

# AMERICAN JOURNAL OF Botany

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Author(s): Patricia L. Goforth and John G. Torrey

Source: *American Journal of Botany*, Vol. 64, No. 4 (Apr., 1977), pp. 476-482

Published by: [Botanical Society of America](#)

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## THE DEVELOPMENT OF ISOLATED ROOTS OF *COMPTONIA PEREGRINA* (MYRICACEAE) IN CULTURE<sup>1</sup>

PATRICIA L. GOFORTH AND JOHN G. TORREY

Cabot Foundation, Harvard University, Petersham, Massachusetts 01366

### A B S T R A C T

Seedling roots of the sweet fern *Comptonia peregrina* (L.) Coult. were excised aseptically and cultured in a modified Bonner-Devirian liquid nutrient medium. Root elongation was very slow in the basic medium which contained inorganic salts, B-vitamins, trace elements and 4% sucrose. The addition of plant hormones including gibberellic acid, indoleacetic acid, and zeatin, alone or in combinations, had little effect on growth. Myoinositol at 10 or 100 ppm doubled the rate of elongation. The effect of this sugar alcohol could not be replaced by scyllitol, D-sorbitol, D-mannitol or by increasing the sucrose concentration. Subcultured root tips showed progressively less elongation in successive transfers. Secondary thickening of the roots, especially in the basal half, occurred in initial passages and in subcultured roots without added hormones. Root buds also occurred spontaneously especially in the basal portions of cultured roots, both in first and in successive passages. An anatomical analysis showed that these buds were endogenous, arising from a secondary cortex of pericyclic origin.

ISOLATED ROOTS of herbaceous species growing in sterile nutrient culture solutions have provided experimental systems for the study of various problems including root nutrition, lateral root and bud formation, secondary thickening and nodule development (Torrey, 1965). Although establishing roots of woody plants in culture has been a difficult process, limited success has been achieved with the following plants: *Acacia melanoxylon* R. Br. (Bonner, 1942), *Robinia pseudo-acacia* L. (Seeliger, 1956), *Pinus* spp. (Slankis, 1947; Barnes and Naylor 1959; Ulrich, 1962), *Acer rubrum* L. (Bachelard and Stowe, 1963) and *Picea abies* L. (Momot et al., 1974).

*Comptonia peregrina* (L.) Coult. (Myricaceae) is a woody shrub commonly found in eastern and central North America. The root system of *Comptonia* consists of a taproot, numerous lateral roots and a few wide-ranging and specialized horizontal roots from which sprouts are readily formed. *Comptonia* propagates itself vegetatively by these root sprouts, forming large clonal populations. Infection of the root by a soil actinomycete-like organism results in the formation of root nodules capable of fixing atmospheric nitrogen (Ziegler and Hüser, 1963). Because of interest in the process of nodule formation in these roots, we made an

effort to establish isolated roots of *Comptonia* in sterile nutrient culture.

**MATERIALS AND METHODS**—Collection and germination of fruits of *Comptonia* followed procedures reported by Del Tredici and Torrey (1976). Root tips used to initiate cultures were taken from seedlings which had been started from seeds germinated on a sterile agar nutrient medium containing 10 ppm gibberellic acid (GA<sub>3</sub>). When the radicle of the germinated seed had reached 2–3 cm in length, the terminal one-centimeter of the radicle was excised aseptically, and transferred to 50 ml of liquid—modified Bonner-Devirian (BD) medium (Torrey, 1956) in a 125-ml erlenmeyer flask. The modified BD medium contained the following components (in mg/l of glass-distilled water): 242 Ca(NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O, 42 MgSO<sub>4</sub>·7H<sub>2</sub>O, 85 KNO<sub>3</sub>, 61 KCl, 20 KH<sub>2</sub>PO<sub>4</sub>, 2.5 FeCl<sub>3</sub>·6H<sub>2</sub>O, 0.1 thiamin HCl, 0.5 nicotinic acid, 0.5 pyridoxine HCl, 1.5 H<sub>3</sub>BO<sub>3</sub>, 1.5 ZnSO<sub>4</sub>·7H<sub>2</sub>O, 4.5 MnSO<sub>4</sub>·H<sub>2</sub>O, 0.25 NaMoO<sub>4</sub>, 0.04 CuSO<sub>4</sub>·5H<sub>2</sub>O, 40,000 sucrose, with the pH adjusted to 5.5 before autoclaving. Any further additions were made after cold sterilization of the solution with a Millipore filter. The root cultures were incubated in the dark at 25 C. The elongation of the main axis of each root was measured and recorded weekly. At the end of a 6–8-wk period of culture one-centimeter tips of the actively growing roots were excised and subcultured. The bases of the actively growing roots were then transferred to a growth chamber with 12 hr of light and 12 hr of dark at 25 C. Mixed white and fluorescent lights with a total intensity of about 260 ft-c were used.

Material for anatomical study was handled by standard paraffin methods; the roots were fixed

<sup>1</sup> Received for publication 25 August 1976; revision accepted 8 November 1976.

The authors are indebted to Peter Del Tredici for providing seeds and seedlings of *Comptonia*, to Kathy Kamo for assistance in culturing roots, to Monica Mattmuller for instruction in histological and photographic techniques. The research was supported in part by research grant BMS 74-20563 from the National Science Foundation and in part by the Maria Moors Cabot Foundation for Botanical Research of Harvard University.

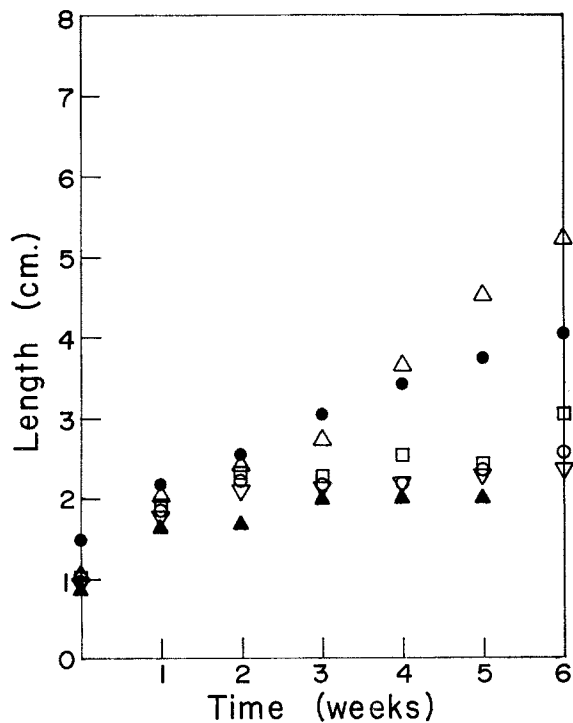


Fig. 1. The effect of various hormones on the rate of elongation of isolated roots of *Comptonia*. ●—BD with no addition; △—10 ppm GA<sub>3</sub>; ▽—1.0 ppm IAA; ○—0.1 ppm IAA; ▲—1.0 ppm zeatin; □—0.1 ppm zeatin.

in formalin-acetic acid alcohol, dehydrated through a tertiary-butyl alcohol series, embedded in Tissueprep, and sectioned at 10 μm. The sections were stained with Delafield's hematoxylin and safranin.

**RESULTS—The effects of plant growth substances on root elongation**—Excised root tips of *Comptonia* grown in BD medium with no added growth substances showed insignificant main-axis elongation, i.e., approximately 4 cm in 8 wk. Tests were made of a number of plant growth substances known to be important in root development (Torrey, 1976). The addition to the BD medium of 10 ppm GA<sub>3</sub>, which was essential for seed germination (Del Tredici and Torrey, 1976), had a slight stimulatory effect (Fig. 1). Indoleacetic acid (IAA) at 0.1 and 1.0 ppm inhibited main axis elongation, but stimulated lateral root initiation. Zeatin at 0.1 and 1.0 ppm also had an inhibitory effect on root elongation.

A dramatic improvement in root elongation was shown upon the addition of 100 ppm myoinositol to the medium (Fig. 2), producing an average elongation of 20 mm/wk, a rate similar to that of roots of pea (*Pisum sativum* L.) at 15 mm/wk (Bonner and Devirian, 1939), but not as rapid as

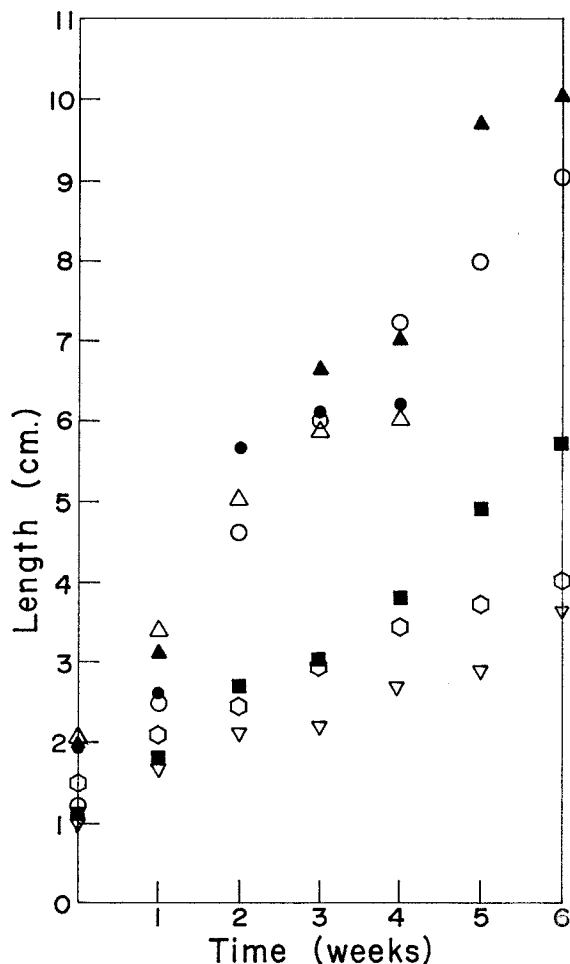


Fig. 2. The effect of various concentrations of myoinositol on the rate of elongation of isolated roots of *Comptonia*. Hexagon—BD with no additions; ●—1,000 ppm myoinositol; △—500 ppm myoinositol; ○—100 ppm myoinositol; ▲—10 ppm myoinositol; ■—1.0 ppm myoinositol; ▽—0.1 ppm myoinositol.

that of *Convolvulus arvensis* L. at 20 mm/day (Bonnett and Torrey, 1965). As the roots matured they became thicker, and formed laterals and root buds (Fig. 4). Different concentrations of myoinositol were tested as a supplement to the BD medium for their effectiveness on root elongation in *Comptonia*. Both 0.1 and 1.0 ppm myoinositol elicited a suboptimal response. Ten ppm and 100 ppm myoinositol produced essentially the same rate of elongation. Higher concentrations of myoinositol were probably supra-optimal. Combinations of myoinositol and other growth substances (GA<sub>3</sub>, IAA, and zeatin) were added to the culture medium (Table 1A). Although GA<sub>3</sub> + myoinositol and zeatin + myoinositol caused a higher rate of elongation than either GA<sub>3</sub> or zeatin alone, neither combination was as

TABLE 1. Mean length in cm  $\pm$  standard deviations (S.D.) of the main axis of cultured roots of *Comptonia peregrina* measured after 6 wk. Each experiment included at least 10 initial root tips. **A.** The influence of combinations of growth substances. **B.** The effect of increased sugar concentration or other sugar alcohols.

Medium	Length in cm ( $\pm$ S.D.)
TABLE A	
BD alone	4.0 $\pm$ 1.4
100 ppm myoinositol	
+	
0.1 ppm IAA	5.0 $\pm$ 3.8
100 ppm myoinositol	
+	
1.0 ppm GA <sub>3</sub>	5.2 $\pm$ 1.5
100 ppm myoinositol	
+	
0.1 ppm zeatin	5.8 $\pm$ 4.0
100 ppm myoinositol	
+	
0.1 ppm zeatin	
+	
0.1 ppm IAA	5.5 $\pm$ 4.5
100 ppm myoinositol	9.1 $\pm$ 3.6
TABLE B	
8% Sucrose	5.1 $\pm$ 3.5
100 ppm D-sorbitol	5.0 $\pm$ 3.3
100 ppm Scyllitol	4.9 $\pm$ 3.2
100 ppm D-mannitol	3.5 $\pm$ 3.2

effective as 100 ppm myoinositol alone. IAA was consistently inhibitory to root elongation at the concentrations tested.

Carbon sources and sugar alcohols other than myoinositol were also tested (Table 1B). When the sucrose level of the basic BD medium, i.e., without added growth substances, was raised from 2% or 4% to 8%, the rate of root elongation increased. This growth rate was not as rapid as in 4% BD plus 100 ppm myoinositol. Of the sugar alcohols tested, scyllitol was structurally most similar to myoinositol. However, 100 ppm scyllitol added to BD medium with 4% sucrose had no effect on root growth. Roots cultured in 4% sucrose BD medium plus 100 ppm mannitol or plus 100 ppm sorbitol had slow growth rates very similar to 4% sucrose BD medium alone.

Attempts were made to establish continuous cultivation of roots of *Comptonia* by repeated subculture as has been done in tomato, *Lycopersicon esculentum* L. (White, 1938), *Convolvulus* (Torrey, 1958) and other roots. At the end of a culture period of 6–8 wk, one-centimeter tips were removed from actively growing roots and were subcultured in BD + 100 ppm myoinositol. This

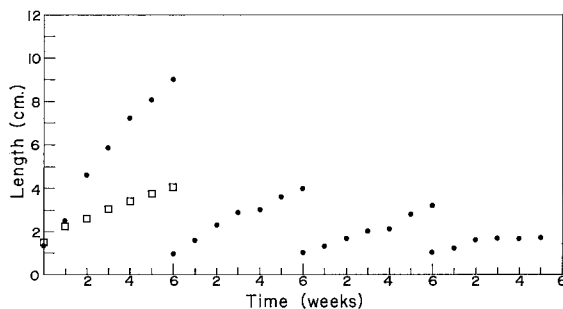


Fig. 3. The rate of elongation of isolated roots of *Comptonia* cultured in BD medium + 100 ppm myoinositol through three successive subcultures of six weeks each. ●—BD + 100 ppm myoinositol; □—BD with no additions.

process was repeated three times in succession (Fig. 3). The subcultured roots declined in root length and in diameter and often in vigor, but maintained the ability to form root buds and appeared to be thickened. Most of the root growth in subcultured roots was in lateral root formation and in the development of elaborately branched roots (Fig. 5). Because only main axis elongation was measured and recorded, an accurate representation of subcultured root growth was not easily obtained from these experiments. It was apparent, however, that indefinite subculture of *Comptonia* roots in BD medium with a myoinositol supplement would not be sustained.

**Secondary thickening**—In the BD culture medium with added myoinositol, *Comptonia* roots underwent secondary thickening, increasing 3–4 times the diameter of the original excised tip. The thickened area extended along a large portion of the proximal half of the root as is seen in Fig. 4. The initial excised tip at the time of inoculation was approximately the diameter of one of the smaller laterals in the photograph. In rapidly elongating roots secondary thickening was initiated before 6 wk. Thickening was also observed in a slow-growing root continually cultured in 4% sucrose medium for 6 months. Roots developed from radicle tips of embryos showed thickening even if they were not elongating, but the thickening process took longer. Actively growing subcultured roots also thickened (Fig. 5), although the thickening appeared more slowly with each successive transfer. There was often visible a longitudinal splitting of the outer cortex, especially on the proximal end of the root. The thickened areas frequently turned green when the cultured roots were placed in low-intensity white fluorescent light.

An anatomical study was made of the thickened roots. Figure 6 shows a cross section of an unthickened portion of a root with primary xylem

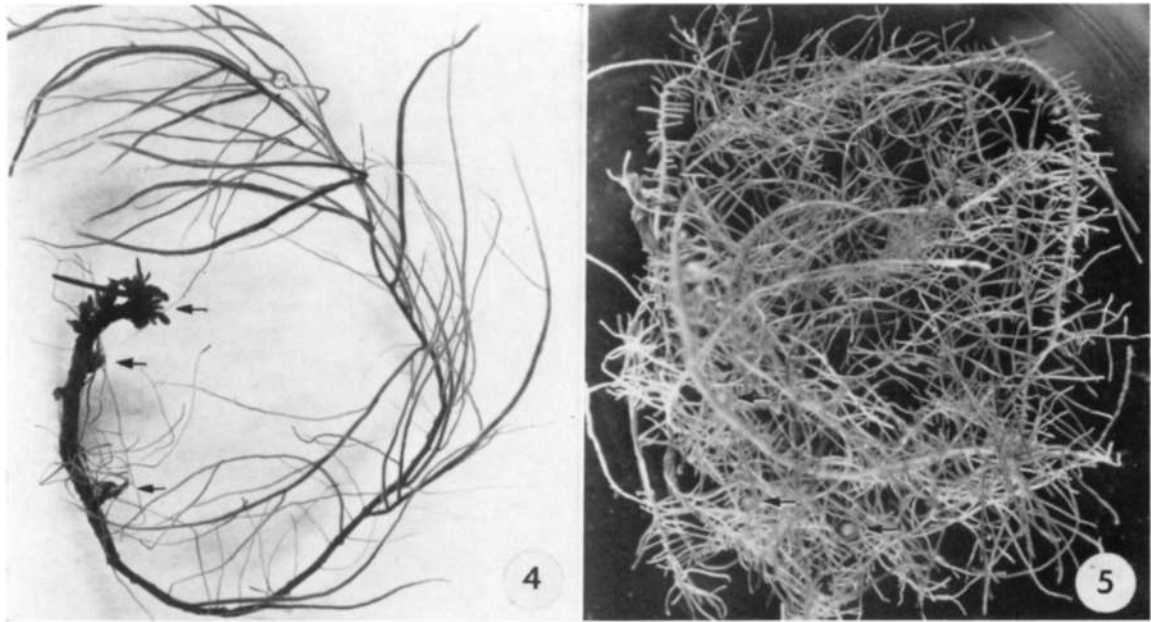


Fig. 4, 5. Cultures of isolated *Comptonia* roots. 4. A root cultured in 100 ppm myoinositol from a radicle tip excised from an embryo. The arrows indicate root buds.  $\times 1.5$ . 5. Three subcultured root tips grown in BD + 100 ppm myoinositol. The arrows indicate root buds.  $\times 1.5$ .

arranged in a tetrarch pattern. The primary cortex is intact and contains many intercellular spaces. A cross section of an older region of the same root is shown in Fig. 7. Secondary xylem formed between the arms of the primary xylem is evidence of cambial activity. The pericycle has undergone one or more divisions, forming the beginning of a secondary cortex. The primary cortex has begun to slough off. In roots which become more thickened, a solid core of secondary xylem is evident (Fig. 8). Radial rows of parenchyma cells form xylem rays in this region. A limited amount of secondary phloem occurs to the outside of the secondary xylem. The secondary cortex is derived from proliferation of the pericycle. The suberized and corky outer layers suggest the activity of a phellogen and the formation of a bark-like outer layer.

**Root bud formation**—Root buds developed on the proximal ends of cultured *Comptonia* roots (Fig. 4). Only four other species of cultured roots, *Robinia pseudoacacia* L. (Seeliger, 1956), *Convolvulus arvensis* L. (Torrey, 1958), *Linaria vulgaris* (Charlton, 1965), and *Isatis tinctoria* L. (Danckwardt-Lillieström, 1957) have been reported to form endogenous root buds regularly.

The root buds first appeared as small primordia along the proximal end of roots cultured in the dark. When placed in light, the shoot elongated and formed leaf-like structures which eventually expanded into small leaves which turned green, then dark burgundy in color.

Figure 9 shows a cross section of a *Comptonia* root with the apical meristem and leaf primordia of the root bud clearly visible in longitudinal section. *Comptonia* root buds appear to originate in the secondary cortex opposite a protoxylem pole. Because the secondary cortex is formed by the pericycle or from pericyclic derivatives, the root buds are endogenous in origin. The vascular connection of the root bud to the main root forms at a later stage than that shown in Fig. 9.

**DISCUSSION**—The successful cultivation of isolated roots of woody species would provide a useful tool for studying a variety of problems in relation to plant form and function. It is interesting to consider why roots of so few species of woody plants have been established in culture. Relatively few attempts may have been made. Difficulties in inducing seed germination may have limited the sources of sterile roots for the initial cultures. Isolated roots of woody species may have had requirements for special factors essential for growth not discovered from studies of herbaceous roots. The requirement for myoinositol by isolated *Comptonia* roots is an example of such a factor.

Myoinositol appears to be an essential component for the growth of isolated roots of *Comptonia* grown in sterile culture. In the first culture period the presence of myoinositol doubles the growth of the roots and thereafter makes possible the subculturing of roots over several passages. The effective concentrations, i.e., 10–100 ppm,

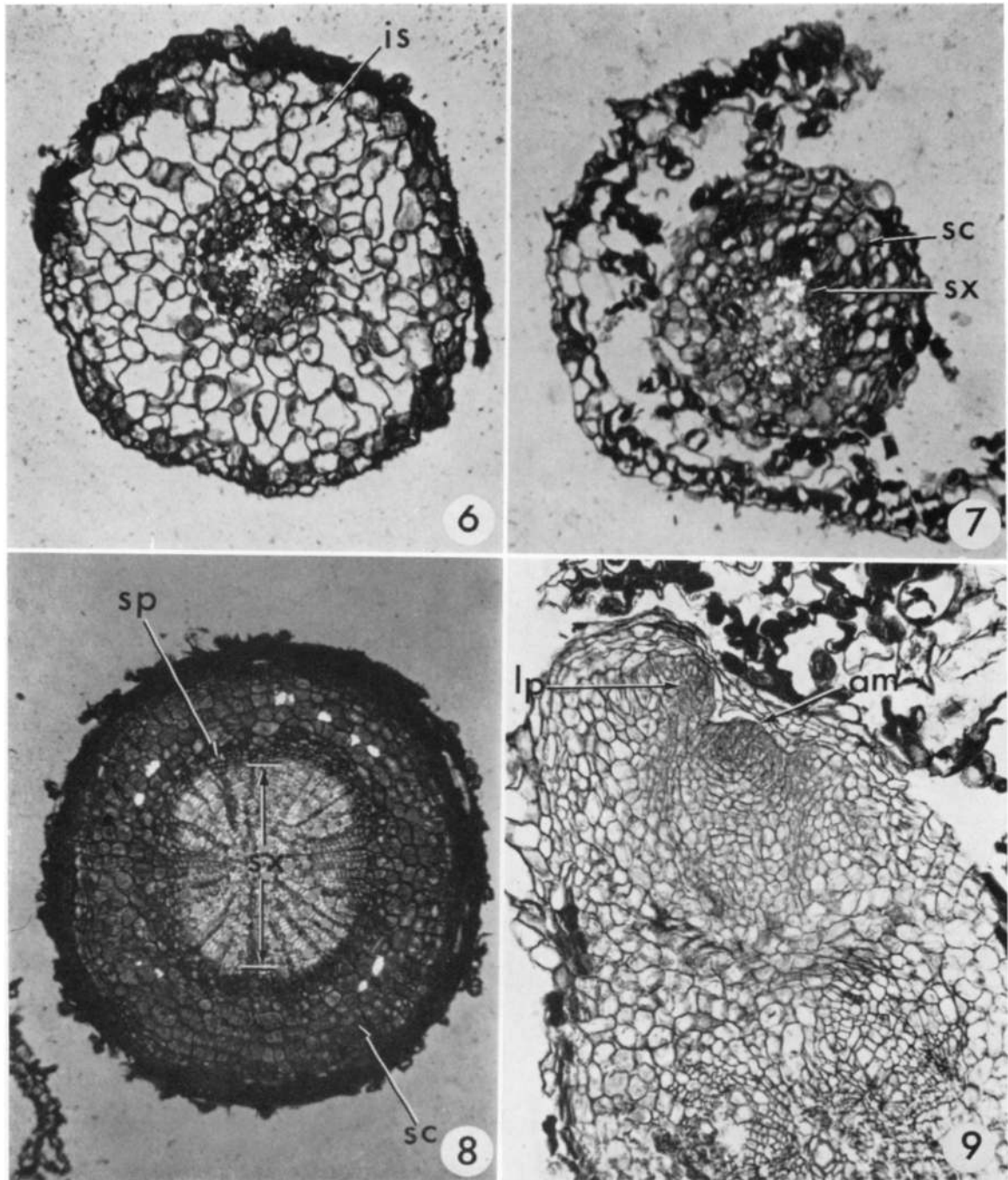


Fig. 6-9. Cross sections of isolated *Comptonia* roots. 6. A cross section of a primary root photographed with polarized light. The birefringence of the primary xylem makes the tetrarch pattern evident. The cortex with many intercellular spaces (is) is intact.  $\times 200$ . 7. A cross section of a more mature region of the root photographed with polarized light. Some secondary xylem (sx) is evident. Divisions have taken place in the pericycle forming a secondary cortex (sc). The primary cortex has enlarged intercellular spaces and is sloughing off.  $\times 200$ . 8. A cross section of a thickened area of a root photographed with polarized light. A central core of secondary xylem (sx) has been formed with rays of parenchyma cells in this region. Some secondary phloem (sp) is evident in the periphery of this region. The secondary cortex (sc) is formed. The brightly birefringent cells in the secondary cortex contain calcium oxalate crystals.  $\times 100$ . 9. A cross section of part of a root with a longitudinal section of a root bud in the secondary cortex. The apical meristem (am) and leaf primordium (lp) are well formed.  $\times 100$ .

indicate that it serves a role intermediate between that of a substrate and a co-factor or vitamin. In this respect, myoinositol behaves in a manner similar to that reported for the growth in vitro of some plant callus tissues (Braun, 1958; Mura-shige and Skoog, 1962; Shantz, Sugii, and Steward, 1967) and for responses such as that of secondary thickening in excised roots of radish grown with basal feeding (Loomis and Torrey, 1964; Torrey and Loomis, 1967a, b). Recently, Kaul and Sabharwal (1975) reported myoinositol to be essential for the survival of callus tissue of *Haworthia* grown in sterile nutrient culture.

The role of myoinositol in plant metabolism is not fully understood. According to Loewus (1971), myoinositol serves as an important substrate for cell wall synthesis, being oxidized to form glucuronic acid which is polymerized to polysaccharides incorporated into the primary cell wall. Jung, Tanner, and Wolter (1972) have presented evidence which demonstrates an important role for myoinositol for the functioning of cell membranes. In either role and possibly others, a limiting supply of myoinositol could limit growth. In *Comptonia* apparently the excised root is unable to synthesize amounts of myoinositol adequate to meet its needs for growth and must be provided the growth factor exogenously.

However, from the growth data presented (Fig. 3), it is clear that BD medium supplemented with myoinositol is not a "complete" medium. Continuous growth of *Comptonia* through repeated subculture has not been possible with this medium and a further search must be made for other factors or conditions to make continuous culture of *Comptonia* roots possible.

Growth in roots of *Comptonia* is difficult to determine quantitatively because of the complexity of the branching habit (Fig. 4, 5). Measurements of linear growth of the main axis presented in Fig. 1-3 and Table 1 do not represent the growth response adequately. Furthermore, with repeated branching, the lateral roots become progressively finer, easily damaged in handling, and difficult to use for subculture. An improved medium may increase the vigor of subcultured roots and their branches so as to obviate these problems.

The formation of secondary tissues in cultured roots is an interesting occurrence and provides another experimental system for studying this developmental process. Dormer and Street (1948) first observed secondary thickening in an isolated tomato root grown in continuous culture for 6 months, a report very similar to our observation of a 6-month-old *Comptonia* root which had thickened after culture in 4% sucrose BD medium. There have been reports of secondary thickening in cultured roots in response to manipulation or addition of growth regulators. Torrey (1951) reported that cultured pea roots would

thicken after root decapitation. In the absence of an apical meristem the lateral root primordia were found to play an active role in determining the differentiation of the vascular tissues. It was assumed that naturally produced auxin was involved in the process.

Loomis and Torrey (1964, 1967a, b) found they could induce isolated radish roots (*Raphanus sativus* L.) to undergo secondary thickening in culture by adding kinetin ( $5 \times 10^{-6}$  M) and indoleacetic acid ( $10^{-5}$  M) to the sucrose and salts in the medium provided to the root base in a vial. The further addition of myoinositol at 100 ppm to the basal feeding medium stimulated root growth and greatly promoted thickening. Myoinositol alone did not induced thickening. Peterson (1973) reported an essentially similar response in cultured roots of turnip (*Brassica rapa* L.).

The initiation of a vascular cambium and the formation of secondary vascular tissues in cultured roots of *Comptonia* are unusual in that no exogenous hormone supply was required. Most of the isolated *Comptonia* roots which were started from an embryonic root tip thickened whether or not they underwent active elongation. Subcultured roots thickened only if root elongation occurred, either in the main axis or in the laterals. This thickening was slower than that in the embryonic root tips. This evidence suggests that there may be some carry over of substances from the embryo which cause thickening. However, thickening in subcultured roots is caused by something synthesized by the root.

Another interesting feature of isolated *Comptonia* roots is their ability to form root buds. Torrey (1958) and Bonnett and Torrey (1966) described in detail the development of root buds in *Convolvulus arvensis* L., which differ considerably from those of *Comptonia*. *Convolvulus* root buds are formed on roots possessing only primary tissues and are initiated by cell divisions in the pericycle opposite a protoxylem pole. A meristematic dome of cells is organized at the outer periphery of the primordium. The first xylem elements are initiated obliquely to the xylem of the root. By two weeks the bud apex and its leaf primordia have penetrated the outer cortex and epidermis of the root.

The root buds on *Comptonia* roots, although not arising from primary tissue, are also endogenous in origin. They arise opposite a protoxylem pole in the secondary cortex which is derived from the pericycle. At the time of bud initiation the root has enlarged by the formation of secondary tissues and the outer primary cortex has been split and sloughed off. The vascular connection of the root bud develops after the formation of the apex and leaf primordia and connects to the secondary xylem of the root.

Root buds play an important role in the vegetative propagation of many plant species. Peterson

(1975) has written an extensive review on the initiation and development of root buds. Root buds allow plants to form large clones and to cover extensive areas. When the root systems are disrupted, the plant has the ability to form new plants, thus maintaining a stronghold in the area. Vegetative propagation of *Comptonia* by root bud formation on horizontally spreading roots may be the primary means of increase in this species.

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