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GROWTH AND CONTROLLED MORPHOGENESIS IN PEA ROOT CALLUS TISSUE GROWN IN LIQUID MEDIA¹

John G. Torrey and Yoshio Shigemura

IN RECENT YEARS considerable interest has centered in the nutritional requirements of isolated fragments of higher plant tissues cultured in vitro. From the early work of White (1943) and of Gautheret (1942), it has been known that tissues derived from the immediate morphological region of the vascular cambium of either stem or root are particularly susceptible to culture in a suitable nutrient medium. Much information has been gained concerning the specific nutritional requirements of a number of callus tissues derived from the vascular cambium region of a wide variety of plants (Gautheret, 1955b).

Most of the callus tissues which have been cultivated in vitro have been derived from secondary tissues of stems. Relatively few callus tissues of root origin have been studied. One notable exception is carrot tissue which has been grown extensively in culture, but even here precise distinction between root and stem (hypocotyl) tissue is difficult to make and frequently has not been made. Only in a few cases have root callus tissue cultures of distinctly root origin been described. Skoog (1944) reported the spontaneous formation of root callus by cultured roots from hybrid tobacco tissues in culture. Jagendorf et al. (1952) describe a callus tissue produced from cabbage roots, Kandler (1950) from sunflower roots in culture, Tryon (1955) from tobacco roots, Nickell (1955) from roots of sweet clover, and Black (1947) and Nickell (1954) the abnormal virus tumor tissue produced by sorrel roots.

In studying problems of plant morphogenesis

using tissue culture methods, careful identification of both morphological and anatomical origin of the tissue may be a matter of considerable importance. Thus, callus tissue derived from roots may be expected to differ fundamentally in inherent morphogenetic capacities from stem callus tissue. These differences may be perpetuated in culture or they may be modified by manipulation of the medium or by the passage of time.

During the past several years, studies have been made of the particular nutritional conditions necessary for continued meristematic activity in the root meristem of isolated pea roots grown in vitro (Torrey, 1954) and for the initiation of cell divisions leading to lateral root formation in isolated pea root segments (Torrey, 1956). Because of our interest in cell division and its biochemical control in pea roots grown in culture, it appeared desirable to us to isolate tissues from the vascular cambium region of pea roots and to study the nutritional conditions necessary for continued cellular divisions in such tissues. Once established in culture as a root callus tissue, it was hoped that the peculiar conditions giving rise to the initiation and continued activity of the secondary meristem in vivo might be discovered and that a study of the inherent morphogenetic capacities of the root callus tissue itself might be made. In the experimental work to be reported here, an account is given of the establishment on a complex nutrient medium of a callus culture from tissues isolated from the vascular cambium region of pea roots. During the course of this study, the callus tissues have been grown in both solidified agar media and in liquid media with constant agitation. Under the latter conditions two morphologically distinct tissues have become evident whose occurrence is dependent upon the constituents of the nutrient medium.

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Establishment of the pea root callus tissue in culture.—Five-mm. root tips excised from 48-hr. germinated pea seeds, variety 'Alaska,' were transferred aseptically to Petri dishes containing a modified Bonner medium (Torrey, 1954) with 0.5 per cent agar and 10^{-5} M indoleacetic acid (IAA) at pH 5.0. In certain cases, the radicle of the pea seed was excised aseptically from the dry unsoaked seed and transferred to the IAA medium. The excised root tips were maintained on the auxin medium in the dark at 25°C. for 5–7 days.

In the auxin medium, elongation of the root tips was markedly inhibited. By the fifth day the basal region of the excised roots had increased in diameter to almost twice the original dimension and lateral roots were evident in this enlarged region (Torrey, 1950, 1951). Histological preparation of such roots showed that the increased root diameter was largely attributable to extensive secondary activity from a well-established vascular cambium. By the seventh day, the vascular cambium, which had originated in typical fashion by periclinal division of cells in the areas between the primary phloem bundles and the primary xylem, was continuous and had formed a triangular central bundle of xylem (fig. 1).

Using a sharp scalpel of the replaceable blade type, the basal end of the root was cut into 1-mm.-thick cross-sections which were placed on the agar medium. The cortical tissues were cut away from the central cylinder tissues under a dissecting microscope and triangular wedges were cut along the radial lines of the primary xylem poles. These wedges included tissues of the primary and secondary xylem, vascular cambium, secondary and primary phloem, pericycle, and endodermis as well as damaged cortical cells. Each wedge-shaped piece was transferred aseptically to a Petri dish of modified Bonner medium containing 0.5 per cent agar and various additions of growth factors and metabolites, and the pieces were cultured in the dark at 25°C.

An alternate procedure which was found equally successful, although much less convenient, was to secure vascular cambial tissues from sections of mature regions of untreated week-old pea root seedlings (see Popham, 1955). In such seedling roots, manipulation is more difficult.

It is a matter of considerable interest that only *initial* root tips taken from the germinating seed respond to auxin-treatment by producing a vascular cambium with extensive secondary activity. Five-mm.-tips excised as *first transfer* tips, i.e., from initial root tips which had been grown on auxin-free medium for one week to an average length of about 60 mm., when placed on a modified Bonner medium containing 10^{-5} M IAA, show no secondary activity and no vascular cambium.

The chemical nature of the starting medium.—The wedge-shaped root pieces containing the vascular cambium did not continue cellular divisions

but turned brown and died if the pieces were maintained on the auxin-containing medium used in the initial treatment which had given rise to the cambium. This medium, free of auxin, is known to support the continued activity of the apical meristem of pea roots through at least a number of weekly transfers during which growth in length averages between 40 and 60 mm. The medium contains as growth factors essential to root elongation the vitamins, thiamin and nicotinic acid as well as the essential micronutrient elements. For continued meristematic activity in the vascular cambium region, however, additional factors apparently originally present in the root tip excised from the germinating seed, must now be provided from the external nutrient medium.

Experiments were made in which the tissue pieces containing the vascular cambium region were tested on a large number of complex media. The medium most effective in producing rapid proliferation of cells which originated in the immediate area of the vascular cambium zone, was a modified Bonner agar medium with 4 per cent sucrose, to which had been added, prior to autoclaving, 1 g./l. Bacto yeast extract (Difco) and 10^{-5} M 2,4-dichlorophenoxyacetic acid (2,4-D). Tissue pieces which showed proliferation maintained a golden yellow coloration and could be seen under the dissecting microscope to manifest a "boiling up" of cells in the cambial zone. Xylem tissue of such pieces tended to turn brown in all media and did not contribute to the growth of the callus culture. Pieces showing proliferation from the vascular cambium region were transferred bi-weekly to the same medium freshly prepared until a large rounded mass of proliferating tissue had been formed (fig. 2). Such callus masses were cut into smaller pieces using a sharp scalpel, and the small pieces were subcultured until a clone of callus tissue sufficient for experimental purposes was established.

Of the auxins tested for effectiveness in establishing the pea root callus tissue in culture, 2,4-D was found to be the most effective. α -naphthalene acetic acid in the presence of 1 g./l. yeast extract, also stimulated callus development. IAA in the presence of yeast extract, was inactive.

Other complex growth factor mixtures were also tested in the presence of 10^{-5} M 2,4-D. Yeast extract was consistently most effective in establishing the callus. Peptone at 1 g./l. when added to the modified Bonner medium in the presence of 10^{-5} M 2,4-D permitted a slow development of root callus tissue from wedge-shaped pieces. Similar slow development was achieved in the presence of 15 per cent autoclaved coconut milk and 10^{-5} M 2,4-D. Because of the consistently better response of the tissues to the medium with yeast extract and 2,4-D, it was decided to use this medium as the basis on which to work toward a synthetic medium. Clonal material was maintained on agar medium contain-

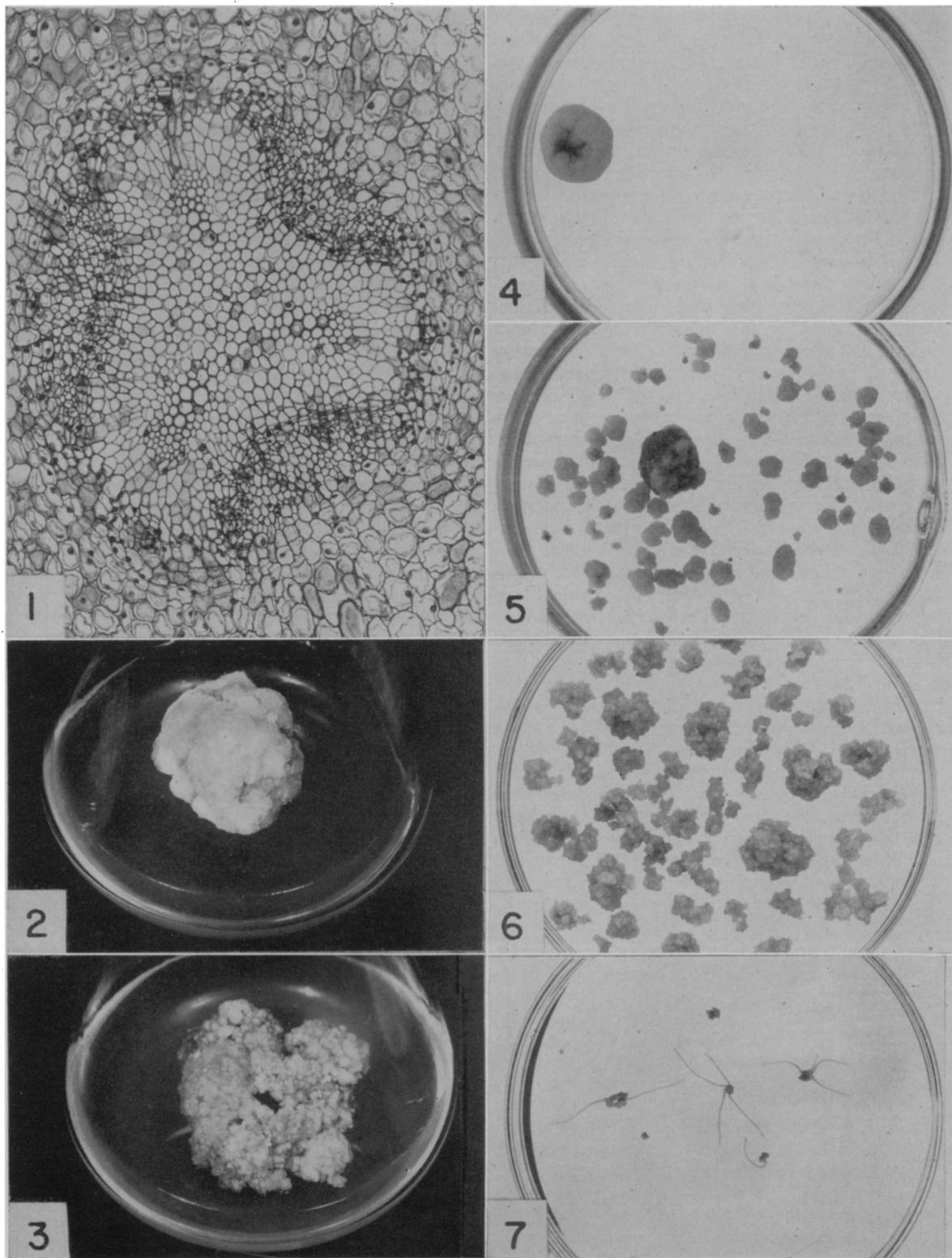


Fig. 1-7.—Fig. 1. Transverse section of isolated pea root grown for 7 days on modified Bonner medium containing 10^{-5} M IAA, showing extensive secondary tissues produced by a vascular cambium. $\times 140$.—Fig. 2. Pea root callus tissue derived from vascular cambium region, after propagation without subdivision for 4 months on yeast extract—2,4-D medium on agar in 125-ml. flask. Note smooth rounded shape of callus mass.—Fig. 3. Callus tissue of the friable

ing 1 g./l. yeast extract and 10^{-6} M 2,4-D with bi-weekly transfer and subculture to fresh medium. The callus tissues grown on agar media were observed to be of two types: either smooth, rounded solid masses of tissue (fig. 2) or loose, friable masses which readily broke apart when handled (fig. 3). Usually, spherical masses of approximately 5 mm. diameter were used as subcultures.

Establishment of clonal material in liquid culture.—For quantitative studies it was desirable to culture the root callus tissue in a liquid medium. Preliminary experiments showed that the callus tissue would continue proliferation in submerged culture in a 50-ml. volume of yeast extract-2,4-D medium per 125-ml. Erlenmeyer flask if the flasks were constantly agitated. Routine culture of the callus in liquid medium was achieved by use of a horizontal-type shaker which produces a vigorous swirling motion of the contents of the flasks. Individual experiments of 200 or more flasks could be run readily on the shaker. Flasks were maintained at 25°C. in the dark with occasional brief periods of white fluorescent light for observations.

Clone 33 which was used throughout the experiments reported here was derived from a single wedge-shaped piece of pea root tissue excised from the cambial region of an IAA-treated root at the 5th day. The callus was established on agar medium containing 1 g./l. yeast extract and 10^{-5} M 2,4-D, was transferred weekly to fresh agar medium for 4 weeks and was then cultured in liquid medium containing 1 g./l. yeast extract and 10^{-5} M 2,4-D. As the initial inoculum grew in size, increasing as a solid, spherical mass of tissue, it was quartered or further subdivided into 3–4-mm. diameter pieces at approximately 4-week intervals until many hundreds of separate pieces were growing in liquid culture. These clonal cultures were routinely maintained with 5–8 pieces per 50 ml. of medium per flask. In order to minimize the lag in growth associated with the wounding effects of the separation procedure, the freshly quartered pieces of inoculum were usually carried in the control medium (1 g./l. yeast extract and 10^{-6} M 2,4-D) for 4 weeks at which time the spherical 5-mm.-diameter pieces were transferred, one per flask, to the medium to be tested. Routinely, twenty flasks were run for each test medium and half were sacrificed at 4 weeks, the second half at 8 weeks—for fresh and dry weight determinations. In certain cases, ten flask samples were taken at 2 week intervals. Fresh weights were determined after blotting away excess surface moisture; dry weights

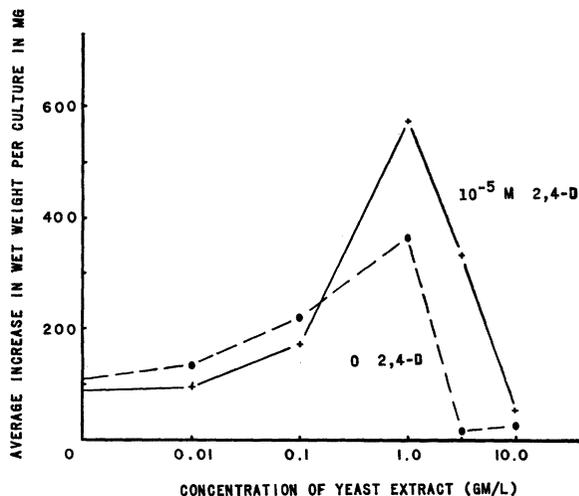


Fig. 8. The average increase in wet weight in mg. of pea root callus tissue grown for 8 weeks in liquid medium containing different concentrations of yeast extract with and without 2,4-D.

were determined after drying at 95°C. to constant weight.

The interaction of yeast extract and 2,4-D in the growth of the callus tissue.—It was apparent quite early that the yeast extract was essential for the maintained growth of the callus in culture and that 2,4-D had a marked synergistic effect in the presence of yeast extract. Experiments were made to determine the optimum concentrations of these two components which would produce maximum growth. In fig. 8 are presented data showing the effect of different yeast extract concentrations on the increase in wet weight in the presence and in the absence of 10^{-5} M 2,4-D. The average increase in wet weight per culture at the end of 8 weeks is presented.

The following facts are evident from these results: (1) In the absence of yeast extract, very little growth occurs whether or not 2,4-D is present. The growth increases as the yeast extract concentration increases up to an optimum of 1 g./l. and then higher levels of yeast extract become toxic. From this fact, one can deduce that some component of yeast extract is essential to the optimum growth of the callus and is limiting at low concentration. (2) In the absence of 2,4-D, yeast extract alone supports some growth of the callus, reaching its most effective concentration at 1 g./l. At 5 g./l. or above, the yeast extract alone is highly toxic. In the presence of 10^{-5} M 2,4-D,

type grown on agar medium for 4 months.—Fig. 4–7. Pea root callus tissue cultured in liquid media in flasks. All photographed in 11-cm. Petri dishes.—Fig. 4. Callus culture of the RS type grown in M6 medium for 4 weeks.—Fig. 5. Callus culture started from single piece of RS type callus, grown for 4 weeks in medium containing 10^{-5} M 2,4-D plus 5 g./l. yeast extract. Note large darkened original inoculum with numerous small friable pieces.—Fig. 6. Callus culture of the friable type propagated on medium containing 10^{-5} M 2,4-D plus 5 g./l. yeast extract. Note numerous rounded protuberances on each piece.—Fig. 7. Pieces of friable callus tissue after transfer to modified Bonner medium lacking both yeast extract and 2,4-D. Note roots which have been formed from the small protuberances.

yeast extract exerts a similar stimulating effect on growth with an optimum at 1 g./l. The auxin alone has essentially no effect on increase in weight. The two components together, however, act synergistically. In the presence of 10^{-5} M 2,4-D, higher levels of yeast extract are tolerated, although the higher concentrations are less effective in producing increased wet weight. At 10 g./l. yeast extract is consistently toxic.

Experiments were then run to establish the optimum 2,4-D concentration in the presence of 1 g./l. yeast extract. In fig. 9 are presented the results of wet and dry weight determinations of callus cultures grown in different concentrations of 2,4-D with a constant yeast extract concentration of 1 g./l. These results are presented as average total weight per culture with an initial inoculum weight of 90 mg. wet and 11 mg. dry weight respectively. It is evident that maximum growth in terms of increase in both wet and dry weight results from a medium containing 10^{-6} M 2,4-D in the presence of 1 g./l. yeast extract. At higher concentrations of the auxin, lower tissue weights occur. In all subsequent experiments the clonal material was maintained in this medium (M6) and the callus grew consistently and well as smooth round solid masses of tissue.

During the course of these experiments, a very disconcerting phenomenon began to show itself. The initial inoculum used in each flask was a spherical piece of callus tissue, quite solid and woody, of golden color, round and smooth in appearance. This type of callus (fig. 4) was designated the RS type and could be reproduced repeatedly with large numbers of uniform pieces when propagated as described previously in the M6 medium.

In the experiments described above, however, in which variations of 2,4-D and yeast extract concentrations were tested, the growth of the callus tissue was not always that of the RS type. Under certain nutritional conditions, the initial inoculum showed a delayed proliferation, which occurred sporadically from localized areas on the surface of the inoculum, so that small nodular pieces of tissue were formed. These pieces increased in size and were frequently broken off from the initial inoculum. At the end of the experiment, such flasks contained many small pieces, each growing separate from the original tissue mass (fig. 5). Thus the tissue had become friable and grew thenceforth in a fundamentally different manner. Such callus tissues were designated the Friable type. In fig. 4 and 5 the two cultures were started from the same RS type inoculum. In the former culture, this type of growth persisted for the duration of the experiment; in the latter, the friable condition has arisen. A rough measure of the friable condition can be made by counting the number of pieces per flask at the end of the culture period. In fig. 6 is illustrated a callus culture produced

from friable tissues only. Wet and dry weight determinations of the friable tissues could be made by determining the aggregate weight of the culture pieces. However, from such friable pieces it has not proved possible to propagate a uniform clone of the RS type, nor even to return to the RS condition. Furthermore, it seems evident that the two types of clonal material respond differently to different nutritional conditions and should in no way be considered identical tissue systems.

Conditions leading to the friable type of callus growth.—Because of the considerable interest in the physiological basis of the friable type of callus growth, an extensive series of experiments was carried out to determine the nutrient conditions leading to friability. It was soon found that the conversion of the RS type callus to the friable callus depended upon the relative concentrations of yeast extract and 2,4-D in the medium. In comparing the effects of the different media on friability it must be borne in mind that the nutritional conditions must be such as to allow some growth to occur. In subsequent experiments, the yeast extract concentration of 10 g./l. was omitted since no growth occurred at any of the 2,4-D concentrations tested (e.g., see fig. 8). An intermediate concentration of 1.5 g./l. yeast extract was introduced. The results of these experiments are summarized in fig. 10 in terms of average increase in wet weight (fig. 10a) and average number of pieces per culture (fig. 10b).

Analysis of fig. 10a shows that the M6 medium (1 g./l. yeast extract and 10^{-6} M 2,4-D) represents the best medium for wet weight increase. A second lesser peak occurs at 1.5 g./l. yeast extract and 10^{-5} M 2,4-D when both components are at a slightly higher concentration than in the M6 medium. When used alone, 1.5 g./l. yeast extract already shows toxicity and no growth occurs at 5 g./l. yeast extract. These data confirm and extend those already presented with respect to the interaction of 2,4-D and yeast extract on growth in terms of wet weight production.

Reference to fig. 10b shows that the peak of wet weight increase is the point of least friability in these experiments. Thus adoption of the M6 medium for the maintenance and propagation of the RS type clone was a happy choice. Further study of fig. 10b shows that friable growth occurs under two nutrient conditions: i.e., at 1 g./l. yeast extract in the absence of 2,4-D and, at the other extreme, at 5 g./l. yeast extract and 10^{-5} M 2,4-D. Neither of these conditions is particularly favorable to growth in weight. In general, one can say that yeast extract in the medium tends to cause friability; 2,4-D acts to prevent the tendency toward friability. At a given 2,4-D concentration, e.g., 10^{-6} M, increasing the yeast extract concentration from 1 to 5 g./l. produces a marked change from the RS type to the friable condition. There is some evidence to suggest that friable tissues,

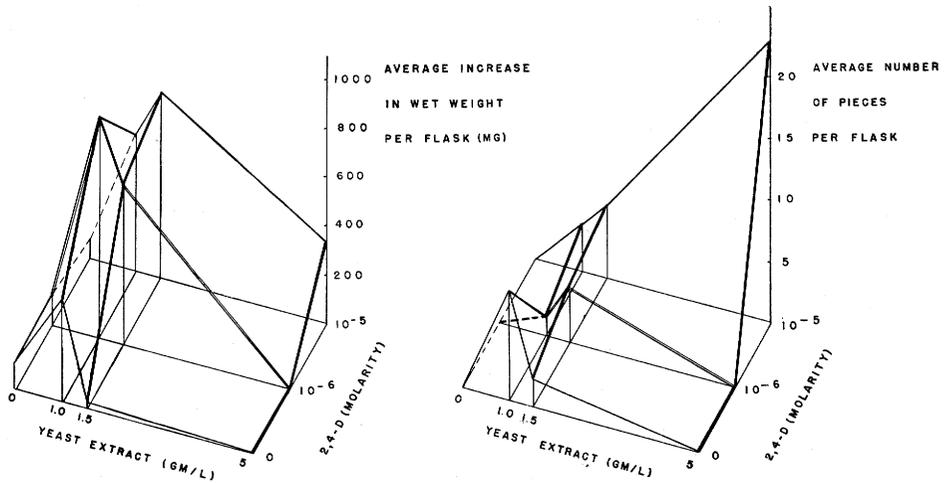
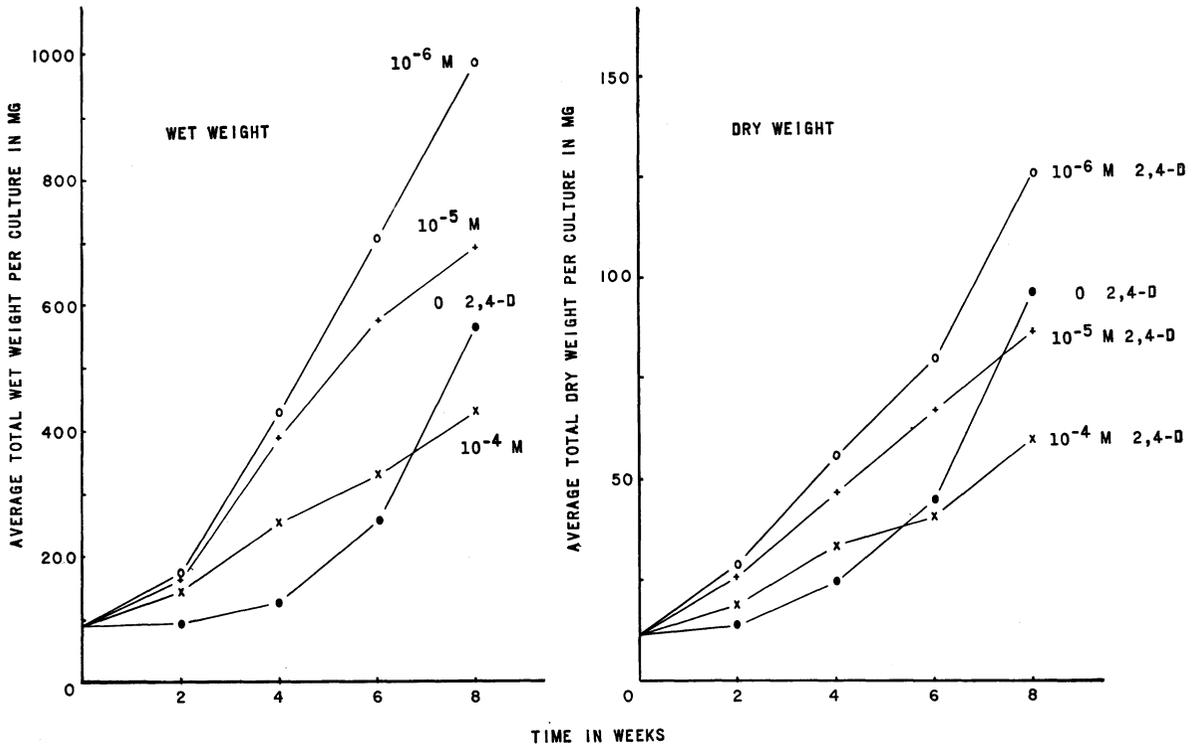


Fig. 9-10.—Fig. 9. (Top) The average weight per culture in mg. of pea root callus tissue grown in liquid media containing 1 g./l. yeast extract in the presence of different concentrations of 2,4-D.—Fig. 9a (left) shows wet weights; initial wet weight was 90 mg.—Fig. 9b (right) shows dry weights; initial dry weight was 11 mg.—Fig. 10. (Bottom) A comparison of the average wet weight increase of pea root callus tissue grown in different liquid media for 8 weeks (fig. 10a, left) with the friability of the cultures as measured by the average number of callus pieces per culture (fig. 10b, right). Horizontal coordinates of the two figures are the same.

once formed, will tolerate and actually be stimulated in their growth (wet weight increase) by higher yeast extract concentrations than will RS callus tissues.

The anatomical basis for differences in the RS and friable type of callus.—In an attempt to discern anatomical differences between the two types of tissues which might help to explain their difference in behaviour, histological preparations were made of the two distinctive tissues. The callus pieces were fixed with aspiration in formalin-acetic acid-alcohol fixative, were dehydrated in an ethyl-butyl alcohol series, embedded in "Tissue-Mat" and sectioned on a rotary microtome at 8μ . Sections were stained with Heidenhain's hematoxylin and safranin.

In the photomicrograph of a section of an RS callus (fig. 11) is evident the unorganized arrangement of cells characterizing the growth of the RS type of callus. The callus piece increased in size as a sphere by random unoriented cell divisions with irregular cellular enlargement and differentiation. The lack of organized development is evident throughout the tissue mass including the peripheral tissues exposed directly to the medium. In fig. 13 is shown at high magnification a region selected at random from the RS type callus where the lack of organization is clearly evident. The tissue is compact and dense with a few isolated scattered areas of tracheids which show secondary thickening and pitted walls.

The histological appearance of the friable type callus tissue is quite different. In fig. 12 is seen a section from a callus originally grown as the RS type and then transferred to a medium of high yeast extract concentration with a subsequent change in anatomical character to the friable type of growth at the periphery of the tissue. The region of friable tissue is most strikingly different in the degree of its organization. Instead of the homogeneous unorganized tissue evident in fig. 11, the friable tissue shows primordium-like areas of organization with extensive rows of cell lineages and structures reminiscent of root apical meristems and root caps. Between these organized structures are regions of sloughed-off cells which serve to separate the peripheral tissue into discrete nodular structures some of which dissociate and fall into the nutrient medium. These separated tissue masses continue cell division and enlargement in a similar, partially organized manner. Thus it appears that the major difference in behaviour of the two tissue types resides in the degree of organization. In the presence of high yeast extract concentration in the medium relative to the auxin concentration, the callus tissue shows a marked tendency toward organization. This tendency toward organ formation is suppressed by high 2,4-D concentration relative to the level of yeast extract in the medium.

That the friable callus tissue, unlike the RS

callus tissue, does, in fact, possess organized root-like structures is evident from the following experiment. Small separate pieces of the friable callus tissue were transferred from medium in which they had developed (5 g./l. yeast extract plus $10^{-6}M$ 2,4-D) to flasks containing either the M6 medium (low yeast extract plus 2,4-D) or the original modified Bonner medium, lacking both yeast extract and 2,4-D. The latter medium had been shown to be optimum for normal root development of excised root tips. In the M6 medium, the tissue continued to grow in the same partially organized manner characteristic of the friable callus. In the Bonner medium, however, the nodular, partially organized structures present in the friable tissues developed into normal, well-organized roots (fig. 7) each projecting from the tissue in the same orientation as the nodular structure from which it was derived.

The formation of cell suspensions from friable callus cultures.—Callus tissue of the friable type, when continued for prolonged periods with regular addition of fresh medium to the flask, produces a culture of many hundreds of small tissue pieces and the culture medium itself becomes a dense suspension of isolated viable cells. The cell suspension may represent a considerable proportion of the weight of the culture i.e., up to 20 per cent of the fresh weight. In fig. 14 is represented under high magnification a sample of this cellular suspension. The cells in the medium vary in size from 20μ up to over 100μ in certain cases. Depending on the age of the culture, a large proportion of the cells appears to be viable with cells usually showing large vacuoles criss-crossed with cytoplasmic strands in which protoplasmic streaming is clearly evident. The nucleus may be surrounded by starch grains and other particulate structures may be present in the cytoplasm. Usually the cell wall is evident and is readily demonstrated by simple plasmolysis of the protoplast. Some plasmolyzed cells can be discerned in fig. 14. No conclusive evidence has been gained that such cells actually divide after they have been separated from the tissue mass from which they were derived.

Any explanation of the friable condition and the separation of cells must ultimately rest at the biochemical level. Analysis of the basis for friability has been hampered by the complexity of the medium. Yeast extract is a complex mixture of organic and inorganic materials and its presence in the medium in quantity is not conducive to a ready clarification of the problem. Work is thus proceeding toward the development of a synthetic medium which will allow manipulation of the nutrition of the tissue under more carefully controlled conditions.

DISCUSSION.—The appearance of morphologically distinct tissue forms in callus cultures has been repeatedly reported in the literature (Gau-

theret, 1955a; Henderson, 1954; Nobécourt, 1955; Reinert, 1956; Tryon, 1956). In agar culture these differences tend to be suppressed and often have been disregarded by tissue culture workers, since the tissue pieces, although of strikingly different appearance, are known to have had a common origin from a particular callus strain. In his review Gautheret (1955b) has discussed certain modifications of this sort which have been studied physiologically. They have been shown to be due to distinct physiological changes as, for example, in the "anergized" (or habituated) tissues which no longer require auxin in the medium and are morphologically distinct from the original tissues. The cause of these tissue modifications is not at all certain, although in the case of auxin anergy it is believed to be a kind of enzymatic adaptation (Gautheret, 1955b). Such a modification may arise in the tissue spontaneously and uncontrollably without deliberate manipulation of the external nutrient medium.

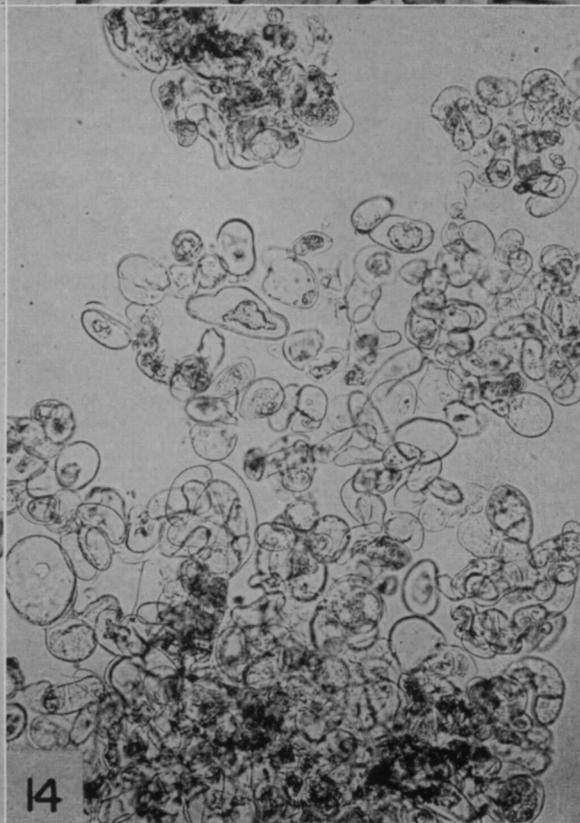
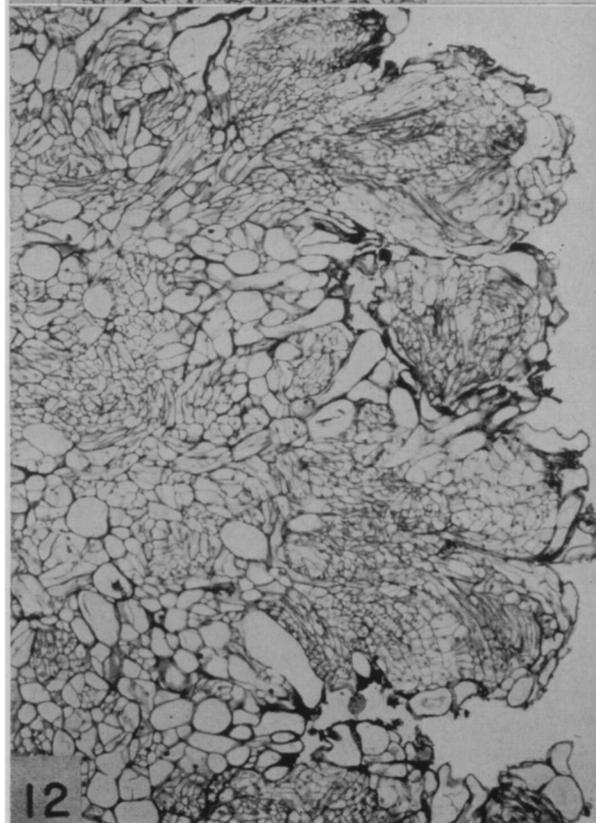
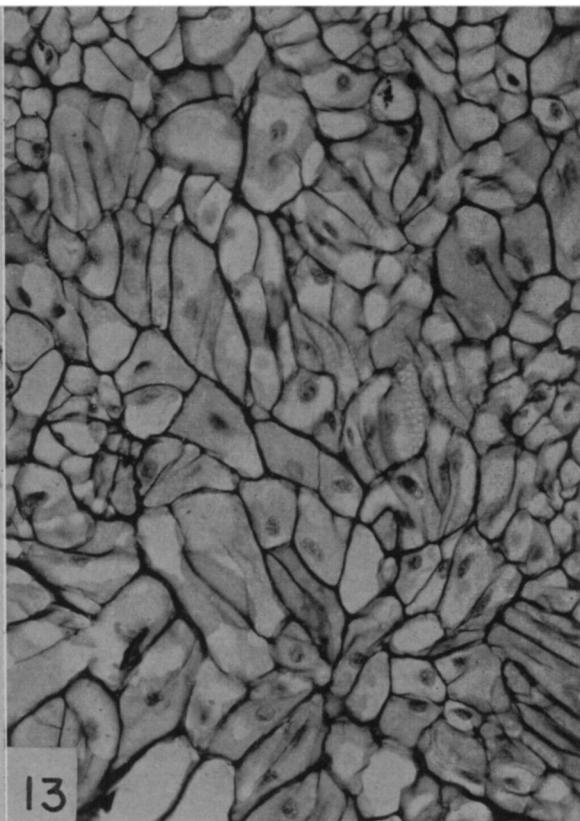
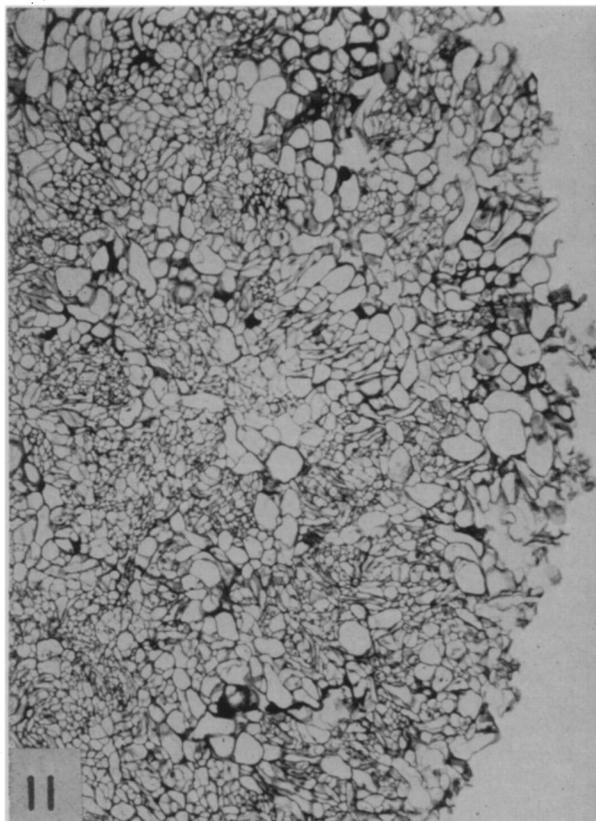
In certain other cases, there are reports of morphologically distinct modifications which are attributable directly to manipulation of the nutrient medium. The best documented case to date is that worked out by Skoog and his associates (Miller and Skoog, 1953; Skoog, 1954) who have shown tobacco pith callus tissue to respond in distinct morphogenetic patterns to manipulation of the nutrient medium, especially of certain components: auxin (indoleacetic acid), adenine (or kinetin), phosphate and amino acids. In the tobacco stem segment test of Skoog, increasing auxin concentration at a constant adenine level caused increasing inhibition of bud formation, whereas at an optimum level of auxin, root initiation was promoted. In most of these studies on tobacco tissue, Skoog did not use clonal callus tissues which had been previously maintained as essentially undifferentiated tissues. His work does show, however, the importance of the nutrient medium in affecting the morphological expression of the tissues in culture.

The pea root callus tissue described here is of particular interest since it represents a tissue system in which the degree of differentiation and/or organogenesis can be controlled in cultured root tissues by manipulation of the components of the nutrient medium. Such a system offers a means of studying the factors controlling the expression of organogenic potentialities in roots. Root callus tissue maintained in an unorganized condition for many months through numerous subtransfers can be made suddenly to form normal, organized root structures by changing the constituents of the nutrient medium. Further, all intergradations of organogenesis from completely organized root structures down to completely disorganized cell suspensions can be produced at will by manipulation of the components of the medium. Thus, the spec-

trum of organization in a genetically homogeneous tissue system is subject to study and analysis.

That 2,4-D suppresses organization in this tissue system seems clear. This behavior of 2,4-D is analogous in certain respects to that described by Skoog (1954) for indoleacetic acid in tobacco stem segments. The suppression of organ formation can be reversed by increased concentrations of yeast extract. Further evidence concerning the nature of the yeast extract effect depends upon the establishment of a synthetic medium capable of maintaining the pea root callus tissue in continuous culture. It seems clear from the present work that the nutrient requirements for continued meristematic activity of the root vascular cambium and its callus derivatives are greater than those of the apical meristem or for the initiation of lateral root meristems. How complex these requirements are remains to be determined. It is also a matter of interest to determine the relationship between the requirements *in vitro* and those *in vivo*. Clearly, 2,4-D is an extraneous stimulator of cell division, but may be replaced in the plant by an auxin which acts together with other factors supplied in the root to initiate the vascular cambium at a particular locus within the plant body.

Several reports of the complex interaction in the growth of callus tissue of auxins of the 2,4-D type and a complex nutrient are of interest. Steward and Caplin (1951) and Steward and Shantz (1956) have reported the synergistic interaction of 2,4-D and coconut milk in the growth of potato tuber callus tissue with a further stimulus to weight production by casein hydrolysate. These authors reported no effect of variation of the components of the medium on organogenesis in the potato tissues, but did indicate differential responses in terms of cell division versus cell enlargement depending upon the structure of the auxin used. In potato tuber tissue, 2,4-D appears to stimulate cell division and enlargement about equally. The active components of the medium remain to be completely determined. Jagendorf (1952) reported the synergistic interaction of *p*-chlorophenoxyacetic acid and yeast extract in "tumor" formation in cabbage roots. The effect of the yeast extract could be replaced by organic nitrogen in the form of urea, asparagine or simply by inorganic nitrogen as ammonium chloride. In this work, studies were made of seedling roots and the tumors were formed on the roots rather than on isolated callus tissues. Jagendorf and Bonner (1953) were able, however, to establish and maintain such callus tissue *in vitro* on the same media. No effects of the different media on the morphology of the callus tissues in culture were described. Tryon (1955) reported the formation of tumorous growths on seedling roots of tobacco grown in a medium containing malt and yeast extract. She has been able to culture these callus tissues *in vitro*. Nickell (1955) has established callus tissues



from roots of the sweet clover, *Melilotus officinalis*, using Jagendorf's technique.

Reinert (1956) has recently described another case in which change in the constituents of the medium has resulted in striking morphological modifications, giving rise to extremely friable tissue. Omission of certain components from the nutrient medium is suggested as a possible cause of the modification in growth form in spruce tissues, *Picea glauca*. Still other reports indicate that the nutrient conditions may affect the morphology of the tissue cultures, giving rise to extreme friability. Muir et al. (1954) reported growing several tissues in the presence of yeast extract with constant agitation, leading to the production of cell suspensions. Similarly, Steward and Shantz (1956) describe a liquid culture method involving large numbers of carrot tissue explants which during culture with constant agitation, produce free floating cells. In this case the friability is perhaps associated with the changing condition of the nutrient medium following periods of culture with large amounts of tissue.

In the pea root callus cultures described here, the friable condition is attributable directly to the constitution of the nutrient medium. Even on agar medium, the friable condition occurs when the medium contains high levels of yeast extract relative to the 2,4-D concentration. With constant agitation this friability becomes most evident and the resulting suspension of innumerable viable cells of higher plant tissue is a remarkable product which should prove of considerable experimental use.

Any explanation of the friable condition must involve the physiology and chemistry of the cell walls or the middle lamellar materials which cement the cells together. The falling apart of the tissue is probably due to breakdown of calcium pectate, the cementing material of the middle lamellae. Such a breakdown could occur either through enzymatic action, e.g., a pectic enzyme capable of dissolving the pectic materials, or by removal of the calcium by chelation with a resultant loss of the cementing properties of the middle lamellae. The present experiments do not give the evidence necessary to decide the issue. Thus far, preliminary experiments do not support the idea of calcium chelation, although this possibility is not to be discarded. Northcraft (1951) has shown that externally supplied chelators such as ammonium oxalate may be used to cause disintegration of higher plant tissues.

Maceration of the compact callus tissues of the

RS type with commercial pectinase preparations using very small tissue pieces has been found possible with the method of Chayen (1952), which points to pectic compounds as the critical binding agents in the root callus tissues. It is suggestive that the conditions leading to partial organization of the callus tissue, e.g., the formation of presumptive root primordia, are the conditions under which friability begins to appear. It is possible that changes in pectic substances usually associated with the dissolution of the middle lamellae in the sloughing of root cap cells in an organized root meristem occur also under the particular nutrient conditions which lead to friability in culture. If such is the case, these changes are suppressed by the presence of 2,4-D. No direct evidence for this possibility has been elicited in the present work. Unfortunately, little is known about the chemistry of the root cell wall components or of the enzymes involved in their metabolism.

SUMMARY

Excised root tips from germinated pea seeds, variety Alaska, were induced to form vascular cambia by treatment with IAA. Tissues isolated from the vascular cambium region of such root tips have been grown as a root callus tissue on a nutrient medium containing the components of a modified Bonner medium: i.e., major salts, vitamins and 4 per cent sucrose and, in addition, powdered yeast extract and 2,4-D. The clone of pea root callus tissue thus established was shown to require both the yeast extract and the auxin for increase in weight with an optimum concentration of 1 g./l. yeast extract and 10^{-6} M 2,4-D. The callus grew on a solidified agar medium or in liquid flask culture if constantly agitated. In liquid culture two morphologically distinct tissue forms were produced in response to different relative concentrations of yeast extract and 2,4-D in the medium. A solid, round, smooth type of callus tissue produced by essentially unorganized tissue growth (RS type) was formed at optimum concentrations for production of increase of tissue weight. At high yeast extract concentrations relative to the 2,4-D concentration, an extremely friable type of tissue was produced which resulted in cultures consisting of many small nodular pieces with heavy suspensions of viable cells in the liquid medium. Anatomical studies of the two morphological types show that in the friable tissues, partial organization of root meristem-like structures had occurred. These organized structures were shown to be capable of developing as

Fig. 11-14.—Fig. 11. Photomicrograph of a section of pea root callus tissue of the RS type showing the homogeneous unorganized nature of the tissue mass. $\times 70$.—Fig. 12. Photomicrograph of a section of pea root callus tissue of the friable type showing on the periphery of the callus to the right the partial organization of the tissue into primordium-like structures. Note the alignment of cells in rows and the discontinuities between primordia. $\times 70$.—Fig. 13. Enlarged section from fig. 11 showing at high magnification the lack of organization of the RS type callus tissue. $\times 270$.—Fig. 14. Photomicrograph of a sample of cell suspension taken from the liquid medium of a friable callus culture. $\times 140$.

roots in auxin-free medium. In the presence of yeast extract, 2,4-D suppresses organ formation, leading to the unorganized development characteristic of the RS type of callus. Once a tissue has changed to the friable type, it has not been possible to date to grow it under conditions in which it will revert to the original RS condition. The

possibilities for study of root morphogenesis offered by the pea root callus which shows such morphological diversity in response to changes in the nutrient medium are discussed.

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