences in

requirement for sugar; and, differences in ability to utilize sugar.

Other Organic Supplements

Numerous investigators using varied tissues, organ-fragments and embryos have reported that the cultured material can assimilate inorganic nitrogen. Nitrates have proven to be the most suitable nitrogen source *per se*. In a few instances ammonium salts have been reported to be as good a source of nitrogen. Generally organic nitrogenous compounds have been assumed to be inadequate nitrogen sources, and even sometimes toxic in culture (Burgeff, 1936; and Knudson, 1932). Brown (1906) reported an increase in dry weight of excised barley embryos grown in media containing asparagine, aspartic acid and glutamic acid.

Spoerl in 1948 noted that in orchid embryos, under certain conditions, arginine and aspartic acid were as effective as ammonium nitrate, if used as a substitute for the latter. However, when used in addition to ammonium nitrate they did not show any stimulation to growth. Spoerl also reported that the effect of amino acids on the growth of orchid embryos depended on such factors, as: concentrations of acids used; light conditions; and age and species of orchid embryos. In "unripe" orchid seeds only arginine supported growth. Eighteen other amino acids tested inhibited growth. In mature embryos,

aspartic acid was a good nitrogen source; glutamine a neutral one; and other amino acids appeared to inhibit growth.

Ziebur et al. (1950) observed that casein hydrolysate when supplied to a medium in combination with phosphate ions and sodium chloride, had an inhibitory effect on the post-germinal development of immature Hordeum embryos, and a stimulating effect on the pregerminal growth of these embryos. They also reported that the excised immature embryos continued their "embryonic growth" but did not germinate. Casein hydrolysate has been categorized by some investigators as a "so-called" embryo factor.

Similar effects have been noted with water extracts of dates, bananas, wheat gluten hydrolysate, and tomato juice (Kent and Brink, 1947). The inhibition of germination is attributed by these authors to the high osmotic pressure (a somewhat controversial point) created by the addition of these extracts and especially by casein hydrolysate in conjunction with sodium chloride. The same authors also assumed that the amino acids present in casein hydrolysate not only act as suppressors of precocious germination of the immature embryos, but also as nutrients helping to increase the rate of embryonic growth.

Sanders and Burkholder (1948) attempted to analyze the growth promoting action of the amino acid complex present

in casein hydrolysate. They made use of postgerminal growth of young Datura embryos as a bioassay. Addition of casein hydrolysate (100-800 ppm) and equal parts of cysteine and tryptophane (6.67 mg. per 100 mg. of cas. hyd.) to the basic medium, resulted in significant growth of the embryos. Similar results were obtained by substituting in the medium a mixture of twenty amino acids, approximating the composition of casein hydrolysate. It was noted that single amino acids or incomplete mixtures of the twenty acids did not promote embryonic growth at rates equal to those obtained by the complex mixture. This led Sanders and Burkholder to conclude, "that the growth stimulus of the combination of the combination of the twenty amino acids, results from a physiological interaction rather than from the summation of effects of the individual acids."

Rijven's (1952) results would seem to contradict those of Sanders and Burkholder. Using Capsella embryos, glutamine proved superior to the amino acid mixture. Two sets of conditions must be taken into consideration when evaluating this apparent contradiction. Rijven cultured immature embryos of Capsella, while Sanders and Burkholder studied Datura. Also, Rijven incorporated glutamine rather than glutamic acid into his medium.

Rijven (1952) suggests that glutamine is significant in the nitrogen metabolism of Capsella. In 1949 Street reported that glutamine was the prevailing amide in crucifer seedlings. Rijven also observed that while the young Capsella embryos apparently can use glutamine as a nitrogen source, asparagine would seem to be of limited value. In addition he noted that the small embryos when grown in a medium with asparagine exhibited significant starch formation, while those grown in a glutamine-containing medium showed negligible starch formation.

The role of compounds with the amino group, in early embryonic development, is somewhat vague. Rijven (1952) reported no detectable improvement when purine derivatives were added to his medium for Capsella embryos. Rappaport et al. (1950) observed that nucleic acids proved inhibitory to postgerminal cultures of Datura. Curtis (1947) and Curtis and Nichol (1948) reported that barbituates in the medium, resulted in unorganized tumor outgrowths from orchid embryos.

It has been noted by numerous investigators that tissue cultures grown on a medium containing only mineral salts and carbohydrates showed only very limited growth. Some authors have referred to this as "residual growth." It has been demonstrated that the promotion of growth in excised materials requires incorporation into the media of varied accessory substances and factors.

Kotte (1922) added "Liebig Fleisch" extract (tomato) to his medium. Robbins (1922) made use of peptone and autolyzed yeast as additives. White (1932a) cultured 0.2 mm. Portulaca embryos on a medium which had in addition to minerals and 2% sucrose, yeast extract. White (1934), Fiedler (1936) and Gautheret (1939) reported, that a "pasteurized" extract of yeast is a useful source of required additional substances. Attempts to identify clearly the nature of these substances has as yet had limited success.

Kogl and Haagen-Smit (1936) considered thiamin and biotin as essential growth factors. They believed ascorbic acid to be a non-essential substance on the basis of lack of stimulation in their experiments. However Bonner and Bonner (1938) reported that ascorbic acid promoted growth in excised pea embryos. Virtanen and Hausen (1950) gave evidence to suggest that the role of ascorbic acid, in the culture of pea and wheat embryos, is that of a reducing agent of nitrates in the medium.

Bonner (1938) found nicotinic acid to be a growth factor for excised pea embryos. Bonner and Axtman (1937) reported that pantothenic acid favored growth in peas.

Van Overbeek et al. (1942) observed that young Datura embryos failed to develop cotyledons on media devoid of supplementary factors. They used an arbitrary mixture of

the "so-called" growth factors, which was composed of: glycine, 3.0 ppm; Thiamine, 0.15 ppm; ascorbic acid, 20.0 ppm; pantothenic acid, 0.5 ppm; nicotinic acid, 1.0 ppm; pyridoxine hydrochloride, 0.2 ppm; adenine, 0.2 ppm; and succinic acid, 25.0 ppm. This mixture was effective in stimulating the growth of Datura embryos, but not of those less than 0.5 mm. in length.

Rijven (1952) reported negative results when the following mixture (in ppm) was added to his medium for Capsella embryos: thiamine, 0.15; nicotinic acid, 1.0; pyridoxine-HCl, 0.2; calcium pantothenate, 0.2; inositol, 0.5; p-amino benzoic acid, 0.5; riboflavine, 0.1; folic acid, 0.01; and biotin 0.0004.

Rytz (1939) found that various varieties of pea embryos did not react in the same way to thiamine treatment.

Coconut milk has been used to stimulate the growth of immature plant embryos. It has been observed frequently that, even when vitamins and other growth factors were added to the media, the embryos failed to grow without the addition of a biological extract.

Van Overbeek et al. (1941, 1942) used coconut milk in their media for immature Datura embryos. When the coconut milk was autoclaved the embryos grew as "an unorganized mass." When the coconut milk was filter-sterilized, "normal" growth of the very young embryos resulted. These embryos developed fr

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from an initial length of 0.15 mm. to 8.0 mm. after seven days. The authors believed that two substances or complexes were to be found in coconut milk. A heat stable one producing cell proliferation, and a heat labile one for "normal" differentiation.

Norstog (1956) and Chang (1957) succeeded, in varying degrees, in culturing relatively undifferentiated barley embryos. Norstog used a medium in which coconut milk was incorporated 90% by total volume. On this medium embryos as small as 0.15 - 0.20 mm. (late proembryos) were grown. Only four such immature embryos were cultured to seedlings, following a precocious form of development. As did other investigators, Norstog believed that coconut milk contains a factor or factors essential for root and shoot development.

Following the technique of Norstog, Chang also incorporated 90% by volume coconut milk into his medium. Thirty-eight embryos with an initial size of 0.5 x 0.30 mm., were grown to an average size of 1.20 mm. x 0.90 mm., after two weeks in culture. He noted that the in vitro embryos differed significantly from those developing in vivo, in that the cultured embryos were larger at all morphological stages of differentiation, and slower in passing through the various levels of development, than those in situ, and in vivo. The cultured embryos never reached a stage comparable to the final level

of differentiation, although seedlings were obtained.

Van Overbeek *et al*. (1942) observed that *Datura* embryos growing on autoclaved coconut milk, did not show any root formation. They assumed that a heat stable substance in coconut milk brings about suppression of root development. Cook and Doyle (1916) found that when germinating coconut embryos, *in vivo*, the developing roots remained in the fibrous outer shell and did not penetrate into the milk.

Other investigators who have made use of coconut milk for various plant parts and embryos, are: Caplin and Steward (1948); Ball (1948); Nickell (1950); Steward and Caplin (1951); Morel and Wetmore (1951); and Steward *et al*. (1952).

Numerous attempts have been made to identify the factor or factors present in coconut milk and responsible for growth promoting activity. Van Overbeek *et al*. (1944) found that with partial purification of the original "sap," they obtained 170 times more activity. Steward and Caplin (1951) and Steward *et al*. (1952) reported the activity in coconut milk was due to a heat stable, water soluble, organic compound. Mauney (1952) with data somewhat contradictory to that of Steward, reported that most of the activity was in the "meat," and not in the milk. After purification he found the factor to be a heat stable, acid and alkali labile, non-volatile, and water soluble organic compound. Mauney

prepared a concentrate 4350 times as active on a fresh weight basis as the raw material.

In many cases coconut milk has been a success in promoting growth which was not deemed otherwise possible. However, in an equally large number of instances it has also been a failure. Van Overbeek et al. (1942) observed that the addition of coconut milk to media for very small (radially sym.) Datura embryos, did not promote growth. Haagen-Smit et al. (1945) reported that coconut milk did not promote any significant growth of immature corn embryos (0.3 mm. in length). Ziebur and Brink (1951) found that the addition of coconut milk to the media did not have a favorable effect on the growth of immature barley embryos (0.3-1.1 mm. in length). The data of Ziebur and Brink do not contradict that of Norstog (1956), nor Chang (1957), in that the latter two investigators used the biological additive at 90% by total volume, while in contrast Ziebur and Brink used 20% by volume.

Van Overbeek et al. (1944) reported that the following exhibited an embryo factor, although not equal to that of coconut milk: yeast extract; wheat germ; almond meal extract; and extracts of Datura ovules.

The value of endosperm as a biological additive to the culture media was noted as early as 1907, by Stingl. Excising embryos of wheat and oats, and then implanting them into

endosperm of their own species, as well as making reciprocal implants into the endosperm of each other, Stingl observed that: wheat embryos grew better in oat endosperm than they did in wheat endosperm; and oat embryos grew better in wheat endosperm than they did in oat endosperm.

Blakeslee and Satina (1944) reported that a powdered malt extract solution was an effective substitute for the culture of immature Datura embryos. However, as with coconut milk, it caused an inhibition of growth after autoclaving. Solomon (1950) reported evidence to the contrary. He found that the growth factor in malt was not destroyed by autoclaving, but rather was masked by the presence of a newly formed growth inhibitor. This inhibitor was water soluble, non-volatile, and heat stable. Dickson and Burkhart (1942) reported that the growth promoting action of malt extract was due to soluble nitrogenous substances present in the extract in the form of amino acids. Sanders (1950) observed that Seitz-filtered malt interfered with the germination of Datura embryos, decreased root formation, and inhibited shoot development.

Van Overbeek (1942) suspected an auxin present in the coconut milk as being responsible for the inhibition of root growth. After testing various extracts of coconut milk he concluded that, "it is the auxin in the medium which keeps

the embryo in the embryonic stage. Lowering of the auxin level causes the embryo to go into the seedling stage."

As one might expect, studies relative to auxins, and their effects on the growth and development of plant tissues and embryos, have yielded a wide variety of results. Went's (1926) publication, "On growth accelerating substances in the coleoptile of Avena sativa," was the forerunner in this field of endeavor.

Sanders (1950) was unable to detect any favorable effect of IAA on Datura embryos. He reported slight improvement in growth of Datura stramonium embryos, with the addition of alpha napthalene acetic acid (0.05 and 0.1 ppm). At a higher concentration of 0.5 to 10.0 ppm an "irregular twisting and bending" of the embryo was observed.

Rijven (1952) reported that the concentrations of IAA 0.001 ppm stimulated the growth of Capsella embryos. Concentrations above 1.0 ppm were inhibitory.

The influence of auxins on the cultures of mature plant embryos is somewhat different. Solacolu and Constantinesco (1936), and Gautheret (1937), working with bean embryos used IAA in concentrations of from 1.0 to 100.0 ppm. They reported, amongst other observations, a swelling of the hypocotyl eventually producing an unorganized mass of cells. Kraus et al. (1936) and Hamner and Kraus (1937), found similar

results with bean seedlings. Lachaux (1944) found that IAA increased respiration of Jerusalem artichoke tissue cultures but was ineffective in carrot tissue.

Physical Factors

Very few investigations have been carried out to clarify the significance of pH. Van Overbeek *et al*. (1944) observed that for "heart shaped" *Datura* embryos, two to four days after excision, a pH of 7 was optimal, and that for later cultures a pH of 5.5 was optimum. Rijven in 1952 was unable to determine a definite optimum for the culture of immature *Capsella* embryos. He felt that "other factors of nutritional nature interfered with the growth of the embryos." Rijven noted that shifts of pH may upset ionic balances of the medium and in this way interfere with normal growth. Street *et al*. (1952) reported that a shift towards alkalinity in White's medium may occur during experimentation, with the resulting precipitation of iron salts, thus rendering the iron unavailable. Street refers to this as a "stalling factor" in growth.

The current literature reports media with a pH range of from 5.7 to 6.0. Some researchers add phosphate buffers, while others apparently rely on phosphates in the biological additives to act as stabilizers.

Van Overbeek et al. (1944) grew Datura embryos at various temperatures. Relative growth was measured at regular intervals for the first five days of the experiment. An optimum of 32°C. was reported. Rijven (1952) found an optimum temperature of 30°C. during the first twenty-four hours of his experiment with Capsella embryos. He points out, however, that over a period of 96 hours, other factors ("probably nutritional") appeared, and rendered difficult an exact determination of non-nutritional factors.

Rijven observed a slight inhibiting effect of light on pregerminal embryonic growth, not significant however until after the fourth day of culture.

As previously noted some investigators place great emphasis on the effects of osmotic pressure. Dieterich (1924) reported significant differences in the developmental pattern of young embryos in culture, when: submerged in the agar medium, embryonic growth was continued; and, when placed on the surface of the agar, precocious germination resulted.

Rappaport (1954) observed that in the culture of Datura embryos, the optimal sugar concentration of the medium varies with the age at the moment of excision. He states that "the younger the embryos the higher must be the sugar concentration." The range of concentrations was from 8.0 to 0.5%.

Ziebur et al. (1950) attributed the inhibition and prolonging of embryonic growth, resulting from the addition of 1% casein hydrolysate to the medium, to the high osmotic pressure created by the addition of amino acids and sodium chloride. They replaced sucrose with mannitol (believed to be nutritionally inactive with barley embryos), maintained a high osmotic pressure, and prevented germination.

Other authors have reported that osmotic pressure to some degree controls behavior of the embryos. Uhvitis (1946) observed that germination of mature alfalfa seed can be inhibited by changing the osmotic pressure of the environment, using sodium chloride or mannitol. Duym et al. (1947) reported that the inhibition of germination caused by extracts of sugar beet balls, can be partly attributed to the osmotic pressure caused by inorganic salts present in the balls.

Pope (1944, 1949) induced barley embryos to germinate while still on the plant, by exposing them in situ, and lowering the osmotic pressure of the environment by applying wads of cotton soaked in water.

Street and McGregor (1952) in their report on the culture of excised roots, relative to sucrose concentration, concluded that "the effect of varying sucrose concentration on the growth of roots is not caused by the resulting changes in the osmotic pressure of the medium."

In 1952 Went suggests, in his paper, "Physical factors affecting growth in plants," that:

> Specific diffusible growth factors, needed for differentiation, are produced within the embryo and have a tendency to diffuse into the surrounding medium. In larger embryos, enough of these substances are accumulated to initiate differentiation, but in the smaller ones these growth factors would diffuse away, and would fail to reach a critical concentration which would cause differentiation.

Possibly this may in part explain why well differentiated embryos can be grown in nutrient media without the addition of growth factors. Relatively undifferentiated embryos it would seem develop satisfactorily only if a complex such as coconut milk, etc. is introduced into the media.

Went concludes that:

> It would appear there is a dependence on the balance between the rates of production of these growth factors not only whether an organism can grow at all in a given medium, but also what its growth rate will be.

Summary

It would seem that there are no real general principles which can be applied per se to the culture of plant embryos, except for the fact that both minerals and an organic carbon source are necessary constituents of the media. The requirements for "immature" embryos frequently differ from those of "mature" embryos. Additives, in addition to the abovementioned, have been demonstrated as being necessary for the

culture of very young embryos. Such additives frequently include biological extracts, which unfortunately, even to the present date, have been poorly delimited.

MATERIALS AND METHODS

As noted in the Introduction, the material used for this study was *Hordeum distichon* L. (Hannchen, C.I. 531). The cultured samples ranged in level of development from the relatively undifferentiated late proembryos on up through the level of late differentiation to "mature" embryos. The "mature" embryos were excised from *in situ*, and *in vivo* ripened caryopses.

One thousand, nine hundred and seventy-eight embryos were used for the investigation. Of this number, 800 were set aside for the exploratory chromatographic studies, as reported in the Appendix. The *in vitro* observations *per se* were based on 1178 embryos.

Levels of Development

As stated in the Introduction: eight arbitrary stages of embryological development can be recognized readily and excised for culture. Extensive studies on the embryogeny of barley have been reported by Merry (1941, 1942), Eunus (1954), and Mericle and Mericle (1957). Mericle and Mericle "arbitrarily divided" barley embryogeny into 13 stages. Their study was based on: 7 proembryo stages (a-g) and 6 stages of differentiating embryo (1-6). The authors describe stage 6 as "being the fully differentiated embryo as found in the

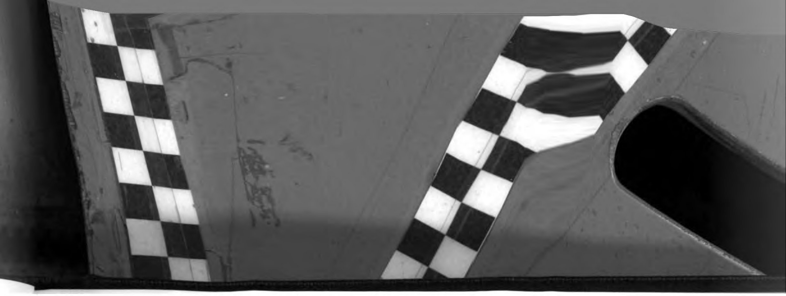

PLATE II. Morphological relationships between the various levels of differentiation. Each representative figure is properly proportioned to the smallest member of the series (x 100).

Fig. 1.	Level I	Late Proembryo	0.12 x 0.09 mm.
2.	Level I	Late Proembryo	0.30 x 0.15 mm.
3.	Level II	Early Differentiation	0.40 x 0.20 mm.
4.	Level II	Early Differentiation	0.60 x 0.30 mm.
5.	Level III	Middle Differentiation	0.80 x 0.40 mm.
6.	Level IV	Late Differentiation	1.30 x 0.85 mm.

PLATE II

seed." These stages were based on characteristic histological and morphological features of the embryo. Barley embryogeny may be divided into two broad categories: proembryos; and differentiating embryos. As reported by Chang (1957) and used in this investigation "the former may in turn be arbitrarily divided into three sub-groups: early, middle, and late proembryos." Additionally the differentiating embryos may be categorized, as: early, middle, and late differentiation.

The youngest embryos successfully excised for culture work were those referred to as late proembryos. For purposes of this investigation the cultured embryos have been grouped into four levels of morphological differentiation. These are: Level I - late proembryo; Level II - early differentiation; Level III - middle differentiation; and, Level IV - late differentiation. This study has been based on the foremen-tioned levels of development. Therefore, for the sake of brevity, morphological manifestations of the cultured embryos are frequently correlated with the various levels: i.e., level I, level II, etc.

Plate II has been designated to show the morphological relationships between the various levels of development. As noted in the descriptive text for this plate, all figures are at a magnification of approximately 100x. Each representative figure is properly proportioned to the smallest member of the series.

The various levels of development were categorized on the basis of two sets of factors: (a) length and width dimensions of the embryo; and (b) characteristic morphological features.

(a) length and width dimensions of the embryos

Level		Reference	Stages
I	Late Proembryo	LP	f-g
II	Early Differentiation	ED	1-2
III	Middle Differentiation	MD	3-4
IV	Late Differentiation	LD	5-6

Level		Dimensions in mm.
I	Late Proembryo	.12 x .09 to .30 x .15
II	Early Differentiation	.37 x .18 to .60 x .30
III	Middle Differentiation	.67 x .35 to .82 x .40
IV	Late Differentiation	.90 x .45 and beyond

(b) characteristic morphological features

Level I (Late Proembryo - Pl. II, Fig. 1-2): Morphologically there are no readily apparent signs of differentiation. Immediately after excision the "youngest" of the late proembryos will be characterized in form by a shape closely approximating that of a prolate spheroid. Towards the end of level I this orbicular shape becomes quite pronounced, tending in some instances to appear slightly obovate.

Level II (Early Differentiation - Pl. II, figs. 3-4): That part of the embryo which is away from the axis of the seed (abaxial), can be observed early in level II as a slight swelling near the distal end of the embryo. The swelling becomes more pronounced towards the end of level II, at which time, this convexity assumes the shape of a semi-circular ridge. This ridge of tissue represents the morphological manifestation of the developing coleoptile (the sheath which surrounds the shoot apex and the developing leaves in the Gramineae). The obovate form of the developing embryo is quite pronounced by the end of level II.

Level III (Middle Differentiation - Pl. II, fig. 5): In this level of development the circular ridge of tissue representing the developing coleoptile is completed. Early in this level the shoot apex can be observed as a pronounced protuberence within the ring of tissue formed by the developing coleoptile. The swelling, denoting the shoot apex, does not completely fill the circle of tissue formed by the coleoptile, rather it takes on the appearance of a "nodule," somewhat centrally located. Towards the end of this level the primordium of the first true leaf can be observed as a delicate fissure "cutting across" the swelling of the coleoptile, and bordering "on the edge" of the forementioned "nodule." The developing radicle (primary root) can be observed histologically, but is not obvious morphologically.

It is within this level that the scutellum (single cotyledon characteristic of Gramineae) first becomes readily apparent. It forms a fan-shaped shield surrounding the coleoptile, and extending out in a distal orientation from the mesocotyl. When compared with the scutellar development in level IV the "fan" is quite thick and "fleshy" in appearance.

Level IV (Late Differentiation - Pl. II, fig. 6): The primordia of two true leaves can be observed within the encircling ridge of the developing coleoptile early in this level. As differentiation proceeds the coleoptile shows a marked extension and takes on the form of a "very stubby tube" at the apex of which can usually be observed a small slit, through which the first true leaf emerges when germinated *in vivo*, and which is generally torn and ruptured by germination *in vitro*. The scutellum is one of the most pronounced features of this level of development. In some instances it has a truly "fan-shaped" appearance. In contrast to level III it becomes almost "paper thin" at its edges. However, in other cases it will be more "shield-shaped" and give the appearance of extending almost to the top of the coleorhiza (sheath encasing the radicle of a monocotyledenous embryo). The coleorhiza is very prominent in level IV and frequently shows a marked swelling at the tip.

Histologically a primary root primordiam and 4-5 seminal root primordia are well developed. Three to four leaf primordia can be observed histologically at this level, although only two are usually discernable morphologically.

Growth of Plant Materials in the Greenhouse

The source material (excluding field-grown crops) was collected from plants prepared for, and grown under the following conditions.

The basic soil mixture consisted of the following: three parts loam; one part sand; and one part peat moss. The seeds were sown in ten inch pots; five plants to a pot. Available stock was maintained by weekly plantings, in a series of five, with a theoretical total of twenty-five plants at harvest time.

In order to obtain relatively uniform embryos, only the main stalks were allowed to develop and the tillers (side "branches") were removed as they appeared. The pots in each linear series were rotated one quarter of a turn daily and changed in position once a week. Such procedure was standard and necessary in order to compensate for varying environmental conditions within the greenhouse, such as: influence of heating pipes; variation in natural light exposure; and position of the pots relative to window vents.

The plants were watered by "flooding" the pots once each week supplemented by intermediate sprinkling when necessary. A regular fertilization schedule was carried out every ten days using the commercial preparation, Vigoro. Disease preventative measures such as spraying with Malathione, dusting with sulfur, and fumigating with "Nicofume," were carried out at regular intervals.

A supplementary light source was supplied daily from 0-1800 hours. Use was made of several one thousand watt incadescent lamps, placed at three foot intervals, and set so as to be a minimum of two feet from the top of the heading plants.

During the fall, winter, and spring the temperature was controlled as closely as the physical set-up and environmental conditions permitted. Daytime temperatures ranged from $75-85^{\circ}$F., and nighttime temperatures from $60-65^{\circ}$. A temperature drop of from $15-20^{\circ}$ was necessary for the production of well-developed viable heads. Lacking this change, or having temperature ranges in excess of the forementioned, invariably resulted in the development of basically sterile heads.

Under the above-mentioned conditions one could, in general, expect to have the relatively undifferentiated late proembryos, on the average, 55 days after sowing. Embryos at the level of late differentiation were usually available on or after the 61st day from the time of planting.

Determination of Embryonic Level of Development (prior to harvesting)

In the culture of plant embryos time is a very critical factor. One must always allow for the fact that a certain number of ovaries will fail to develop, with resultant sterility. The pure mechanics of excision is susceptible to human error. A number of embryos will be injured or destroyed, even with very careful technique. In general the terminal and proximal four caryopses of any given head of _Hordeum distichon_ L., var. Hannchen, are frequently somewhat out of phase with the remainder. These eight caryopses are therefore lost for experimental purposes. The longer the elapsed time between harvesting and the actual excision and inoculation of the embryos, the greater is the chance for deterioration of the material, and a change in the level of development which would render it useless for a given series of treatments.

A procedure for determining the level of differentiation of the embryo, while _in situ, in vivo_ was worked out. On the whole this has proven to be most satisfactory for the variety of material studied, particularly for plants cultivated in the greenhouse during the late fall, winter and early spring,when environmental conditions can be reasonably controlled.

The technique used to ascertain the level of differentiation of the embryos, before microscopic examination, and pr

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practical for rapid analysis in the greenhouse was based upon the time of emergence of the awns (bristle-like appendage, occurring on the lemma* of each floret) from the "boot" (vernacular description of the terminal leaf sheath enveloping the immature head). It was observed that there was an apparent correlation between the level of differentiation of the embryos and the number of days elapsed after the awns first emerged from the boot.

Elapsed Time	Level of Development	
6 - 7 days	I	Late Proembryo
8 - 10 days	II	Early Differentiation
11 - 12 days	III	Middle Differentiation
13 and beyond	IV	Late Differentiation

Sterilization of Living Material

The procedure which produced the most satisfactory results (relative to effective sterilization with an absence of readily recognizable injury) was as follows:

A.

1. **Palea, lemma and glumes intact. Place material in a vial containing a 1% solution of Kromet***

*Lemma - the lower of the two bracts inclosing the flower in the grasses, formerly called the flowering glume.

**Palea - tiny upper bract which with the lemma incloses the flower in the grasses.

***Kromet - a commercial complex of sodium hypochlorite, with wetting agent, supplied by the Wyandotte Chemical Corporation, Detroit, Michigan.

(2 1/2 min.), with intermittent agitation: shake 1/2 min.; rest 1 min.; shake 1/2 min.; and rest 1/2 min.

2. Decant Kromet solution. Rinse rapidly in three changes of sterile basic salt solution (in triple distilled water).

B.

1. Remove: glumes; palea, lemma, and awn.
2. Place material ("naked" fruits) in a 1% solution of Kromet and agitate as above.
3. Decant Kromet solution, and rinse rapidly three times in basic salt solution, as above.

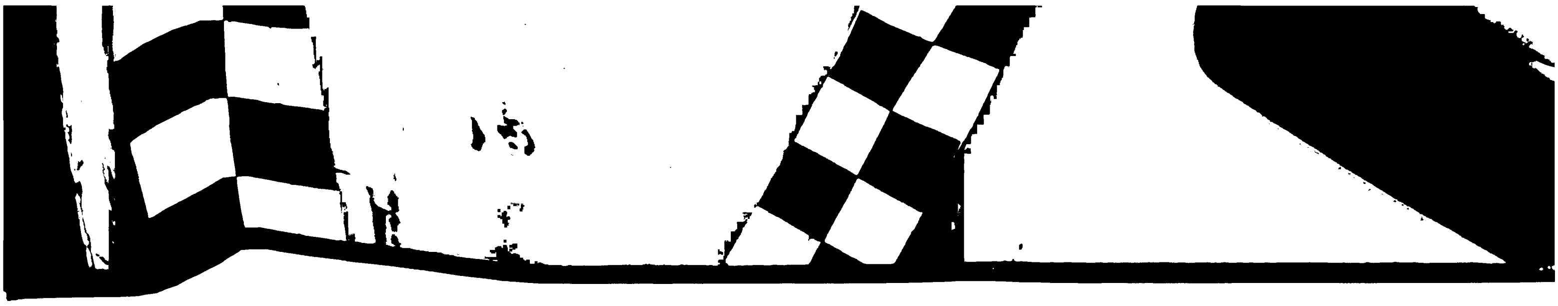
C. Place sterilized material (preparatory to excision) in a sterilized petri dish containing filter paper moistened with basic salt solution. Spread the material evenly, permitting no layering.

Note: total elapsed time in the disinfectant = 5 min.

Contamination

The degree of contamination was relatively low. The cultures were regularly 95-100% free of contaminants. Organisms most frequently encountered in the few samples which had to be discarded, were: Aspergillus sp.; Penicillium sp.; and Mucor sp. Fungal infection almost invariably preceded bacterial contamination.

Material cultured during the late fall, winter and early spring exhibited only negligible traces of contamination. Embryos excised and cultured from samples collected in the greenhouse during the summer months, were likewise low in their degree of infection. However, since conditions in the greenhouse during the summer months were frequently unfavorable to



good heading, it was often necessary to make use of samples collected from field-grown crops. It was from such material that the greatest amount of contamination developed. The techniques of sterilization and excision were standard throughout. The fungus growth was characteristically initiated at the point of inoculation of the embryo, usually on the surface of the embryo <u>per</u> <u>se</u>. One might therefore conclude that the contaminant was already within the embryonic tissue, and accordingly not affected by ordinary means of sterilization. Undoubtedly more drastic techniques would have eliminated this variable. Unfortunately such methods probably would also have been detrimental to the inoculum.

Culture "Chambers"

Three different types of culture chambers were used in this investigation. The type of chamber used is noted separately for each experiment, and their descriptions are as follows.

I. Plastic cups (Plate III): in a set of four, with depressions 15 mm. in diam., and a depth of 10 mm.

II. Round-bottom vials (Plates X-XIV): 25 mm. in diam. x 100 mm. in length; cotton stoppered, and "sealed" with Sargent, grade M, Parafilm.

III. Test tubes (Plate IX): 30 mm. in diam. x 195 mm. in length; cotton stoppered, and "sealed" with Parafilm.

PLATE III. Culture chamber formed by placing plastic cups in a petri dish ("sealed" with masking tape) in the bottom of which are two moistened filter papers.

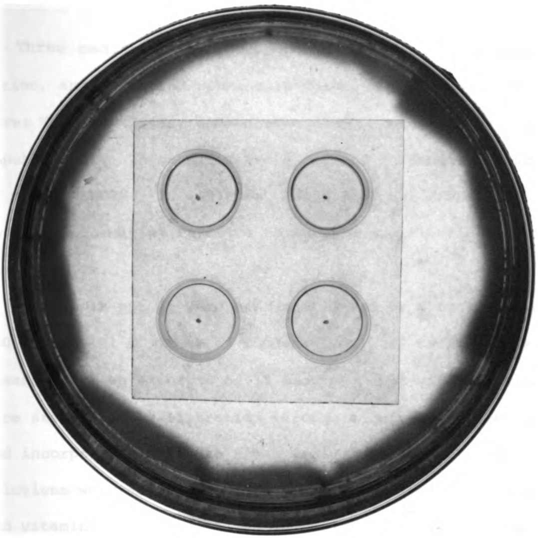


PLATE III

Media

Three media were used during the course of the investigation, and are noted separately for each experiment. These were: White's (1943), Gautheret's (1950), and LaRue's (1955, unpublished). These media are described in detail in Table 1 of the APPENDIX. In addition, Table 3 of the APPENDIX gives further information relative to LaRue's modification of White's Basic Medium.

The media *per se* were heat-sterilized by autoclaving at 250°C. (or 121°C., Heat of Condensation), 15 to 17 pounds pressure for an exposure of 15 minutes. The vitamin solutions were sterilized by filtration through a bacterio-sinter filter, and incorporated into the final medium. Separate stock solutions were maintained for the: basic salts, trace elements, and vitamins. Such additives as auxins (i.e., IAA) were prepared freshly and heat sterilized along with the basic medium. The pH for all media was adjusted to 5.7 by means of 0.1 N KOH.

Cultural Conditions

With the exception of the first Preliminary Experiment, all cultures were maintained in the dark at from 75-80°F. One experiment was carried out in a "growth control laboratory." Embryos excised at level IV (Late Differentiation) and cultured on White's unmodified basic medium in test tubes (Plate IX), were grown under the following conditions, as per Mericle and Mericle (1957): day temperature, 72°F.; night temperature, 60°F.; and light, 1200 ft.-candles during an 18-hour day.

EXPERIMENTAL RESULTS

The investigation *per se* was based on eight experiments. Studies 1-7 were concerned with the *in vitro* activity of various levels of embryonic differentiation. Experiment #8 (Chromatographic Studies), although certainly purposeful, was exploratory in nature and is reported on and discussed in the APPENDIX.

The seven cultural studies of excised barley embryos were grouped into the following three categories: preliminary; intermediate; and final experiments.

As noted earlier in MATERIALS AND METHODS a total of 1,978 embryos were used for the investigation. Of this number 800 embryos were used for the chromatographic studies.

The following is a numerical breakdown of the number of embryos upon which the results for the remaining seven experiments were based.

I. Preliminary Experiments (Culture medium: White, 1943)

#1.	10 embryos
2.	60 embryos
3.	60 embryos
	130

II. Intermediate Experiments (Culture medium: Gautheret, 1950)

#4.	40 embryos
5.	48 embryos
	88

III. Final Experiments (Culture medium: LaRue, 1955)

#6.	384 embryos
7.	576 embryos
	960

No observations were reported for contaminated specimens. Where an individual within a set (with reference to embryos cultured in plastic cups as a set of four) became contaminated, the whole set was discarded and that portion of the experiment repeated. The embryos cultured in plastic cups were grown in very close proximity to one another. Under such circumstances it was felt that the presence of an infected sample in the immediate vicinity of "healthy" embryos would introduce a variable which might be significant in an interpretation of the results.

The mechanics of the experimentation was a limiting factor in the number of samples used at any one time. This involved such considerations as: availability of samples at the proper level of differentiation; degree of sterility within the heads; and the time factor (elapsed time) between harvesting, sterilization, excision and inoculation. The most satisfactory results can be obtained only when a small number of fruits are processed at any one given time. Permitting the sterilized material to "lie around" in a moist chamber for an "excessive" period of time introduces, at the very least, two new variables: deterioration of the material; and significant changes in the level of development.

The entire investigation must be classified as "exploratory." It was therefore considered that the studies, at this stage, would be most useful if a wide range of treatments were carried out using a limited number of samples per treatment; and in particular a small number of "replicates," rather than attempting to concentrate on any one particular area.

TABLE 1. Summary of the responses in vitro, relative to the level of differentiation of the embryo at the time of inoculation

Level I.	Late Proembryo	Reference LP	Stage f-g
Level II.	Early Differentiation	ED	1-2
Level III.	Middle Differentiation	MD	3-4
Level IV.	Late Differentiation	LD	5-6

No responses were observed with embryos cultured at level I.

Level II	Level III	Level IV
highly aberrant plantlet	less aberrant plantlet than II	seedling rather than plantlet
partial germination common	"complete" germination more frequent than II, but not as common as IV	generally "complete" germination
shoot usually restricted to an extension of the coleoptile	shoot not restricted to an extension of the coleoptile	"normal" shoot development common
1st true leaf occasionally emerges from coleoptile	1st true leaf frequently emerges from coleoptile	1st true leaf regularly emerges from coleoptile; up to three true leaves (*in vitro*)
primary root rarely emerges from coleorhiza; no seminal roots observed	primary root frequently emerges from coleorhiza, but not as common as IV; seminal roots infrequent	primary root regularly emerges from coleorhiza. seminal roots abundant
callus a regular occurrence	callus prevalent, but not as common as in II	no callus observed at any time
extensive callus development; embryo per se often "obscured"	callus not as extensive as in II. rarely "obscures" the embryo; frequently concentrated in mesocotyl and coleoptile regions	
formation of callus apparently a "pre-requisite" for subsequent development	callus does not seem to be as significant as in II	
responses characteristic; relatively predictable under expt'l conditions	responses more variable than in II	responses characteristic and predictable under most conditions

PLATE IV. Aberrant plantlet on the 17th day of culture. Produced by an embryo inoculated at level II (Early Differentiation). Note: coleoptile protruding from a substantially callused and swollen mesocotyl region; the outline of the first true leaf can be seen vaguely within the coleoptile (x130).

PLATE IV

PLATE V. Aberrant plantlet on the 15th day of culture. Produced by an embryo inoculated at level III (Middle Differentiation). Note: callus development in the mesocotyl region, though not as substantial as in Plate IV; a deterioration at the tip of the coleoptile; the first true leaf clearly visible within the sheath; and the second leaf visible vaguely at the edge of the callus formation (x75).

PLATE V

PLATE VI. Aberrant plantlet on the 20th day of culture. Produced by an embryo inoculated at level II (Early Differentiation). Note: the pronounced collar formed by the coleoptile at the base of first and second true leaves; the darkened shield-like formation below the coleoptile is the scutellum bending upward; and the loose and shaggy callus development (x70).

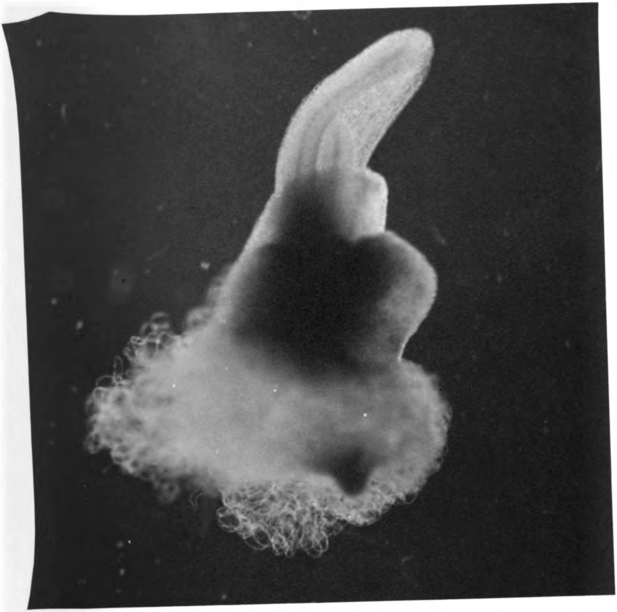


PLATE VI

PLATE VII. Aberrant plantlet on the 15th day of culture. Produced by an embryo inoculated at level III. Note: the coleoptile is represented by a vestigial "ring" at the base of the first true leaf; characteristic callus; and the vague outlines of the recurved second leaf approximately 3.5 cm. from the tip of the coleoptile (x85).

PLATE VII

The EXPERIMENTAL RESULTS of the investigation are summarized in Tables 1 and 2.

General Morphological Development

Table 1 summarizes in detail the morphological characteristics of the developing embryo and subsequent seedling (or plantlet) in vitro, relative to the level of differentiation at the time of excision and inoculation.

Both the shoot and the root arising precociously from embryos cultured at levels II and III generally have a somewhat abortive appearance. To this aberrant type of development I have applied the term "plantlet," rather than seedling. Only the latter can properly be applied to the young plant which has evolved from embryos placed in culture at level IV (Late Differentiation). Plates IV, V, VI, and VII are examples of aberrant plantlets. A detailed description of these is included in the legend for each plate.

Attention should be drawn to the four, somewhat different, manifestations of the coleoptile, as shown in the forementioned plates. Plate IV shows a coleoptile protruding from a substantially callused and swollen mesocotyl region. As in Plate IV, in Plate V the coleoptile also has emerged from an extensively callused mesocotyl region, though it is much more pronounced in its development and the first true leaf can be observed readily within the coleoptile. As noted in MATERIALS AND METHODS, the first true leaf as it emerges from the coleoptile during precocious germination does not pass through the "emergent" pore at the tip of the sheath. In precocious germination the first true leaf generally emerges by ripping and tearing the tip of the coleoptile as it comes out. Sometimes there is a shredding and partial deterioration of the tissue at the extremity of the coleoptile even before the first true leaf has reached this point (Plate V).

In Plate VI the coleoptile forms a pronounced "collar" at the base of the first and second leaves. In contrast, in Plate VII the coleoptile is represented only by a vestigial "ring" at the base of the first true leaf.

When placed in a moistened paper towel, germination from _in situ_, _in vivo_ ripened caryopses usually occurs within 48 hours (Plate I). Under favorable conditions, embryos excised and cultured at level IV (Late Differentiation) generally germinate in from 48 to 72 hours. Germination of embryos cultured at earlier levels of differentiation is not as clear cut. Precocious germination of early differentiating embryos (level II) is usually 10-15 days from the time of inoculation. Embryos inoculated into culture at level III will germinate, if at all, by the 10th day.

PLATE VIII. Characteristic callus formation of level II (Early Differentiation), 12th day of culture. Note: the more or less spherical shape; masking of the morphological features; pronounced swelling of the coleoptile; and the shoot apex represented as a slight, lateral protrusion. (x180)

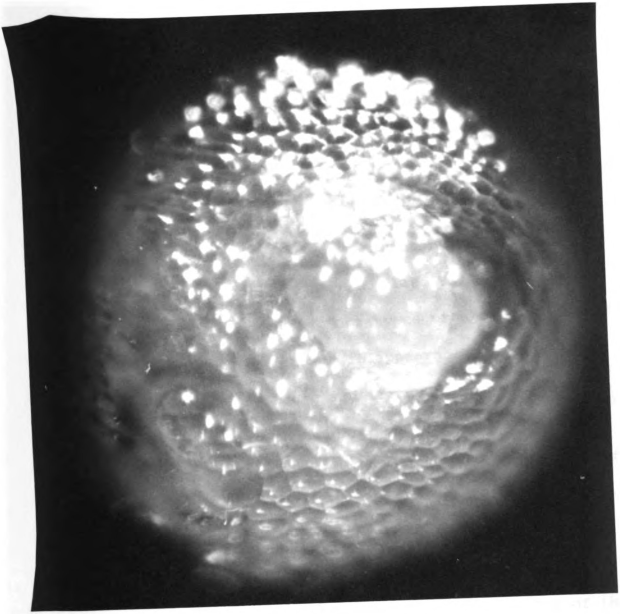


PLATE VIII

Roots produced at levels II and III frequently are poor in root hair development. Root hairs derived from embryos inoculated at level IV will vary from sparse to dense pubescence. In some cases epidermal hairs can be observed on the surface of the "tips" of the coleorhizae of embryos inoculated at level IV.

The callus formation so very characteristic of level II (Early Differentiation) is shown in Plate VIII. The callus development may be of two distinct types or a combination of both. It may be highly compact in appearance, as in Plate VIII, or it may be very loose and irregular, as in Plate VI. The compact type of growth tends to assume a more or less spherical shape. As noted in Table 1, during the process of callus development, the characteristic morphological features of the embryo are frequently all but obliterated.

Table 1, level III (Middle Differentiation), shows responses which are more variable than level II. All of the embryos used in the study have been very carefully selected to fit one of the four levels of development. However in level III, even with these precautions, the samples often act in the fashion of either level II or level IV.

PLATE IX. Seedling produced by a "mature" embryo, excised from an <u>in vivo</u> ripened caryopsis. Culture chambers exposed to 18 hours of illumination daily. Note: the three leaves in Figure 8, and the abundant root hair development throughout.

Using Figure 1 as the standard (with an arbitrary value of 1), the scales of the remaining seven figures are shown in the form of a ratio.

Fig. 1	2 days	
2	4 "	1:2
3	6 "	1:2
4	8 "	2:5
5	10 "	3:10
6	12 "	2:5
7	14 "	2:5
8	20 "	1:5

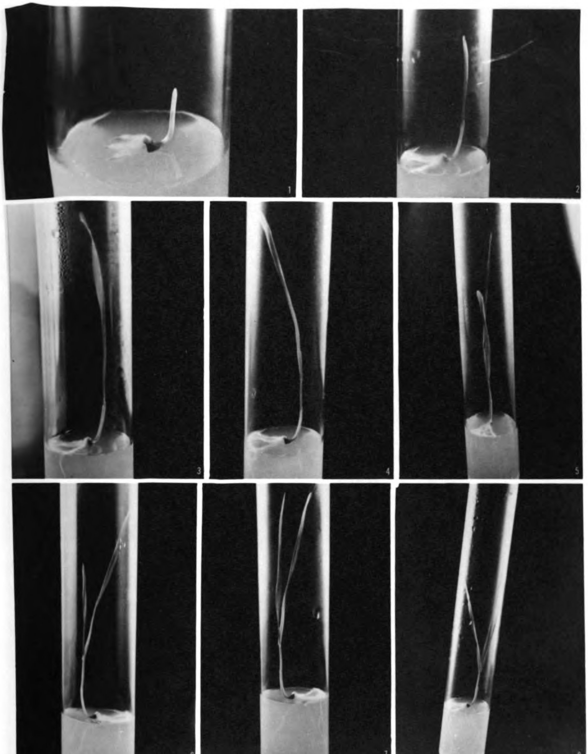


PLATE IX

PLATES X - XIV. Embryos at level IV (Late Differentiation) are cultured (in the dark) on media in which the concentrations of sucrose are: 1, 2, 3, 4, 5, and 6%. The figures can be read directly in concentration of sugar (i.e., Fig. 1 = 1%).

This series of plates emphasizes the response of the late differentiating embryos to a sugar concentration of 3% (x1.7).

Plate X	5 days
XI	7 days
XII	11 days
XIII	14 days
XIV	20 days

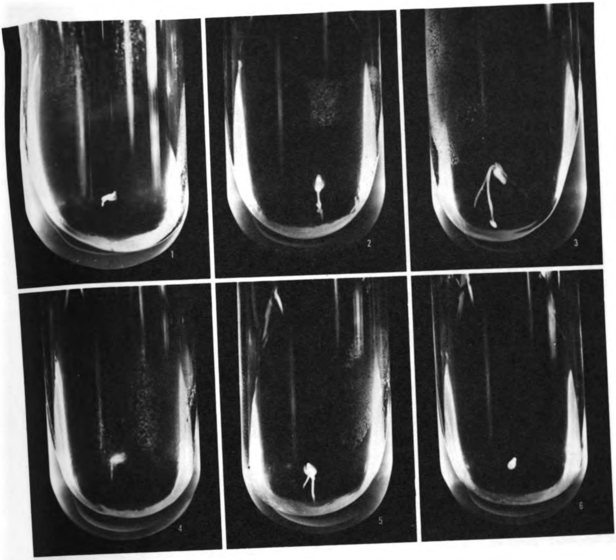


PLATE X

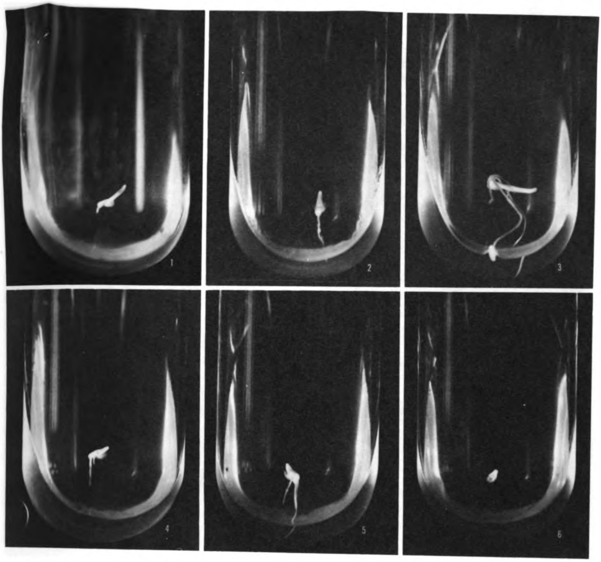


PLATE XI

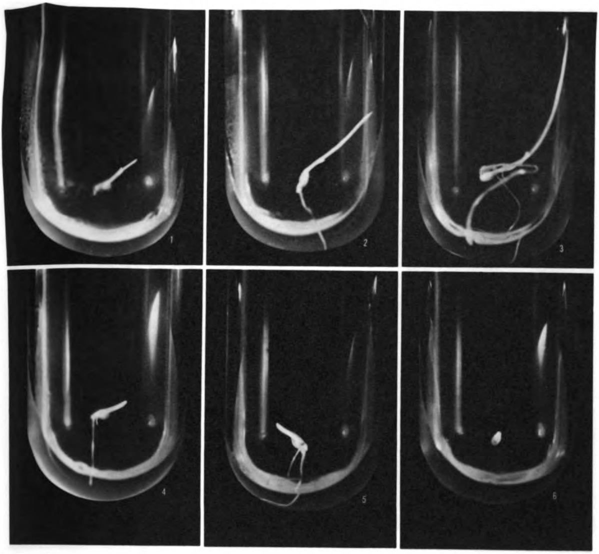


PLATE XII

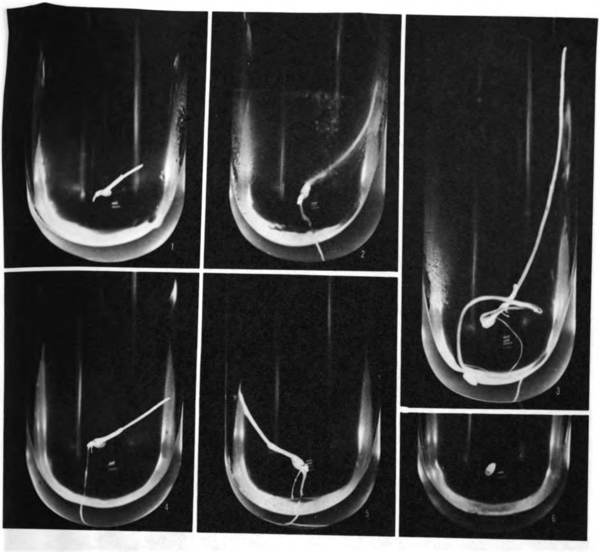


PLATE XIII

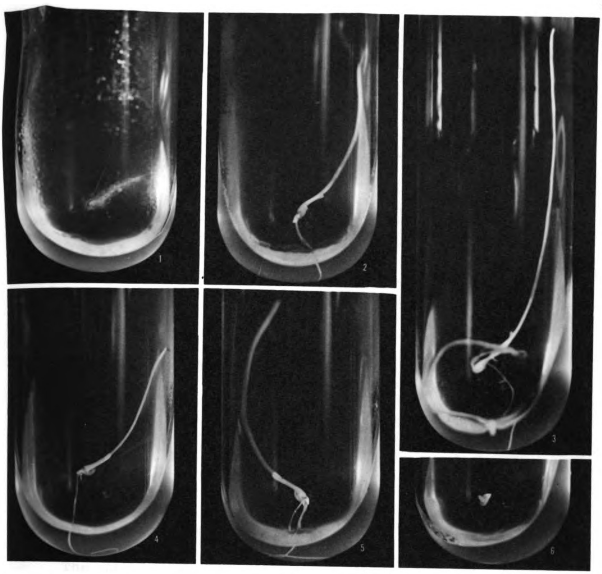


PLATE XIV

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Responses to Organic Carbon Sources

Thirteen sugars and seven intermediate (glycolytic) respiratory products were used as primary organic carbon sources. These compounds included the following: four pentoses; five hexoses; four di-hexoses; four hexose-phosphates; a triose; a triose phosphate; and pyruvic acid.

The thirteen sugars were used as the primary organic carbon source for all four levels of development. The intermediate respiratory products were used only for level II (Early Differentiation) in Intermediate Experiments 4-5.

Preliminary Experiments

Experiment 1: Embryos excised at level IV (Late Differentiation) were cultured on White's unmodified basic medium in test tubes (Plate IX).

The culture chambers for experiments 2-3 were round-bottom vials.

Experiment 2: Late differentiating embryos were cultured on a modified version of White's medium, in which the organic carbon source, sucrose, was incorporated into the media at 1, 2, 3, 4, 5, and 6% (Plates X-XIV).

Experiment 3: The purpose of this experiment was to compare the responses *in vitro* of embryos excised and inoculated at level IV into media (White's) which contained sucros

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sucrose as the organic carbon source, or media in which there was a combination of fructose and glucose.

The following treatments were used:

a. sucrose 3%
b. fructose 3
c. glucose 3
d. fructose 1.5
e. glucose 1.5
f. fructose + glucose (1.5 each, for a total of 3% sugar)

Intermediate Experiments

The purpose of experiments 4-5 was to study the effect on embryos cultured at level II (Early Differentiation) when various intermediate (glycolytic) respiratory products were used as the sole carbon source. In a secondary series of treatments these compounds were used in combination with sucrose (3%).

The embryos used for experiments 4-5 were cultured in plastic cups on Gautheret's medium, modified by the incorporation of the various intermediate products.

Experiment 4: Pyruvic acid as the primary organic carbon source was incorporated into the media at: 2000, 1000, 500, 100, and 50 ppm. A secondary series of treatments made use of the forementioned (in the same series of concentrations) in combination with sucrose.

Experiment 5: The following intermediate products were incorporated into the media at a concentration of 500 ppm

(0.05%): glucose-1-phosphate; glucose-6-phosphate; fructose-6-phosphate; fructose-1,6-diphosphate; glyceraldehyde; and phosphoglyceric acid. As above, a secondary series of treatments made use of the intermediate products in combination with 3% sucrose.

Final Experiments

The final series of experiments (6-7) had three purposes: to study the in vitro responses of all four levels of development on media containing sucrose as the primary organic carbon source; to see if a variation in the concentration of agar would affect the cultured embryos; and to study the responses of all four levels to sugars, other than sucrose.

The embryos used for experiments 6-7 were cultured in plastic cups on LaRue's medium, modified by: various sugar concentrations; varied sugars; and various concentrations of agar.

Experiment 6: Sucrose was incorporated into the media at the following concentrations: 4, 3, 2, 1, 0.5, and 0.25%. The following concentrations of agar were used: 0.8, 0.75, 0.70, and 0.65%.

Experiment 7: Using an agar concentration of 0.8%, twelve sugars, other than sucrose (as listed in Table 2), were incorporated into the media as the primary organic carbon source.

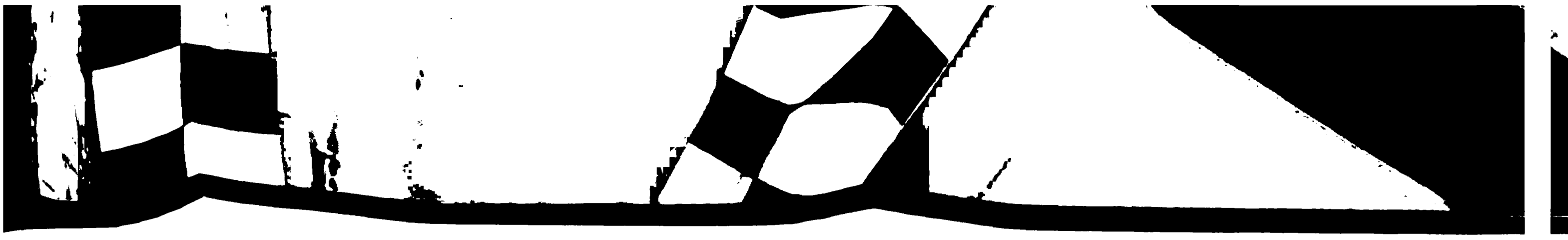

TABLE 2. In vitro responses of barley embryos to various organic carbon sources

Organic carbon source	Level I #	:Resp	:Opt	:Rng	Level II #	:Resp	:Opt	:Rng	Level III #	:Rsp	:Opt	:Rng	Level IV #	:Rsp	:Opt	:Rng
sucrose	96	--		.25-4	96	+7	4	.25-4	96	+7	2	.25-4	176	+7	3	.25-6
D(+) glucose	12	--		2-4	12	+5	4	2-4	12	+5	2	.5-3	32	+5	3	.5-3
D(-) fructose	12	--		2-4	12	+4	4	2-4	12	+4	2	.5-3	32	+5	3	.5-3
cellobiose	12	--		2-4	12	+4	4	2-4	12	+4	2	.5-3	12	+4	3	.5-3
maltose	12	--		2-4	12	+3	4	2-4	12	+3	2	.5-3	12	+3	3	.5-3
L(+) arabinose	12	--		2-4	12	+2	4	2-4	12	+2	2	.5-3	12	+2	3	.5-3
D(+) galactose	12	--		2-4	12	+	4	2-4	12	+	2	.5-3	12	+	3	.5-3
lactose	12	--		2-4	12	+	4	2-4	12	+	2	.5-3	12	+	3	.5-3
D(+) mannose	12	--		2-4	12	±	4	2-4	12	±	2	.5-3	12	±	3	.5-3
D ribose	12	--		2-4	12	--		2-4	12	--		.5-3	12	--		.5-3
D(-) lyxose	12	--		2-4	12	--		2-4	12	--		.5-3	12	--		.5-3
D(+) xylose	12	--		2-4	12	--		2-4	12	--		.5-3	12	--		.5-3
L sorbose	12	--		2-4	12	--		2-4	12	--		.5-3	12	--		.5-3
glucose + fructose																
(@ 1.5% each)													10	+2		
pyruvic acid					20	±	.05	.005-2								
glucose-1-phosphate					4	--										
glucose-6-phosphate					4	+										
fructose-6-phosphate					4	--										
fructose-1,6-diphosphate					4	+										
glyceraldehyde					4	--										
phosphoglyceric acid					4	--										
pyruvic acid + sucrose																
(sucrose @ 3%)					20	+		.005-2								
glucose-1-PO_4 + s					4	--										
glucose-6-PO_4 + s					4	+3										
fructose-6-PO_4 + s					4	+2										
fructose-1,6-diPO_4 + s					4	+2										
glyceraldehyde + s					4	--										
phosphoglyceric acid + s					4	--										

Explanation of Table 2

1. Abbreviations

Explanation of Table 2

1. Abbreviations

   a. # - number of embryos cultured and evaluated
   b. Resp - "graduated" ratings of the responses of barley embryos to the various organic carbon sources
   c. Opt - optimum concentration of carbon source, expressed in %, when a range was tested
   d. Rng - range of concentrations tested, expressed in %
   e. s - abbreviation for sucrose, when used in combination with the various intermediate respiratory products

2. Rating System

   The evaluation of the responses of barley embryos to the various treatments (organic carbon sources) *in vitro*, is recorded in the form of an "arbitrary" rating scale. The scale ranges from (--) no observable response, to (+7), the most favorable rating.

3. Although the rating scheme is the same for each level the responses were evaluated for each level of differentiation separately.

4. Where only a single concentration was tested, as for the intermediate respiratory products, a response is noted, but no optimum is recorded.

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The sugar concentrations for levels I (Late Proembryo) and II (Early Differentiation), were: 4, 3, and 2%. For levels III (Middle Differentiation) and IV (Late Differentiation), the concentrations were: 3, 2, and 0.5%.

Table 2 gives a tabulation of the in vitro responses of barley embryos to various organic carbon sources.

As Table 2 indicates, the responses to the various carbon sources are characteristic of the level of differentiation, regardless of the sugar or intermediate respiratory compound incorporated into the medium. The response is primarily quantitative rather than qualitative. A carbon source that is unsatisfactory for one level is also unsatisfactory for the other two. The significant difference between the levels is to be found in the optimum concentration of sugar: level II - 4%; level III - 2%; and level IV - 3%.

In general, pentoses are unsatisfactory carbon sources. Although responses in varying degrees were observed for the intermediate respiratory products, they too were poor carbon sources. Even though responses of +2 and +3 were observed, they were for treatments in which sucrose was incorporated into the medium along with the intermediate compound.

It should be noted that the combination of fructose and glucose was not as effective as sucrose or even as either sugar when used alone as the sole organic carbon source.

PLATE XV. Embryos cultured during late differentiation often exhibit two trends. During the first ten days following inoculation the most satisfactory responses may be in media with exceedingly low concentrations of sucrose. A "plateau" is frequently attained by the 10th day, after which greater responses can be observed on media with sugar concentrations of 2-3%; and in the final stages of incubation (21 days) 3% is superior.

This plate shows seedlings, on the 10th day of culture, exhibiting the forementioned "plateau."

Fig. 1	4% sucrose	(Scale: each unit equals 1 mm.)
2	3% "	
3	2% "	
4	1% "	
5	0.5% "	
6	0.25% "	

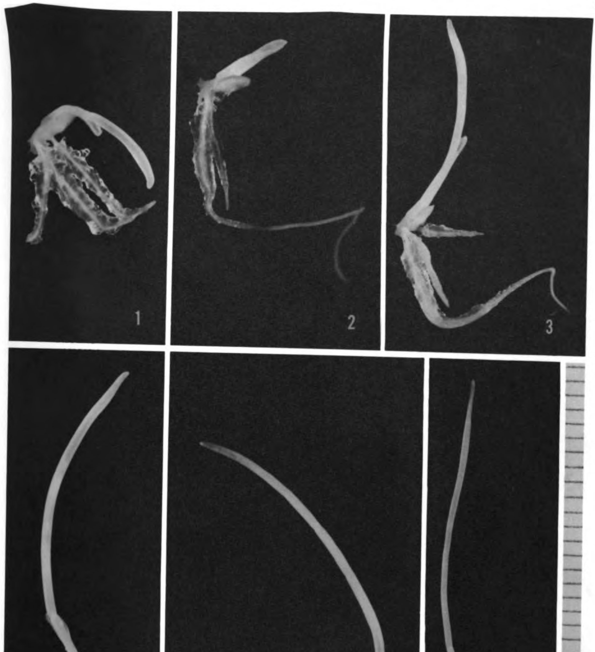

PLATE XV

PLATE XVI. Late differentiating (level IV) embryos cultured on a medium with an agar concentration of 0.65%; fourteen days after inoculation, germination having occurred on the tenth day in culture. Note: aborted shoot development and the absence of roots (x1.6).

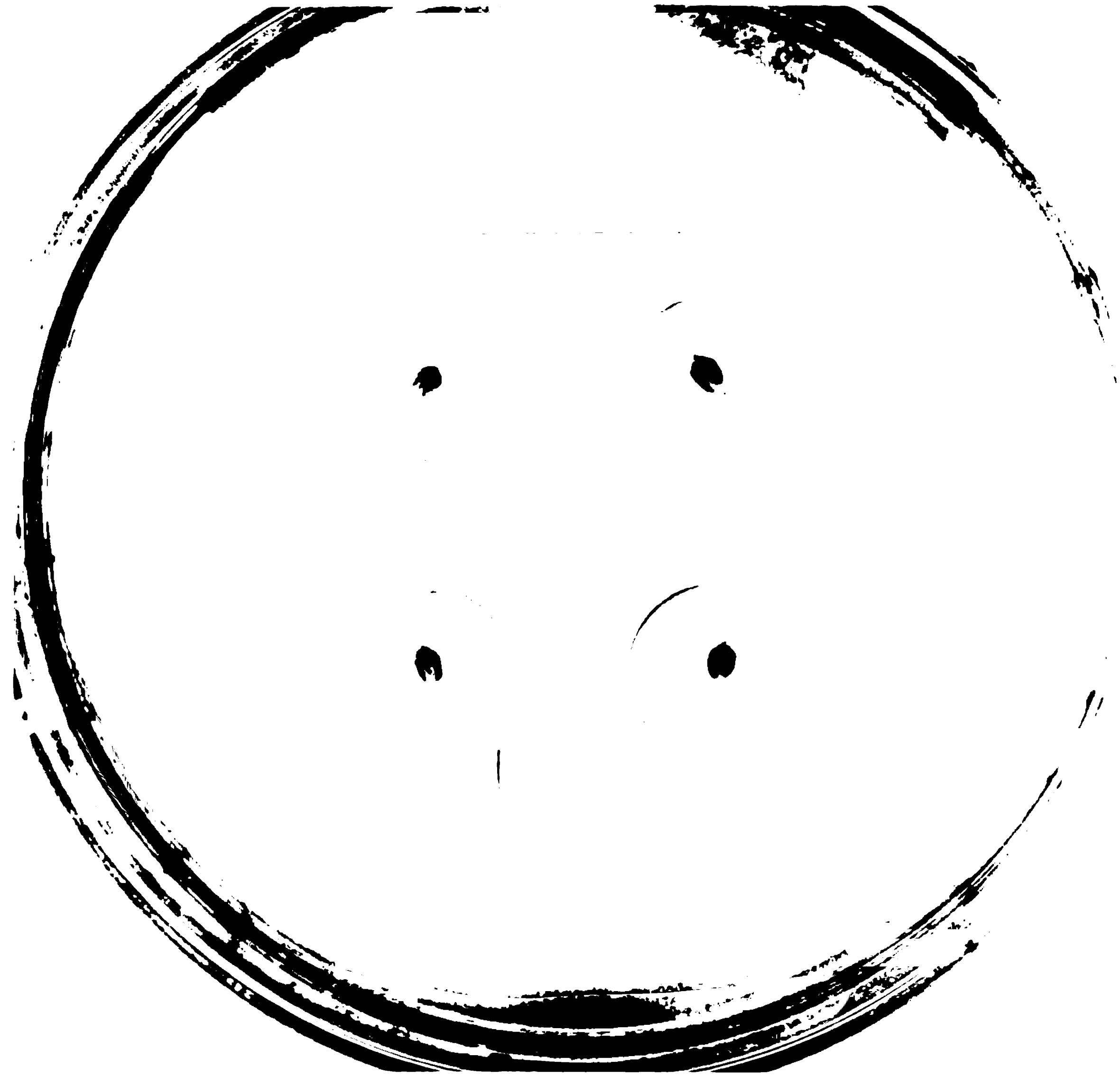

PLATE XVI

At level IV (Late Differentiation) glucose and fructose were rated as being equally effective, although in levels II and III glucose was considered to be slightly superior.

Embryos cultured during late differentiation often showed two trends. When using a series of treatments in which the concentrations of sucrose ranged from 4.0 - 0.25%, the lower concentrations of 0.25, 0.5, and 1.0% produced the greatest response of the seedlings during the first ten days after inoculation (Plate XV). After this period, those embryos cultured at 2 and 3% not only equalled but surpassed the development of cultures at the lower concentrations. In the final stages (21 days) sucrose at 3% was definitely superior.

Agar

The responses of the various levels of development would appear to be somewhat related to the concentration of agar in the media. As the agar becomes less solid (lowered concentration) the growth responses of the three levels were decreased. At a concentration of 0.7%, levels II and III exhibited very erratic growth, and showed no response at 0.65%. Level IV responded over the entire range (0.8 - 0.65%); however as can be seen in Plate XVI, at a concentration of 0.65% the shoot development was aborted and root development rare.

DISCUSSION

To develop a meaningful modification of previously reported culture techniques, three sets of facts are required: well-defined components of the media; the concentrations used; and the level of differentiation of the embryos cultured.

In order to study embryological development in vitro, one must be able to culture an embryo from its relatively undifferentiated state. Few investigators have been able to do this. Those who have succeeded have had only limited success. When they did succeed in getting very young embryos to grow, they did so by incorporating biological extracts, such as coconut milk, into the medium (Van Overbeek et al., 1941, 1942; Norstog, 1956; and Chang, 1957). The use of such an additive presents two problems: the components of the extract are not delimited; and no two "batches" of the coconut milk will be precisely the same. The environmental conditions must be such that they can be "duplicated" throughout a series of treatments. If one desires to study the effects of a given treatment on cultured material there is no room for unknown variables in the medium, which of course eliminated the use of coconut milk in this investigation.

A superficial examination would lead one to conclude that the concentrations used in the various media are rather well established. The basic minerals can be incorporated into the media over a rather wide range of concentrations with seemingly no deleterious effects; for other components this is not true. Concentrations used for the various carbon sources, and in particular the sugars have been reported as being relatively "critical" (LaRue, 1936b). A number of investigators have indicated that the satisfactory development of the very young embryos in culture required a high sugar concentration, while in contrast, the older embryos did better on media containing a low concentration (LaRue, 1936b; Tukey, 1938; Lammertz, 1942). There would seem to be a variance of opinion as to what is high and what is low. For example: a concentration of 2% sucrose will be considered as being low by one individual (when compared with 6%); and yet another will consider it as being high (when contrasted with 0.5%).

There has been a tendency to describe the cultured embryos as being either mature or immature with a paucity of parameters, morphological or otherwise, to delimit one from the other. It would seem that what is not mature (a term at best, very difficult to define) is immature. The detailed embryological development of barley has been extensively studied by such investigators as Merry (1941, 1942), Eunus (1954), and Mericle and Mericle (1957). This study has been based on the work of Mericle and Mericle.

Since no dependable culture technique was available, one had to be developed, and therefore the technique became the problem. From the problem evolved certain fundamental observations which may in the future permit one to use the technique of plant embryo culture more effectively as a means of studying the responses of cultured barley embryos to various treatments.

Although late proembryos (level I) showed no response in culture, barley embryos at the level of early (II), middle (III), and late (IV) differentiation can be cultured on well-defined media devoid of biological extracts. The morphological responses of the cultured embryos are characteristic for the level of differentiation at the time of inoculation. Embryos cultured at level IV readily germinate to produce "normal" seedlings, while embryos cultured at levels II and III germinate precociously to form aberrant plantlets. The development of a callus is also characteristic of levels II and III.

Sucrose is by far the most satisfactory carbon source for the culture of barley embryos. The responses to the various sugars are characteristic of the level of differentiation at the time of inoculation and the variation between carbon sources is a matter of degree rather than type of response. The well differentiated embryos of level IV respond most favorably to a concentration of 3%, and the relatively undifferentiated embryos of level II develop best at a concentration of 4%.

The responses of the various levels of development would appear to be somewhat related to the concentration of agar in the media. The most satisfactory concentration for the three culturable levels was 0.8%.

The variation in the responses to agar may in part be due to the fact that agar is a colloid, and as such has distinctive adsorptive properties. The effects of such adsorption may be either harmful or beneficial. Adsorption might very well be effective in altering the available ionic concentrations of a nutrient medium. It is possible that the agar could "remove" toxic metabolic biproducts by adsorption. It is a semi-solid base in which there could be significant build up between ions and organic substances in the nutrient, and metabolic products removed from the living tissues.

The experiments were designed to determine whether or not carbon sources other than those commonly used would improve the growth of excised embryos either qualitatively or quantitatively. No investigation of the metabolic use of the various carbon sources was attempted as the experimental system was not considered suitable for this purpose.

Sucrose, glucose and fructose were the most satisfactory carbon sources. All three sugars are directly or indirectly

involved in glycolysis. In general the pentoses and the intermediate respiratory products are unsatisfactory carbon sources. Relative to the carbon sources and in particular the more satisfactory sugars there were really no unexpected results. It would seem that the carbon source is not too important, as long as it is functional and will give a response. The differential response of the various levels of development, was a matter of degree, and could be directly related to an optimum concentration.

It is possible that the quantitative differences were in part due to variations in osmotic pressure; the older embryos developing on a lowered sugar concentration and the younger ones at a higher concentration. A determination of osmotic pressure, and in particular an evaluation of its significance, is at best rather nebulous. Osmotic pressure can be determined by physical means for a given medium. However, this technique does not take into account the conditions within the living cells, which are certainly involved in any osmotic relationship.

The Chromatographic Studies, as reported in the APPENDIX, did indicate that the relatively undifferentiated embryos of level II contained a high percentage of reducing sugars. In contrast, the older well differentiated embryos of level IV contained a very low percentage. There may very well be a

significant relationship between the storage and availability of sugar and the level of development which is not readily apparent.

The responses exhibited by the embryos, when cultured at various levels of differentiation, is I feel quite important. Although many of these responses are abnormal when compared with <u>in vivo</u> development, the fact is that they do occur with a high degree of regularity and are characteristic for a given level under the experimental conditions. An awareness of the characteristic responses, under well-defined conditions, permits one to extend the investigation and to study the effects of various treatments.

Previous investigators have undoubtedly noted the same callus development in culture as observed in this work. Upon cursory examination, however, the cultures might be discarded as being highly abnormal, as indeed they are when compared to <u>in vivo</u> development. But for embryos cultured at the level of early differentiation this is a highly characteristic response. An embryo is an organism; as such, it is composed of organs, tissues, and cells. The embryo has both potential and capacity. It produces new cells and the cells can differentiate into tissues. Its growth and development is dependent on both external and internal environment; an unbalance in either or both will cause a reaction.

Two facts are well known: cell replication and cell differentiation do not occur simultaneously (Fischer, 1946); and the requirements for differentiation appear to differ from those for cell replication. The level of early differentiation is precisely what it says; a phase during embryogeny in which very little differentiation (into tissues and organs) has taken place. Excising the embryo from a well balanced and well controlled environment, and placing it in at best a "poor imitation" is bound to upset the normal sequence of events. As long as the embryo is living it will continue to grow and develop. If the environment is less than optimum, it is not illogical to assume that the response will be in the direction of least resistance and least demanding requirements - cell replication. If one assumes that the concept of "tissue induction" can also be applied to plants, then it is very possible that until such time as an internal environment favorable to differentiation is established, growth and development will be largely due to cell replication and cell enlargement. A concentrated effort in this direction could well result in callus formation - which is so extensive and characteristic of embryos inoculated at early differentiation. Before some manifestation of a shoot is observed at level II, a callus develops. If there is no callus formation there is no emergence of a shoot. However, a callus may develop

without subsequent emergence of a shoot. One might infer from the foregoing that during the development of the callus an internal environment favorable to further differentiation has been established.

Level III exhibits less callus formation than does level II. Differentiation in level III is already well advanced, and it may very well be that at this level the organism "adapts" itself more rapidly and with greater ease. The root primordium is not well developed until level III, and as noted in Table 1, the primary root frequently emerges in level III, but rarely emerges from embryos inoculated at level II. It is possible that in level II the embryo "exhausts" much of its potential in first developing an internal environment favorable to differentiation, and then in producing a shoot.

Level IV (Late Differentiation) germinates and produces a seedling with relative ease. Although the culture medium may be somewhat less than optimum, it is possible that the embryo at this level of development may very well have an extensive food supply within the scutellum upon which it can draw until such time as the seedling has become well established.

Aberrant plantlets are characteristic of both levels II and III. As with the callus development in culture, this is a regular and characteristic occurrence. Aberrant plantlets when compared with seedlings are both abortive and abnormal. However, if any environmental conditions favorable to normal seedling development are lacking, one might expect that should a young embryo germinate, the resultant product will be both aberrant and abortive.

Cultured embryos, therefore, do not follow their normal developmental sequence. When evaluating this "failure" one should take into consideration our present inability to "duplicate" the _in vivo_ conditions. It is possible that the _in vivo_ conditions. It is possible that the media were too complex. Just as a deficiency of a given factor, or factors, could be detrimental to growth and development; so could an excess of one or more factors be toxic or even lethal.

The constancy of the medium is a problem which has been little studied. The natural environment is not statis; it is kinetic. As the embryo develops _in vivo_ its requirements undoubtedly change; both qualitatively and quantitatively. Some substances are conducted to the developing embryo - others are conducted away. _In vitro_ the standard procedure is to place the embryo on a comparatively fixed medium. It remains there for the period of the incubation. Some substances are removed from the substrate, and others added to it. It is most likely that deficiencies, which may have a pronounced effect on growth and development, will occur in the immediate vicinity of the embryos. Also exudations may be built up to the point of being toxic or even lethal.

In nature the developing head is subject to temperature fluctuations. In cultures, however, the embryos are generally maintained at a constant temperature. This neglect to vary the environmental conditions during the culture period is possibly a contributing factor in the failure to culture the embryonic material successfully through a normal developmental sequence.

SUMMARY AND CONCLUSIONS

Summary

1. Under the experimental conditions of this investigation, the morphological responses of embryos cultured on well-defined media, are characteristic, and directly related to the level of embryonic differentiation at the time of inoculation into culture.

2. The cultured embryos did not follow the normal sequence of *in situ* and *in vivo* development. Though late pro-embryos could not be cultured, under the experimental conditions and with the media used, embryos excised and placed in culture at early and middle differentiation germinate precociously to form aberrant plantlets, and embryos cultured at late differentiation germinate readily to produce "normal" seedlings.

3. It would seem that the development of callus is characteristic of embryos cultured at early and middle differentiation. In cultures of early differentiating embryos the callus formation is extensive and apparently under the experimental conditions a forerunner to subsequent development.

4. Sucrose is the most satisfactory organic carbon source for the culture of barley embryos under the conditions

of this investigation. The responses exhibited by the cultured embryos to the various carbon sources are characteristic of the level of differentiation at the time of inoculation. The variation between the carbon sources is a matter of degree rather than the type of response. Level IV responds most favorably to a sugar concentration of 3%; level III to 2% and level II to 4%.

5. It would seem that the responses of the various levels of development are somewhat related to the concentration of agar in the media. Over a tested range of from 0.65 to 0.8% agar, the most satisfactory concentration for the three culturable levels, was 0.8%.

Conclusions

1. It would seem that the morphological responses of cultured barley embryos, <u>Hordeum</u> <u>distichon</u> L. (Hannchen C.I. 531), under the experimental conditions of this investigation, are characteristic of, and directly related to the level of differentiation of the embryo at the time of excision and inoculation into culture.

2. The responses of the cultured embryos to the various carbon sources appear to be quantitative rather than qualitative; and, directly related to the level of embryonic differentiation.

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APPENDIX TABLE 1. Media used in the investigation

Nutrient	LaRue (1955)*	Gautheret (1950)**	White
	Parts per million (or mg./l.)		
A. Macroelements			
$MgSO_4$	360.0	25.0	360.0
$Ca(NO_3)_2$	200.0	100.0	200.0
Na_2SO_4	200.0	.....	200.0
KNO_3	80.0	25.0	80.0
KCl	65.0	.....	65.0
NaH_2PO_4	165.0	.....	16.5
KH_2PO_4	.....	25.0	.....
B. Microelements			
$FeSO_4$	.....	5.0	2.5
$FeC_6H_5O_7$	10.0	.....	.....
H_2SO_4 (SG 1.83)	0.915	1.83	.....
$MnSO_4$	3.0	2.0	4.5
$ZnSO_4$	0.5	0.1	1.5
H_3BO_3	0.5	0.1	1.5
$CuSO_4$	0.025	0.05	.....
Na_2MoO_4	0.025	.....	.....
$CoCl_3$	0.025	0.05	.....
KI	.....	0.5	0.75
$Ti(SO_4)_3$	.....	0.2	.....
$NiSO_4$	.....	0.05	.....
$BeSO_4$	.....	0.05	.....
C. Amino acids, auxins, vitamins			
Glycine	15.0	.....	3.0
Cysteine-HCl	.....	10.0	.....
Thiamine-HCl	0.5	1.0	0.1
Niacin	2.5	.....	0.5
Pyridoxine	0.5	.....	0.1
Biotin	.....	0.1	.....
Inositol	.....	100.0	.....
I.A.A.	1.0	.....	.....
N.A.A.	.....	0.3	.....
Ca Pantothenate	0.5	0.1	.....
		Grams per liter	
D. Agar, sugars			
Sucrose	20.0	.....	20.0
Glucose	.....	30.0	.....
Agar	8.0	6.0	7.5

*LaRue (1955) composed of: White's (1943) modified stock solution; Nitch's (1951) modified trace elements; and White's (1943) modified vitamin solution.

**Gautheret (1950) composed of: Knop's (1884) modified salt solution; and Berthelot's (1934) modified trace elements.

APPENDIX TABLE 2. Components of three media for plant embryo cultures

Nutrient	Rijven (1952)*	Randolf and Cox (1943)**	Tukey (1934)***
	Parts per million (or mg./l.)		
A. <u>Macroelements</u>			
KCl	.....	65.0	680.0
$CaSO_4$	.....	.....	185.0
KNO_3	149.0	85.0	135.0
$Ca(NO_3)_2$	168.0	236.8	.....
$Ca_3(PO_4)_2$	.....	.....	185.0
$Fe_3(PO_4)_2$	.....	.....	185.0
KH_2PO_4	23.0	.....	.....
$MgSO_4$	101.0	36.0	185.0
B. <u>Microelements</u>			
$MnSO_4$	0.4	.....	.....
H_3BO_3	0.4	.....	.....
$ZnSO_4$	0.2	.....	.....
$CuSO_4$	0.1	.....	.....
$(NH_4)_2MoO_4$	0.05	.....	.....
$FeC_6H_5O_7$	50.0	.....	.....
$FeSO_4$	.....	2.0	.....
$Na(PO_3)_n$ Calgon	.....	10.0	.....
C. <u>Agar, sugars</u>		Grams per liter	
Sucrose	80.0	20.0	.....
Glucose	.....	.....	5.0
Agar	8.0	7.0	6.5

*Rijven (1952): <u>Capsella</u> embryos

**Randolf and Cox (1943): <u>Iris</u> embryos

***Tukey (1934): embryos of deciduous fruits

APPENDIX TABLE 3. LaRue's modification of White's Basic Medium, composed of: White's modified stock solution, Nitch's modified trace elements, ferric citrate, White's modified vitamin solution, indoleacetic acid, sucrose, agar, and triple distilled water (LaRue, 1955)

Nutrient ingredient	Stock concn.	-------- Medium concentration --------			
	ppm.	ppm.	mg./l.	gm./l.	gm./l
$MgSO_4$	3600	360.000	360.000	0.36	$36,000 \times 10^{-5}$
$Ca(NO_3)_2$	2000	200	200	0.2	$20,000 \times 10^{-5}$
Na_2SO_4	2000	200	200	0.2	$20,000 \times 10^{-5}$
KNO_3	800	80	80	0.08	$8,000 \times 10^{-5}$
KCl	650	65	65	0.065	$6,500 \times 10^{-5}$
$NaH_2PO_4 \cdot H_2O$	1650	165	165	0.165	$16,500 \times 10^{-5}$
H_2SO_4 SG 1.83	915	.915	.915	0.000915	91.5×10^{-5}
$MnSO_4 \cdot 4H_2O$	3000	3	3	0.003	300×10^{-5}
$ZnSO_4 \cdot 7H_2O$	500	0.5	0.5	0.0005	50×10^{-5}
H_3BO_3	500	0.5	0.5	0.0005	50×10^{-5}
$CuSO_4 \cdot 5H_2O$	25	0.025	0.025	0.000025	2.5×10^{-5}
$Na_2MoO_4 \cdot 2H_2O$	25	0.025	0.025	0.000025	2.5×10^{-5}
$CoCl_3$	25	0.025	0.025	0.000025	2.5×10^{-5}
Ferric citrate	2500	10	10	0.01	$1,000 \times 10^{-5}$
Glycine	1500	15	15	0.015	$1,500 \times 10^{-5}$
Niacin	250	2.5	2.5	0.0025	250×10^{-5}
Thiamin HCl	50	0.5	0.5	0.0005	50×10^{-5}
Pyridoxine	50	0.5	0.5	0.0005	50×10^{-5}
Ca Pantothenate	50	0.5	0.5	0.0005	50×10^{-5}
Indoleacetic acid		1	1	0.001	100×10^{-5}
Sucrose		20000	20000	20	
Agar		8000	8000	8	

Chromatographic Studies

The purpose of the exploratory investigation was to determine if there are quantitative and qualitative differences in sugars extractable by means of an 80% ethanol-reflux which might be useful in further characterizing the various stages of embryological development.

Eight hundred embryos were used for this study; 200 for each level of development. That portion of the caryopsis remaining after the embryo had been excised and samples of the shoot material remaining after the removal of the heads were also extracted and analyzed.

An ascending chromatographic technique was used for the separation of the various alcohol-soluble sugars. The reagents were: resolving solution - a freshly shaken mixture of 500 ml. of equal volumes of water and n-butanol to which is added 60 ml. of glacial acetic acid - using only the upper layer; and developing solution - 0.93 g. of aniline and 1.66 g. of pthalic acid in 100 ml. of water-saturated n-butanol. (See: R. J. Block, E. L. Durrum, and G. Zweig, 1955. A manual of paper chromatography and paper electrophoresis, Chapter VI, Carbohydrates. Academic Press. New York.)

The chromatograms were scanned photospectrometrically in a Spinco Model RB Analytrol. The 13 sugars reported on in

Table 2, were chromatographed and scanned for use as standards of comparison with the extracted material.

The quantitative results are recorded in Appendix Table 4. Glucose, fructose, sucrose and maltose were the only sugars sufficiently resolved to be definitely identified. They were present in the samples tested at all four levels of development.

There may very well be a significant relationship between the storage and availability of sugar and the level of development which is not readily apparent.

APPENDIX TABLE 4. Percent sugar (on a dry weight basis) at the various levels of development: extracted in an 80% ethanol-reflux; chromatographed; and evaluated photo-spectrometrically.

Level of development		-------Percent sugar-------		
		Embryo	*Fruit	**Shoot
I	Late Proembryo	83.7	8.1	1.7
II	Early Differentiation	55.3	5.1	2.0
III	Middle Differentiation	9.0	1.0	1.8
IV	Late Differentiation	4.5	0.64	1.3

*Fruit - that portion of the caryopsis remaining after the embryo has been excised

**Shoot - shoot material corresponding to the appropriate level of development