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THE CULTURE OF EXCISED OVULES,
ENDOSPERM AND EMBRYOS
OF CERTAIN GRASSES

by
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THE CULTURE OF EXCISED OVULES, ENDOSPERM
AND EMBRYOS OF CERTAIN GRASSES

by Knut J^{sen} Norstog

The study deals with various problems of grass embryogenesis in vitro. Attempts were made to obtain differential growth of embryonic structures and continued growth of cultured embryos beyond that which takes place in vivo. The possibility of culturing excised immature ovules of certain of the grasses also was investigated.

Part I. Various modifications of White's medium were used in cultures of excised embryos of oats, wheat, barley and rye. Cultures of mature embryos failed to produce the desired results except that in oats, extreme coleorhizal elongations were observed. It was determined that these elongations occurred as a result of lowered oxygen tension. Numerous cultures of immature embryos were made. Most of these were of uniform size (0.8 mm.) although some cultures were made using embryos of barley as small as 0.15 mm. It was found that a medium containing 90 per cent coconut milk allowed embryonic differentiation to continue in vitro. In no instance, however, did such growth exceed that occurring in vivo. An embryo 0.15 mm. in length when excised eventually grew into a well developed seedling on this medium. Other substances were also tried. Indole-3-acetic acid was ineffective. Yeast extract was not as good as coconut milk. Adenine sulfate produced coleorhizal elongations in oat and barley embryos, but did not noticeably affect the growth of other parts of the embryos. Other development on the part of embryonic tissues and organs are noted and discussed in this study.

Part II. Cultures of excised immature ovules of wheat, barley, rye and English rye grass (Lolium perenne L.) did not continue to grow in vitro except in two instances. Several ovules of barley grew for a time. The endosperm of rye grass continued to grow and was subcultured many times. This tissue was cultured on various media. It showed an increase in dry weight of 1678 per cent when cultured three weeks on White's agar containing 0.50 per cent yeast extract and 1.0 mg./l. Indole-3-acetic acid. Growth also was obtained on coconut milk media. The effects of temperature, light, dark, and pH on the growth of the tissue were determined. The most rapid growth occurred at 26⁰ C. and pH 7. There was no difference between the growth in light and in dark. The tissue continued to store starch in vitro. Chromosomal aberrations were frequent. The basic chromosome number of the cultured tissue was 21 (3n). Larger numbers were observed. Chromosome bridges also were frequent. These aberrations (hyperploidy and bridge) were associated with the formation of large starch storage cells. The tissue did not differentiate other than in chromosome configurations and starch storage. Tracheids did not occur nor did organization take place.

Attempts to culture embryos in contact with the endosperm tissue or on extracts of endosperm tissue failed to produce conclusive results.

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INTRODUCTION

An interesting aspect of plant tissue cultures is that tissues do not behave in culture as they do in the living plant. This characteristic may sometimes be turned to advantage, especially when it is desired to study the growth of simple tissues such as often occur in endosperm cultures, crown gall tissues, and other cultured callus types in which little if any organization or differentiation takes place. Anomalous growth may serve a useful purpose in that sometimes knowledge may be gained of the factors responsible for normal embryonic development.

Culture techniques have advanced to the stage where it is possible to grow a wide variety of plant tissues, organs and embryos, and this gives promise of important advances in the understanding of morphogenesis. The present study is centered about the problem of morphogenesis in the grass embryo. In the development of the plant embryo, final form may result from such influences as mechanical pressures exerted by the physical environment within the ovule, from the genic as well as cytoplasmic constitution of the zygote and its derivatives, from the physiological and biochemical factors present in the ovule, and from the environment of the entire plant. In the investigations to be described here, particular attention has been directed toward the following problems of embryogenesis: Can immature and relatively undifferentiated grass embryos be induced to continue their embryonic development in vitro? To what extent is the final

form of the embryo determined by mechanical pressures exerted by surrounding tissues? What are the effects of various biochemical substances on morphogenesis in the embryo? What are the influences of physiological gradients of substances essential to the normal embryo development? To what extent are embryonic structures plastic and amenable to changes in form within the restrictions imposed by the hereditary gene constitution?

Initial experiments consisted of attempts to extend the period of embryonic growth of naturally matured embryos through various culture techniques. With the exception of certain responses noted in developments of the coleorhiza and epiblast, experiments to obtain further increase in size in these embryos were not successful. Later experiments utilizing immature embryos were more successful in that continued differentiation and differential responses in the development of embryonic organs were realized.

A number of methods have been used in the latter studies, including the culture of excised grass ovules containing immature embryos. One of the surprising developments in the latter series of experiments has been the continued growth in culture of the endosperm of English Rye Grass, Lolium perenne. The tissue has been grown on various media and in several controlled environments. The resulting data have been incorporated into the body of the thesis, not only because of the novelty of the tissue culture, but also because the contrast between the growth of endosperm and embryo represents one of the principle problems in morpho-

genesis--that of organization and disorganization in tissues having an identical hereditary constitution. Immature embryos have also been cultured on masses of rye grass endosperm tissue and on extracts of the cultured endosperm.

Experimental morphology and tissue culture

In light of the complete reviews of the history of plant tissue culture given by Gautheret (1942) and White (1943), both of which are readily available, it seems necessary here only to call attention to those investigations which are pertinent to the present study, or which are so recent as not to be included in the reviews of these two authors.

The investigations of Haberlandt (1902), and his theory of cellular totipotency are intriguing to those who work in tissue culture. The original experiments in which he attempted to obtain growth and regeneration from single cells of green plant tissues, while failing to produce the desired results, may be considered as pioneer efforts in the field. White (1939), Gautheret (1939) and Nobecourt (1939), working independently, all achieved the unlimited growth in vitro of excised plant tissues. Since then a number of investigators have successfully isolated and cultured tissues from all of the major groups of vascular plants.

Wetmore and Morel (1951_a, 1951_b) have cultured tissues derived from the gametophytes of Lycopodium and Selaginella.

Fern tissue cultures have been obtained from gametophytes of Pteridium by Steeves and Sussex (1952) and from prothallia of Osmunda by Morel and Wetmore (1951_a). Neither of these

tissues was reported to differentiate to any extent other than the formation of tracheids.

LaRue (1954) cultured female gametophytes of the gymnosperms, Cycas and Zamia. He observed the regeneration of both shoots and roots in these tissues. Ball (1950) has cultured callus of Sequoia, and Tulecke (1952) obtained a tissue culture derived from the male gametophyte of Ginkgo. Loewenberg and Skoog (1952) describe methods for the culture of Pinus callus from seedlings grown on media containing malt extract.

Only a few tissues of monocotyledonous plants have been cultured. LaRue (1949) cultured the endosperm of Zea mays and obtained a tissue which is still growing after five years. Morel and Wetmore (1951_b) observed the regeneration of buds and roots in tissue cultures from tubers of an aroid, Amorphophallus. Recently, Archibald (1954) has reported the growth of stem callus of Cacao in culture. These three and a tissue derived from the endosperm of Lolium perenne in the course of the present investigation are the only tissue cultures of monocotyledons known to the writer.

A great many tissues of dicotyledonous origin have been successfully cultured. Most of these have been obtained either from stems, storage roots, or crown galls, as were those of White (1939), Gautheret (1939), Nobecourt (1939), and Henderson, Durrell and Bonner (1952). References will be made to these and others in succeeding pages where they are pertinent to the present study. The only endosperm of a dicotyledon to be cultured in vitro thus far is that of

Asimina (Lampton, 1953). A particularly significant recent advance is the successful culture of single isolated cells of marigold crown gall callus and normal tobacco stem callus in vitro (Muir, Hildebrandt and Riker, 1954). These cells have given rise to tissues.

A review of the history of embryo culture is given in the next section and will not be included here. Particularly significant to the present study are references by Wardlaw (1952) to research in tissue and embryo culture and his suggestions that these fields of investigation may render valuable assistance in the discovery of underlying factors responsible for the development of form in plants. His aim is to focus the attention of investigators in all fields on the problem of morphogenesis.

DEVELOPMENTAL MORPHOLOGY OF THE GRASS EMBRYO

In order to provide a background for data and discussions relating to the development of excised grass embryos in culture, there follows a brief outline of the developmental morphology of the grass embryo in vivo. This is based on the works of Kennedy (1897), Cannon (1900), Sargent and Arber (1915), Worsdell (1916), Souèges (1924), Avery (1930), McCall (1934), Arber (1934), Randolph (1936), Yung (1938), and Merry (1941).

Morphology

Most authors agree on features of embryogenesis in the grasses. However, many of them disagree about the homologies

of certain structures peculiar to grass embryos.

In morphology and anatomy the embryos of oats, barley, wheat and rye are very similar, differing only in minor aspects. A generalized diagram of these embryos is shown in plate I, fig. 1. The embryo may be considered as an axis consisting of a stem apex separated from a primary root and two or more lateral roots by a region of vascular transition, the scutellar plate. The roots are enclosed in a sheath of several cell layers, the coleorhiza. The shoot is similarly enclosed by the coleoptile. Subtending the coleoptile in oat, wheat and rye embryos are two lateral structures, the larger of which is the scutellum (cotyledon). The smaller structure is the epiblast. In the mature barley embryo the epiblast is absent, but it is sometimes present in immature embryos.

From the scutellar plate, which is a region of vascular transition between the root and the shoot primordia of the embryonic axis, vascular traces extend down into the root primordia and upwards into the coleoptile and first and second leaf primordia. A vascular bundle also extends into the scutellum after making a slight loop as shown in plate I, fig. 1.

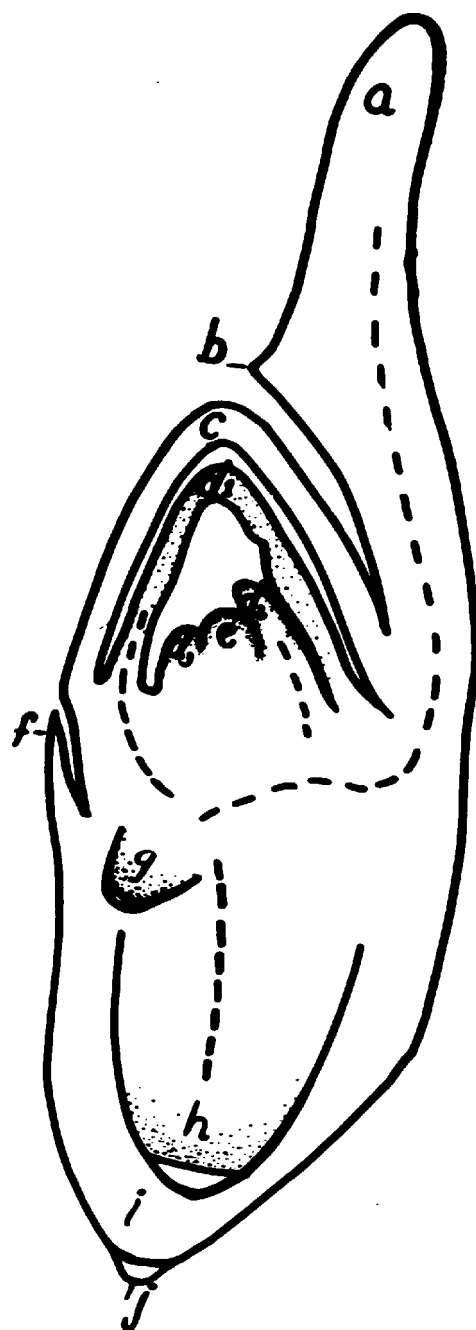
Avery (1930) reports, in a general review of the literature dealing with the comparative morphology of grass embryos, that the scutellum is variously considered to be (a) the lateral limb of the cotyledon of which the coleoptile is the ligule, (b) the single cotyledon of the grass embryo, or (c) the first cotyledon, when the epiblast is considered

to be the second cotyledon. The coleoptile has been considered to be (a) the true cotyledon, (b) the second foliage leaf of the plant with the scutellum as the first, (c) the third foliage leaf when the scutellum is thought to be the second and the epiblast the first, or (d) the ligule of the cotyledon. The epiblast has been said to represent (a) the second cotyledon, (b) an appendage of the scutellum, (c) an outgrowth from the coleorhiza, and (d) the first leaf of the axis.

The coleorhiza is generally considered to be a downward growth from the scutellar node without phylogenetic significance. However Paskov (1951) believes that the coleorhiza is the main axial root of the embryo which has lost most of its characteristics except the ability to form root hairs. He regards the functional embryonic roots as lateral roots, one of which has taken over the function of the primary root. There are objections to this theory, however, since the epiblast is a tubular structure without a terminal meristem or any trace of vascularization.

A number of observations support the view that the coleoptile and the scutellum are separate and distinct structures and are the first and second leaves of the axis, or at least two of the first three leaves. Kennedy (1897), Avery (1930), and Yung (1938) all report the presence of buds in the axils of coleoptiles of various species of grass. Arber (1934) mentions that the scutellum of Spartina townsendii reportedly is green and contains chloroplasts. Reeder (1953) found that in the embryo of Streptochaeta, a

PLATE I



- a. scutellum
- b. ventral scale, or
ligule of scutellum
- c. coleoptile
- d₁. first leaf primordium
- d₂. second " "
- d₃. third " "
- e. apical meristem
- f. epiblast
- g. lateral root meristem
- h. primary root meristem
- i. coleorhiza
- j. suspensor

Figure 1. Diagram of mature grass embryo, sagittal section.
The dotted lines indicate vascular traces.

primitive grass, the coleoptile is an open, sheathing structure having a midrib as well as lateral bundles, instead of the tubular form with only two lateral bundles, common to other grass embryos.

Embryogenesis

The following description of embryogenesis is taken from Merry (1941) who has given a complete account for the embryo of barley. Cannon (1900), Avery (1930), Souéges (1924), McCall (1934), and Randolph (1936) give similar descriptions for other grasses.

In barley the first divisions of the zygote occur within two days after fertilization, and after a few days a club-shaped proembryo has been formed. Seven days after fertilization the proembryo has begun to differentiate and has grown to a length of between 0.2 and 0.3 mm. It now consists of a proximal stalk-like region, the suspensor, which holds the embryo fast to the nucellus, and of a broader, distal portion, the dorsal surface of which has become slightly flattened. According to Cannon (1901) the suspensor later becomes detached and the embryo is no longer joined by connective tissue to any part of the ovule.

The first signs of lateral differentiation in the embryonic axis are seen in the flattening of the distal (chalazal) end of the embryo to form the scutellum and in the appearance of a protuberance, the shoot primordium, which is partly encircled by a crescent-shaped ridge, the coleoptile. This occurs at eight days after fertilization, when the

embryo is about 0.5 mm. in length.

At ten days (about 0.6 mm.) a clearly recognizable grass embryo has differentiated. It has a well-defined scutellum, and a stem apex, which bears the primordium of the first foliage leaf and is completely encircled (but not enclosed) by the coleoptile. The coleorhiza, or root sheath, encloses the newly formed primary root. The suspensor, the cells of which have ceased to divide, apparently is non-functional at this stage.

When the embryo is twelve days old and about 1.1 mm. in length, the first pair of lateral roots make their appearance. At this time the coleoptile is no longer a circular ridge. Except for a small pore at its apex, it completely encloses the shoot primordia. Two other pairs of lateral roots are produced at approximately three day intervals. The second leaf appears at 13 days, the third at 16 days. After 28 days the embryo is fully developed, having three leaves, three pairs of lateral roots, and a scutellum which has elongated, broadened and developed a palisade-like layer of epidermal cells on the surface in contact with the endosperm. A ridge on the face of the scutellum, termed the "ventral scale", forms a semicircle about the free end of the coleoptile. Only a vestige of the suspensor remains attached to the distal end of the coleorhiza of the mature embryo.

The epiblast is not normally present in barley embryos, but in oats, wheat and rye it makes its appearance at about the same time as the first lateral roots, when the embryo is

half grown. Eventually it attains a length of about 0.2 mm. in oats and 0.1 mm. in wheat and rye.

Most of the immature embryos cultured in the following experiments corresponded to the 11 day old embryos figured by Merry. They measured 0.8 mm. in length and contained single leaf and root primordia. However, embryos of barley as small as 0.16 mm. grew in culture and developed leaves and roots.

PART I

THE CULTURE OF GRASS EMBRYOS

Introduction

Most investigations in embryo culture are concerned either with the nutritional aspects of embryo growth in vitro or with the production of seedling plants from excised, hybrid embryos which for one reason or another fail to mature in the seed. Comparatively few are centered on the problem of embryogenesis.

Hännig (1904) was the first to obtain a measure of success with cultures of immature embryos in synthetic media. Although he failed to obtain growth of cruciferous embryos in embryo sac liquid, he succeeded in growing excised embryos to mature plants in media containing minerals and cane sugar. He made several interesting observations on embryogenesis in vitro, the most remarkable of which was that immature embryos do not continue to grow as embryos in culture but develop immediately into small seedling plants.

The earliest successful culture of excised grass embryos was made by Stingl (1907), who succeeded in obtaining germination of embryos of several cereals by making reciprocal transplants of mature embryos and endosperms. Thus he showed the embryos of different species of grasses have common nutritional requirements. Similar studies were made by Buckner and Kastle (1917) and Andronescu (1919). The former grew excised, mature embryos of Lima Bean on nutrient agar after removal of the cotyledons, and obtained normal seedlings. Andronescu cultured excised maize embryos on nutrient media

and also obtained normal seedlings. Both of these studies were important in that they showed the embryo to be capable of normal growth independently of the natural food reserves. However they give very little information on embryogenesis, since only mature embryos were cultured. Knudson's (1922) classical study on the culture of orchid seeds in the absence of certain micro-organisms thought to be essential for the germination and early growth of orchid embryos also belongs in this category, since the problem was primarily nutritional.

The first, and still one of the few investigations to be focused directly on problems of experimental morphogenesis (using the techniques of embryo culture) is Dietrich's (1924). He, in a sense, took up Hännig's work and amplified it, using a larger number of species and simpler media. He found that the form of the embryo in the seed is determined only superficially by the spatial factors in the ovule; that embryos cultured on agar media germinated immediately to produce small seedlings, but that this premature germination could be suppressed through subsurface culture. Dietrich's work, though of considerable interest in the manner of approach, was greatly hampered by the lack of a suitable medium. He found, for instance, that he could not grow embryos smaller than one third of their mature size. Embryos at that stage are usually quite well differentiated and thus do not permit the study of the early stages of embryogenesis.

Laibach (1929) first obtained seedlings from certain hybrid embryos which normally die in the seed. He cultured differentiated, though somewhat anomalous, embryos of flax

on bits of cotton moistened with a sucrose solution, and eventually obtained seedling plants.

LaRue (1936) grew immature, excised embryos of a number of plants in order to determine the minimum size which could be cultured, and also to investigate the effects of such culture on the developmental morphology of the embryos. He was particularly successful in culturing excised embryos of a smaller size than any previously grown, and also in observing that cultured embryos often grow in such a way as to suggest atavistic behavior. LaRue and Avery (1938) compared the development of excised, immature embryos of Zizania in vitro with their normal development in vivo. Similar studies were made by Merry (1942) using barley embryos with much the same results, since these investigations showed that excised embryos undergo what the former authors term "precocious germination" in vitro and do not continue to develop as normal embryos.

Tukey (1933, 1934, 1938) refined the methods of hybrid embryo culture to the point that hybrid embryos, which normally abort, could readily be grown into seedling stages in vitro. He, too, found that immature embryos did not continue to differentiate as embryos in culture but developed as seedlings if they grew at all.

The first real success in attempts to grow immature embryos and to obtain their continued development as embryos was that of van Overbeek, Conklin and Blakeslee (1941, 1942) who obtained the growth and continued differentiation of very small embryos of Datura in media containing coconut milk.

They ascribed their success to a substance or combination of substances present in coconut milk which they termed "embryo factor." Subsequently a number of studies by these and other investigators have been conducted using Datura embryos and a variety of accessory substances reputed to contain the "embryo factor".

In the years following these advances, excised embryos of many other species have been cultured. In the gymnosperms, embryos of Pinus and Keteleeria were cultured by Loo and Wang (1943) and those of Larix by Sterling (1949). Excised embryos of Gingko had been cultured at an earlier date by Radforth (1937). These cultures all were made with pro-embryos. In all three studies the embryos were reported to have grown for a time, forming undifferentiated masses of cells. The cultures, however, failed to produce either normal embryos or tissues capable of being maintained in vitro. Cultures of excised embryos of angiosperms have been more successful.

The mature embryos of Solanum were grown in vitro by Hall (1943). Randolph and Cox (1943) were able to obtain germination of mature, excised embryos of Iris in culture, thus eliminating a prolonged period of dormancy that occurs in naturally developing seeds. Smith (1944) cultured excised embryos of a hybrid of Lycopersicon. Lofland (1950) obtained seedlings from cultures of differentiated embryos of Gossypium but was not able to grow smaller embryos of this plant. Lee (1952) grew immature, excised embryos of Carex and found that the seedlings produced in vitro were smaller

and weaker than those growing naturally.

Other studies in vitro have been directed at the problem of discovering the factors responsible for normal embryogenesis. Rijven (1952) investigated some of the nutritional and environmental requirements of very small embryos of Capsella. He makes some pertinent observations in connection with differences between embryonic growth in vitro and the premature germination of excised embryos so often observed in culture. These are discussed in another section of this report. An aspect of the differentiation of embryos was reported by Curtis (1947) who found that the addition of barbiturates to the media used in cultures of orchid embryos resulted in the production of undifferentiated masses of tissue rather than seedling plants.

Particularly pertinent to the present investigation are a number of studies dealing with embryo culture in the grasses. These are in addition to those previously cited. Brink, Cooper and Ausherman (1944) grew a hybrid embryo, the product of a cross between a wild species of barley and domestic rye, into a mature plant using embryo culture methods. Haagen-Smit, Siu and Wilson (1945) attempted the culture of immature, excised embryos of maize but failed to obtain growth of very young embryos even though they used coconut milk as a source of "embryo factor".

Kent and Brink (1947) cultured immature excised barley embryos using tomato juice and other substances, and obtained continued embryonic development. Later Ziebur (Nancy Kent Ziebur), Brink, Graf and Stahman (1950), Ziebur and Brink (1951),

obtained much the same results using casein hydrolysate and other substances in a continuation of the earlier investigations. They were particularly interested in discovering the nature of the "embryo factor" reported to be present in such natural substances as coconut milk, malt extract, casein hydrolysate, and excised barley endosperm, and in determining the extent to which relatively undifferentiated embryos might be induced to prolong their embryonic growth and differentiation.

Konzak, Randolph and Jensen (1951) obtained seedlings from excised embryos of hybrids between common barley and a wild, perennial barley.

Pieczur (1952) found that immature embryos of maize could be cultured more effectively when masses of cultured tissue derived from maize endosperm were included in the culture tubes. This last study is perhaps the first instance of an attempt to produce an "artificial seed" in vitro using a cultured rather than an excised endosperm.

The knowledge of the plant embryo in vitro which has accumulated over the years appears to be very substantial, yet thus far no one has been able to reproduce the normal development of the embryo. The present status of embryo culture is reviewed by Rappaport (1954). His discussion is especially directed toward research in the physiology of the embryo, but also includes important contributions to the study of embryo morphology.

In the present investigations, as previously stated, excised embryos of oats, barley, wheat and rye have been

cultured in order to study the morphogenetic effects of various factors in vitro.

Material and Methods

The original supply of seeds used in the following experiments was obtained from the North Dakota State Agricultural Experiment Station at Fargo, North Dakota. The species represented were Avena sativa variety Andrew, Hordeum sativum variety Moore, Secale cereale variety Dakold, and Triticum vulgare variety Mida.

In preparing cultures of mature embryos, the seeds were sterilized in a solution of 20 per cent Clorox and water for 20 minutes and rinsed in sterile distilled water. Then they either were transferred directly into culture tubes or petri dishes, or were opened aseptically in order that the embryos might be removed and cultured.

A number of excised, mature embryos was grown on the media listed in table II. Many more were cultured intact in seeds which were immersed in sterile water with and without added substances. Others were placed on filter paper moistened with water or a solution of water and IAA. In one series of experiments the effects of various levels of oxygen on the growth of coleorhizae and epiblasts of mature oat embryos were investigated. Oat seeds were placed on moist filter paper in bottles from which the oxygen had been removed with pyrogallol and in the apparatus shown in plate II which gave different mixtures of air and nitrogen.

In another experiment a few coleorhizae of oats were

removed from mature embryos and cultured separately. Some were slit open and flattened out on nutrient agar with either their inner or outer surfaces exposed to the atmosphere. Isolated cells from the coleorrhizae of wheat embryos were also cultured on nutrient agar.

All dissections, whether of mature or immature seeds, were made under a dissecting microscope at a magnification of 17 diameters. The sterilized seeds were placed on a sterile microscope slide and the embryos were removed by means of sterile dissecting needles. The lengths of immature embryos were measured with an ocular micrometer.

Seeds containing immature embryos were obtained from outdoor and greenhouse plantings of the cereal varieties previously listed. Oats, wheat and barley were sowed at weekly or biweekly intervals in order to insure a constant supply of young embryos. Since Dakold Rye is a biennial winter variety, only one crop, harvested in the spring, was grown. Collections of immature seeds of oats, wheat and barley were made more or less regularly from July, 1953 to August 1954, but rye embryos were available only for about a week during the spring of 1954.

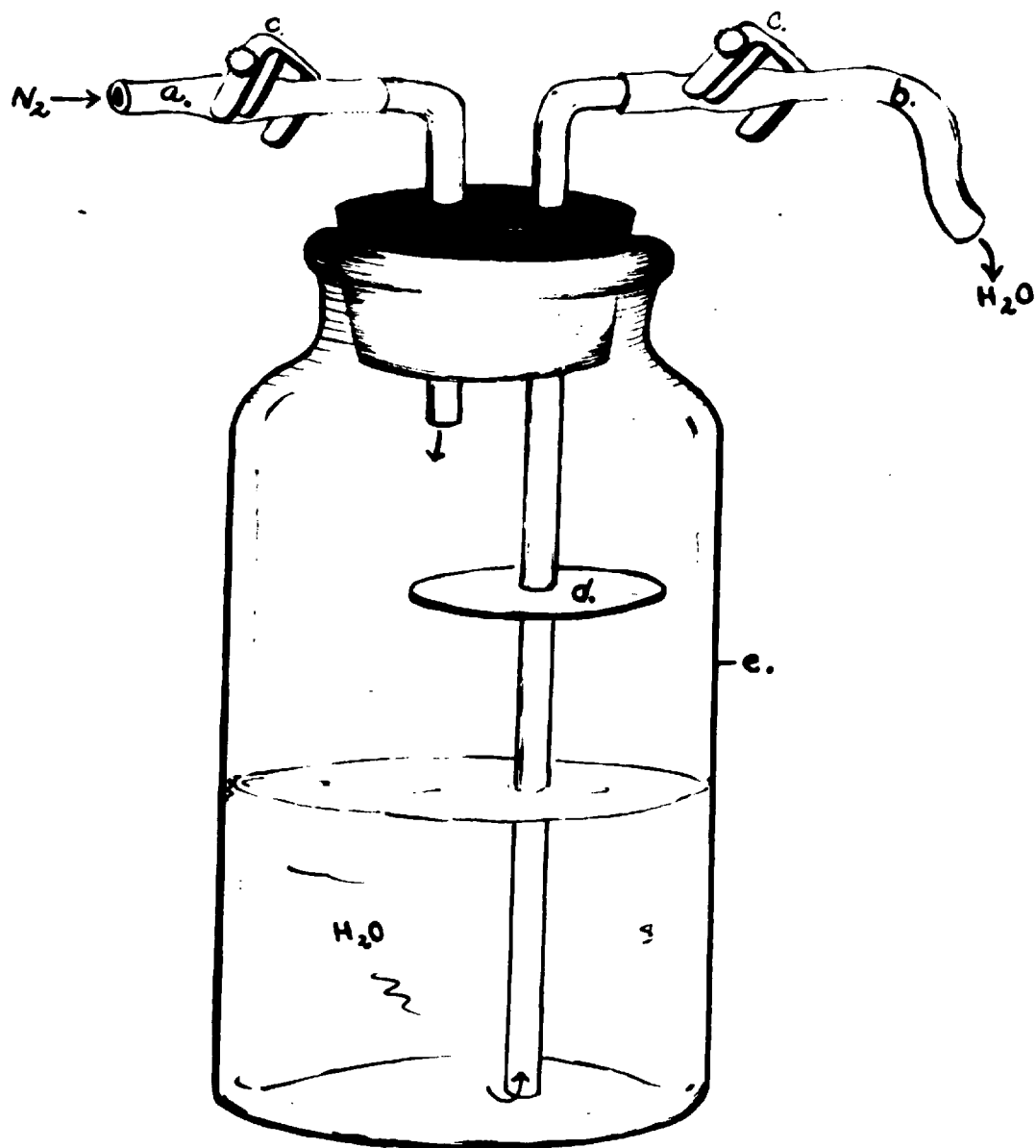
The unripened inflorescences were removed from the plants when examinations of one or two spikelets in each indicated that the embryos within were about the right size for excision and culture. Preliminary cultures had shown that embryos of 0.8 mm. total length were the smallest that could be cultured successfully on all of the media listed in table II, and that the embryos of all the four cereals were

PLATE II

Figure 1. Apparatus used in germination of oat seeds in various dilutions of air.

Nitrogen gas, N_2 , from a pressure cylinder connected to tube (a) was used to force water from the bottle (e) through tube (b) after which the two screwclamps (c) were closed to seal the bottle. Sterilized seeds had previously been placed on a platform (d) covered with wet filter paper. For a 50 per cent dilution of air, 250 cc. of water were placed in the 500 cc. bottle and then after the seeds were in place the water was forced out with nitrogen gas. The dilutions used were only approximate since allowances were not made for the displacement of the tubes, platform and the seeds in the bottle.

PLATE II



at about the same stage of development at that size and were not so well differentiated as to preclude further development and differentiation in culture. With the exception of still smaller barley embryos which were cultured in order to discover the size limitations on embryo growth in vitro, all of the embryos used were 0.8 mm. in length.

The immature seeds were sterilized in a 5 per cent Clorox solution for five minutes, the maximum time and concentration which could be used without injury to the young material. Following sterilization, the seeds were rinsed in sterile distilled water and the embryos excised as previously described.

A uniform series of replicates was not used in these experiments since the supply of embryos at a particular time could not be anticipated. Embryos of the proper size were placed in culture as they were obtained. An attempt was made to secure about 30 embryos of each species for culture on each of the media used. Nearly every set of data for a particular medium and species contains measurements recorded from several culture periods.

Cultures of mature embryos were kept both in the light and in the dark at about 26° C. for various lengths of time. Cultures of 0.8 mm. embryos were kept in darkness at room temperatures (about 26° C.) and after 7 days were removed from the media and measured. At this time, in addition to total length, the lengths of the coleorhiza, suspensor, coleoptile, epiblast (when present), scutellum, roots (when present) and the width of the scutellum were measured by

means of the ocular micrometer and recorded. Also recorded were observations of the presence of coleorhizal hairs, general appearance of the embryo and its parts, and particularly any peculiarities in form which differed from the usual. At the end of each experiment the data were assembled and averages were calculated for all of the measurements. Following the measurements, camera lucida drawings were made in some instances and the embryos were placed in a formalin, acetic acid, ethyl alcohol formula (FAA) or in a weak chromic acid, acetic acid, formalin solution (CRAF) for preservation and later reference. Subsequently, embryos of the size at excision and from mature seeds as well as those representing certain types of growth in vitro, were removed from the killing and fixing solutions, dehydrated, and imbedded in paraffin by means of the ethyl, tertiary butyl alcohol series of Johansen (1940). The embryos were sectioned at 12 microns by rotary microtome, mounted serially on slides, and stained with safranin and fast green.

Because this study has been chiefly concerned with embryonic growth, no special attention has been given to prolonged growth of excised embryos beyond the 7-day period. Moreover, the many measurements taken of embryos at the end of culture periods precluded the maintenance of asepsis in the cultures. For these reasons, relatively few data were obtained with regard to the later, seedling growth of excised embryos.

Media: Throughout these experiments the basal medium has been a modified White's solution. The changes in the

original formula were the substitution of Nitsch's (1952) trace elements for those of White (1943), of ferric citrate for ferric sulfate, and doubling of the concentrations of accessory substances. When effects other than those given by the basal medium were desired, other substances were added to it. As a result, a number of media were used which differed from the basal solution in that they contained one or two and at most three additional components. These media are listed in tables I and II.

The media were prepared by placing the aqueous solution of minerals, vitamins, sucrose (and indole-3-acetic acid, adenine sulfate, agar, when used) in a leveling bulb equipped with a pinch clamp and a cotton plug. The bulb and its contents were then sterilized by autoclaving. The media were tubed while hot in 10 ml. aliquots poured into each of the 20 ml. screw cap vials used as culture tubes. Yeast extract and coconut milk, if included, were sterilized by Seitz filtration and added to the autoclaved media prior to tubing. The pH of the basal medium and those containing indole-3-acetic acid or adenine sulfate was adjusted to about 5.6 using HCl and KOH to raise or lower the pH to the desired level. The pH of media containing coconut milk or yeast extract was slightly higher, about 6.1, owing to the natural buffering effect of these substances.

Definitions

To avoid misunderstanding in the use of several terms, the following definitions are included.

TABLE I
Components of the Basal Medium

Ingredients	mg./l.
major elements	
MgSO ₄ . 7H ₂ O	360.0
Ca(NO ₃) ₂ . 4H ₂ O	200.0
Na ₂ SO ₄	200.0
KNO ₃	80.0
KCl	65.0
NaH ₂ PO ₄ . H ₂ O	16.5
FeC ₆ H ₅ O ₇ . 5H ₂ O	10.0
Nitsch's trace elements	
MnSO ₄ . 4H ₂ O	3.0
ZnSO ₄ . 7H ₂ O	0.5
H ₃ BO ₃	0.5
CuSO ₄ . 5H ₂ O	0.025
Na ₂ MoO ₄ . 2H ₂ O	0.025
CoCl ₂ . 6H ₂ O	0.025
H ₂ SO ₄ sp. gr. 1.83	(0.0005 ml.)
accessory substances	
Glycine	7.5
Niacin	1.25
Thiamine HCl	0.25
Pyridoxine	0.25
Ca pantothenate	0.25
Sucrose	(20.0 gm.)
H ₂ O	1 liter
Bacto agar*	(7.5 gm.)

* not used in all media

TABLE II

Media Used in the Culture of Excised Embryos

Medium*	Components				
A	modified White's solution (with or without agar)				
B	A plus indole-3-acetic acid 0.1 mg./l.				
C	A	"	"	1.0	"
D	A	"	adenine sulfate	20.0	"
E	A	"	"	40.0	"
F	A	"	"	80.0	"
G	H	"	"	40.0	"
H	A	"	20 per cent coconut milk by volume		
I	A	"	90	"	"
J	G	"	indole-3-acetic acid 0.1 mg./l.		
K	C	"	0.25 per cent yeast extract by weight		

* Medium A henceforth will be referred to in the text as White's medium. Media, B - K, will be referred to in terms of the additional substances they contain. Indole-3-acetic acid will be abbreviated to IAA. The letter symbols will, however, be used in subsequent tables.

References will be made from time to time to the seedling growth or germination of excised embryos in culture as opposed to their embryonic growth. As has been stated, immature embryos in culture usually do not continue to exhibit meristematic growth in those organs which are, strictly speaking, specific parts of the embryo of determinate growth such as the scutellum, epiblast, coleoptile, suspensor and coleorhiza, but instead grow as in normal germination through the enlargement of existing cells and the meristematic activity of the stem and root meristems. This distinction is made very clearly by Rijven (1952) who says: "Germination is defined here to start at the moment that the embryonic tissue--in a state of cell division and plasmatic growth--becomes separated by an intercalated section of incipient cell elongation. The appearance of cell elongation marks off a new phase of the life cycle, as it affects the nature of growth and structure essentially. So we emphasize the necessity of distinguishing between pregerminal and post-germinal cultures, both being quite different objects of investigation."

When the growth of the cultured embryos resulted wholly or in part from the meristematic activity in any or all of the embryonic organs, this behavior will be termed embryonic growth, development, or differentiation. The growth of immature embryos in culture consisting largely of activities of apical and root meristem and cellular enlargement, will be referred to as seedling growth, germination, or more properly, premature germination.

CULTURES OF MATURE EMBRYOS

Results

In cultures of mature embryos on nutrient agar the results were invariably the same. Within a few days mature, excised embryos developed into seedlings of normal appearance. In none of the cultures did any of the embryonic structures exhibit signs of renewed meristematic activity. The coleorhizae and epiblasts in all of the mature embryos cultured on agar and in seeds germinated on moist filter paper enlarged to some extent during the first 24 hours but did not undergo further growth in length thereafter. Within a short time the roots broke through the coleorhizae, tearing them apart more or less completely. However, in the first 24 hours it was observed that hairs grew out from the epidermal cells of the coleorhizae of wheat, oats and rye. Those of oats are illustrated by fig. 5, plate III. These hairs resembled root hairs in every way and were a constant feature of the embryos germinated in moist air. They were never observed in cultures in liquid media. Instead, when excised embryos were cultured in nutrient liquids the coleorhizae and epiblasts grew to a much greater extent than those grown on nutrient agar or on filter paper.

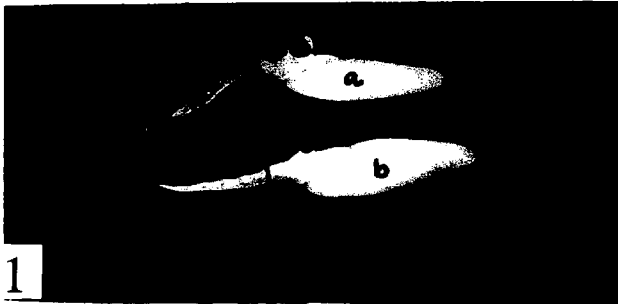
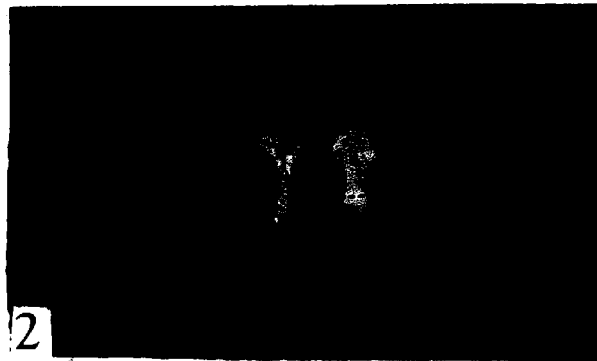
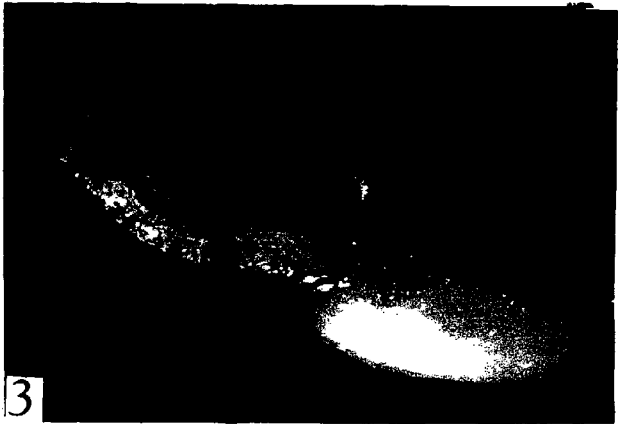
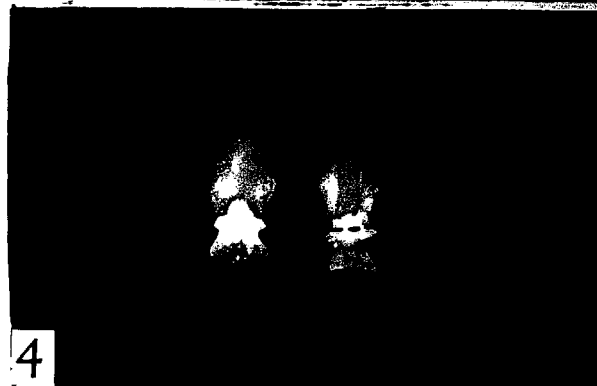
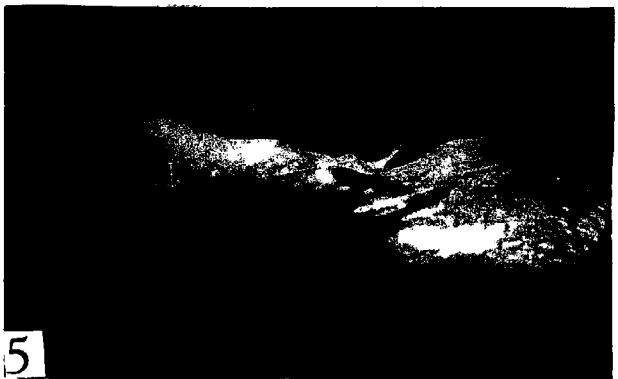
As long as the embryos remained immersed in the solutions, germination was inhibited, but as the coleorhizae enlarged, gas collected within and the embryos floated to the surface and germinated. Attempts to fasten embryos to the bottom of culture tubes were abandoned in favor of culturing intact seeds which sank in the solution and remained on the

PLATE III

Cultures of Mature Embryos.

- Figure 1. Coleorhiza and epiblast growth in seeds of oats (a) cultured in solution of H_2O and 0.1 mg./l. IAA and (b) in H_2O alone. Dotted lines indicate the approximate length attained by coleorhizae of seeds germinated in air.
- Figure 2. Coleorhizae of rye seeds after five days culture in sterile water. Dotted line shows upper limit of the coleorhiza.
- Figure 3. Oat seed after five days culture in sterile water. Arrow shows the epiblast. a - coleorhiza. c - coleoptile.
- Figure 4. Coleorhizae of wheat after five days culture in sterile water. Dotted line shows upper limit of coleorhiza.
- Figure 5. Hairs of coleorhiza and epiblast of oat seed germinated on moist filter paper after soaking in solution of H_2O and 10.0 mg./l. IAA.
- Figure 6. Coleorhizae of barley seeds after five days culture in sterile water. Dotted line shows upper limit of coleorhiza.

PLATE III



bottom of the tube. Later it was found that the same developments of coleorhizae and epiblasts were produced in simpler solutions (table III) and the elaborate nutrient solutions were dispensed with.

While germination of all embryos cultured in liquid was inhibited, that of oats and barley seemed to be less than wheat and rye. Oat and barley embryos would sometimes grow out of the solutions and form seedling plants.

Barley, wheat and rye. The coleorhizae of wheat, barley and rye all grew to about the same extent in liquid culture although their appearance differed (plate III, figs. 2, 3 and 4). Those of wheat were spatulate, while those of rye and barley were awl-shaped. The epiblasts of wheat and rye doubled in size after five days of culture but grew no more after that. Barley embryos have no epiblasts and none developed in this series of cultures. The coleorhizae of all three species attained an average length of about 2.5 mm. after five days of culture. There was no further elongation after that time.

After a week or so in culture, cells near the tips of the coleorhizae began to separate from one another. This was most apparent in wheat. Eventually the coleorhizae disintegrated into flocculent masses of cells. Some of these cells are shown in plate III, figs. 1 and 2. They remained alive in nutrient liquids and in a solution of sterile water and sucrose (4 per cent), but were never observed to divide or increase in number even when removed and placed on nutrient agar. Some of these cells appeared to have developed

PLATE IV

Cultures of Mature Embryos.

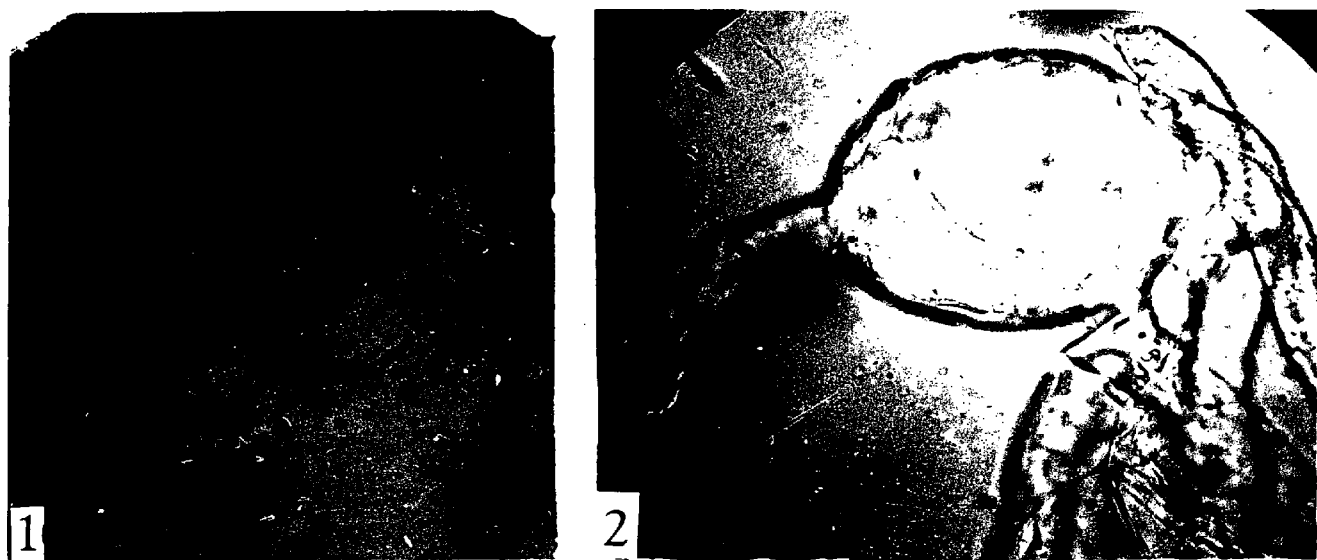
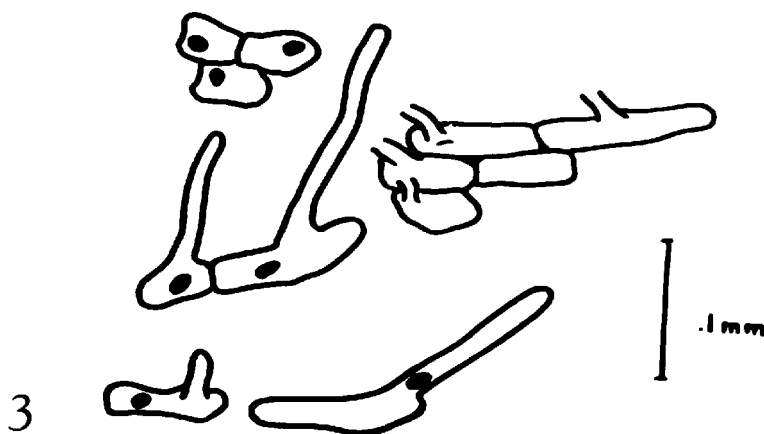


Figure 1. Isolated cells from the coleorhiza of a wheat embryo cultured in nutrient liquid.

Figure 2. Isolated coleorhizal cell of a wheat embryo cultured in nutrient liquid. Two root hair-like outgrowths are visible.

Figure 3. Camera lucida drawings of coleorhizal hairs of oats after 24 hours culture in moist air.

finger-like projections somewhat resembling root hairs except that often more than one outgrowth appeared on a single cell.

Oats. Mature embryos of oats when cultured in nutrient solutions gave rise to much longer coleorhizae and epiblasts than wheat barley and rye. Oat coleorhizae did not disintegrate as did those of the other three species. Because of the relatively great length attained by the oat coleorhizae, they were used exclusively in further experiments. The results of these are summarized in tables III, IV, V, and VI. Data taken from tables III and V were used to prepare plate V.

TABLE III

Coleorhizal Elongations in Embryos of Oats Cultured in Liquid Media for Five Days, in Darkness, at Room Temperature.

Medium	No. of embryos	Average coleorhizal length		Increase
		<u>initial</u>	<u>final</u>	
H ₂ O	48	0.8 mm.	6.2 (8)mm.	675%
H ₂ O+0.4 Ad.*	15	0.8	6.3 (8)	687%
H ₂ O+0.1 IAA**	29	0.8	7.2 (10)	800%
H ₂ O+1.0 IAA	29	0.8	7.1 (9)	787%
H ₂ O+5.0 IAA	32	0.8	5.7 (8)	612%
H ₂ O+10.0 IAA	33	0.8	5.5 (8)	587%

* Milligrams per liter of adenine sulfate

** Milligrams per liter of indole-3-acetic acid

Note: Figures in parenthesis in tables III - VI

denote the maximum length attained by coleorhizae in each set of cultures. The initial lengths in these tables are averages obtained from both fresh, excised embryos and fixed and sectioned embryos.

The greatest increases in coleorhizal length were noted in cultures containing lower concentrations of IAA. The greatest increase was obtained with 0.1 mg./l. of IAA. Higher levels of IAA produced smaller increases in length.

Adenine sulfate markedly stimulated coleorhizal elongation in some cultures of immature embryos. This is reported in a later section. However, it was of little benefit in liquid culture of mature embryos and cultures on agar media E (40 mg./l. adenine) and F (80 mg./l. adenine) as well as a few at higher concentrations (400 mg./l.) also produced no elongations.

TABLE IV

Effect of pH on Coleorhizal Elongation in Mature Embryos of Oats Cultured for Five Days in Darkness at Room Temperature.

Medium	No. of embryos	Average coleorhizal length		Increase
		<u>initial</u>	<u>final</u>	
H ₂ O pH 7	25	0.8 mm.	6.4 (8)mm.	700%
H ₂ O pH 6*	25	0.8	6.2 (8)	675%
H ₂ O pH 5*	19	0.8	6.6 (8)	725%
H ₂ O pH 4*	26	0.8	6.0 (7)	650%

* Adjusted with HCl and KOH.

Coleorhizal elongation was not greatly influenced by pH within the ranges from 4 to 7. A slight increase at pH 5 was observed but the maximum lengths attained at pH 5, 6 and 7 were the same (8.0 mm.). A slightly smaller average length was noted in cultures at pH 4.

TABLE V

The Growth of Coleorhizae of Mature Oat Embryos in Moist Air and at Various Dilutions of Air with Nitrogen. Cultured on Filter Paper, in Light at Room Temperature.

Medium	No.	Culture period	Average coleorhizal length		Increase
			initial	final	
Air 100%	20	48 hrs.	0.8 mm.	2.6 (3.2)mm.	225%
Air 100%	21.	48	0.8	2.5 (3.2)	212%
+ IAA*					
Air 50%	16	48	0.8	3.2 (3.8)	300%
Air 20%	20	72	0.8	3.7 (4.0)	362%
Air 10%	20	72	0.8	5.0 (5.6)	525%

* Soaked overnight in a solution of water and 10.0 milligrams per liter of IAA prior to culture.

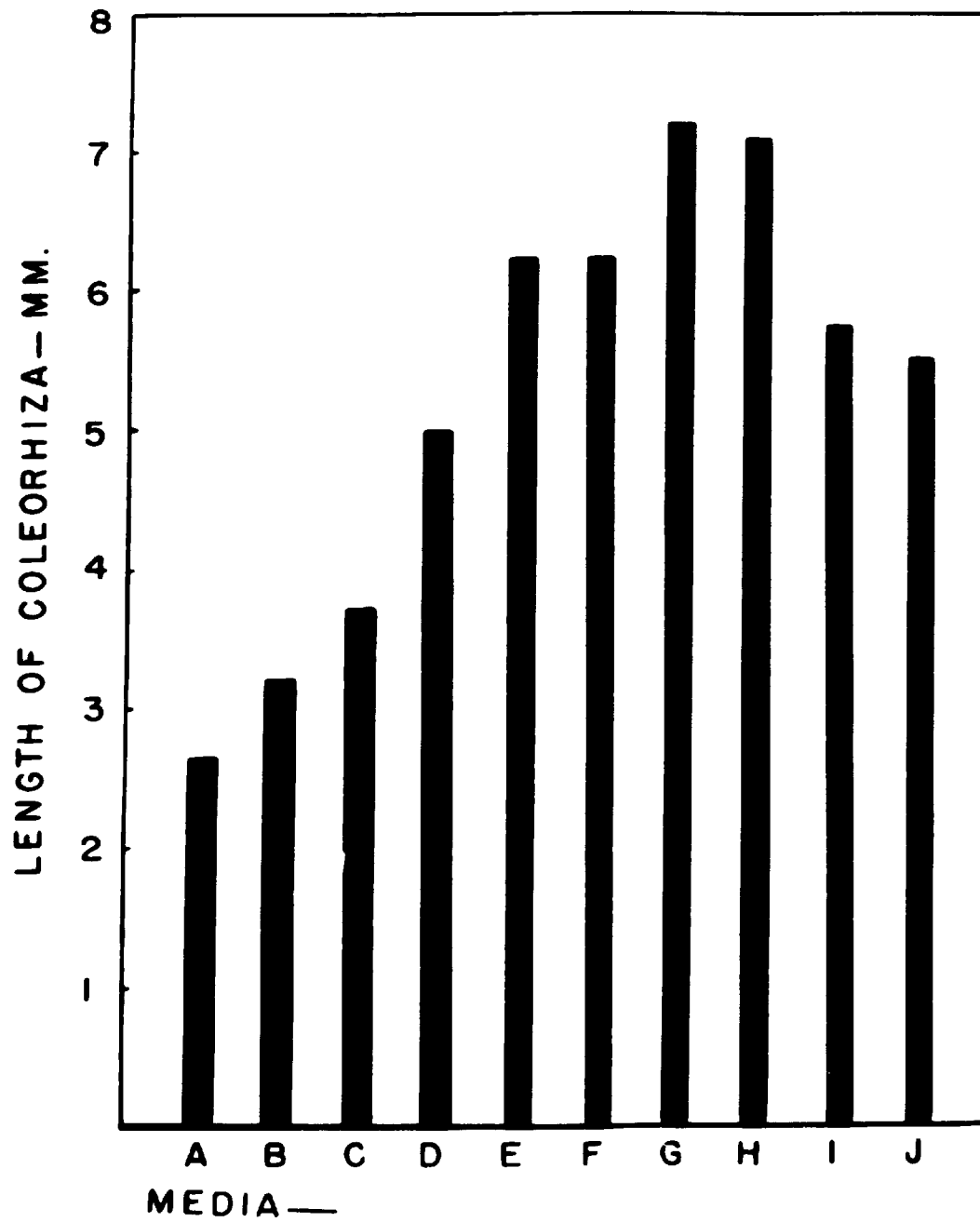
Additional seeds of oats were placed on moist filter paper in a bottle from which the oxygen had been removed with pyrogallol. Germination of these seeds was completely inhibited. Little if any coleorhizal elongation took place but since measurements were not taken the data were not judged to be conclusive enough to be included in table V.

PLATE V

Figure 1. The growth of coleorhizae of oats in various media.

- A- moist air
- B- 50 per cent air, 50 per cent N₂.
- C- 20 per cent air, 80 per cent N₂.
- D- 10 per cent air, 90 per cent N₂.
- E- sterile distilled water.
- F- sterile distilled water and 4.0 mg. per liter adenine sulfate.
- G- sterile distilled water and 0.1 mg. IAA per liter.
- H- sterile distilled water and 1.0 mg. IAA per liter.
- I- sterile distilled water and 5.0 mg. IAA per liter.
- J- sterile distilled water and 10.0 mg. IAA per liter.

PLATE V



Little difference between the elongation of coleorhizae of seeds which were presoaked in IAA and those placed directly in moist air was observed. The difference in the final average lengths was only 0.1 mm.

In cultures made in air diluted with nitrogen gas, the coleorhizal length increased inversely to the concentration of air. Those cultured in 10 per cent air became nearly as long as those cultured in water (table III). In 100 per cent air and in 50 per cent air the maximum lengths were attained in 48 hours. In 10 and 20 per cent air the maximum lengths were reached after 72 hours of culture.

Observations on the formation of coleorhizal hairs were recorded for all of the cultures reported in tables III, IV and V. The relative amounts of coleorhizal surface from which hairs grew were recorded in terms of the percentage of coleorhizal surface covered with hairs. The average coleorhiza cultured in moist air was 37.5 per cent covered. Those cultured in moist air after presoaking with IAA averaged 50 per cent. Three out of the 16 embryos cultured in 50 per cent air had sparse hairs. The remainder had none. One of the embryos cultured in 20 per cent air had sparse hairs. None of the embryos cultured in 10 per cent air or in any of the liquid cultures formed hairs. Embryos cultured in 100 per cent air developed hairs which in some instances covered nearly all of the coleorhiza. This was most evident in the embryos which had been treated with IAA. In some of these hairs not only covered the coleorhiza but were also present on the epiblast (plate III, fig. 4).

The hairs appeared to be capable of arising from the epidermal cells in all parts of the coleorhiza. Some were fully formed within 24 hours after the seeds or embryos had been placed in culture. Cells near the distal end of the coleorhiza were the first to form hairs, followed in most cases by those toward the proximal end. Hairs first became visible as small papillae forming near the distal ends of epidermal cells. Within a few hours these grew out into structures that looked like root hairs. Various stages of hair formation are shown in plate IV, fig. 3.

A few coleorhizae were isolated from mature embryos of oats and placed in nutrient agar. They failed to grow in length but they also formed hairs. When some of these coleorhizae were slit open and flattened on agar with their inner surfaces in contact with the medium, hairs were formed on the exposed epidermal surfaces. When the orientation was reversed and the coleorhizae were flattened on agar with the epidermis in contact with the medium the cells of the inner surfaces, thus exposed to the air, did not form hairs. In these instances the epidermal cells which were in contact with the medium also failed to produce hairs.

The growth of the epiblast in embryos of oats cultured under the conditions described in table VI was much like that of the coleorhizae as shown in tables III and IV. However, the relative increases of epiblast lengths were somewhat greater than those of the coleorhizae. The epiblasts also were less inhibited by higher concentrations of IAA. As has been stated, in several embryos which had been presoaked in

TABLE VI

Growth of Epiblasts of Mature Embryos of Oats in Culture.

Medium	Culture period	No.	Average length of epiblast		Increase
			<u>initial</u>	<u>final</u>	
H ₂ O	5 days	23	0.2 mm.	1.4 (2.4)mm.	600%
H ₂ O+0.4 Ad.*	5	15	0.2	1.6 (2.0)	700%
H ₂ O+0.1 IAA**	5	29	0.2	2.8 (4.0)	1300%
H ₂ O+1.0 IAA	5	29	0.2	2.4 (3.6)	1100%
H ₂ O+5.0 IAA	5	32	0.2	1.9 (3.2)	850%
H ₂ O+10.0 IAA	5	33	0.2	1.7 (3.6)	750%

Air 100%	48 hours	20	0.2	0.6 (1.0)	200%
Air 100%	48	21	0.2	0.7 (1.1)	250%
+ IAA***					
Air 50%	48	16	0.2	0.6 (1.0)	200%
Air 20%	72	20	0.2	0.9 (1.3)	350%
Air 10%	72	20	0.2	1.4 (1.6)	600%

* milligrams per liter of adenine sulfate.

** milligrams per liter of indole-3-acetic acid.

*** presoaked overnight in a solution of water and IAA (10.0 mg./l.).

IAA and placed in moist air, hairs formed on the epiblasts.

The epiblast in oats, when well developed, has a sickle-like form. The basal stalk resembles the handle of a sickle with a semicircular blade. The blade is bifurcate at the tip (plate III, figs. 1 and 2).

CULTURES OF MATURE EMBRYOSDiscussion

Seeds of cereals ordinarily undergo a period of dormancy before they are able to germinate. The excised embryos of cereals, however, usually will germinate immediately if placed in the proper medium. Atwood (1915) was able to obtain germination of newly ripened seeds of Avena fatua by wounding the seed coats, by placing the seeds in higher concentration of oxygen, and by combinations of both procedures. It appears then that dormancy in the cereals may be due, at least in part, to the interference by the seed coat with the uptake of oxygen by the seed.

Excised embryos of oats, wheat, rye and barley do not grow very well in nutrient liquids. The growth of rye and wheat embryos was completely inhibited, while that of oats and barley was greatly reduced. This inhibition evidently is due to the low oxygen content of the media. Such embryos as floated to the surface of the liquid media after a time, germinated and grew very well. The similarity between this situation and natural dormancy is obvious. Morinaga (1926) studied the underwater germination of seeds of a number of species and found that seeds of oats and wheat did not germinate under water. Taylor (1942) reports that wheat seeds are not able to germinate in the absence of oxygen. In the present investigation, oat and barley seeds in some cases germinated under water after a few weeks if the cultures remained free of contamination.

For a time the writer thought that if excised, mature

embryos were cultured in nutrient liquids they might be induced to resume their embryonic growth, since germination is inhibited with this technique. This did not occur. The coleorhizae and epiblasts continued to grow for a time as reported, but only through enlargement of those cells already present and not by cellular division. Howarth (1927) states that in germination the growth of the coleorhiza is due to the vacuolation of existing cells. Stingl (1924) reports unusual elongations of oat coleorhizae in subsurface cultures of excised embryos. Nishimura (1922) found that the coleorhizae of Poa, Phleum and Setaria elongated to some extent in water culture and that their form was distinctive in each species. This has also been true of the four cereals cultured in the present investigation. (plate III, fig. 1, 2, 4, and 6). The coleorhizae did not expand simply by a random enlargement of their cells but attained a distinctive form in each species through what must have been a correlated growth of all the cells.

The eventual disintegration of the coleorhizae observed in cultures of wheat is interesting in that cells thus separated from one another continued to differentiate to some extent. Some became extremely attenuated, resembling root hairs. Others were rounded with finger-like outgrowths from several points along the cell walls. Gautheret (1943) figures cells of similar appearance, which were isolated from cortical layers of cultured roots.

The greatest elongations of oat coleorhizae occurred in water containing 0.1 mg./l of IAA. Higher concentrations

of IAA were inhibiting. An effect of IAA on plant tissue is that of increasing water uptake and thus producing cellular enlargement. There are a number of views as to the manner in which this takes place. Gordon (1953) lists several, including increased plasticity of the cell wall, higher respiratory activity of the cell, increased permeability of the cell membrane, increased cyclosis, greater uptake of water and solutes even against osmotic gradients, increased starch hydrolysis within the cell, and increased protein synthesis. Burström (1951) suggests that cellular elongation is due in part to an active extension of the cell wall rather than a passive stretching. In either case cell elongation is accompanied by increased water uptake.

Thimann (1951) considers that auxin acts in some metabolic process common to all plants and is capable of producing various results depending on the influences of other factors on the process. Hackett and Thimann (1952, 1953) found that IAA induced a slight increase in the uptake of water by cells of potato tissue. This water uptake was inhibited by a reduction in oxygen. Higher concentrations of IAA are inhibitory to the growth of some cells, notably in buds and roots. Still higher auxin concentrations are toxic as reported in a review of plant growth hormones by Thimann (1948).

The view that IAA is less effective in increasing the uptake of water at lower oxygen tensions seem difficult to reconcile with the effects observed on the coleorhiza of oats. However, it may be that this structure has a more

efficient respiratory system at lower oxygen concentrations than some other plant tissues.

The elongation of the coleorhiza in water culture is due to lowered oxygen tension rather than to complete water saturation. This is demonstrated in the series of experiments using various dilutions of air, in which the elongation appears to be inversely proportional to the concentration of oxygen.

When coleorhizae of grass embryos are exposed to moist air, their epidermal cells form outgrowths which are identical to root hairs in their development and appearance. This has been reported by numerous authors, among them Howarth (1927), Nishimura (1922), Percival (1921), LaRue (1936), Dietrich (1924), and Avebury (1892). Both Howarth and Nishimura considered that the coleorhizal hairs serve to supply water to the embryo during germination, and Nishimura found that in Poa and Phleum the hairs were alive and functioning 30 days after germination. In oats the hairs were formed at the distal ends of epidermal cells in all parts of the coleorhiza. Reeder and von Maltzahn (1953) classify root hairs, formed in the grasses, into the festucoid and the panicoid types. In the panicoid type, found in members of the subfamily, Panicoideae, the root hairs originate from the middle of the cell and grow straight out from the root at an angle of 90 degrees, whereas in the festucoid type the hairs originate near the distal end of the cell and project forward at an angle of about 45 degrees. The coleorhizal hairs of oats conform to the festucoid type as would be expected, since the tribe Aveneae is a member of the Festucoideae.

Coleorhizal hair formation also appears to be accentuated by IAA. Table III shows that hair formation was increased by previous soaking of seeds in water containing 10 mg. per liter of IAA. The optimum concentration for this effect was not determined since the amount of IAA taken up during the soaking period was unknown. Borgström (1939) found that by growing Allium roots in tap water containing IAA at concentrations of 1.0 and 0.1 mg. per liter, root hairs were produced in greater abundance than on roots growing without IAA. These hairs showed no localization but appeared along the entire root. He concluded that auxin is a fundamental factor in root hair formation.

Cormack (1944) was able to repress root hair formation in Phleum and Sporobolus through culture in tap water and redistilled water. He reports that root hair production was increased in water containing calcium sulfate. Calcium sulfate was not used in the present experiments but a number of cultures were made in nutrient liquids containing calcium nitrate. Coleorhizal hairs were not produced in any of these.

If IAA is responsible for the elongation of coleorhizal cells in liquid and for coleorhizal hair formation in air, it must determine the degree rather than the direction of growth. The latter appears to be influenced by the amount of oxygen present. Coleorhizal growth in normal germination takes place in two stages. The first is an elongation induced by low oxygen tension coupled with uptake of water. This elongation effects the rupture of the seed coats and, in barley and oats, the penetration of the enclosing lemma. The

second stage is seen in the formation of coleorhizal hairs which may function in the same manner as do the regular root hairs.

Epiblast development in the present series of cultures paralleled that of the coleorhiza. This tends to support the hypothesis reported by Avery (1930) that the epiblast is an outgrowth of the coleorhiza. The well developed epiblasts observed in liquid cultures regularly exhibited dichotomy. Though they lacked vascular tissue they appeared to be divided into stalk and blade. In these characteristics they were leaflike.

CULTURE OF IMMATURE EMBRYOS

Results

Data were obtained and tabulated from the culture of 1,091 immature embryos of oats, barley, wheat and rye, all of which were of uniform size when excised and placed in culture. The results from the growth of an additional number of smaller embryos of barley in vitro also are included in this section.

For comparative purposes the data for each organ of the cultured embryos have been tabulated separately. Overall increases in the size of cultured embryos also have been included in separate tables for each species.

All of the media listed in table II were used to culture these embryos, although those of some of the four species were grown on fewer kinds of media than others, due either to the lack of sufficient numbers of embryos or to poor results with

certain media.

Unless otherwise stated, all of the data presented in the following tables are averages of measurements recorded for individual 0.8 mm. embryos cultured for one week in total darkness at room temperature (about 26° C.). All of the measurements were recorded in micrometer units (1 unit=0.08 mm.) and converted to millimeters for tabulation.

Overall growth of cultured embryos: The growth in width of cultured embryos paralleled their increases in length in most cases. However, since the scutellum is the widest part of the embryo, the widths are included in the section dealing specifically with the growth of that organ.

In cultures of immature embryos of all four species it was discovered that the embryos did not develop normally unless they were placed with the ventral surface of their scutella in contact with the nutrient agar. For the sake of uniformity, care was therefore taken to insure placement of embryos with scutella in contact with agar. The reaction of embryos cultured upside down was interesting nevertheless, particularly with respect to scutellar development. These observations are reported in the section dealing with the scutellum.

Oats: Immature embryos of oats grew readily in most of the media used, and the seven day culture period proved to be adequate to allow comparisons of the growth on the different media. The results from the cultures of 386 embryos are presented in table VII.

The increases in embryo length obtained from cultures

PLATE VI

Camera Lucida Drawings of Oat Embryos

- Figure 1. Immature oat embryo at time of excision (0.8 mm. in length). Surface view.
- Figure 2. Immature embryo cultured on medium A (basal medium) for seven days. Surface view.
- Figure 3. Immature embryo cultured on medium E (40 mg./l. adenine sulfate) for three weeks. Side view.
- Figure 4. Immature embryo cultured on solid medium H (20% coconut milk) for one week. Side view.
- Figure 5. Immature embryo cultured in nutrient liquid H for one week. Surface view. Little coleorhizal elongation in evidence.
- Figure 6. Same conditions as for figure 5. Note the elongation of the coleorhiza. (See text p. 64)
- Figure 7. Mature oat embryo. Surface view.
- Figure 8. Embryo cultured on medium G (20% coconut milk, 40 mg./l. adenine sulfate) for one week. Side view.

a- coleoptile
b- coleorhiza
c- scutellum
d- coleorhizal hairs
f- primary root
g- epiblast
h- suspensor

PLATE VI

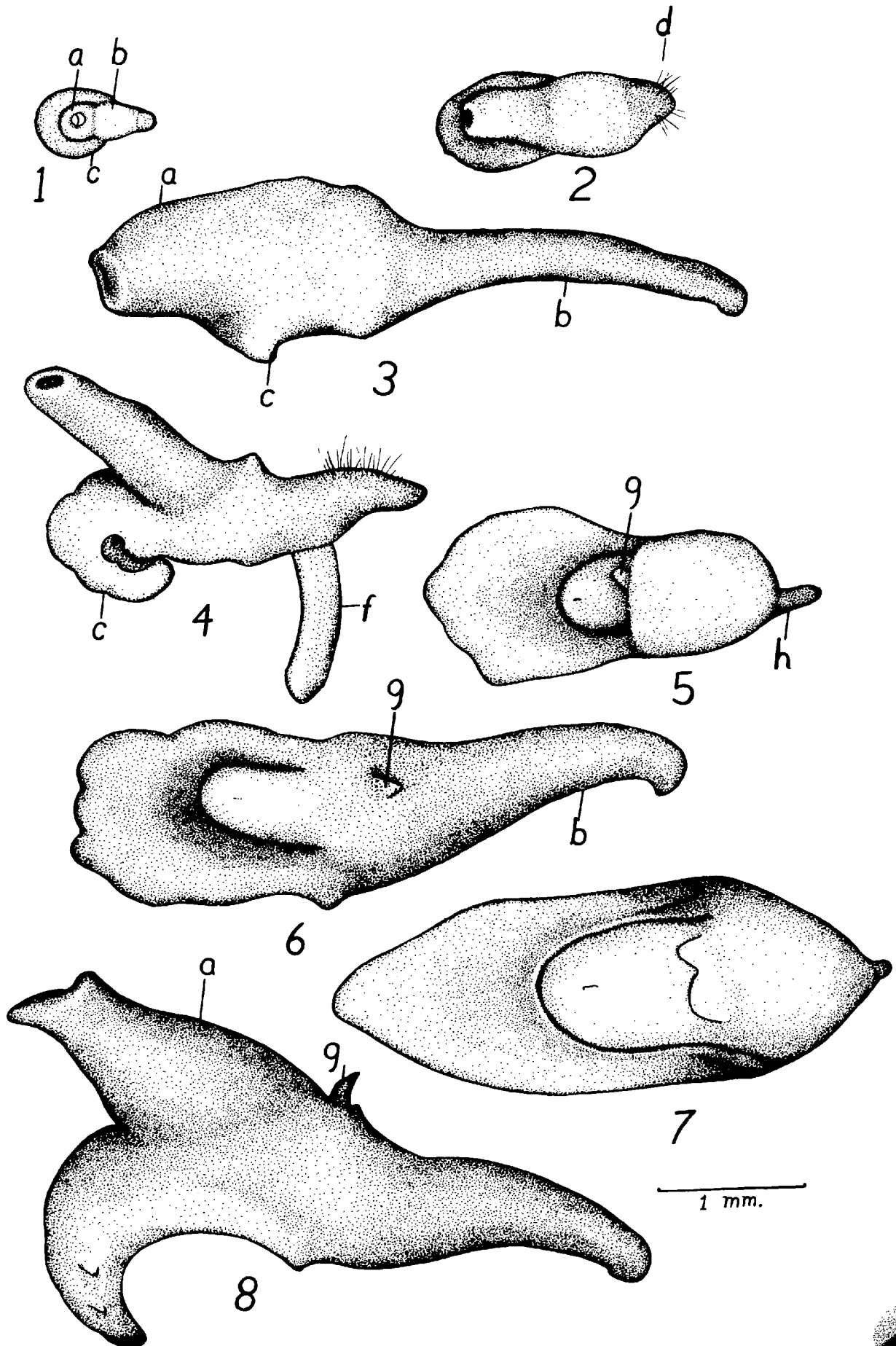


TABLE VII

Growth in Length of Immature Embryos of Oats in vitro.
Cultured Seven Days in Darkness at Room Temperature.

Medium (see p.25):	A	B	C	D	E	G	H	I
<u>Agar media</u>								
No. of embryos	46	23	30	27	28	21	59	27
Length - initial	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Length - final	1.8	1.8	1.6	1.8	2.7	3.7	2.2	2.3
Per cent increase	125	125	100	125	237	362	175	187
<u>Liquid media</u>								
No. of embryos	25			19	30		28	23
Length - initial	0.8			0.8	0.8		0.8	0.8
Length - final	1.7			1.6	1.9		2.7	2.0
Per cent increase	112			100	137		237	150

Note: In tables VII - XXIII all lengths and widths are averages of data recorded for the number of embryos listed. Length and width are tabulated in millimeters in all except table XIX.

on agar with medium A (White's agar), B (0.1 mg./l. IAA), and D (20 mg./l. adenine sulfate) were of the same magnitude. Growth on C (1.0 mg./l. IAA) was slightly less. The greatest average increase in length was observed on medium G (20% coconut milk, 40 mg./l. adenine sulfate). Smaller increases occurred on E (40 mg./l. adenine sulfate), H (20% coconut milk) and I (90% coconut milk). However, the relatively large increases in length of embryos cultured on media E and G

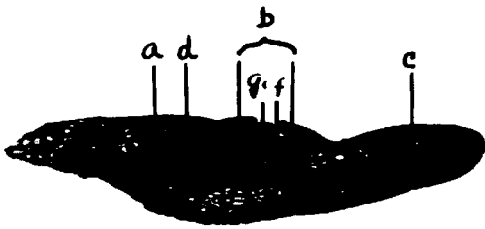
PLATE VII

Sectional Views of Embryos of Oats Cultured on Various Media

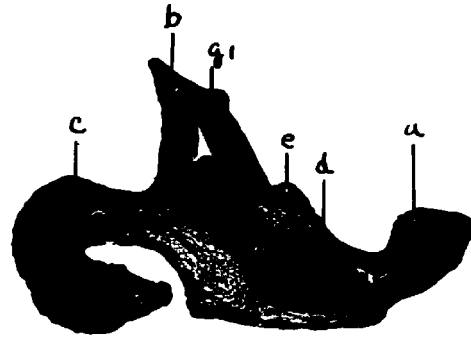
- Figure 1. Embryo at time of excision. X 70.
- Figure 2. Embryo cultured on medium I (90% coconut milk)
one week. X 25.
- Figure 3. Embryo cultured on medium G (20% coconut milk,
40 mg./l. adenine sulfate) one week.
Lateral to midsection. X 25.
- Figure 4. Same as in figure 3 but sagittal. X 25.
- Figure 5. Embryo cultured on liquid medium H (20% coconut
milk) for one week. X 35.
- Figure 6. Same as in figure 2 but lateral to midsection.
X 25.

- a- coleorhiza
- b- coleoptile
- c- scutellum
- d- primary root meristem
- d₁- first lateral root meristem
- e- epiblast
- f- apical meristem
- g₁- first leaf primordium
- g₂- second leaf primordium

PLATE VII



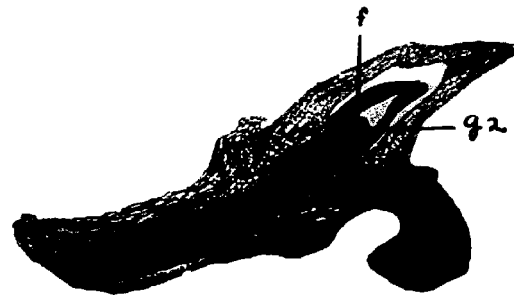
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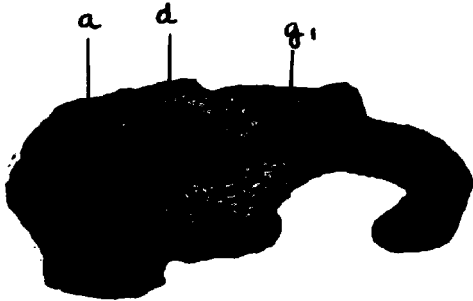
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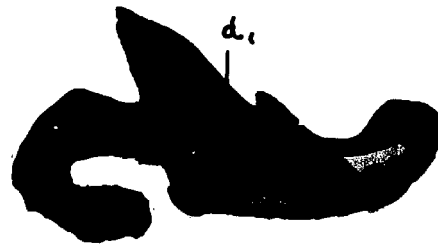
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PLATE VIII

Sectional Views of Embryos of Oats.



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Figure 1. Embryo cultured on solid medium H (20% coconut milk) for one week. X 25.

Figure 2. Embryo cultured on solid medium A (White's solution) for one week. X 35.

Figure 3. Embryo normally matured in the seed. Lateral to midsection showing secondary roots. X 25.

Figure 4. Same as in figure 3, except sagittal. The two leaf primordia and apical meristem are visible. X 25.

(both containing adenine sulfate) were due in part to unusual coleorhizal elongations. If this effect is discounted, the best overall growth was obtained on medium I. This is apparent if one compares figs. 1, 2, 4, and 6 in plate VII, in which it may be seen that the embryonic growth and differentiation on medium I was somewhat more advanced than on the other media.

Figures representative of the different growth responses in embryos of oats cultured on the various media are shown in plates VI, VII and VIII. Organ by organ comparisons of growth on the different media will be given later. Nevertheless, even a casual observation of the figures in these three plates demonstrates that the growth in vitro of excised, immature embryos of oats did not approach their normal development in vivo.

Cultures in liquid media were successful mainly in the inhibition of germination. Most of the increases in size came from cellular enlargement rather than through continued cellular division and differentiation of organs. The few embryos which were allowed to remain in the culture tubes beyond the one week period usually grew into small and underdeveloped seedling plants except on medium E (40 mg./l. adenine) and in liquid media. In the latter, germination was completely inhibited.

Barley: A total of 331 embryos of barley were cultured. These, for the most part, continued to differentiate in vitro to a greater extent than did those of oats. Reference to the figures in plates IX, X and XI shows that in embryos cultured

PLATE IX

Camera Lucida Drawings of Barley Embryos.

- Figure 1. Immature embryo of barley at time of excision (0.8 mm. long).
- Figure 2. Embryo cultured on medium A (White's agar) for one week.
- Figure 3. Same as in figure 2 except after two weeks. (Note development of leaf-like coleoptile).
- Figure 4. Embryo cultured on medium I (90% coconut milk) for one week.
- Figure 5. Embryo cultured on medium H (20% coconut milk) for one week. This embryo is also illustrated in sectional view in Plate XI, fig. 3.
- Figure 6. Mature embryo, excised from seed.
- Figure 7. Embryo cultured for two weeks on medium H. Some embryos in this particular set of cultures showed unusual scutellar developments.

a- coleoptile
b- coleorhiza
c- suspensor
d- primary root
e- shoot (first foliage leaf)
f- scutellum
g- epiblast
h- scutellar concavity or sheath

PLATE IX

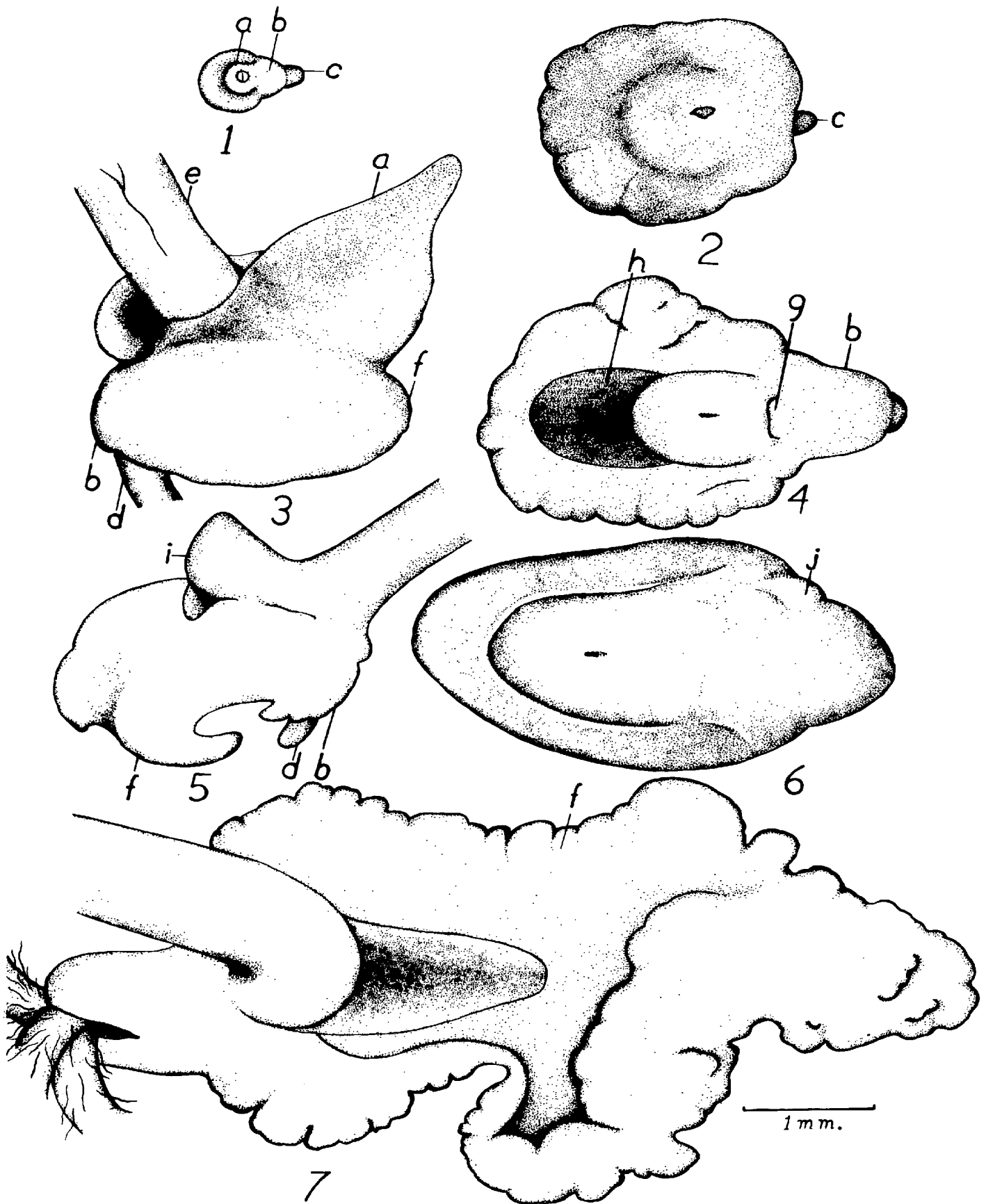


TABLE VIII

Growth in Length of Immature Embryos of Barley in vitro.
Cultured Seven Days in Darkness at Room Temperature.

Medium (see p. 25):	A	B	C	E	G	H	I	J	K
<u>Agar media</u>									
No. of embryos	38	32	32	34	34	43	29	14	23
Length - initial	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Length - final	1.3	1.3	1.1	1.4	2.6	1.8	2.3	2.0	1.7
Per cent increase	62	62	37	75	225	175	187	150	112
<u>Liquid media</u>									
No. of embryos	25					27			
Length - initial	0.8					0.8			
Length - final	1.4					1.5			
Per cent increase	75					87			

on medium I (90% coconut milk) with agar added, differentiation and growth in some parts of the embryos equaled and even surpassed that of normally developed embryos.

As in cultures of embryos of oats, the results with barley on media A (White's agar) and B (0.1 mg./l. IAA) were much the same, but embryos grown on medium C (1.0 mg./l. IAA) were smaller than any of the others. A small increase in the size of embryos cultured on medium E (40 mg./l. adenine sulfate) was noted, and the remaining embryo cultures on agar media G (40 mg./l. adenine sulfate, 20% coconut milk), H (20% coconut milk), I (90% coconut milk), J (G plus 0.1 mg./l. IAA) and K (0.25% yeast extract, 1.0 mg./l. IAA) were all benefited

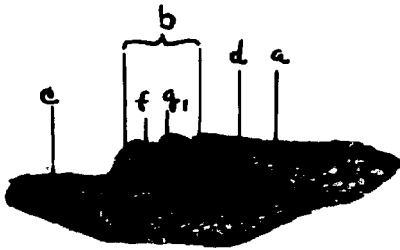
PLATE X

Sectional Views of Embryos of Barley

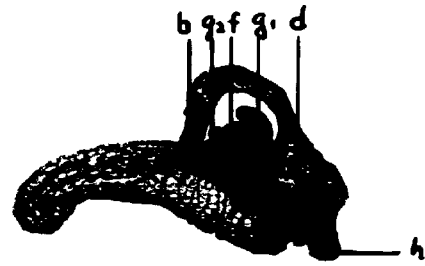
- Figure 1. Embryo at time of excision (0.8 mm. long).
X 60.
- Figure 2. Embryo cultured on medium A (White's medium)
for one week. X 35.
- Figure 3. Embryo cultured on medium I (90% coconut milk)
for one week. Sagittal section. X 25.
- Figure 4. Same as in figure 3 except lateral to mid-
section. X 25.
- Figure 5. Mature embryo excised from seed. Sagittal
section. X 25.
- Figure 6. Same as in figure 5 but lateral to midsection.
X 25.

- a- coleorhiza
- b- coleoptile
- c- scutellum
- d- primary root primordium
- d₁, d₂, etc.- lateral root primordia
- e- epiblast
- f- apical meristem
- g₁- first leaf primordium
- g₂- second leaf primordium
- g₃- third leaf primordium
- h- suspensor

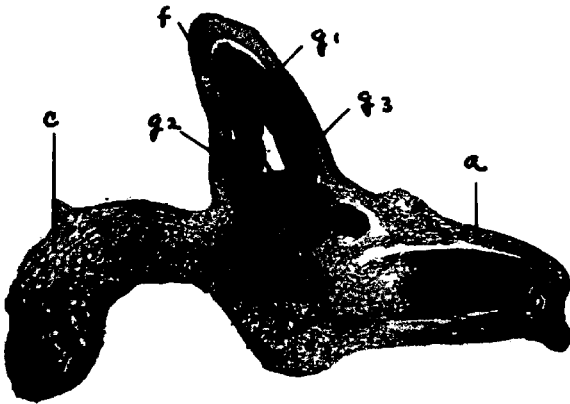
PLATE X



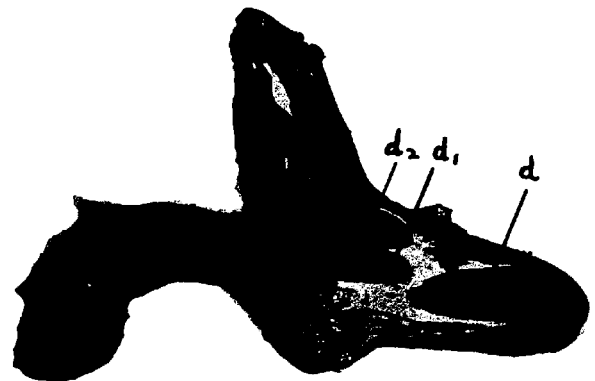
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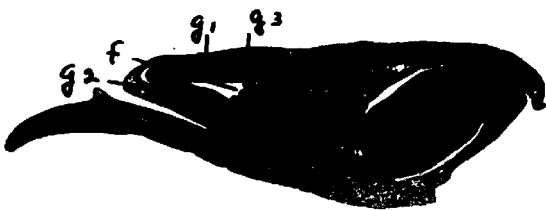
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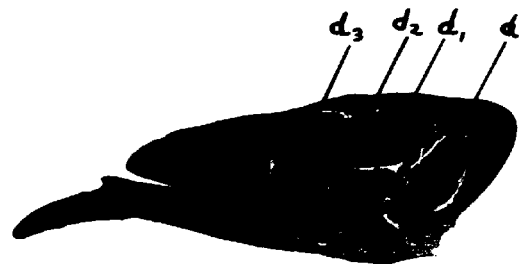
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PLATE XI

Sectional Views of Embryos of Barley

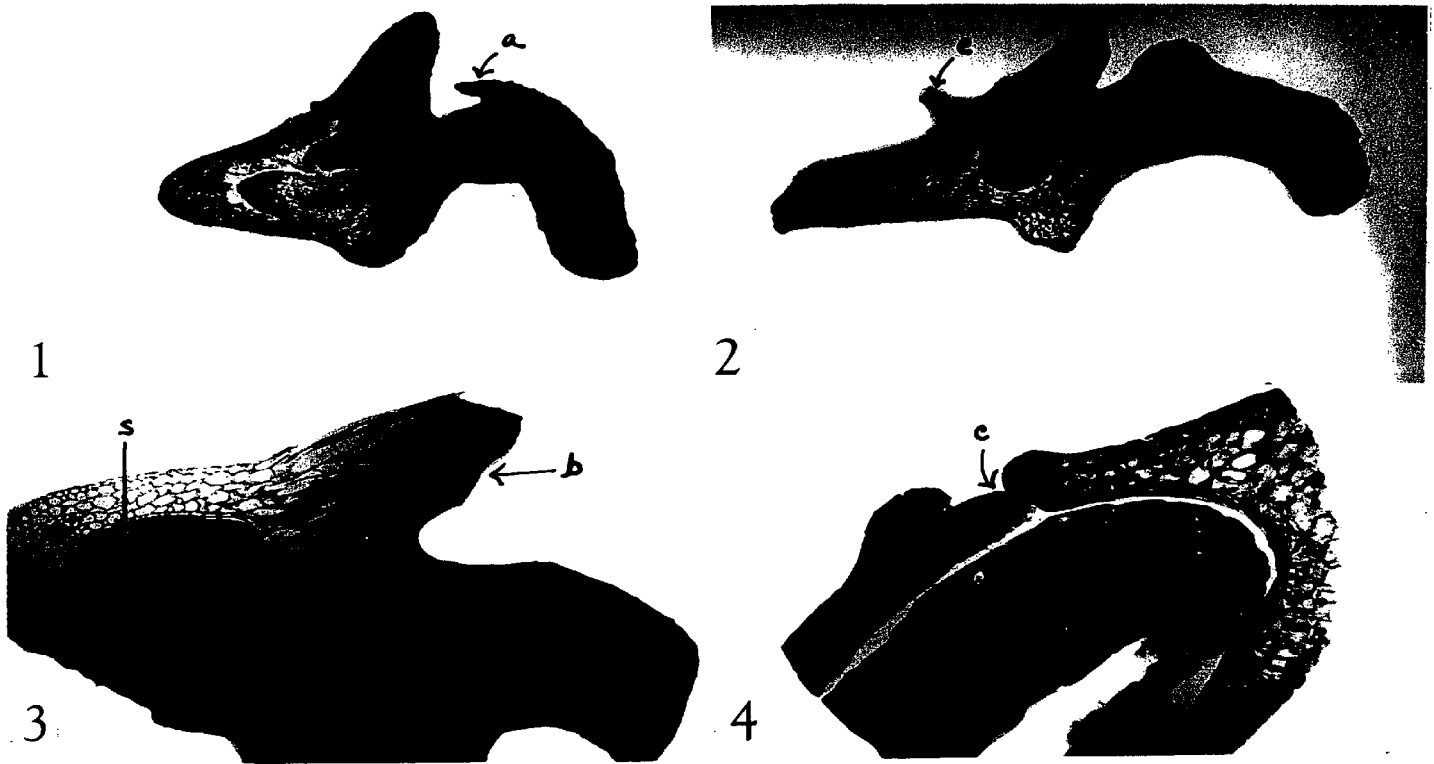


Figure 1. Embryo cultured on medium I (90% coconut milk) for one week. Note development of the ligule of the scutellum (a). X 25.

Figure 2. Embryo cultured on medium I for one week. A well developed epiblast (e) is present. X 25.

Figure 3. Embryo cultured on medium H (20% coconut milk) for one week. The shoot (s) is shown in transverse section since it grew out laterally in this particular embryo. An outgrowth (b) from the axil of the coleoptile is shown. X 35.

Figure 4. A portion of the coleoptile of an embryo cultured for one week on medium I. Note the proliferation of cells along the margin of the coleoptilar pore (c). X 70.

by substances included in these media.

Seedling growth was not inhibited completely in embryos grown on any of the media, although some of those cultured on medium I grew and differentiated without germinating prematurely throughout the seven day culture period and actually became larger than mature embryos in the seed (plate X, figs. 3 to 6). The growth of embryos of barley on all media containing coconut milk has been rather variable, particularly on media containing 20 per cent coconut milk. A few embryos cultured on one lot of this medium developed unusually large scutella. One of these, shown in plate IX, fig. 7, had a scutellum which measured nearly six millimeters in length.

The color and texture of cultured embryos do not lend themselves easily to tabulation and were not recorded in such a way that they could be included in tables. In general, the majority of embryos except those grown on medium I exhibited a watery, translucent appearance which is typical of growth by cellular enlargement rather than by division. Embryos on I and some on G, H, and J, had an opaque, ivory color which also is characteristic of embryos that have matured in the seed.

The increased growth of embryos on medium H, containing 20 per cent coconut milk, and on medium E (40 mg./l. adenine sulfate) as compared to that on the basal medium, led to the combination of these two substances in medium G. A number of embryos were cultured on this medium with the results shown in table VIII. The work of Skoog (1944), Skoog and Tsui (1948, 1951), and Miller and Skoog (1953) has shown that IAA

counteracts adenine sulfate in its effects on differentiation of buds in tobacco callus cultures. Therefore, a medium made of 20 per cent coconut milk, 40 mg./l. adenine sulfate and 0.1 mg./l. IAA was tried. The results, given under J in table VIII, indicate an inhibition of growth as compared to the results on G.

Only two sets of cultures in liquid were carried to completion since it was discovered that, in barley, nearly as many embryos died in culture as grew. For example, of 51 embryos cultured in the basal liquid medium A, 25 grew and 26 died. Very little difference was noted between cultures in liquid media A and H. The embryos which did grow were poorly developed and were almost transparent in appearance.

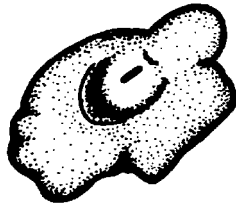
A number of embryos of barley smaller than 0.8 mm. were cultured on 90 per cent coconut milk agar and on White's agar in order to discover to what extent the growth and development of very young embryos could be influenced. A comparison between the effects of autoclaved and Seitz-filtered coconut milk was also made. The results of these cultures may be summarized as follows: On the white's agar alone, embryos as small as 0.5 mm. formed undifferentiated masses of callus (plate XII, fig. 2). Smaller embryos failed to survive. Embryos as small as 0.3 mm. continued to grow and differentiate on medium I which contained 90 per cent coconut milk. Embryos which were 0.6 mm. in length appeared to grow as well on autoclaved coconut milk as on filtered coconut milk for the first week. However, later growth on autoclaved coconut milk was inhibited. In particular, a complete lack of root

PLATE XII

Cultures of Very Small Embryos of Barley

- Figure 1. Immature embryo of barley after two weeks culture on medium I (90% coconut milk). Embryo was 0.5 mm. in length at excision. X 15.
- Figure 2. Immature embryo of barley after two weeks culture on medium A (White's medium). Embryo was 0.5 mm. in length at excision. X 15.
- Figure 3. Seedling of barley with two shoots, two coleoptiles (x) and 10 roots. This seedling was grown from an embryo measuring 0.16 mm. at excision. Cultured four weeks on medium I and one week in soil. X 1.
- Figure 4. Embryos in all of the culture tubes shown were 0.6 mm. in length at excision. The upper row of tubes contained media with added Seitz filtered coconut milk. The lower row contained autoclaved coconut milk media. In both sets of culture the concentration of coconut milk was 90 per cent by volume. Note the lack of root development in embryos on autoclaved coconut milk medium.

PLATE XII



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development appeared on autoclaved media (plate XII, fig.4).

Two embryos out of five, measuring 0.3 mm. at time of excision, grew into apparently normal seedlings on Seitz filtered, 90 per cent coconut milk, medium I. Five embryos, 0.4 mm. in size, developed normally. Six, smaller than 0.3 mm. in length and apparently undifferentiated when excised, grew for a time without differentiation to form small balls of callus. Later two of these, both less than 0.2 mm. in length when placed in culture, produced leaves and roots. One of the two subsequently developed what appeared to be two coleoptiles, two normal leafy shoots, and ten roots. It was transplanted to soil and flourished for a week or so, when it was removed and the photograph in plate XII, fig. 3. was taken.

Wheat: Embryos of wheat grew well on medium I (90% coconut milk agar) but rather poorly on the other media. The results of cultures on media A, D, E, and H were similar. Embryos grown on medium C were most reduced in size and development. The response to liquid culture was about the same as in barley. Figures representative of a number of these embryos are to be found in plates XIII and XIV. Data resulting from the culture of 229 excised embryos of wheat are included in table IX.

Embryos of wheat cultured on I and in liquid, remained dormant and were not observed to germinate even after a month in culture. Nearly normal differentiation occurred in the former but not in the liquid cultures. Premature germination took place on all of the other media and, as in oats and

PLATE XIII

Camera Lucida Drawing of Embryos of Wheat

- Figure 1. Embryo at time of excision (0.8 mm. long).
- Figure 2. Embryo after one week of culture in liquid medium A (White's liquid).
- Figure 3. Embryo after one week's culture on medium I (90% coconut milk).
- Figure 4. Embryo after one week of culture on solid medium A (White's agar).
- Figure 5. Embryo after one week of culture on medium H (20% coconut milk).
- Figure 6. Mature embryo, excised from seed.

a- first leaf primordium
b- ligule of scutellum
c- epiblast
d- suspensor
e- coleorrhiza

PLATE XIII

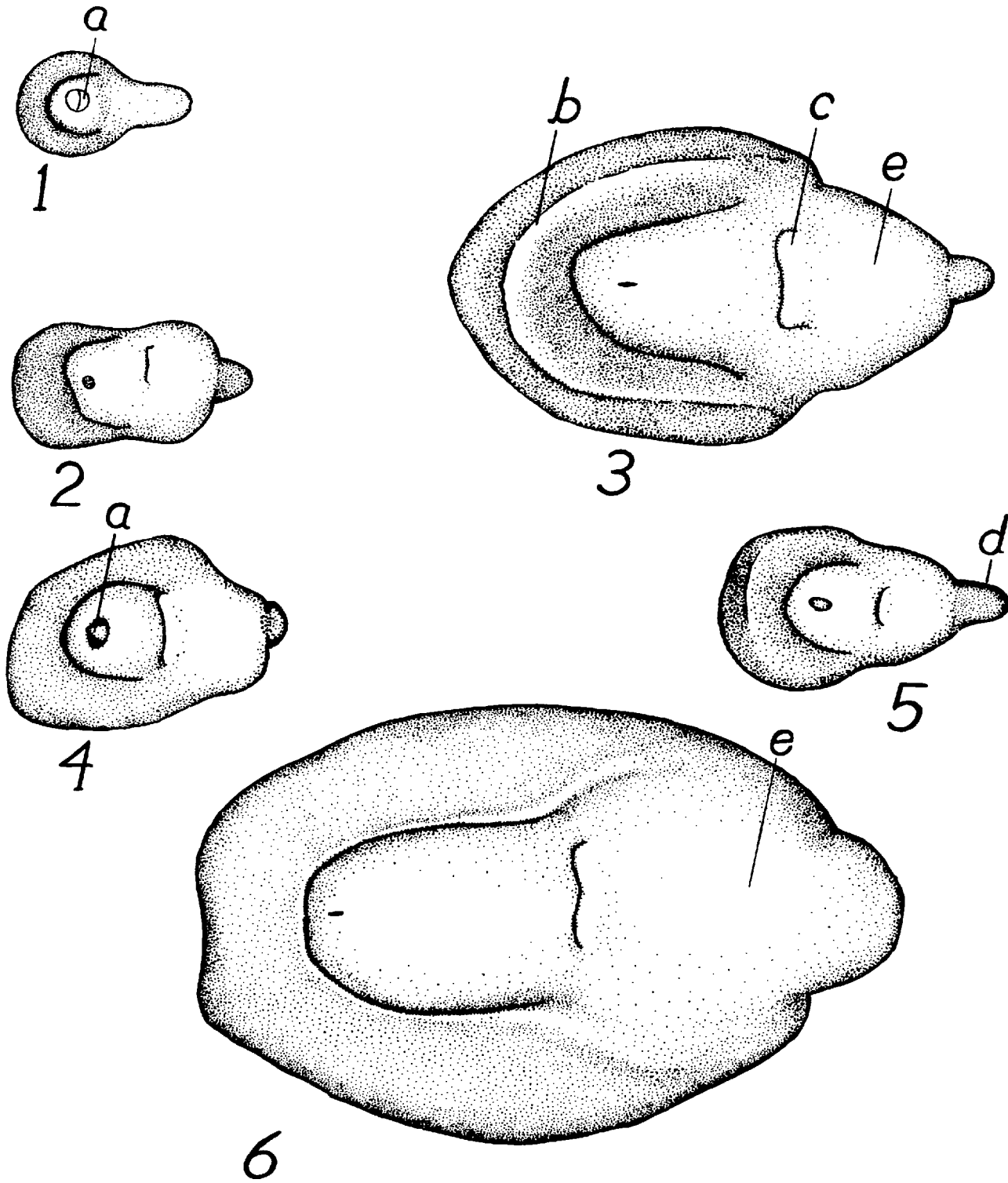


PLATE XIV

Sectional Views of Excised Embryos of Wheat

Figure 1. Embryo at time of excision (0.8 mm. long). X 60.

Figure 2. Embryo cultured on medium I for one week.

Section lateral to midsection. X 25.

Figure 3. Embryo cultured for one week on medium A

(White's agar). X 35.

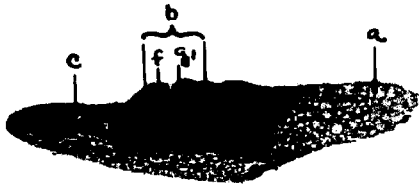
Figure 4. Same as in figure 2 except sagittal section.

Figure 5. Mature embryo excised from seed. Three leaf primordia are present although more appear to be, due to the overlap of the margins of the well developed first leaf. An epiblast is present in the mature wheat embryo but did not appear in these sections. X 25.

Figure 6. Same as in figure 5 except lateral to midsection.

- a- coleorhiza
- b- coleoptile
- c- scutellum
- d- primary root primordium
- d₁- first lateral root primordium
- d₂- second lateral root primordium
- e- epiblast
- f- apical meristem
- g₁- first leaf primordium
- g₂- second leaf primordium
- g₃- third leaf primordium

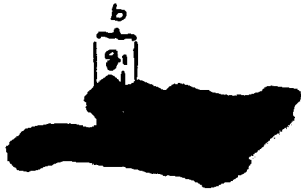
PLATE XIV



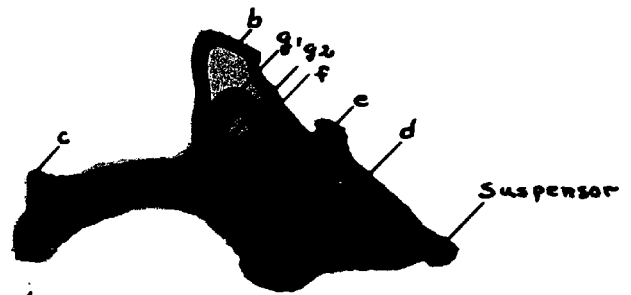
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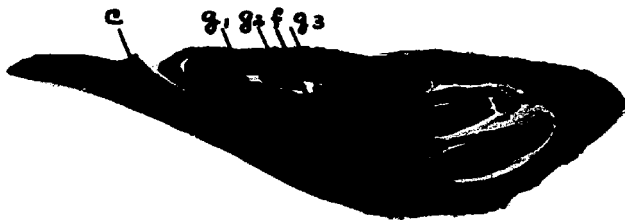
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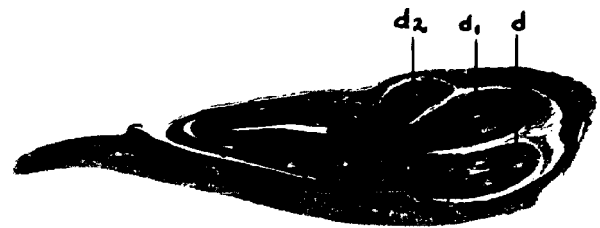
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barley, produced small seedling plants.

TABLE IX

Growth in Length of Immature Embryos of Wheat in vitro.

Medium (see p.25):	A	C	D	E	H	I	A	D
	<u>Agar media</u>						<u>Liquid</u>	
No. of embryos	37	26	29	25	29	29	32	22
Length - initial	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Length - final	1.2	1.0	1.3	1.1	1.1	1.7	1.0	1.0
Per cent increase	50	25	62	37	37	112	25	25

Rye. The results from the culture of 145 embryos of rye are given in table X.

TABLE X

Growth in Length of Immature Embryos of Rye in vitro.

Medium (see p.25):	A	G	H	I	H	I
	<u>Agar media</u>				<u>Liquid</u>	
No. of embryos	31	21	30	27	19	17
Length - initial	0.8	0.8	0.8	0.8	0.8	0.8
Length - final	1.0	1.2	1.3	1.7	1.3	1.7
Per cent increase	25	50	62	112	62	50

Again the best results were obtained with medium I on which embryonic growth approaching that in vivo took place,

PLATE XV

Camera Lucida Drawings of Embryos of Rye

- Figure 1. Embryo at time of excision (0.8 mm. long).
- Figure 2. Embryo after one week of culture in liquid medium H (20% coconut milk). Note lateral proliferations of scutellum.
- Figure 3. Embryo after one week of culture in liquid medium I (90% coconut milk).
- Figure 4. Embryo which had grown floating upside down on liquid medium H. Note the abnormally large scutellum (f).
- Figure 5. Embryo after one week of culture on solid medium A (White's agar). The first foliage leaf had grown through the coleoptilar pore which had failed to close properly.
- Figure 6. Embryo after one week of culture on solid medium H. The failure of the coleoptile to form a closed structure is illustrated.
- Figure 7. Embryo after one week of culture on solid medium I.
- Figure 8. Mature embryo, excised from the seed.
- Figure 9. Same as in figure 7 except after two weeks of culture.

- a- coleoptile
- b- first leaf primordium
- c- suspensor
- d- epiblast
- e- ligule of scutellum
- f- scutellum
- g- pore of coleoptile
- h- swelling produced by lateral root

PLATE XV

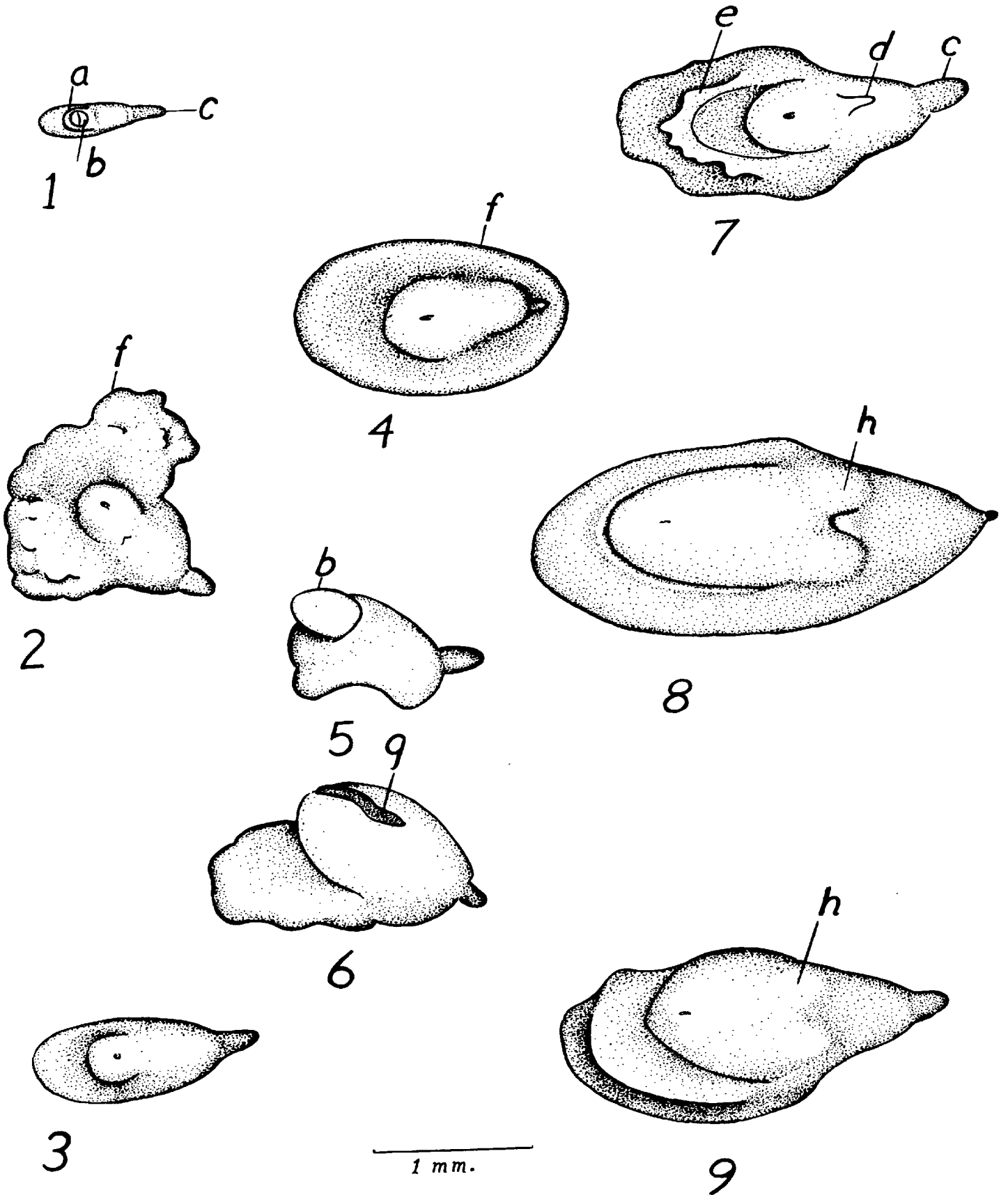


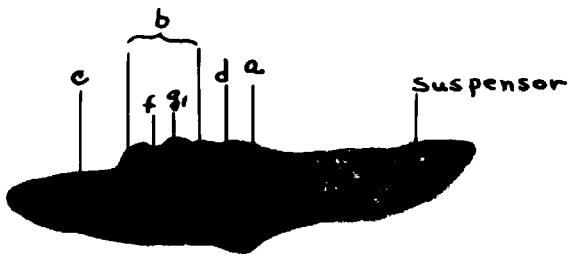
PLATE XVI

Sectional Views of Excised Embryos of Rye

- Figure 1. Embryo at time of excision (0.8 mm. long).
X 70.
- Figure 2. Mature embryo excised from the seed. X 25.
- Figure 3. Embryo after one week of culture on medium I
(90% coconut milk). Lateral to midsection.
X 35.
- Figure 4. Same as in figure 3 except sagittal section.
X 35.
- Figure 5. Same as in figure 4 except X 70.
- Figure 6. Same as in figure 2 except X 35.

- a- coleorhiza
- b- coleoptile
- c- scutellum
- d- primary root meristem
- d - first lateral root meristem
- e- epiblast
- f- apical meristem
- g₁- first leaf primordium
- g₂- second leaf primordium
- g₃- third leaf primordium

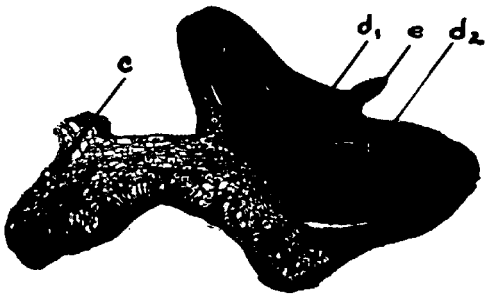
PLATE XVI



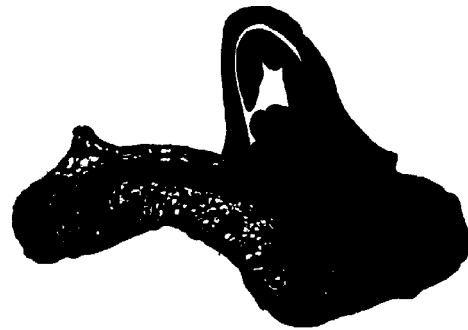
1



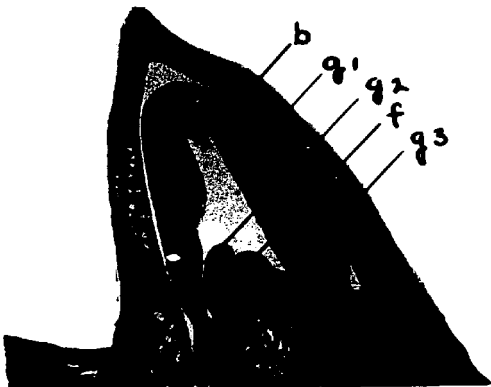
2



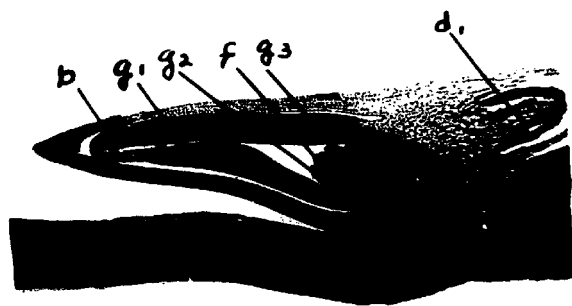
3



4



5



6

as is shown in plate XV, figs. 7 and 9. At the end of thirty days' culture, some of these embryos were just beginning to germinate. Embryos cultured on the other media differentiated only slightly, and germinated to produce weak and poorly developed seedlings (plate XV, figs. 2, 3, 5 and 6). Those in liquid culture did not germinate at all.

Growth of the coleorhiza in vitro: Data pertaining to the development of coleorhizae of cultured embryos of oats, barley, wheat and rye are reported in the following pages. With a few exceptions, all of the results were obtained from embryos measuring 0.8 mm. in length at time of excision. They were cultured for one week in darkness at room temperature.

Oats: Effects noted in the development of coleorhizae of 386 oat embryos are summarized in table XI.

TABLE XI

Growth of Coleorhizae of Oat Embryos in vitro

Medium (see p.25):	A	B	C	D	E	G	H	I
<u>Agar media</u>								
No. of embryos	46	23	30	27	28	21	59	27
Length - initial	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24
Length - final	0.74	0.84	0.79	0.74	1.58	1.96	0.85	0.72
Per cent increase	208	250	229	208	558	717	254	200
<u>Liquid media</u>								
No. of embryos	25			19	30		28	23
Length - initial	0.24			0.24	0.24		0.24	0.24
Length - final	0.82			0.67	0.87		0.96	0.86
Per cent increase	242			179	262		300	258

Two types of coleorhizal growth were encountered. Some elongated to an approximate length of 0.8 mm. without producing more than a few hairs from the epidermal cells (plate VI, figs. 2 and 4). Others continued to elongate to a final length of nearly 2.0 mm without producing hairs (plate VI, fig. 3). As in similar elongations observed in mature oat embryos, the growth appeared to be a result of cellular elongation rather than cell division.

The presence of coleorhizal hairs was noted in embryos grown on all of the solid media except those containing adenine sulfate. On the latter, coleorhizal elongation occurred.

Irregular results were obtained on media containing adenine. The first cultures, on medium E (40 mg./l. adenine), produced unusually long coleorhizae in all of the 28 embryos grown. Subsequent attempts to obtain this effect failed. Both medium E and F (80 mg./l. adenine) were used. The results of these trials were simply recorded as negative and are not tabulated. In the spring of 1954 the elongation effect was again observed in 21 embryos cultured on a medium containing 20 per cent coconut milk and adenine sulfate (medium G), but as before further attempts to repeat these results failed.

Although mature and even half grown embryos of oats produced elongated coleorhizae in liquid culture, embryos 0.8 mm. long did not do so except in a single instance. One embryo cultured in liquid medium H developed a lengthened coleorhiza (plate VI, fig. 6).

Barley: In most respects the growth in vitro of the

coleorhizae of immature barley embryos was similar to that of oats. Coleorhizal development on media A, B, C and E was almost completely negative.

TABLE XII

The Growth of Coleorhizae of Barley Embryos in vitro.

Medium (see p. 25):	A	B	C	E	G	H	I	J	K
<u>Agar media</u>									
No. of embryos	38	32	32	34	34	43	29	14	23
Length - initial	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24
Length - final	0.26	0.18	0.17	0.17	0.58	0.34	0.46	0.39	0.32
Per cent increase	08	-	-	-	142	42	92	62	33
<u>Liquid media</u>									
No. of embryos	25					27			
Length - initial	0.24					0.24			
Length - final	0.31					0.34			
Per cent increase	29					42			

Apparent decreases in length occurred on media B, C and E. Actually these reductions came about because of the forward growth of the coleoptiles which caused a downward displacement of the coleorhizae. This is shown in plate IX, figs. 3 and 5. The coleorhizae of embryos cultured on media G, H, I, J (all containing coconut milk) and K (0.25% yeast extract, 1.0 mg./l. IAA) were better developed. The combination of coconut milk and adenine sulfate (G) produced coleorhizal elongations that were nearly twice as great as on any other

media. Thus the results with barley were similar to those with oats. The addition of IAA to this medium resulted in shorter coleorhizae in the 14 embryos cultured using this combination of substances (medium J).

The growth of coleorhizae in liquid media A and H was no better than on agar media A and H.

Coleorhizal hairs were not observed in any of the immature embryos of barley. This also was true of the mature embryos cultured in the earlier experiments.

Wheat: The only medium on which coleorhizal growth in embryos of wheat was noticeably increased was that containing 90 per cent coconut milk, medium I. Coleorhizal hairs were not observed in any of the cultured embryos although they are often present in mature embryos.

TABLE XIII

The Growth of Coleorhizae of Wheat Embryos in vitro.

Medium (see p. 25):	A	C	D	E	H	I	A	D
	<u>Agar media</u>						<u>Liquid</u>	
No. of embryos	37	26	29	25	29	29	32	22
Length - initial	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24
Length - final	0.30	0.30	0.31	0.30	0.30	0.39	0.30	0.34
Per cent increase	25	25	29	25	25	62	25	42

Rye: There was very little difference observed in the growth of the coleorhizae of rye in any of the cultures. Coleorhizal hairs did not develop although mature embryos often produce them in abundance.

TABLE XIV

The Growth of Coleorhizae of Rye Embryos in vitro.

Medium (see p. 25):	A	G	H	I	H	I
	<u>Agar media</u>				<u>Liquid</u>	
No. of embryos	31	21	30	27	19	17
Length - initial	0.24	0.24	0.24	0.24	0.24	0.24
Length - final	0.32	0.29	0.30	0.31	0.35	0.24
Per cent increase	33	21	25	29	46	0

Growth of the coleoptile in vitro: In some instances the coleoptiles of cultured embryos grew, as in germination, by the enlargement of existing cells. At other times their growth involved cellular division. Growth of the coleoptile by cell division is present during embryogenesis, while coleoptilar growth by cell enlargement alone is indicative of premature germination as well as being characteristic of normal germination. In cultures in which premature germination was partially blocked, both kinds of development were observed.

Oats: Both division and elongation of cells in the coleoptiles of immature oat embryos occurred in all cultures on agar. Those in liquid media appeared to be inhibited in both aspects of growth although they enlarged slightly. The closing of the apical pore of the coleoptile, which is significant in that it indicates at least a certain amount of embryonic differentiation, occurred in all cultured embryos. This may be seen in plates VI and VII.

TABLE XV

The Growth of Coleoptiles of Oat Embryos in vitro.

Medium (see p. 25):	A	B	C	D	E	G	H	I
<u>Agar media</u>								
No. of embryos	46	23	30	27	28	21	59	27
Length - initial	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24
Length - final	0.90	1.02	0.58	1.02	1.26	1.09	1.19	0.92
Per cent increase	275	325	142	325	425	354	396	283
<u>Liquid media</u>								
No. of embryos	25			19	30		28	23
Length - initial	0.24			0.24	0.24		0.24	0.24
Length - final	0.51			0.75	0.60		0.56	0.56
Per cent increase	112			212	150		133	133

Barley: The most striking examples of the two types of development (pregerminal and postgerminal) in coleoptiles of cultured embryos were observed in barley. On media A, B, C, E and to a lesser extent on G, H, J and K the coleoptiles failed to develop normally; that is, to form a closed sheath. Instead they grew in length without closing. The apical pore also stretched to form a slit. A blade-like coleoptile was thus formed (plate IX, fig. 3). The apical pore closed normally in embryos cultured on 90 per cent coconut milk agar (I), often by a curious proliferation of cells (plate XI, fig. 4).

TABLE XVI

The Growth of Coleoptiles of Barley Embryos in vitro.

Medium (see p. 25):	A	B	C	E	G	H	I	J	K
<u>Agar media</u>									
No. of embryos	38	32	32	34	34	43	29	14	23
Length - initial	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24
Length - final	0.40	0.46	0.42	0.67	4.22	1.99	2.62	3.19	0.51
Per cent increase	67	92	75	179	1658	729	992	1229	112
<u>Liquid media</u>									
No. of embryos	25					27			
Length - initial	0.24					0.24			
Length - final	0.46					0.33			
Per cent increase	92					37			

While a number of embryos cultured on 90 per cent coconut milk developed elongated coleoptiles, in some longitudinal growth did not appear to be greatly stimulated. (plate IX, fig.4; plate X, figs. 3 and 4). The greatest increases in length were observed on medium G (20 per cent coconut milk, 40 mg./l. adenine sulfate). Lesser, though still impressive, elongations occurred in cultures on media H (20 per cent coconut milk) and J (G plus IAA). Growth was reduced in both sets of liquid cultures.

Wheat: The development of the coleoptiles of immature wheat embryos was not remarkable except on 90 per cent coconut milk agar. Extreme elongations did not occur in

coleoptiles of wheat during the period of observation (plate XIII).

TABLE XVII

The Growth of Coleoptiles of Wheat Embryos in vitro.

Medium (see p. 25):	A	C	D	E	H	I	A	D
	<u>Agar media</u>						<u>Liquid</u>	
No. of embryos	37	26	29	25	29	29	32	22
Length - initial	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24
Length - final	0.35	0.25	0.29	0.31	0.29	0.46	0.26	0.28
Per cent increase	46	04	21	29	21	92	08	17

It is significant that the growth was reduced in media containing 1.0 mg./l. IAA (medium C). This was also true in the preceding examples. The coleoptiles of embryos cultured on White's agar (A) grew mostly by cell enlargement. Failures in closure of the apical pore were observed in many of these cultures (plate XIII, fig. 4; plate XIV, fig. 3).

Rye: The rye coleoptile responded to culture in much the same manner as wheat. The lack of normal differentiation on media A and H may be seen in plate XV, figs. 5 and 6. This was true, although to a lesser degree, of cultures on medium G. Growth of the rye coleoptile in liquid culture was very much reduced. The nearly normal development of the coleoptiles of embryos cultured on medium I (90 per cent coconut milk) is shown in plate XV, figs. 7 and 9, and also in plate XVI, figs. 4 and 5.

TABLE XVIII

The Growth of Coleoptiles of Rye Embryos in vitro.

Medium (see p. 25):	A	G	H	I	H	I
	<u>Agar media</u>				<u>Liquid</u>	
No. of embryos	31	21	30	27	19	17
Length - initial	0.24	0.24	0.24	0.24	0.24	0.24
Length - final	0.46	0.48	0.46	0.53	0.34	0.32
Per cent increase	92	100	92	121	42	33

Growth of the epiblast in vitro: Epiblast growth was observed in all of the cultured embryos although it did not occur with the same frequency in all species or on all media. Since the embryos at excision were too small to have the epiblast developed, such growths of this structure in vitro are significant. The data from these cultures were not extensive enough to be tabulated by species but were assembled in table XIX.

Epiblasts developed consistently in cultured embryos of oats, less frequently in wheat and rye and scarcely at all in barley. Epiblasts normally are absent in the latter, even in the mature embryo. In oats the epiblasts appeared on media E and H (40 mg./l. adenine and 20 per cent coconut milk respectively). They attained their greatest size on media G (coconut milk and adenine) and I (90 per cent coconut milk).

Epiblasts were present only on a few barley embryos cultured on medium I. However they were unmistakeably present

in these instances (plate IX, fig. 4; plate X, fig.3; plate XI, fig. 2).

TABLE XIX

Growth of Epiblasts of Oat, Barley, Wheat and Rye Embryos.

Medium (see p. 25):	A	B	C	D	E	G	H	I
<hr/>								
<u>Average length of</u>	<u>Agar media</u>							
<u>epiblast of:</u> Oats	0.1	0.4	0.0	0.2	0.7	1.5	0.6	1.1
Barley	0.0	0.0	0.0		0.0	0.0	0.0	+
Wheat	0.0		0.0	0.2	0.4		0.4	1.4
Rye	0.0					0.5	0.2	1.2
<hr/>								
	<u>Liquid media</u>							
Oats	0.0			0.0	0.3	0.0	0.0	0.0
Wheat	0.1			0.1				
Barley and Rye	- - no growth in liquid media							

Note: The figures in this table are recorded in micrometer units. 1 unit = 0.08 mm.

In wheat also the largest epiblasts were found on embryos grown on medium I. Those grown on D (20 mg./l. adenine sulfate), E, and H also gave rise to epiblasts, although these were not well developed.

Epiblasts of rye attained their greatest increase on medium I. There was a slight growth of epiblasts in medium G and medium H.

In liquid culture, only embryos of wheat and oats grown in media containing adenine exhibited epiblasts.

Growth of the Scutellum in vitro: The growth of scutella were recorded both in terms of length and width. Scutellar measurements were often difficult due to curling or other distortions of this organ.

Normally the scutellum grows more in length than in width, but in embryo cultures the opposite is often true, particularly on such media as A, B, C and D which do not contribute much toward embryonic differentiation.

Previously it was mentioned that when embryos were placed "face down" on the agar, the root-stem axes did not develop. Instead, these embryos produced large, callused scutella. Scutella of embryos cultured "face up" developed in a more normal manner. It is significant that the position of the embryo in culture has so marked an effect on the development of the scutellum. It seems likely that this effect is due to oxygen differentials. Callused scutella were developed when exposed to the air instead of in contact with the agar surface of the media. However, the possibility cannot be discounted that the effect may be due either to contact alone or to diffusion gradients of substances other than oxygen.

Oats: The scutella of oats in culture often grew in an abnormal manner. The cells of the lower surface did not grow as rapidly as those of the upper surface, consequently the scutella became recurved (plates V, VI and VII).

Although the development of most embryonic organs were inhibited in liquid culture, the scutella of oats grew better submerged than on surface culture (1.78 mm. long in liquid as compared to 1.53 mm. long on the surface).

TABLE XX

The Growth of Scutella of Oats in vitro.

Medium (see p. 25):	A	B	C	D	E	G	H	I
<u>Agar media</u>								
No. of embryos	46	23	30	27	28	21	59	27
Length - initial	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.48
Width - initial	0.52	0.52	0.52	0.52	0.52	0.52	0.52	0.52
Length - final	0.97	0.74	0.78	0.92	1.05	1.53	1.26	1.42
Width - final	0.84	0.72	0.78	0.79	0.89	1.18	1.00	1.26
Length - increase	102%	54%	62%	92%	119%	219%	163%	196%
Width - increase	62%	39%	50%	52%	71%	126%	92%	142%
L/W*	1.6	1.4	1.2	1.8	1.7	1.7	1.8	1.4
<u>Liquid media</u>								
No. of embryos	25			19	30		28	23
Length - initial	0.48			0.48	0.48		0.48	0.48
Width - initial	0.52			0.52	0.52		0.52	0.52
Length - final	0.96			0.98	0.87		1.78	1.19
Width - final	0.81			0.80	0.74		1.28	1.14
Length - increase	100%			104%	81%		271%	148%
Width - increase	56%			54%	42%		146%	119%
L/W*	1.8			1.9	1.9		1.9	1.2

* Ratio of increases, length/width.

The longest scutella were produced by embryos cultured in liquid medium H, followed by those on solid media G and H, and liquid medium I. The smallest scutella in this series were grown on agar media B and C. These also had the lowest length/width ratio indicating an inhibition of longitudinal growth.

There was a parallel in the growth on medium G (20 per cent coconut milk and adenine sulfate) and liquid medium H (20 per cent coconut milk). Scutellar increases were greater on these two media than on any other.

The scutella of embryos cultured on agar medium I were larger than on many of the other media. However, they later became shrunken and suberized. A brown exudate, probably of tannins, discolored the agar beneath the embryos. After two weeks of culture the scutella had become completely scabrous.

Barley: Unusual scutellar developments were observed in cultures of barley. The average increases in length and width were highest in embryos cultured on media G, I and J but extra large scutella were found on embryos cultured on one lot of medium H (20 per cent coconut milk agar). The largest of these (6.0 mm. long), shown in plate IX, fig. 7, was three times as long as any produced on other media. This embryo and one shown in fig. 4 of the same plate also show a common phenomenon seen in many barley embryos, the callusing of scutellar margins. Aceto-carminic smears of this marginal tissue showed mitotic figures. Apparently these areas are meristematically active in cultured embryos.

The development of a concavity in the scutellum which in the normally matured embryo enfolds the lower half of the coleoptile, also develops in vitro (plate IX, figs. 4 and 7). The coleoptiles of cultured embryos grew erect (plate X, figs. 3 and 4). Coleoptiles of embryos in the seed are horizontal in orientation (plate X, figs. 5 and 6). The depression in the scutellum thus appears to be formed as a result of

scutellar differentiation instead of coleoptilar pressure as one might think from observation only of the mature embryo in the seed. This is a feature which may be modified in vitro. It occurred regularly on medium I (90 per cent coconut milk), sporadically on the other media containing coconut milk (G, H and J) but never on the rest of the media.

TABLE XXI

Growth of the Scutella of Barley Embryos in vitro

Medium (see p. 25):	A	B	C	E	G	H	I	J	K
<u>Agar media</u>									
No. of embryos	38	32	32	34	34	43	29	14	23
Length - initial	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.48
Width - initial	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.56
Length - final	0.95	0.85	0.77	0.92	1.82	1.27	1.43	1.38	1.22
Width - final	1.29	1.35	1.12	1.30	2.13	1.61	1.98	1.98	1.66
Length - increase	98%	77%	60%	92%	280%	165%	198%	187%	154%
Width - increase	130%	141%	100%	132%	280%	187%	254%	254%	196%
L/W*	0.7	0.5	0.6	0.7	1.0	0.9	0.8	0.7	0.8
<u>Liquid media</u>									
No. of embryos	25	27							
Length - initial	0.48	0.48							
Width - initial	0.56	0.56							
Length - final	0.97	1.07							
Width - final	1.14	1.30							
Length - increase	102%	123%							
Width - increase	104%	132%							
L/W*	1.0	0.9							

*Ratio of increases, length/width.

Another effect observed in some scutella in cultures on

medium I was the hyperdevelopment of the ventral scale (fig.1, plate XI). This structure, located at the margin of the scutellar concavity, is sometimes compared to the ligule of the grass leaf.

The widths of scutella exceeded their lengths at the beginning of the culture period. This condition persisted throughout the culture period in most of the embryos, probably because of the meristematic activity in the marginal tissues previously mentioned. In barley as in oats this was most obvious in embryos cultured on media B and C (L/W - 0.55 and 0.60).

Scutellar growth in barley, unlike that in oats, was not increased in liquid culture.

TABLE XXII

The Growth of Scutella of Wheat Embryos in vitro.

Medium (see p. 25):	A	C	D	E	H	I	A	D
	<u>Agar media</u>						<u>Liquid</u>	
No. of embryos	37	26	29	25	29	29	32	22
Length - initial	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.48
Width - initial	0.52	0.52	0.52	0.52	0.52	0.52	0.52	0.52
Length - final	0.88	0.66	0.67	0.77	0.74	1.21	0.71	0.70
Width - final	0.82	0.68	0.68	0.74	0.84	1.21	0.74	0.72
Length increase	83%	37%	40%	60%	54%	152%	48%	47%
Width increase	58%	31%	31%	42%	62%	133%	42%	38%
L/W*	1.4	1.2	1.3	1.4	0.9	1.1	1.1	1.2

*Ratio of increases, length/width.

Wheat: The lateral growth of wheat scutella kept pace

with, but did not exceed, their growth in length. Their greatest increases were on medium I (90 per cent coconut milk). Results on other media, both agar and liquid, were uniform with the exception of medium A. Growth on the latter was somewhat reduced.

The ventral scale was well developed only on medium I (plate XIII, fig. 3).

Rye: The results from cultures of rye embryos were about the same as those of wheat.

TABLE XXIII

Growth of Scutella of Rye Embryos in vitro.

Medium (see p. 25):	A	G	H	I	H	I
	<u>Agar media</u>				<u>Liquid</u>	
No. of embryos	32	21	31	27	19	17
Length - initial	0.48	0.48	0.48	0.48	0.48	0.48
Width - initial	0.24	0.24	0.24	0.24	0.24	0.24
Length - final	0.58	0.86	0.92	1.12	0.90	0.93
Width - final	0.56	0.74	0.82	1.04	1.01	0.74
Length - increase	21%	80%	92%	133%	87%	94%
Width - increase	133%	208%	242%	333%	321%	208%
L/W*	0.2	0.4	0.4	0.4	0.3	0.5

*Ratio of increases, Length/Width.

Scutellar development on agar medium H (20 per cent coconut milk) was alike for both wheat and rye. Embryos of wheat were not grown in H and I liquid cultures but in rye results were obtained which were interesting from a morphogenetic standpoint. The scutella of embryos cultured in H

were widened by callus-like outgrowths from the marginal tissues, but this did not occur in the higher concentration of coconut milk in medium I. Representative embryos grown in these two liquids are shown in plate XV, figs. 2 and 3.

The greater increases in width, in terms of per cent, exhibited by all of the cultures are not so indicative of greater final width as they are of a higher rate of lateral growth, since the embryos were relatively narrow to begin with (plate XV, fig. 1). For example, on medium I the initial ratio of length/width was 0.48/0.24 or 2.0. The final length/width ratio was 1.12/1.04 or 1.1. The ratio of increases was 133/333 or 0.4. This is indicative of a higher rate of lateral growth.

Plate XV, fig. 4, is an illustration of an embryo which had been cultured in liquid and had remained floating upside down instead of sinking to the bottom of the tube. In several such embryos, the scutella grew to such an extent that they exceeded the embryonic axes in length.

Development of the shoot in vitro: Although measurements were made only of the external structures of cultured embryos, internal developments were observed in sectioned embryos. The results are summarized in Tables XXIV and XXV.

The development of the shoot apex in vitro, in terms of the number of leaf primordia formed, shows that media containing coconut milk and agar allow nearly normal growth and development of leaf primordia, but that on media not containing this substance, or in liquid media, further embryonic differentiation of shoot structures did not take place. These are

illustrated in embryos pictured in plates VII, VIII, X, XII, and XIV.

TABLE XXIV

Development of the Shoot in vitro.

Number of leaf primordia present after seven days in culture.

Medium (see p. 25):	A	E	G	H	I	M*
<u>Agar media</u>						
Oats - No. leaf primordia	1	2	2	2	2	2
Barley- " " "	1	-	2	2	2	3
Rye - " " "	1**	-	-	-	3	3
Wheat - " " "	1	-	-	2	2	3
<u>Liquid media</u>						
Oats - " " "	1	-	-	1	-	2
Wheat - " " "	1	-	-	-	-	3

*Embryos matured in the seed.

**Through an oversight, embryos grown on A were not preserved for sectioning; however, their later growth in culture indicated the presence of one and at most two primordia.

Root differentiation in vitro: Except in media containing coconut milk, differentiation of lateral root primordia did not occur, but elongation of the primary root took place. In embryos that were maintained on media which did contain coconut milk, the lateral root primordia were formed. This was particularly evident in cultures on I. Here again the difference between the two types of response of embryos grown in vitro is illustrated. Embryos on some media grew as seedlings, mainly through elongation of existing

organs, while on other media differentiation of structures continued in much the same way as in normal embryogenesis.

TABLE XXV

Development of Root Primordia in vitro.

Number of root primordia present at the end of seven days' culture.

Medium (see p. 25):	A	E	G	H	I	M*
<u>Agar media</u>						
Oats - No. root primordia	1	1	1	3	3	3
Barley- " " "	1	-	-	1	3	7
Wheat - " " "	1	-	-	1	3	5
Rye - " " "	1**	-	-	-	3 (5)#	-
<u>Liquid media</u>						
Oats - " " "	1	-	-	1	-	3
Wheat - " " "	1	-	-	-	-	-

*Embryos matured in the seed.

**Observed in vitro. Through an oversight embryos grown on A were not preserved for sectioning; however their later growth in culture indicated the development of the primary root only.

#Observed in embryo cultured two weeks.

CULTURE OF IMMATURE EMBRYOS

Discussion

General effects: An interesting aspect of plant embryo culture is precocious germination. This phenomenon has been observed by numerous investigators both in monocotyledons and in dicotyledons. Dietrich (1924) observed that embryos of oats, wheat and barley were able to grow in culture when but a third of their mature size. However, their development in vitro was not one of further differentiation of embryonic

structures but consisted of premature outgrowth of the roots and shoot. Nearly twenty years previously, Hännig (1904) had commented upon this same anomalous response in cultures of excised immature embryos of several members of the Cruciferae. LaRue and Avery (1938) who grew excised embryos of Zizania, termed this exhibition of seedling characteristics "precocious germination".

In the present investigation this phenomenon occurred on defined media, such as White's solution with and without IAA and adenine sulfate; differentiation of new roots and shoot primordia did not occur.

For some time very immature embryos could not be grown in vitro. Since premature germination consists of the outgrowth of the roots and the shoot apices already formed, it is clear that a barrier to the growth of smaller embryos or proembryos is simply the absence of roots or shoot primordia from which to grow.

Up to the present time the most successful cultures of undifferentiated embryos have been made with embryos of the genus Datura. So far as the writer knows, his culture of barley embryos as small as 0.3 mm. is the first record of such, in members of the family Gramineae.

The problem of whether embryos are plantlets of determinate growth in the embryonic state or whether they are capable of indefinite development, if certain conditions are met which allow a continuation of the pregerminal growth and differentiation, is one of great interest to the experimental morphologist. LaRue (1951) speculates that if excised embryos

of the grasses can be induced to continue to grow as embryos in vitro, abnormally large embryos may result. Embryos so large, in fact, that superior plants might eventually be produced. Since the reduced shoot apices of immature embryos which germinate prematurely in vitro produce small plants with leaves and vascular bundles, the opposite might also hold true. Larger embryos with hyperdeveloped apices should produce large seedlings with numerous leaves and vascular bundles. Kent and Brink (1947) actually were able to obtain embryos of barley which were larger than normal using such substances as casein hydrolysate and tomato juice, and higher (up to 12.5%) sucrose levels. However, the authors make no mention of the seedling characteristics of the plants produced.

In the cultures of embryos in the present study, continued embryonic differentiation in vitro often was observed. Some of the embryos approached, and in some instances equaled, the developments of roots and shoot of the mature embryos in the seed. In none of these cultures, however, did the development in vitro of root and shoot primordia exceed that in vivo. The eventual sizes of excised, immature embryos grown on some media often was greater than that of naturally matured embryos. This apparently was due to the greater growth in the size of the cells of the cultured embryos.

These results were obtained with a medium containing 90 per cent coconut milk. The efforts of others who have used coconut milk in the culture of immature embryos of the grasses have not been as successful. Haagen-Smit et al (1945) tried coconut milk in cultures of immature maize embryos but

were not able to ascertain any benefit from its use. Ziebur and Brink (1951) also had little success with coconut milk in concentrations of 20 to 50 per cent in cultures of immature embryos of barley. In cultures of dicotyledonous embryos, other than Datura, similar failures have been reported. Smith (1944), Hall (1943), and Lofland (1950) all tried coconut milk in cultures of various species without success.

In the cultures of the four cereals used in the present investigations, coconut milk has produced somewhat variable results, particularly at the lower concentration (20 per cent). This is probably due in part to the lack of uniformity in the milk, since the coconuts used varied in the length of time they had been stored, in the amount of milk they contained, and perhaps in other factors as well. On 90 per cent coconut milk, embryonic differentiation and the inhibition of premature germination have been observed in all of the cultured species. The germination of immature embryos of oats and barley were delayed to some extent, while in rye and wheat embryos a prolonged period of dormancy occurred.

The difficulties which other workers have had in the use of coconut milk may stem from their not having used the higher concentrations. The writer also has observed that some investigators do not make a distinction between what might be termed true embryo culture, the culture of immature embryos in order to obtain continued embryonic growth, and seedling culture, the culture of excised embryos in order to obtain seedling plants directly. Those who have objected to the use of coconut milk because it interferes with, or inhibits,

normal seedling growth, have obviously failed to grasp the significance of the effect since such inhibition is precisely the effect which the use of coconut milk is intended to produce.

Embryo factor has been found in other solutions as well as in coconut milk. Blakeslee and Satina (1944) found that in cultures of young Datura embryos, malt extract could be substituted for coconut milk. Van overbeek et al. (1944) tested extracts, such as of Datura ovules, coconut meat, wheat germ, and yeast, and found that they were active, although not to the extent of coconut milk. Ziebur and Brink (1951) tried extracts of barley endosperm, malt, potato tuber, dried peas and beans, frozen peas and sweet corn, fresh peas, and found that positive results were obtained only with extracts of barley endosperm. With excised barley endosperm, which was placed on the agar surrounding the excised embryos of barley, they were able to obtain growth and eventually seedlings from differentiated embryos as small as 0.5 mm. Sanders and Burkholder (1948), Paris and associates (1953), obtained differentiation of young Datura embryos on casein hydrolysate and found that certain amino acids could be effectively substituted for casein hydrolysate. The latter make an interesting comment on the appearance of thriving embryos on media containing "embryo factor". They say that on casein hydrolysate media the embryos were white and opaque, while on the control media, without this source of "embryo factor", the embryos were nearly transparent. Ziebur and Brink (1952) found this to

be true under similar conditions in the culture of barley embryos. In the present study this aspect has been mentioned in connection with the difference between embryos grown on 90 per cent coconut milk and those grown on the other media.

There are probably a number of factors involved in the "embryo factor" effects. It has been known for some time that higher osmotic pressure in culture media inhibits germination of excised embryos. Dietrich (1925) obtained better results with 5 per cent sugar than with 1.5 per cent. Reitsema and associates (1953_b) report that the optimum sucrose concentration for growth of very young Datura embryos was 8 per cent, and that lower concentrations were better for the growth of older embryos. Nearly mature embryos had an optimum sucrose requirement of 0.1 to 1.0 per cent. They also found that smaller embryos did not grow on media containing .2 per cent sucrose as a carbohydrate source. The embryos, however, would grow if the osmotic pressure of the medium was raised using manitol, a substance not utilized by the embryos as a carbohydrate source. Ziebur and associates (1951) and Ziebur and Brink (1952) found that the higher osmotic pressures obtained with casein hydrolysate prevented germination in older barley embryos, but when this substance was used in cultures of younger embryos it proved to be inhibitory. They substituted 12.5 per cent sucrose in order to obtain the desired osmotic pressures in their medium but found that this alone was not enough to produce differential growth in very small embryos. They then used excised barley endosperms to supplement the effects of the higher sucrose level, and thus

obtained a measure of differentiation and growth of relatively undifferentiated embryos.

Rijven (1952) obtained good results from various amino acids but concluded that something still was missing from his medium, since undifferentiated embryos could not be grown. Van Overbeek et al (1944) tested a number of pure substances including sugars, purines, amino acids and vitamins, without finding "embryo factor" activity in any of them. IAA has been used in a number of investigations but does not seem to induce differentiation in immature embryos, although at levels below 0.1 mg./l. it appears to have a slightly stimulating effect on growth (Reitsema et al 1953_a). Higher concentrations are inhibiting, as demonstrated by the results of cultures on the medium containing 1.0 mg./l. of IAA in the present series of experiments.

The majority of the investigators cited in the preceding pages have tried cultures in both liquid and solid media. The results have been conclusive. Embryos grow much better exposed to air than in liquid. However, Dietrich (1924) found that nearly mature embryos could be grown without germination when cultured just under the surface of nutrient agar. Van Overbeek and his associates, in the cultures of Datura embryos previously cited, have also found that they obtained better results with cultures of embryos just under the surface of the agar. Our own experiments indicate that liquid culture is effective in inhibiting premature germination, but that such cultures are almost completely ineffective in the maintenance of embryonic differentiation.

There is general agreement that pH and temperature are not limiting within the range 5-7 and 20-30°C. The present experiments were conducted within these ranges.

"Embryo factor" therefor does not seem to be a single substance but rather the product of interactions between a number of substances. It is present in coconut milk and is effective to a point in the promotion of embryonic differentiation of embryos of some species, at least. With 90 per cent coconut milk, differentiation and growth of apparently undifferentiated proembryos of barley have been induced. The smallest of these, an embryo of barley 0.16 mm. in length, developed into a seedling but the growth seemed to have been a regeneration from a callus rather than normal embryogenesis, and the plant consisted of two shoots rather than one as well as having an extra cotyledon and numerous roots. The development of abnormal and supernumerary organs in undifferentiated or nearly undifferentiated embryos in vitro, has been reported by Rijven (1952), McLean (1946), Van Overbeek et al. (1942), and Sanders and Burkholder (1948). It apparently is associated with such cultures. Rijven suggests that the induction of form in proembryos may be a result of concentration gradients of growth substances within the ovule. Such gradients have not been duplicated in vitro. The morphogenetic effects of oxygen gradients in embryos cultured upside down and right side up on agar media and on embryos cultured in nutrient liquids indicate that diffusion gradients cannot be ignored in the culture of excised embryos.

Present results indicate that grass embryos may develop

in near normal fashion at all stages of development including those in which the first signs of external differentiation are visible, but that smaller, undifferentiated embryos first produce callus-like masses from which roots and shoots are produced later. Such differentiation and organization may be a result either of regeneration or from outgrowths of initials which somehow have become dislocated.

If it is true that embryonic differentiation depends on an interaction of factors as the preceding account indicates, then it becomes a necessity that further studies of differential responses on the part of immature embryos to various morphogenetic factors be undertaken. The present study does reveal some such responses in the developments of excised embryos, particularly to such substances and factors as IAA, adenine sulfate, oxygen tension and "embryo factor". These will now be discussed as they affect the development in vitro of individual organs and tissues of the four species of grasses studied.

The coleorhiza: The growth of coleorhizae in the cultured embryos ranged from apparent decreases in length, observed in barley, to increases of nearly 1000 per cent in coleorhizae of oats. Very often the epidermal cells of oat coleorhizae produced hairs when cultured on nutrient agar. The coleorhizae of all species, when cultured on 90 per cent coconut milk medium, continued to differentiate, at least to some extent. From these examples it is apparent that various morphogenetic effects can be induced in vitro.

The elongation of coleorhizae of mature embryos of oats

has already been shown to be due to reduced oxygen tension effected either through culture in liquid media or dilute air. That this same effect should appear in the coleorhizae of immature oat embryos, cultured not in liquid media but on agar to which adenine sulfate had been added, would seem quite puzzling except for a parallel phenomenon observed in some cultures of tobacco callus. White (1939) reported that stem callus tissue of tobacco grew in an undifferentiated state when cultured on agar but that if it was allowed to grow partly immersed in nutrient liquid, buds and roots were produced. Skoog (1944) also reported this and Skoog and Tsui (1948, 1951) and Miller and Skoog (1952) later reported that similar results were obtained on nutrient agar to which adenine sulfate had been added. The optimum concentrations (40 mg./l.) markedly increased bud formation in the tobacco stem callus. They further discovered that this induction could be reversed with small amounts of IAA (0.0005 mg. IAA counteracted the effect of 40 mg. adenine). It would seem from this that in some way adenine sulfate is able to produce the same response in plant tissue as that effected by reduced oxygen tension and that this is responsible for the outgrowth of coleorhizae on media containing adenine sulfate. These results were obtained in but two sets of cultures. However, in these two instances all of the embryos cultured exhibited the effect. Attempts to reproduce the results of the first successful cultures met with failure, although repeated trials were made with fresh media made up from new ingredients. Finally the same effect was again obtained on media containing

adenine sulfate and coconut milk, but as before subsequent attempts to obtain positive results were unsuccessful. Since IAA appears to act in opposition to adenine sulfate, the possibility of auxin contamination in the media cannot be overlooked. It appears more likely, however, that the failure lies with the embryos. Coleorhizal elongation occurred in only one of the 0.8 mm. embryos of oats which were cultured in nutrient liquid. Since elongation occurred so irregularly in liquid culture, perhaps one should not expect embryos cultured on agar media containing adenine to respond at all times. Older embryos which did produce coleorhizal elongation in liquid should also have responded to adenine treatment on agar, but did not. Germination took place within a matter of hours and the coleorhizae became torn by the rapidly expanding seminal roots. In older embryos auxin may also be produced by the rapidly growing meristems in amounts sufficient to counteract the effects of adenine sulfate. Skoog and Tsui (1951) report that in tobacco callus in which budding had been initiated through the use of adenine, further bud induction was inhibited by the auxin produced by the buds already formed.

Because of the unpredictability of results the relationship between IAA and adenine in coleorhizal elongation was not tested. It could not be ascertained whether failure to elongate with mixtures of adenine and IAA was due to the effects of IAA or to the same factors which resulted in a lack of response on adenine media alone.

In cultures of barley embryos similar coleorhizal

elongations were noted on adenine sulfate media although they were not of the same magnitude as those in oats. Barley embryos grown on a mixture of adenine and IAA did not respond in this way. The sample was small (14 embryos) but is at least indicative of a relationship between IAA and adenine such as has been reported by Skoog.

The coleoptile: Various morphogenetic responses in coleoptiles of cultured, immature embryos have been observed. Coleoptiles of all species cultured on 90 per cent coconut milk agar developed more or less as in normal embryogenesis. The coleoptiles grew by cellular elongation on most of the other media. Probably the most conspicuous feature of the lack of continued differentiation in vitro is the failure of the apical pore to close. The result is the formation of a bladelike coleoptile rather than a tubular one. This was most apparent in cultures of barley embryos on White's agar which lacks the necessary substances to promote embryonic growth. In these cultures the leaflike coleoptile was open on the side opposite the scutellum (plate IX, fig. 3; plate XV, fig. 6). It was alternate in relation to the first foliage leaf and followed the alternate distichous arrangement of successive leaves.

The similarity to the arrangement in succeeding leaves is so obvious as to lead to the conclusion that it is in reality a modified leaf. This appears to be of some phylogenetic significance in light of a recent paper by Reeder (1952) on the coleoptile of a primitive grass, Streptochaeta, which is discussed on page 9. The well known observation

that juvenile appendages in plants, in particular the seedling leaves, tend to display a kind of atavism might also be applied to the development of the coleoptile in premature germination.

The epiblast: Perhaps the most interesting observation regarding the formation of epiblasts in immature embryos in vitro is that in embryos of barley, which normally do not possess this structure at maturity, rather well developed epiblasts appeared in some cultures on 90 per cent coconut milk. Barley seeds are invested with tough, adherent lemmas and paleas. The embryo appears to be somewhat compressed, which may account for the failure of epiblasts to develop in nature. If so, the development in vitro is partly due to the continued differentiation induced by the coconut milk and also to the freedom from the pressures of seed coat and husk. Epiblast development in oat, rye and wheat embryos was also stimulated by 90 per cent coconut milk. Adenine sulfate appeared to stimulate epiblast growth in some instances. This probably is part of the same phenomenon reported in the case of coleorhizal elongation.

The scutellum: In immature embryos of the size cultured in the preceding experiments, the scutella are wider than they are long, except in those of rye, which are rather narrow. The scutella of the mature embryo in the seed are usually quite elongate. Their growth in vitro, whether of the pre-germinal or postgerminal type, did not resemble normal embryogenesis since they continued to grow as much laterally as longitudinally. The result was that the scutella after a period of culture often were as broad or broader than they

were long. This was most noticeable in embryos of barley but also occurred in wheat and rye. The scutella of oats generally became more elongate, but since they at the same time became recurved in most cultures, their resemblance to normal scutella was often not very apparent. This continued lateral growth in vitro may be a result of freedom from the pressure of the seed coat, but more probably it is due to a failure of the scutella to differentiate normally, perhaps because of the lack of proper concentration gradients in the artificial substratum. On certain lots of coconut milk media, the scutella of barley elongated enormously. The scutella of rye embryos expanded laterally when cultured on 20 per cent coconut milk liquid, but did not do so in 90 per cent coconut milk liquid. Scutellar growth in oat embryos on 90 per cent coconut milk is inhibited. The possibility exists that certain concentrations of substances present in the coconut milk (and by inference in the endosperm of the seed) may be essential for the development of form in scutella.

Oxygen gradients too seem to influence the development of scutella. Embryos which were inadvertently cultured upside down, with the lower scutellar cells exposed to the air instead of in contact with the media, responded by developing large, cushion-like pads of callused scutellar tissue which in some cases completely outstripped the development of the embryonic axes. Recurved scutella were observed in all cultured immature embryos but were most evident in those of oats. These developments apparently were due to the failure of the lower epidermal cells to grow apace with the cells

above. These cells give rise to the palisade-like lower epidermis of the scutellum and are rather specialized in comparison with those of the upper epidermis. It is probable that they have certain requirements which are not met in the environment in vitro. The greater growth of the lower epidermal cells in embryos cultured upside down seemed to be of the nature of callus and was a response to higher oxygen tensions. For a time it appeared that the greater growth of upper scutellar cells, compared to the reduced growth of the lower epidermis in contact with the agar, was a result of lower oxygen tension at the surface of the media. Later this same type of growth was observed in scutella of oat embryos grown in liquid culture in which the concentration gradients of substances presumably were equal at all surfaces; yet the lower scutellar cells still did not grow at the same rate as the upper cells, and curved scutella were also produced. These observations seem to support the theory that concentration and diffusion gradients, probably of nutritive substances, are necessary for normal development of the scutellum.

Coconut milk appears to contain substance which may accelerate growth and differentiation of scutella at certain concentrations. Van Overbeek et al. (1942) observed that abnormally large and anomalous cotyledons were developed by Datura embryos cultured in coconut milk media. They postulate a special cotyledon factor in coconut milk and refer to the tremendous cotyledonary development in the germinating coconut embryo which often completely fills the lumen of the coconut kernel. The present investigation indicated that the substance

or substances responsible for this stimulation of cotyledonary growth is more effective at lower concentrations than at higher levels, thus tending again to substantiate the theory that concentration gradients are responsible for normal development of the scutellum.

LaRue (1936) observed abnormal developments of scutella of maize embryos in vitro and later (1952) cultured scutella of maize from which the embryonic axes had been dissected. In the latter experiments he obtained scutella which grew to twice the size of those of the mature seed. Some of the larger barley scutella similar to the one pictured in plate IX, fig. 7, were removed from the embryos and allowed to remain in culture, but they did not continue to grow. These observations indicate that the scutellum is an organ of rather indeterminate size and form, but one incapable of continued growth in vitro--at least with present techniques.

In embryos which have matured in the seed there is a depression in the upper surface of the scutellum. This depression or concavity appears to have been formed as a result of downward pressure from the coleoptile. In excised immature embryos cultured on 90 per cent coconut milk agar the concavity also appeared, but in this case it was apparent that the coleoptile, which in vitro is usually more erect than in vivo, plays no part in the formation of its depression. This was most apparent in barley. In addition, the ventral scale sometimes became larger in vitro than in vivo. The development of these two features in culture are suggestive of the leaf sheath and ligule present in the foliage leaves

of the grasses.

Immature barley embryos cultured on 20 per cent coconut milk agar produced outgrowths from the axils of coleoptiles in some instances. Buds have been reported to be formed in the axils of coleoptiles of several grasses by Kennedy (1897), Avery (1930), and Yung (1938). For a time it was thought that the structures observed here in barley might be axillary buds, but sections showed that they contained only parenchymatous tissue. Due to this lack of organization such foliar relationships can only be surmised.

The shoot: It has been shown that a seedling type of growth took place in cultures of immature embryos on agar media which lacked the substances necessary for further differentiation. In cultures on liquid media, neither germination nor development of new organs occurred. Embryos cultured on 90 per cent coconut milk agar continued their embryonic growth. Differentiation of leaf primordia proceeded in essentially normal fashion. In oats and rye, the same number of primordia were formed as in normally matured embryos. One less than the normal complement were formed in wheat and barley. Even in smaller embryos of barley (less than 0.8 mm.) in which no primordia were visible at time of excision and culture, normal shoots were produced.

In rye embryos which remained dormant on 90 per cent coconut milk agar, the possibility was considered that over a longer period of time supernumerary leaf primordia might develop. Free hand sections were made of rye embryos which had remained in culture for two months without germinating.

No more than three leaf primordia were observed in any of these. The possibility exists that under some conditions further differentiation of leaf primordia might occur, but on the basis of the present observations their embryonic differentiation must be regarded as determinate.

The root primordia: The development of roots in cultures on various media was similar to that of other organs. Sometimes the growth was of a postgerminal nature in that it took place through activity of the root meristem and elongations in existing cells. At other times it was of the pregerminal type and consisted of the differentiation of new root primordia. A third type of development also was observed. It was the combination of both embryonic growth and later germination. Only in the oat embryo, which normally possesses three seminal roots at maturity, did root development in vitro equal that in vivo. In the others, fewer roots were formed in vitro than in vivo (three as compared to five in wheat and rye, five as compared to seven in barley). In barley embryos, some of which were very small when placed in culture, seminal roots in numbers up to seven were produced after germination had taken place. In one instance more than this were seen. One embryo measuring only 0.16 mm. in length when excised had ten roots after a month in culture.

Conclusion: On the basis of the results obtained in the preceding experiments, an attempt should be made to answer the questions which were raised in the statement of the problem contained in the introduction to this study.

As to the continuation of pregerminal differentiation

in vitro, it now seems clear that a measure of success has been obtained through the use of media containing 90 per cent coconut milk.

The effect of outside pressures exerted by seed coats and husks upon the development of the final form of the grass embryo in vivo also appears to have been determined, at least to some extent. In these cultures the excised embryos have shown certain morphogenetic responses as a result of their freedom from restricting envelopments. Fundamentally, however, the form of embryos grown in culture was much the same as in vivo. Thus it appears that the final form is either impressed upon the embryo in some way in its early developmental stages, or is present from the first divisions of the zygote and cannot be radically altered through culture. Nevertheless, within this "framework" of form it has been found that various biochemical substances and physiological factors may effect pronounced variations in the morphogenesis of cultured embryos. This was particularly evident in the developments in embryonic structures such as the coleoptile, coleorhiza, epiblast and scutellum.

PART II

CULTURES OF OVULES, ENDOSPERM AND EMBRYOS OF SOME GRASSES

Introduction

In a search for methods of culturing immature grass embryos in vitro, the work of Harlan and Pope (1926) on the ripening of immature seeds of barley after harvesting came to the attention of the writer. These authors discovered that seeds of barley eventually produced viable seeds even though harvested just five days after pollination. Immature embryos continued to grow and differentiate for a time after the parent plants had been cut down if the seeds were allowed to remain on the culms, but embryos in naked seeds which were removed from the culms and placed in damp chambers failed to mature. This work suggested that unripened ovules of grasses, if removed from the seeds and placed on nutrient agar, might continue to grow and develop. There were precedents for this hypothesis. LaRue (1942) had cultured flowers of a number of species on nutrient agar and had obtained the development of fruits in some instances. Nitsch (1951) cultured the excised ovules of several species and obtained the development of fruits which contained viable seeds.

Immature, excised ovules of barley, wheat, rye and English rye grass, Lolium perenne L. were cultured with some success in the present investigation. The ovules of oats proved to be quite difficult to remove intact and so were not used.

A significant development in these experiments was the

outgrowth of a tissue from the endosperm of rye grass which could be cultured indefinitely. Embryos of wheat, rye and barley grew in this type of culture, though not as well as had been hoped. Additional embryos of barley and rye grass also were cultured in contact with the endosperm derived tissue of Lolium and also on a medium containing an extract of the cultured endosperm. However, since the successful culture of an endosperm tissue seemed to be the most significant result of these experiments, this section of the thesis is devoted primarily to it.

Material and Methods

The procedures used in connection with the harvest of immature seeds of the four cereals, together with their descriptions and the source of supply, have already been discussed in part I and require no further elaboration. English Rye Grass is a common European meadow grass which has become naturalized in the United States. The plants used in the present investigation were found on the campus of the University of Michigan.

Media: Several media were used in culturing excised ovules, embryos and endosperm. Except for slight modifications, they were identical to those used in part I, and reference should be made to pages 23-26 for a description of the media and their preparation. Exact descriptions of the media are made in the following pages wherever applicable.

Culture methods: Immature seeds of barley, wheat, rye and rye grass were removed from the plants and placed in a

ten per cent solution of commercial Clorox and water. After ten minutes of sterilization the seed coats were removed. The ovules then were placed in screw cap vials containing a modified White's agar with added coconut milk and IAA (medium II). Medium II, except for the addition of 1.0 mg./l. of IAA, was the same as medium H (see page 25) used in the culture of immature embryos. Cultures of intact seeds were made at the same time as those of excised ovules. These cultures were established as controls.

The ovules of wheat, rye and barley, when excised, contained embryos that were about 0.4 mm. long. Rye grass ovules contained embryos measuring between 0.2 and 0.4 mm. in length. The size of the embryo was determined by making a small opening in the integuments of the ovule and measuring the exposed embryo with the aid of an ocular micrometer. The cultures of both ovules and intact seeds were placed in darkness at room temperature where they remained for five days. At that time they were removed for examination and any changes that had taken place in either the ovules or embryos were recorded.

Later on, cultures of excised ovules were made on White's medium without IAA (medium A, table III) and on a medium containing 0.25 per cent yeast extract and 1.0 mg./l. IAA (medium III, table XXVII). The tissue obtained from the endosperm of rye grass was cultured on a number of media, both in light and darkness and at various temperatures and levels of pH. Finally, excised embryos of barley and rye grass were cultured adjacent to bits of endosperm tissue. Some also were

cultured on a medium containing a water extract of the tissue.

Results

Cultures of excised ovules: After five days of culture the ovules of wheat, barley, rye and rye grass had undergone certain changes both in the nature of the endosperm and embryos. In the first three species the results were disappointing. The growth of the embryos was quite variable. In a few instances the embryos had continued to grow and differentiate without germinating but in most the embryos had formed small masses of undifferentiated cells. In no case did the growth of embryos in ovules surpass that of excised embryos cultured on 90 per cent coconut milk.

In most of the excised ovules the endosperm has grown to some extent. Intumescences from the endosperm projected inwardly partially filling a lumen which had formed in the ovules. Occasionally small callus outgrowths of endosperm were observed along the margins of the openings previously made in order to measure the embryos. Attempts to obtain further growth of these bits of endosperm failed. Most of the intact seeds, used as controls, became distended with water. The embryos within either had died or else had not grown. In a few instances the seeds had continued to mature, were filled with endosperm, and contained well developed embryos.

Callus-like outgrowths of endosperm tissue were obtained from excised rye grass ovules on both coconut milk and yeast extract media. The time required for this development was not noted for the coconut milk cultures; however, in

the cultures on yeast extract the callus outgrowths became microscopically visible in five days' time (see fig. 1, plate XIX). About twenty per cent of the endosperms in culture produced tissue masses large enough to be subcultured. The ovules at time of excision were nine or ten days past pollination.

Growth of rye grass endosperm in vitro: In order to determine the proper time for subculturing the tissue, data were obtained relative to the rate and nature of its growth on 0.25 per cent yeast extract medium. This was done by establishing five sets of cultures, each set consisting of a number of tubes (usually forty) among which a known weight of tissue was distributed in approximately equal amounts (about 100 mg. per tube).

At the end of five days the tissue contents of the first set were pooled and weighed, then dried and reweighed. At the end of 10 days, 15 days, 20, and 25 days, the remaining sets were similarly handled. All sets were run in duplicate. The results of these experiments are shown in table XXV and plate XVII.

A lag period in the growth of the tissue persisted for about five days after inoculation. The growth was most rapid during the 10 to 15 day period with a maximum increase in dry weight at 20 days of more than 1600 per cent. Wet and dry weights taken on the 25th day of culture indicated that little growth took place after the third week on media containing yeast extract. Microscopic examinations of tissues of this age revealed many dead cells and a decrease in stored starch.

Subcultures made with tissues of this age usually failed to grow.

It was discovered that the average dry weight, based on the results of a number of cultures, after 20 days on the medium and under conditions cited in the heading of table XXV, was 3.5 per cent of the wet weight of the tissue. This figure has been used throughout these experiments to calculate the dry weight of the inocula.

Growth on various media, at different temperatures, in light and darkness: When the nature of the growth of the endosperm in culture had been established, it was decided to determine what the culture requirements were for the optimum growth of the tissue. To this end, the tissue was grown on various media as well as at several levels of pH and temperature and in light and darkness. The methods of inoculation and culture were the same as before, except that the cultures were grown only for twenty days. Both wet and dry weights were recorded for each set of cultures. The results of these cultures are shown in tables XXVI, XXVII, XXVIII, and XXIX.

Growth on White's medium: The growth of endosperm on the modified White's medium (I--table XXVI) was a great deal less than on any other media used in these experiments. The maximum increase in wet weight of 82 per cent and in dry weight of 127 per cent was half that attained by the tissue in ten days' culture on medium III (0.25 yeast extract, see table XXV). However, the tissue still was alive after twenty days and might have grown more if cultured over a longer period of time.

TABLE XXVI

The growth rate of rye grass endosperm, cultured on a medium containing 0.25 per cent yeast extract, 2 per cent sucrose, and 1.0 mg./l. IAA, in darkness at constant 26° C.

Days Set		Initial weight gms.		Final weight gms.		Increase	
		WET	DRY	WET	DRY	WET	DRY
5	1	4.873	0.169	5.185	0.210	6%	24%
5	2	<u>4.072</u>	<u>0.142</u>	<u>4.202</u>	<u>0.172</u>	<u>3%</u>	<u>21%</u>
Ave.		4.472	0.155	4.693	0.191	4%	22%
10	1	3.876	0.136	16.061	0.531	312%	290%
10	2	<u>3.260</u>	<u>0.114</u>	<u>12.178</u>	<u>0.410</u>	<u>273%</u>	<u>280%</u>
Ave.		3.568	0.125	14.119	0.470	292%	285%
15	1	3.604	0.126	43.752	1.192	1114%	846%
15	2	<u>4.075</u>	<u>0.143</u>	<u>35.043</u>	<u>1.256</u>	<u>760%</u>	<u>778%</u>
Ave.		3.839	0.134	39.397	1.224	937%	812%
20	1	1.863	0.065	30.384	1.103	1531%	1597%
20	2	2.035	0.071	34.416	1.180	1591%	1561%
20	3	<u>3.985</u>	<u>0.139</u>	<u>75.920</u>	<u>2.266</u>	<u>1805%</u>	<u>1530%</u>
Ave.		2.628	0.092	46.907	1.516	1642%	1563%
25	1	1.961	0.067	37.535	1.253	1814%	1770%
25	2	<u>1.933</u>	<u>0.068</u>	<u>24.658</u>	<u>1.224</u>	<u>1693%</u>	<u>1700%</u>
Ave.		1.947	0.067	36.096	1.238	1753%	1735%

Note: Days - days in culture.

PLATE XVII

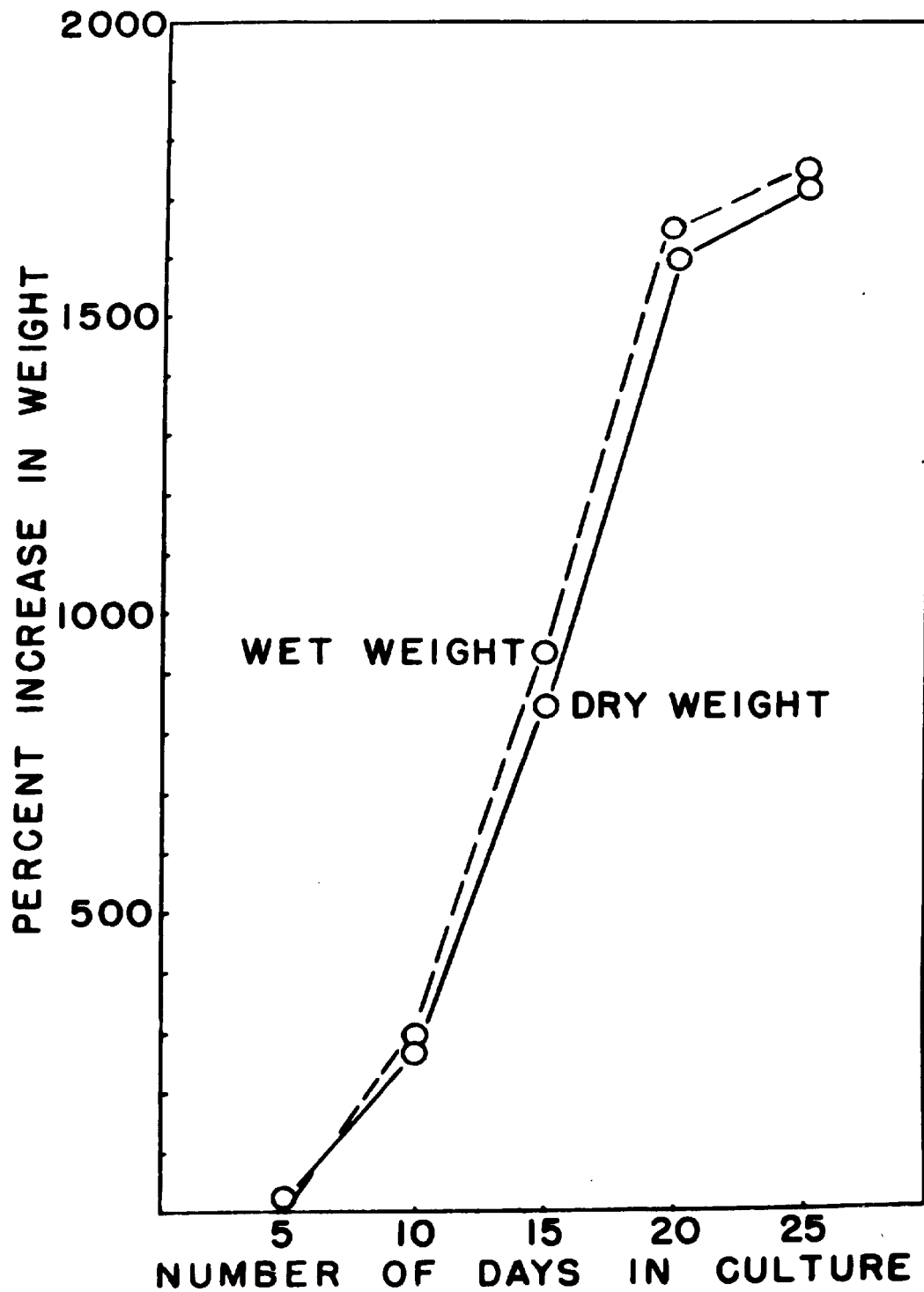


Figure 1. Growth rate of rye grass endosperm in vitro.
Data from table XXV.

TABLE XXVII

Growth of rye grass endosperm cultured on various media for 20 days (except as noted) in darkness at constant 26°C.

Medium Set		Initial weight mgs.		Final weight mgs.		Increase	
		WET	DRY	WET	DRY	WET	DRY
I	1	3.975	0.139	7.225	0.300	82%	116%
	2	3.680	0.127	6.327	0.293	72%	127%
	Ave.	3.827	0.133	6.776	0.296	77%	121%
II	1	3.348	0.130	37.052	1.702	1067%	1355%
	2	4.024	0.141	52.908	2.169	1215%	1438%
	Ave.	3.686	0.135	44.980	1.935	1141%	1396%
III	Ave.*	2.628	0.092	46.907	1.516	1642%	1563%
IV	1	3.973	0.139	52.534	2.014	1222%	1349%
	2	3.911	0.137	49.661	2.012	1170%	1367%
	Ave.	3.942	0.138	51.097	2.013	1196%	1358%
	3**	3.877	0.136	70.114	3.285	1708%	2315%
V	1	3.837	0.134	52.125	1.687	1256%	1159%
	2	3.996	0.140	60.629	1.877	1417%	1241%
	Ave.	3.917	0.137	56.377	1.782	1326%	1200%
VI	1	3.506	0.123	72.411	2.080	1965%	1691%
	2	3.873	0.135	84.999	2.384	2094%	1666%
	Ave.	3.689	0.129	78.705	2.232	2029%	1678%
VII	1	4.010	0.140	41.023	1.266	924%	804%
	2	4.213	0.147	39.513	1.358	838%	828%
	Ave.	4.111	0.143	40.268	1.312	881%	816%

Explanation: *From table XXV--twenty days' culture.

**Cultured for 25 days.

Medium I White's medium with 1 mg./l. IAA.
 Medium II White's medium with 1 mg./l. IAA and 20 per cent coconut milk.
 Medium III 0.25 per cent yeast extract, 1 mg./l. IAA, 2 per cent sucrose.
 Medium IV Same as III except 4 per cent sucrose.
 Medium V Same as III except no IAA.
 Medium VI Same as III except 0.50 per cent yeast extract.
 Medium VII Same as III except 0.75 per cent yeast extract.

PLATE XVIII

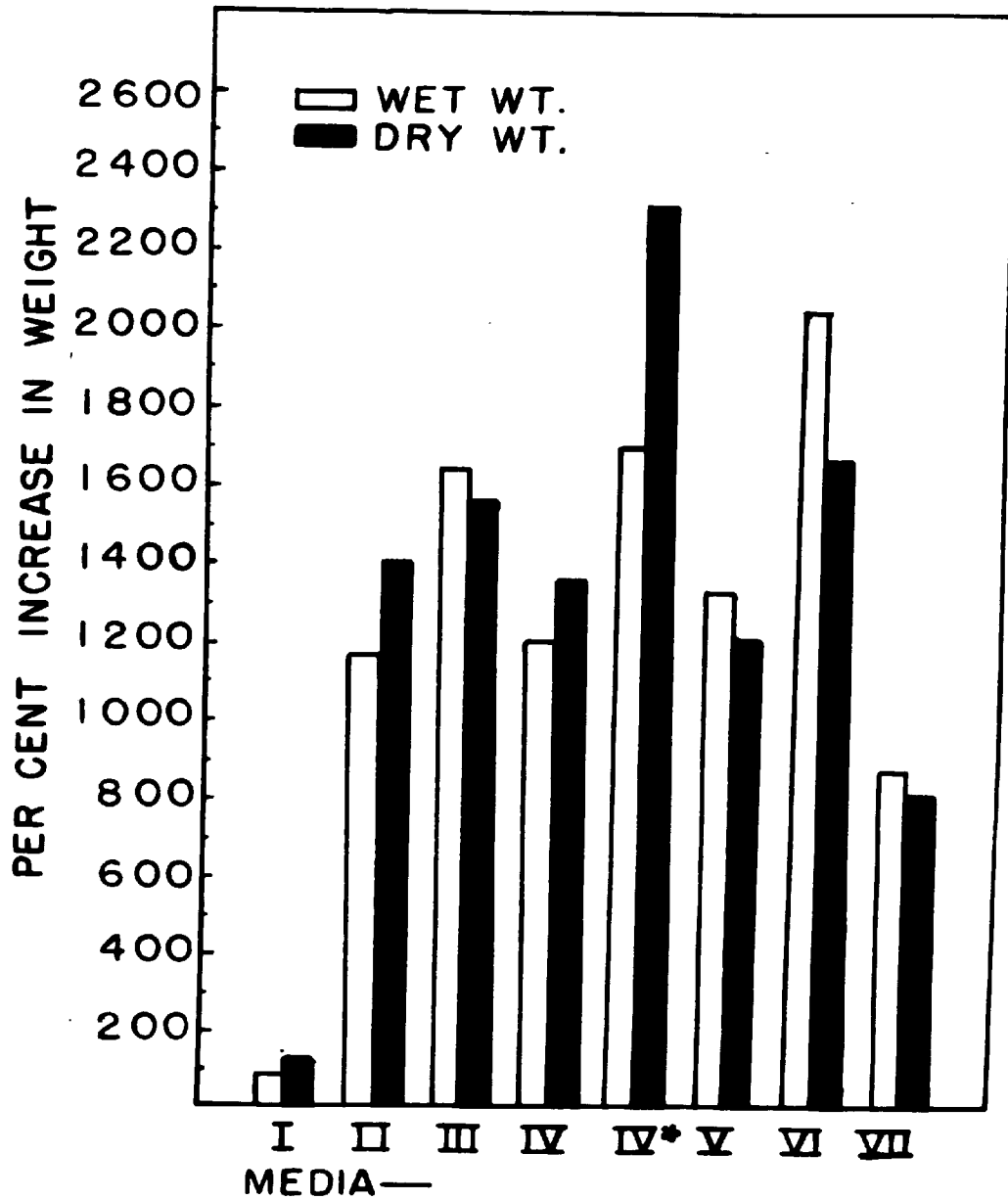


Figure 1. Growth of rye grass endosperm cultured 20 days on various media. Data from table XXVI.

* cultured 25 days.

Growth on coconut milk media: The tissue grew quite well on medium II. It did not form as much new tissue in twenty days as on some of the other media, but it grew for a longer period of time so, in the end, a greater mass of tissue was produced. A visual comparison between the growth of endosperm on coconut milk and yeast extract media may be made by looking at plate XIX, fig. 4. Successful subcultures have been made from tissues which have remained on this medium as long as 60 days. A few cultures were made on media containing 90 per cent coconut milk and on pure coconut milk and agar alone. Although quantitative determinations of the growth on these media have not been made, it is quite evident that it proceeds rather slowly.

Growth on yeast extract: Medium III, containing 0.25 per cent yeast extract, has proved to be capable of producing a high rate of growth in rye grass endosperm cultures, as is shown in table XXV. An increase of the yeast extract concentration to 0.50 per cent (medium VI) gave only a small increase in growth, while a concentration of 0.75 per cent appeared to be inhibiting of growth. Concentrations lower than 0.25 per cent were not tried.

Sucrose: The 2 per cent concentration of sucrose in medium III gave the maximum increase in both wet and dry weights for the twenty-day period. Cultures made on medium IV (0.25 per cent yeast extract, 4 per cent sucrose) showed a somewhat smaller increase in dry weight and a considerably smaller increase in wet weight as compared to cultures on medium III. The higher sucrose concentration, however,

appeared to prolong the period of active growth of the tissue to a degree, as indicated by a comparison between the twenty and twenty-five day old cultures on medium III (table XXV) and those on medium IV (table XXVI).

Effect of pH on the growth of endosperm tissue: The pH of media containing yeast extract is in the neighborhood of 6.1, due to the natural buffering activity of this substance. The pH levels in two sets of media were adjusted to 7.3 and in two other sets to 5.1, using KOH and HCL respectively. The results of these experiments are shown in table XXVIII.

TABLE XXVIII

The growth of rye grass endosperm on media at pH 5.1, 6.1 and 7.3 during twenty days' culture in darkness at constant 26°C.

pH	Set	Initial weight mgs.		Final weight mgs.		Increase	
		WET	DRY	WET	DRY	WET	DRY
5.1	1	3.034	0.106	45.065	(lost)	1385%	-
	2	4.121	0.144	56.253	1.846	1265%	1184%
	Ave.	3.577	0.125	50.659	1.846	1325%	1184%
6.1	Ave.*	2.628	0.092	46.907	1.516	1642%	1563%
7.3	1	3.903	0.137	46.783	1.579	1099%	1053%
	2	3.887	0.136	48.857	1.557	1157%	1045%
	Ave.	3.895	0.136	47.820	1.568	1128%	1049%

*from table XXV--20 days' culture

The pH of the media was again checked at the end of the culture period and found to be 5.2, 5.7, and 5.8 in the media which had originally been 5.1, 6.1, and 7.3 at the start of the experiment. All determinations were made with a Beckman pH meter.

A pH at or near 6.1 appears to be optimum in terms of greatest amount of weight increase in the tissue over the twenty day culture period.

Effect of temperature and light: Cultures of rye grass endosperm on medium III were kept for twenty days at constant temperatures of 20° C., 26° C., and 32° C., in order to determine the effect of temperature on growth. One set of 40 cultures was kept in total darkness and another set of the same number of tubes was exposed to continuous light from a fluorescent fixture. Both of these sets are included in the sample for culture at 20° C.

TABLE XXIX

The growth of rye grass endosperm, cultured 20 days on a medium containing 0.25 per cent yeast extract, 2 per cent sucrose and 1.0 mg./l. IAA at various controlled temperatures and in darkness except as noted.

Temp.	Set	Initial weight mgs.		Final weight mgs.		Increase	
		WET	DRY	WET	DRY	WET	DRY
20° C.	1	3.946	0.138	51.056	1.601	1194%	1060%
	(light)2	3.987	0.139	47.278	1.163	1086%	1076%
26° C.	Ave.*	2.628	0.092	46.907	1.516	1642%	1563%
32° C.	1	4.041	0.141	6.930	0.278	71%	97%
	2	4.177	0.146	12.145	0.550	198%	276%
	Ave.	4.109	0.143	9.537	0.414	134%	186%

*Average of cultures for twenty day period from table XXV.

It is apparent from table XXIX that the optimum temperature for the growth of rye grass endosperm is in the neighborhood of 26° C., and the lower temperatures are not as inhibiting of growth as the higher. The effect of light on

the growth of the tissue appears to be insignificant.

Morphology and cytology: The initial outgrowths of the tissue in culture appear to originate in the outer cell layers of the endosperms of excised rye grass ovules. This is illustrated in plate XIX, fig. 1. The growth in most cultures was quite rapid. The tissue from the first had a nodular appearance which seems to be a characteristic of endosperm cultures. Examination of sectioned nodules of tissue shows that the cells tend toward a radial arrangement (plate XIX, fig. 2). Proliferation takes place by the division of the peripheral cells. Those cells which are near the centers of larger nodules differentiate into starch storage cells which are greatly enlarged and contain large numbers of multiple starch grains such as are also present in the natural endosperm. Many of these central cells contain large, often lobed, nuclei. These features are illustrated by figures 2 and 3, plate XIX. The starch content of the endosperm during its most active period of growth (10 to 20 days after inoculation) is quite high (plate XIX, figures 5 and 6), but diminishes after four weeks of culture on yeast extract media containing 2 per cent sucrose. On media containing coconut milk or higher sucrose levels, the amount of starch remains high for longer periods of culture.

Aside from the differences observed in the size and shape of its cells and the formation of starch storage cells, the tissue remains relatively undifferentiated in culture. Tracheids, which are found in many other tissue cultures, have never been observed in the rye grass endosperm cultures.

The cells occur in clusters of from four to several thousands and the entire tissue mass which is a granular accumulation of these clusters, has the texture and consistency much like that of soft corn bread. As the culture ages, the tissue becomes progressively softer and more watery, while its starch content diminishes and disappears almost entirely after thirty days in culture. It is almost impossible to subculture the tissue after it reaches this stage. The color of the active tissue is a translucent, off-white which yellows as the culture ages.

Aceto-carmine preparations of the endosperm tissue in its most active phase of growth showed many mitotic figures. Numerous metaphase figures showed the triploid chromosome number of 21 (plate XX, figures 1 and 2). A number of chromosomal aberrations were observed, the most common one being hyperploidy (plate XX, figs. 4, 5 and 6). Several hexaploid metaphases were counted (plate XX, fig. 4), while larger numbers of chromosomes, estimated in some instances to be several hundred within a cell, were frequent. Such highly polyploid sets of chromosomes probably are associated with the large lobed nuclei seen in starch storage cells. Chromosomal irregularities of other kinds also were present. Of 70 anaphase figures observed in rye grass endosperm tissue cultures, 29 contained single chromosome bridges, five had double bridges, and one showed a triple bridge. The remaining 35 figures appeared to be normal. Some of these aberrations are shown in plate XX, figs. 3, 5 and 6.

PLATE XIX

Cultures of Rye Grass Endosperm

- Figure 1. Excised endosperm of rye grass after five days culture. Arrows indicate margins of new tissue formed in vitro. X 70
- Figure 2. Sectioned nodes of endosperm tissue. Large cell at center of cluster is a starch storage cell. X 70
- Figure 3. Starch cell of cultured endosperm tissue. Arrow points to the margins of a compound starch grain. X 700
- Figure 4. Culture tubes containing cultured rye grass endosperm. Tubes at the ends contained 90 per cent coconut milk media. The center tube contained 0.25 per cent yeast extract medium. The cultures shown are representative of the maximum amount of tissue produced per tube on each kind of medium.
- Figure 5. Mass of cultured endosperm stained with iodine to show localization of starch storage areas. X 70.
- Figure 6. Cultured endosperm of rye grass stained with iodine solution. Mass at left had been cultured 30 days. That at right 15 days. Illustrative of the loss of starch in older cultures. X 1

PLATE XIX

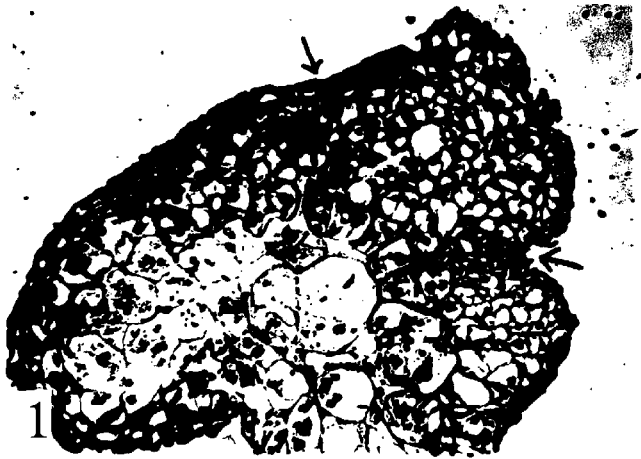
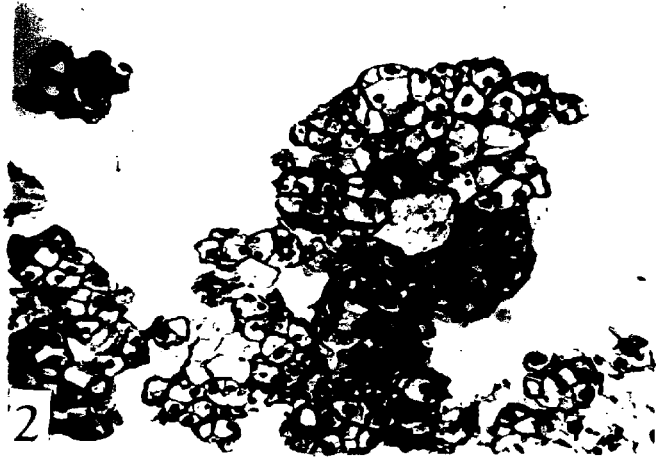
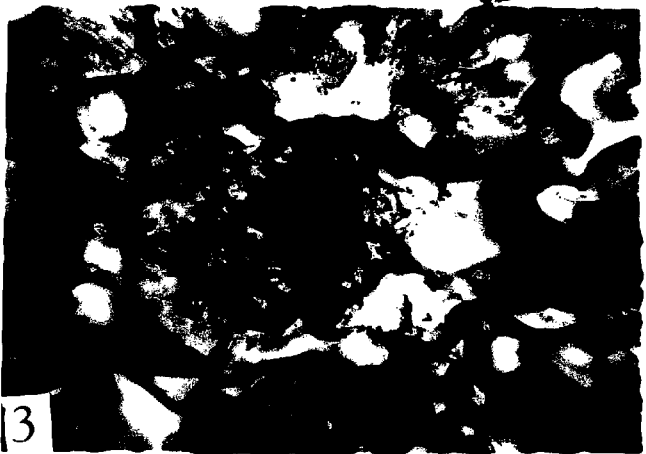
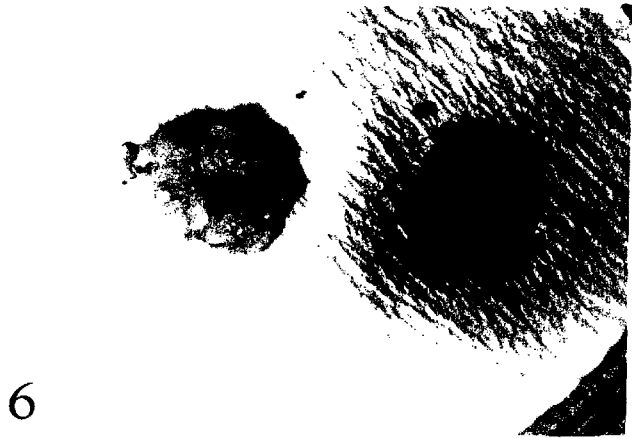


PLATE XX

Chromosome Studies of Rye Grass Endosperm in vitro

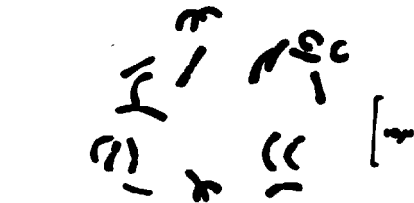
- Figure 1. Triploid chromosome number in metaphase figure from rye grass endosperm tissue culture. Aceto-carmin preparation. Photomicrograph X 1600
- Figure 2. Same as in fig. 1 except camera lucida drawing.
- Figure 3. Anaphase from cultured rye grass endosperm showing a double chromosome bridge. X 1600
- Figure 4. Camera lucida drawing of a hexaploid metaphase in cultured endosperm of rye grass. X 2200
- Figure 5. Two aberrant mitotic figures in cultured rye grass endosperm. At the left is an early telophase with a single chromosome bridge. At the right is an anaphase which is apparently hexaploid and shows fusion of chromosomes (arrow). X 1600
- Figure 6. Highly polyploid anaphase (possibly 6 n) with two chromosome bridges. X 2200

PLATE XX

1



2



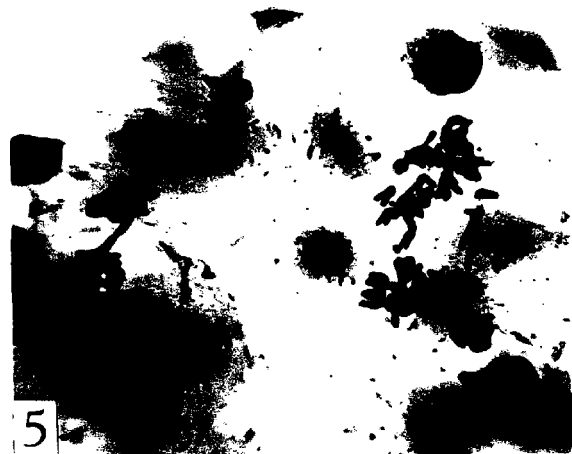
3



4



5



6



The growth of rye grass and barley embryos on media

with rye grass endosperm: Since endosperm has been reported by Ziebur and Brink (1951) and also by Pieczur (1952) to exert a beneficial influence on the growth of immature embryos in vitro, it was decided to investigate the effect of rye grass endosperm on immature embryos of rye grass and barley. In a preliminary experiment, bits of endosperm tissue were placed on White's agar adjacent to excised embryos. As controls, small bits of yeast extract media on which the endosperm was grown were used in embryo cultures in the same manner as the pieces of endosperm.

The results of this experiment were inconclusive, although a slightly increased growth was observed in some embryos of both species on the media with endosperm tissues. A second experiment was then made using a water extract of cultured endosperm in place of the actual tissue.

A mass of cultured rye grass endosperm weighing 50 grams, wet, was transferred aseptically into 200 cc. of sterile distilled water. After 24 hours this mixture was Seitz filtered and the filtrate was added to 300 cc. of White's agar and tubed. Embryos of rye grass and barley, measuring 5 and 10 micrometer units in length, respectively, (0.4 and 0.8 mm.), were cultured on this medium and on the medium used to grow the endosperm (0.25 per cent yeast extract, 2 per cent sucrose and 1.0 mg./l. IAA). The results of this experiment are presented in table XXX. In this experiment there was no appreciable difference in the average final length of rye grass embryos cultured with endosperm extract and on yeast

extract. Embryos of barley grew larger on the endosperm extract than on the yeast extract medium, but were not nearly as well developed as in similar cultures made previously on a medium containing 90 per cent coconut milk.

TABLE XXX

The growth of immature embryos of rye grass and barley on media containing an extract of cultured rye grass endosperm and on a control medium containing yeast extract and IAA. The average increases in length of embryos cultured for one week in darkness at room temperature are shown.

Media	Species	No. embryos	Initial length	Final length	Increase
A*	rye grass	25	0.4 mm.	1.2 mm.	200%
B**	rye grass	34	0.4	1.2	200%
A	barley	29	0.8	2.1	162%
B	barley	23	0.8	1.7	112%

*White's agar and rye grass endosperm extract

**White's agar and 0.25% yeast extract, 1.0 mg./l. IAA

Discussion

Isolation and culture of rye grass endosperm: A number of aspects of the culture of the endosperm of Lolium perenne are noteworthy. It is one of the few cultures of monocotyledonous tissue to be successfully established and maintained in vitro, as reported in the introduction to the present study, and it is one of only three endosperms to be cultured. The others are the endosperm of maize isolated by LaRue (1947,1949) and that of Pawpaw grown by Lampton (1953). Furthermore, it

has consistently demonstrated an almost complete lack of differentiation and organization in vitro. This has been observed only in the other two species of cultured endosperms, in a tissue obtained from the microgametophyte of Gingko by Tulecke (1953) and in tissues derived from the gametophyte of Selaginella by Wetmore and Morel (1951). All other tissues differentiate to some extent in vitro, at least to the extent of forming tracheids. Its rate of growth has been rather rapid in comparison to that of many other cultured tissues, and it has been possible to subculture the tissue successfully at frequent intervals without apparent diminution in its growth.

The rate of growth: On the basis of wet and dry weights taken at five-day intervals over a twenty-five day period, it is possible to state that the tissue undergoes a lag period of about five days after inoculation and then grows very rapidly for the next fifteen days, after which its growth declines sharply. Only a slight increase in wet and dry weights was observed after 20 days' culture on the medium containing 0.25% yeast extract, 2% sucrose and 1 mg. IAA. The maximum increase in dry weight of the tissue grown under these conditions was over 1600 per cent. Straus (1953) made an intensive study of the growth of maize endosperm in vitro and reported very nearly the same rate of growth as observed here for rye grass endosperm.

Growth on various media:

White's medium: The growth of endosperm on White's medium was so poor as to be almost nonexistent in comparison with some of the other media. The onefold increase in wet and dry

weights after 20 days' culture may be due to residual growth substances taken up by the inocula from the yeast extract media on which the stock cultures were maintained. The need for such accessory growth stimulators as yeast extract and coconut milk are clearly demonstrated.

Yeast extract media: The medium containing .25% yeast extract is capable of producing a high rate of growth in endosperm cultures. Increase of the yeast extract to 0.50% results in a slight increase in growth, while a concentration of 0.75% yeast extract appears to be inhibiting. Concentrations lower than 0.25% were not tried. Straus (1953) obtained the best growth of maize endosperm on a medium containing 0.50% yeast extract but no IAA. Lolium endosperm seemed to grow slightly better when the medium contained IAA.

The greatest increase in wet and dry weights of endosperm occurred on media containing 2 per cent sucrose, but tissues cultured on 4 per cent sucrose continued to grow for a longer period of time. The higher concentration of sucrose was reflected in lesser increases in wet weight and probably was due to higher osmotic pressure which would interfere with the water uptake of the tissues. From the data at hand it appears that in cultures on yeast extract, the sucrose level is growth-limiting.

Coconut milk: Rye grass endosperm grew well on 20% coconut milk media. The increase in its weight over the 20 day culture period, though less than on most yeast extract media, was still more than tenfold. More importance should be attached to the duration of growth with coconut milk than to

the rate of increase. The endosperm continued to grow for a much longer time than on yeast extract media containing 2 per cent sucrose. Higher concentrations of coconut milk (up to 100%) produced even slower growths, but at the same time increased the longevity of the culture and in the end resulted in the production of much larger masses of tissue as may be seen in plate XIX, fig. 4. Since sucrose appears to be limiting of growth on yeast extract media, it would also seem that the slow but longer lasting growth on coconut milk media is largely due to its higher carbohydrate content. It is also apparent that coconut milk contains a substance essential to growth of endosperm not supplied by the minerals and accessory substances in White's medium, but present also in yeast extract. The stimulating effect of coconut milk on tissue cultures is well known. Duhamet (1951_a, 1951_b) reports that coconut milk media greatly accelerated the growth of crown gall tissue of salsify, tobacco, grape and Jerusalem artichoke. The virus tumor tissue of Rumex has been grown by Nickell (1950) on coconut milk media and found to be greatly stimulated by this substance. Nickell states that the tissue also grew well on pure coconut milk. Caplin and Steward (1952) found that the growth of carrot callus was greatly increased on media containing coconut milk, and that the callus could also be grown on just coconut milk and sucrose. Archibald (1954) found that the highest rate of growth in Cacao callus was obtained on a medium of pure coconut milk. Henderson et al. (1952) used 100 per cent coconut milk media in sunflower tissue cultures with good results. The use of coconut milk in the culture of

immature embryos has already been discussed; however, an interesting fact about its use in embryo and tissue cultures is that it becomes inhibiting to embryos upon being autoclaved, whereas in tissue cultures autoclaving has no such effect. For this reason some authors are inclined to think that the embryo factor is not the same factor as promotes the growth of tissues in culture.

Effect of pH on growth: Straus (1953) found that in cultures of maize endosperm the pH of the medium was not limiting within the range 6.1 to 8. As may be seen in table XXVII, Lolium endosperm cultures grew at pH levels 5.1, 6.1 and 7.3, but demonstrated the greatest increase in weight on media of pH 6.1.

Effect of temperature and light: The results of cultures in light and darkness and at different temperatures have already been reported and require little further discussion. Similar results were obtained by Straus (1953) who obtained the best growth in maize endosperm at 25° C.

Morphology and Cytology: Straus (1952) describes the initial outgrowths of endosperm-derived tissues of maize as originating in the surface layers of excised endosperm in which the cells have a definite cambium-like arrangement. In rye grass endosperm, the planes of division occur more or less at random although the overall appearance is somewhat suggestive of a cambium and, as in maize, the initial outgrowths appeared to originate in the outer cell layers of the endosperm.

It is interesting that the endosperm of Lolium continues to store starch, when cultured, in much the same manner as it

does in the seed, and also that chromosomal abnormalities occur in the cells in culture which are also reported to be present in at least some endosperms growing naturally. Clark and Copeland (1940) describe the chromosomal aberrations in the developing endosperms of several mosaic lines of maize and report abnormalities which occurred at varying frequency depending upon the strain. They state that the most frequent aberration was the occurrence of single bridges. Double bridges and occasionally triple bridges also were observed. Straus (1953) found chromosome bridges in about 30 per cent of the anaphases he saw in maize endosperm cultures. These observations are in agreement with those of the rye grass tissue.

McClintock (1941) reports the bridge phenomenon in cell divisions of maize endosperm in vivo and gives evidence that a regular fusion-bridge-break cycle may persist in such divisions. She further states that in maize this cycle is peculiar to endosperm and gametophytic divisions rather than to sporophytic divisions. Since most of the bridges observed in rye grass endosperm cultures appear to be of a secondary nature, not accompanied by unattached chromosome fragments, this may be evidence that the cultured tissue was not greatly altered after passage through a number of subcultures.

Many of the cells retained the triploid number of chromosomes which in Lolium perenne is 21, as ascertained by Evans (1926).

A marked similarity may be seen between sections of cultured endosperm and the endosperm of the seed which are

illustrated in plate XIX, figs. 1 and 2. In both the meristematic portions of the tissues the peripheral and starch cells are centrally located. Again the growth in vitro appears to resemble that in vivo.

As reported earlier, the complete absence of differentiated cellular elements such as tracheids and other vascular structures was observed in cultured endosperm of rye grass. In this it is like the cultured endosperms of maize and pawpaw. One wonders whether this repression of differentiation and organization is due to some deficiency in the cytoplasm or whether it is an expression of some inhibition impressed upon it in the embryo sac. It would not seem to be due to the triploid chromosome number, since triploid sporophytes of Lolium are known to occur in nature (Myers, 1944).

Growth of embryos with rye grass endosperm: The absence of any stimulation of rye grass embryos in cultures on media containing extract of Lolium endosperm, together with the apparent increased growth of barley embryos on this same medium is disturbing. The lack of good controls in these cultures also places the matter of positive influence of cultured endosperm on excised immature embryos in a dubious light. Had spectacular increases in embryos cultured on endosperm extract been observed, one could state with confidence that the use of the extract was of benefit. Since the endosperm can only be grown on media containing accessory growth substances which are also known to stimulate the growth of embryos in culture, and the amount of these substances remaining in the tissue extract was not determined, it is apparent that a precise

control medium could not be developed. It was felt that this difficulty was partly solved through the use of a control medium identical with that used in the culture of the endosperm from which the extracts were made. Pieczur (1952) found that the use of maize endosperm was of benefit in the culture of immature maize endosperm, but in her investigation no allowance was made for the effect on the embryos of accessory substances in the media which might have been taken up by the endosperm.

The writer feels that a carefully controlled experiment on the effect of cultured endosperm on growth in excised embryos must wait until the endosperm can be grown on a defined medium.

SUMMARY

Mature embryos of oats, barley, wheat and rye were cultured in liquid media and on solid media. Those placed on nutrient agar did not undergo further embryonic differentiation but germinated to form normal seedling plants. In liquid media germination of excised embryos was inhibited. At the same time, the coleorhizae and epiblasts in embryos of all four species enlarged to a greater extent than those in embryos grown on solid media. Oat coleorhizae which commonly attained a length of about 2.5 mm. during germination and developed epidermal hairs, sometimes became 10 mm. long without attendant hair formation when cultured in liquid media. Elongation was found to be proportional to the reduction in the amount of air surrounding the embryos. However, coleorhizal elongations did not occur in cultures from which all oxygen had been removed. The growth of epiblasts and coleorhizae of oats in liquid cultures was increased through addition of small amounts of IAA to the media. In surface cultures, on the other hand, IAA was found to increase the amount of coleorhizal hair formation without stimulating the elongation of coleorhizae and epiblasts.

The coleorhizae of wheat embryos cultured in nutrient liquids often disintegrated after a week or so. The resulting detached, but still living, cells in some instances had differentiated to the extent of forming root hair like outgrowths, but were never observed to undergo division.

A total of 1091 immature embryos of oats, barley, wheat and rye were cultured on various media. The embryos all were of a uniform size (0.8 mm.) when excised, and were cultured in

darkness at room temperature for a period of one week, after which measurements were made in order to determine the amount of growth which had occurred in the embryo and its parts. Lengths of coleorhizae, coleoptiles, epiblasts, and scutella were recorded in addition to total lengths and scutellar widths. These data were tabulated and showed that various treatments of cultured embryos resulted in differential growth of embryos and their organs.

The coleorhizae of barley and oats were stimulated to greater elongations on media containing 40 mg. per liter of adenine sulfate. This is thought to be a response comparable to that of coleorhizae of mature embryos which were grown in liquid cultures or at reduced concentrations of air.

The coleoptiles of immature embryos responded in several ways to different treatments in vitro. Those cultured on defined media, such as White's solution, did not continue to grow by cellular division but became elongated as in normal germination. Since they often were incompletely formed at the time of excision, their resulting developments were abnormal. In barley, the coleoptiles sometimes became open and leaflike, which suggested the possibility that atavistic tendencies were involved since such characteristics have been reported to be primitive. The growth of coleoptiles in media containing 90 per cent coconut milk resembled that occurring in normal embryogenesis. However, in barley, curious proliferations of cells at the margins of the coleoptilar pore were observed.

Epiblast developments in vitro were observed in all the species cultured, particularly on media containing 90 per cent

coconut milk. Well-formed epiblasts were observed in a few cultured embryos of barley, which is remarkable in that mature embryos of barley in the seed do not possess epiblasts.

Scutellar developments in vitro were unlike those occurring in natural embryogenesis. Generally in cultured embryos, increases in the length of scutella were less than normal. Failures of the palisade-like lower epidermis to develop at the same rate as the upper epidermal cells resulted in the formation of abnormal curvatures in these organs. Some barley embryos, cultured on coconut milk media, produced very large scutella, but even in these instances scutellar growth appeared to be determinate since prolonged meristematic activity did not take place. The scutella of all embryos displayed a position effect in that they became callused and enlarged in embryos cultured "face down" on the surface of the media. A response also was observed to concentration gradients of a substance or substances in coconut milk which affected lateral proliferation of cells in the scutella of rye embryos cultured in liquid media. The development of other scutellar features also was observed and discussed.

It was found that through the use of media containing 90 per cent coconut milk, relatively undifferentiated embryos of oats, barley, wheat and rye could be induced to continue embryonic differentiation and growth without undergoing premature germination. The development of shoot primordia on this medium was quite similar to that occurring in the seed. Root primordia also continued to differentiate although these did not equal the number developed in vivo except in oats.

Embryos of barley, as small as 0.15 mm. were successfully grown in vitro. The smallest of these produced a curious compound seedling which had two coleoptiles, two shoots, and ten roots. The production of supernumerary organs in immature and externally undifferentiated embryos may be a result of regeneration from undifferentiated callus rather than a continuation of normal embryogenesis.

As a result of the present investigation, it appears that various morphogenetic variations may be induced in embryos of grasses through different treatments in vitro, but that the degree to which these developments are developed appears to be limited by factors within the embryo, since unlimited expressions of differential growth were not observed. For example, in some cases cultured embryos exceeded the size of mature embryos in the seed but never in the number of leaf and root primordia.

Excised ovules of oats, barley, wheat, rye and English rye grass were cultured and at least limited growth of embryos and endosperms were observed in all. The proliferation of a callus-like tissue from the endosperm of rye grass was the most significant development in this experiment. That tissue, which resembled the normal rye grass endosperm in many ways, proved capable of potentially unlimited growth in vitro. Its rate of growth was such that a better than 16-fold increase in dry weight was observed after three weeks' culture. The cultured endosperm proved to be amenable to culture on various media, providing either yeast extract or coconut milk was included in the substrata.

Various chromosomal aberrations were observed in Lolium tissue cultures, particularly anaphase bridges. The frequent occurrence of single bridges in anaphase figures indicated that bridge-fusion-break cycles comparable to those reported for maize endosperm in vivo might also be present in rye grass endosperm in vitro.

Growth of excised embryos of rye grass on media containing either bits of cultured rye grass endosperm or an extract of such endosperm, were not noticeably stimulated. However, barley embryos grew to a greater average length on media containing the endosperm extract than they did on a control medium. Due to difficulties in establishing suitable controls, and also to the lack of a clearly defined effect of endosperm extract on embryo growth, the results of these experiments were not conclusive.

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