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ANATOMY AND BUD FORMATION OF SUBTERRANEAN PARTS OF

LEAFY SPURGE (EUPHORBIA ESULA L.)

BY

CHARLES A. BEASLEY

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in partial fulfillment of the requirements for the
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of Agronomy, South Dakota State
College of Agriculture and
Mechanic Arts

1964

ANATOMY AND BUD FORMATION OF SUBTERRANEAN PARTS OF LEAFY SPURGE (<u>EUPHORBIA</u> <u>ESULA</u> L.)
Abstract

CHARLES A. BEASLEY

Under the supervision of Professor Lyle A. Derscheid

In order to obtain plant material for <u>in</u> <u>vitro</u> bud formation studies, many preliminary experiments involving germination and seedling growth were conducted. Anatomical studies were conducted to establish the identity of underground parts.

Cyclic temperature changes of 20 C and 30 C (12 hours each) provided 97, 80, and 21% germination for mottled, grey, and purple seeds in 12 days. Mottled seeds require only 2 cycles of alternating temperature to approach or equal the germination percent obtained with 10 additional temperature alternations. Two cyclic temperature changes provided about one-fourth of the maximum germination percent of grey and purple seeds obtained with 10 additional cycles. Hot water extracts of seeds inhibit seedling growth. Inhibition was less from extracts of more mature seeds (mottled) than from less mature seeds (grey and purple).

Maximum growth of isolated root tips was obtained under alternating temperature conditions, when 20 mg per liter adenine sulfate had been added to the media. The greatest portion of the increase was due to the cyclic temperature changes. Kinetin was a potent inhibitor of seedling and isolated root tip growth, in culture, at concentrations as low as 10^{-4} mg per liter.

Root segments from a plant grown on sucrose-based media were induced to maximum lateral root initiation by 1 mg per liter IAA. This same concentration of IAA allowed no buds to form. Maximum stimulation of bud formation was observed at 10^{-1} mg per liter kinetin. On root segments from a plant grown on sucrose-based media supplemented with adenine sulfate, IAA only induced callus. Bud formation was negligible or non-existent in both kinetin-treated and untreated segments.

Maximum stimulation of bud formation on hypocotyl segments occurred at 10^{-2} mg per liter kinetin, and this effect was removed by 10^{-4} mg per liter IAA. The callus formation observed on the basipetal ends of untreated segments or segments treated with 10^{-4} mg per liter IAA was diminished as the concentration of included kinetin increased.

The main axis of a mature plant of leafy spurge was composed of aboveground stem(s), a hypocotyl region, varying from a few millimeters to a few centimeters, a transition zone, and the primary root. This vertical primary root produced lateral roots, feeder roots, and adventitious buds. The lateral roots, and roots of other orders, produced additional lateral roots, feeder roots, and adventitious buds. The adventitious buds extended, forming vertical, underground, and aboveground shoots. In established patches of leafy spurge, about 90% of the aboveground shoots arose from adventitious buds on lateral roots. Crowns of buds, at and just below the soil surface, were formed both from vertical stems and from the collet or hypocotyl region of the main axis.

The transition zone was found in the collet (lower hypo-cotylary swelling). The transition from exarch, radial stele of the root to endarch, collateral stele of the upper collet was complete in approximately 2500 microns. All root primordia had endogenous origin from pericycle or pericylic tissues. Buds arose endogenously in roots but exogenously in the region of the hypocotyl with endarch protoxylem. In many cases, primordia were identified as roots or shoots prior to their emergence from the main axes.

ANATOMY AND BUD FORMATION OF SUBTERRANEAN PARTS OF

LEAFY SPURGE (EUPHORBIA ESULA L.)

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Doctor of Philosophy, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

_____   Nov 26, 1963
        Thesis Adviser   Date

_____   Nov 26, 1963
   Head, Agronomy Department   Date

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INTRODUCTION

Leafy spurge, like many other weeds, is extremely variable; genetically, morphologically, and ecologically. Some of the morphological and ecological variants have been treated as separate species by many authors. Bakke (3) cites five authors' attempts to distinguish between Euphorbia esula L. and Euphorbia virgata Wald. and Kit., but concludes that the two plants are conspecific. In light of Bakke's treatment of the "Esula" controversy, the plant in this study will be referred to as leafy spurge, Euphorbia esula L.

Control of leafy spurge is difficult, due primarily to the fact that it is a prolific producer of vegetative buds, both on vertical underground stems and upon horizontal and vertical roots. It is postulated that more effective measures of control might be conducted if the natural chemical factors responsible for initiation and development of these vegetative buds were elucidated.

While it was known that the primary organ derived as a result of the extension of the radicle of a seedling would be root, the exact extent or location of the transition zone was not known. Neither was the identity known of the various underground parts giving rise to numerous buds. Anatomical studies were conducted to answer these unknowns. These studies were broadened in efforts to determine the origin of lateral organs.

It was thought that seedlings would provide tissue of known age and uniformity suitable for studying the effects of growth substances on bud formation. However, seed germination and seedling growth were

extremely non-uniform. Efforts were then expended to ascertain the conditions necessary for rapid and complete germination of seeds. Subsequent attention was directed to establishing the proper media and external environment essential for uniform growth of seedlings and isolated plant parts.

Having established proper conditions for the germination and growth of leafy spurge, and knowing the exact nature of the tissue selected for experimentation, work could be conducted attempting to elucidate some of the chemical factors involved in vegetative bud formation on subterranean parts of leafy spurge.

The development of techniques used in this study, and recorded in this writing, may present useful information to persons conducting further in vitro studies on the physiology of growth in leafy spurge.

REVIEW OF LITERATURE

Germination and Seedling Growth

Germination studies of leafy spurge seeds were first presented in the literature by Hanson and Rudd (26). Seeds were found to germinate best at alternating temperatures of 20 C to 30 C, and better germination was obtained at 30 C than at 20 C on unsorted lots. Similar data were given by Brown and Porter (6). Sorting seeds according to weight demonstrated that the heaviest seeds germinated more readily than the lightest seeds.

Several workers have sorted seeds by color and/or weight and some disagreement exists as to which seeds are most mature. Bakke (3) selected and tested brown and grey seeds. It was found that brown seeds weighed more, imbibed more water, and had a higher germination percent than did grey seeds. Selleck and Coupland (52) also reported that brown seeds germinated more readily than did grey seeds but, in a later paper, Selleck, Coupland, and Frankton (53) present the information that grey seeds consistently imbibed more water than did mottled seeds. The change in term from brown to mottled was initiated by Wicks and Derscheid (67), prior to the later paper by Selleck et al. Wicks and Derscheid correlated seed color with maturity by examining seeds from capsules of known age. Yellow seeds and yellow seeds with brown tips are the least mature, followed by seeds with brown ends and a narrow yellow band, brown with an orange band, and reddish-brown seeds. These seeds are considered non-viable by Wicks and Derscheid, with viable seeds, in order of increasing maturity,

being brown, grey brown, grey, and mottled. Under conditions used by
Wicks and Derscheid, alternating temperatures (8 hours light, 30 C,
and 16 hours dark, 20 C) gave highest germination of all classes of
seeds with grey seeds being slightly higher in germination percent
than mottled seeds.

The gibberellins have been shown to break the dormancy of seeds,
induced by such agents as darkness, osmotic effects, high temperature,
coumarin, and gamma radiation. Stimulation of germination by gibberel-
lic acid (GA) is found in both light-requiring and light-indifferent
seeds (Koller, Mayer, Poljakoff-Mayber, and Klein (35)). Numerous
citations by Koller et al. result in the statement that "the possible
significance of gibberellic acid as a natural factor in germination
has been increased by the detection of gibberellin-like substances in
a number of seeds." Reports by Miller (38) and Strong (58) indicate
that kinetin may substitute directly for red light, but a later study
by Miller (39) points out that kinetin will enhance germination of
lettuce seeds, but only in combination with an amount of light which
is too small to promote germination by itself. Koller et al. (35)
carefully point out several differences between the effects of kinetin
and gibberellic acid on tobacco seeds, notably that "kinetin is effec-
tive only in combination with light while gibberellic acid may effect
germination in both light and darkness." Crocker and Barton (11) cite
work in which no correlation was found between auxin content of seeds
and promptness of germination. Other work cited presents the informa-
tion that "loss of auxin is not related to loss of vitality." Ricard

and Nitsch (50) conclude that indoleacetic acid (IAA) is not the
natural promoter of wheat coleoptile growth during the first stages
of development. Two acidic compounds, which stimulated isolated wheat
coleoptile growth, were separated from germinating wheat grains. The
amount of the active complex increased during germination and was not
inhibitory at high concentrations.

That diurnal thermoperiodicity is essential to maximum germina-
tion of leafy spurge seeds is readily seen by a review of the litera-
ture (3, 6, 26, 53, 67) as well as reports presented later in this
paper. Koller et al. (35) state that the requirement of thermoperio-
dicity usually disappears upon removal of the seed coat, but this has
not been found to be the case with leafy spurge as presented by Sel-
leck et al. (53). Crocker and Barton (11) cite many works demon-
strating both diurnal and seasonal periodicity requirements for
maximum seed germination. Stotzky, Cox, and Goos (57) present the
information that seeds of the banana, Musa balbisiana Colla, may be
induced to germinate in 5 to 6 days of alternating temperatures, but
additional temperature changes are required for normal seedling devel-
opment. Citations of literature presented by Koller et al. (35) pre-
sent 3 possible theories for the favorable effect of alternating
temperatures. First, the possible creation of a balance of the inter-
mediate materials of respiration during the high temperature part of
the cycle, which would induce germination during the lower temperature
cycles. Second, a temperature induced modification of a macro-
molecular compound, which would give rise to initiation of germination.

Third, that the fluctuating environment, or even single shifts or impulses, may synchronize endogenous daily rhythms which are out of phase with each other, in component parts of the seed or even in different cells.

Seed color, as an index to promptness of germination, is not restricted to leafy spurge. The information is presented in Agricultural Research (64) that dark colored seed of both alfalfa and clover have lower germination than light colored seed after two years of storage. Water extracts from different colored seeds exhibited chemical differences. Three separate components of a water extract of dark seeds were found. The leachable inhibitor found in dark seeds will prevent germination of light colored seeds. This same natural inhibitor is also blamed for poor seedling development of alfalfa, white, crimson, and Ladino clovers. Miyamota, Tolbert, and Everson (42) cite 6 references indicating that varieties of wheat having red kernels are more resistant to sprouting than those with white kernels. Employing a wheat embryo bioassay, Miyamota et al. found that post harvest dormancy of wheat seeds was caused by inhibitors which were located in the seed coat. Loss of dormancy by the time seeds were mature was correlated with the natural inactivation of the inhibitors. Two catechin-tannin fractions, one alkaloid, and a potent, unidentified fraction, were extracted from the seed coat. The inhibitory effect induced by the unknown fraction on wheat embryo germination was reversed by gibberellin A_3. The inhibitory effect on lettuce seed was not, however, reversed by gibberellin A_3. Some early work is cited by Crocker

and Barton (11), which places the cause of dormancy of immature corn seeds, on the quality of the endosperm. This assumption was made since excised embryos of unripe seed germinated more readily than intact, unripe seeds. Also endosperm juice of unripe seeds inhibited germination. Crocker and Barton (11) also review the work of van Overbeek and associates, dealing with the "embryo factor" of coconut milk. Three factors or complexes present in coconut milk which affect the growth of embryos are listed. These are: (1) a thermolabile factor causing both growth and differentiation, (2) a heat stable factor causing in some cases a callus-like growth but no differentiation, and (3) a heat stable factor which inhibits root growth and which may be related to auxin.

In his detailed review of plant growth substances, Audus (2) deals at great length with natural growth inhibitors. The blasto-cholines as defined by Audus are "simple molecules of diverse chemical affinities, but all possessing the property of preventing germination in relatively low concentrations." Audus cites work in which it was shown that blastocholines in fruit flesh are present in 33 genera, of 16 families, from 16 widely spaced orders of plants. In addition they are often found in fruit juices and in fruit and seed coats. Audus lists coumarin as one of the widely occurring, known blastocholines. Crocker and Barton (11) list "Blastokolin" as being a separate "substance" from other naturally occurring germination inhibitors such as ammonia, hydrocyanic acid, essential oils, alkaloids, and glycosides.

Wareing and Villiers (65) present the complex picture of dormancy as found in the seeds of _Fraxinus excelsior_. Dry seeds contain no inhibitors; however, a metabolically produced inhibitor is present in the endosperm and embryo after the seed has been allowed to imbibe water for 24 hours. Leaching the embryo for 48 hours removes dormancy. A chilling period of 4 to 5 months at 0 C to 1 C does not appreciably reduce the level of inhibitor found in the embryo. On the other hand, extracts of chilled embryos contain a germination promoter which is capable of overcoming dormancy of unchilled embryos.

Ching (7) demonstrates that presoaking of Douglas fir seeds in a 1% H_2O_2 solution for 36 to 48 hours stimulates germination. One of the four suggested mechanisms, for this stimulation, is that a peroxidase functions with the H_2O_2 to oxidize growth inhibitors.

An early report by Hanson (25) states that leafy spurge seedlings are weak and easily destroyed by ordinary methods of cultivation. Hanson and Rudd (26) present the information that by the 15th of May in North Dakota less than 10% of the seedlings were alive under their field conditions. Selleck et al. (53) report that maximum emergence of leafy spurge seedlings occurred at depths of 0.5 to 2 inches, but germination was possible at 4 inches. Vegetative shoots emerged earlier than did seedlings. An accurate description of young seedling development is also presented by Selleck et al. Imbibition of water causes rupture of the testa in 12 to 24 hours. The radicle may then appear after about 12 hours. The radicle develops to 1.9 cm within ten days after the seed coat has broken and root hairs are initiated during this time.

Exposure of leafy spurge seedlings to even the slightest amount of light results in a pink colored hypocotyl, when the hypocotyl is in the first stages of elongation. Increasing amounts of light result in a change from pink to green in the upper part of the hypocotyl within a day or two. This same sequence is reported by Selleck et al. (53). The seedling is green above the ground and dull reddish brown at the soil surface. This reddish brown area is the region immediately above the union of root and hypocotyl and appears to have this characteristic color whether at the soil surface or just below the soil surface. If below the soil surface, however, it is more typically brown.

Aseptic culture of both intact and isolated roots has proved a valuable research tool in recent years. While there are certain limitations and disadvantages of this technique, it has been successfully used to study the role of macronutrient and micronutrient element requirements, auxin status of excised roots, biosyntheses of alkaloids, apical dominance, and the effect of chelating agents on the uptake of iron and other elements, as cited by Guinn (24). While White's medium, employing sucrose as the carbohydrate source, is most commonly used, Guinn cites work demonstrating that roots of some monocots actually do as well or better with glucose. Also, galactose and mannose were shown to be inhibitory and, in general, organic acids and sugar alcohols were ineffective sources of energy.

Galston and Purves (22) state that "the mechanism of auxin action is not yet known," and cite that this subject had previously been reviewed 13 times. Despite the obvious fact that much remains to

be known about the mechanism of auxin action, it is of interest here that auxin is known to have marked effects on many aspects of sugar metabolism. Details of the sucrose-auxin interaction in pea epicotyl section growth was studied by Purves and Galston (47). The inhibition of elongation, caused by high concentration of IAA (10^{-4}M), was only apparent in the presence of sucrose. Also, 10^{-4}M IAA, in the presence of 2% sucrose, is markedly inhibitory if applied during the first 3 hours of the growth period; relatively inactive if applied during the fourth to sixth hours; and strongly promotive after the seventh hour of the growth period. This change from inhibition to promotion corresponds to the length of the lag period required for maximum stimulation by sucrose when applied alone.

Experiments by Brown and Gifford (5), on pine embryos, demonstrate that sucrose, when supplied through the cotyledons, exerts a much greater beneficial effect on the rate and duration of root growth than when supplied to the embryo directly by way of the root. Roots of embryos in 2% sucrose medium, but with cotyledons in a plain medium, attained a length of about 7 mm in 30 days. Roots of embryos in plain medium, but with cotyledons placed in 2% sucrose medium, attained a length of about 60 mm in 30 days. The inhibitory effects of 0.1, 1.0, and 10 mg per liter IAA were somewhat modified when sucrose was supplied through the cotyledons.

In addition to the different growth responses of root and shoot caused by application of various sugars or concentrations of IAA; GA (Dycus (17)); kinetin (Wittwer and Dedolph (70)); and temperature

(Davis and Lingle (14)) have been shown to effect the shoot/root ratio
of plants. Galston and Warburg (20) have interpreted the GA-IAA in-
teraction as requiring a third factor, which is apparently limiting in
etiolated pea stem tissue but abundant in green tissue. Kefford and
Goldacre (32) dwell on a theory of the triple interaction of IAA, gib-
berellin, and kinin and propose that "auxin is a correlative regulator
of plant growth, which predisposed cells to change, the exact nature
of the change being determined by other chemical regulators such as
gibberellin and kinin." The complexity of plant growth regulation ap-
pears to be increasing, especially in light of the paper by Purves,
Kato, and Glenn (48). A substance "tentatively assumed to represent a
new class of plant growth regulators" has been isolated from cotyle-
dons of cucumber, squash, and watermelon. The water-soluble growth
substance stimulates elongation of excised stem segments of the cucur-
bits and is neither an auxin nor a gibberellin. Elucidation of struc-
ture is pending.

Resultant effects of cotyledon removal are extreme and variable,
depending on experimental conditions and plant species tested. Kidd
and West (31) cite work in which cotyledon removal reduces the number
of secondary roots formed. Torrey (62) presents the fact that an ac-
tive vascular cambium is initiated by IAA, but only in root tips ini-
tially excised from the pea seedling. Subculturing the initial root
tip removes this effect of cambium initiation and subsequent secondary
tissue formation. It is suggested that essential factors for cambial
activity, usually available from the cotyledons, are depleted on

continued subculture. Torrey cites work in which a mixture of amino acids and adenine improved the development of decotylized pea seedlings. Knowles and Zalik (34) found that removal of the cotyledons of Viburnum trilobum Marsh. resulted in the breaking of epicotyl dormancy. The seedlings tested were grown in vermiculite with no mineral nutrients or carbohydrate source added. Explanations offered for the cotyledon-controlled epicotyl dormancy are (1) that a competitive relationship exists between the cotyledons and the epicotyl for food reserves, (2) that the cotyledons inhibit the epicotyl in a manner similar to that by which apical buds inhibit the growth of laterals, or (3) that a combination of these two may be operative.

It is well known that high concentrations of IAA will induce the formation of root initials, and that all or nearly all of the IAA must be removed for subsequent root development. Many papers cling to the idea of, and present support for, a root-forming hormone (rhizocaline) stored in the cotyledons of young seedlings. Audus (2) presents many pros and cons of the hypothetical calines. Primary among the pros is the theory of Bouillene whereby a "cell factor" (specific enzyme), in addition to auxin and the mobile rhizocaline, is essential to root initiation. If such an enzyme were confined to certain tissues; e.g., the pericycle, it would explain why only these tissues are induced to form roots. In Sinnott's discussion (55) of auxin transport, specific reference is made of the fact that when primary roots are bent, lateral roots grow chiefly from their convex side. This phenomenon has been interpreted as "the result of a transverse

polar gradient in a root forming hormone." Torrey (61) demonstrates that extracts of seedling pea roots contain substances, which completely inhibit IAA induced lateral root formation on pea root segments in vitro. In more recent studies by Torrey (63), it has been shown that kinetin can either stimulate or inhibit cell division leading to lateral root initiation in pea roots, depending on its concentration or that of IAA and adenine sulfate. A brief summary of the lateral root initiation system as presented by Torrey is (1) IAA is required for lateral root initiation, (2) kinetin at low concentrations increases the effectiveness of IAA, when the auxin is already maximally effective, but higher concentrations of kinetin antagonize the stimulatory effect of IAA, (3) adenine sulfate effectively prevents the inhibitory action of added kinetin over a range of intermediate concentrations of the latter, but at higher concentrations does not prevent the kinetin inhibition. Galston and Hand (21) have shown that adenine may produce effects in the etiolated pea plant, ascribed to all three of the postulated calines: (1) caulocaline—formed in the roots and essential for stem elongation and bud growth; (2) rhizocaline—stored in the cotyledons and essential for root initiation; and (3) phyllocaline—stored in the cotyledons and essential for leaf growth. Equally interesting is the fact that the pea stem sections showed a marked temperature optimum at ca. 30 C. The thermal inhibition of growth at higher temperature was largely overcome by small amounts of adenine. Galston and Hand imply a parallelism between the response of etiolated peas and the temperature-sensitive Neurospora mutant.

Certain mutants of <u>Neurospora</u> are able to synthesize adequate adenine for their growth needs at temperatures of 28 C or lower but require exogenous adenine when grown at elevated temperatures.

Bud Formation and Development

Capacity for Reproduction

That leafy spurge can be controlled without soil sterilization has been amply proven by Derscheid, Wallace, and Nash (15), and Derscheid, Wicks, and Wallace (16). Control is difficult, however, due to the production of numerous vegetative buds and the ability of these buds to develop into shoots, even from very small root cuttings. Early work by Hanson (25) reported pink buds on roots as deep as 42 inches in the soil. Bakke (3) told of buds found as deep as 7 feet on a root system extending to a maximum depth of 15 feet, 8 inches. Robbins, Crafts, and Raynor (51) state that shoots may arise from buds as deep as 20 inches. More recently, Coupland and Alex (8, 9) and Coupland, Selleck, and Alex (10) have discussed the distribution and regenerative capacity of vegetative buds on the underground parts of leafy spurge. Monson (43) and Monson and Shafer (44) stated that new buds are initiated subsequent to new vegetative growth in the spring. Also, bud dormancy occurs during late summer and early fall, followed by release of dormancy by low temperature in the fall and winter. Similar work by Wicks (66) indicated that in 1956 and 1957 "a killing frost is instrumental in causing complete dormancy." Dormancy was not complete after a killing frost in 1962, however, as found by Dosland of South Dakota State College (personal communications). Transplanting

methods differed between these two workers; Wicks (66) transplanted into soil in the greenhouse, while Dosland placed the root segments, containing buds, into non-nutrient agar at constant 25 C in the dark.

There seems to be no disagreement concerning the point that existing buds (axillary buds on stems and adventitious buds on roots) are maintained in a quiescent state by apical dominance. If the top growth is intact the buds are quiescent, but if the top growth is removed, either by mowing or chemical killing, the buds become active. One or two of these buds (usually the uppermost) develop faster than the others and assume apical dominance over those basipetal buds. Switzer and Bibbey (59) summarized that translocation of herbicides in leafy spurge appears to stop at the "crown," resulting in the breaking of apical dominance due to injured top growth.

Chemical Factors

Shafer and Monson (54) have shown that GA, when applied to leafy spurge foliage, will stimulate the growth of dormant crown buds, cause new shoot development on old shoots, and stimulate emergence of new buds. Some interactions of GA and IAA have previously been mentioned (17, 20, 32).

The cell division factor, kinetin discovered by Miller, Skoog, Okumura, Van Saltza and Strong (41), provided great impetus to those plant physiologists concerned with tissue differentiation and morphogenesis. This is not meant to detract from the works concerning the "coconut milk factor" by Van Overbeek and associates, and Stewart and co-workers, as cited by Strong (58). In 1956, Strong (58) reviewed

the history, isolation and characterization procedures, analogs, and the biological action of kinetin. The literature concerning kinetin and related compounds was more recently reviewed by Miller (40) in 1960. It suffices to say then that no all-inclusive review of the kinetin or the postulated natural kinins may be included here. Some works concerned with the effects of adenine and kinetin have already been discussed under the section Germination and Seedling Growth.

It has been amply demonstrated by Geissbühler and Skoog (23), Skoog and Miller (56), and Strong (58) that in excised tobacco pith, a relatively low kinetin/IAA ratio promotes root formation; an intermediate ratio leads to undifferentiated growth; and a high kinetin/IAA ratio promotes bud formation development. Growth stimulation decreases when kinetin exceeds 2.0 mg per liter. Kinetin alone is without effect and IAA alone leads only to masses of giant cells. Also from treated tobacco pith tissue, Das, Patau, and Skoog (13) made the following observations: no IAA - no mitoses; with IAA - a few mitoses and even fewer cell divisions; with IAA plus kinetin - many mitoses, virtually always followed by cytokinesis. It is suggested that a natural kinin is essential for both mitosis and cytokinesis. Patau, Das, and Skoog (45) later dealt with the effects of IAA and kinetin on another phase of cell division, that of deoxyribonucleic acid (DNA) synthesis. DNA doubling was induced by IAA alone, kinetin alone, but most effectively by the combination of both.

Keitt and Skoog (30) found that 2,3,6-trichloro-, 2,6-dichloro- and 2,5-dibromo-benzoic acids were highly effective in causing apolar

distribution of callus growth in excised tobacco stem segments. Un-
treated segments were able to polarly transfer many times the level of
endogenous IAA. Also, their data present presumptive evidence for con-
tinued auxin synthesis in the cultured stem segments. IAA was also
shown to be basipetally transferred in bean hypocotyl segments, with
polar distribution prevented by 2,3,5-tri-iodobenzoic acid (TIBA).
Wickson and Thimann (68), by floating second node and internode pea
segments in IAA-C^{14} solution containing 1% sucrose, found that although
IAA entered at both cut ends, it was transported away from the apical
end. Entry of IAA-C^{14} into the bud was delayed for about 9 to 12
hours, but at 48 hours, the level of activity reached the same order
as that of the stem. When IAA was applied to the apical end (the seg-
ments being supported on horizontal glass rods), appreciable amounts
reached the base, but not vice versa. Additional studies with kinetin
by Wickson and Thimann show that kinetin markedly limits uptake of
IAA-C^{14} into the pea stem sections in the light. This effect was also
seen with intact plants placed in identical solutions (3 mg per liter
IAA-C^{14} and 6 mg per liter kinetin). The three conditions which fa-
vored bud growth on the pea sections were also those which reduced
radioactivity in the sections; namely, exposure to light, simultaneous
treatment with kinetin, or decapitation of the plants several days be-
fore the sections were subjected to the various tests.

Wickson and Thimann (68) reported that externally supplied
kinetin at 4 to 5 mg per liter will completely remove the bud inhib-
iting effect of from 0.3 to 5 mg per liter IAA on pea epicotyl

sections. More kinetin was required to remove the effect of IAA in the absence of sucrose. Kinetin alone had only a slight stimulatory effect. Miller and Skoog (37), on tobacco stem sections, demonstrated the same response with adenine that is often observed with kinetin; namely, that the purine (40 to 80 mg/l) stimulated bud formation and that low concentrations of IAA (0.002 mg/l) reversed this stimulation. Note the same response by kinetin and adenine, but the great difference in concentrations. The theory that kinetin is a highly effective adenine appears to be attractive to Fox and Miller (19). Miller and Skoog (37) also observed that callus formation and cell enlargement promoted by IAA are inhibited by 2,6-diamino purine, this inhibition being reversed by adenine.

Howell and Skoog (27) obtained data which support the caulocaline theory and suggest that probably adenine is a part of the complex. Coconut milk at 10 and 15% of the media greatly stimulates the growth of excised pea epicotyls, and it appears that this effect is enhanced by adenine. Adenine alone shows some stimulation. By extracting certain data, it was shown that only the epicotyls which formed roots grew appreciably and that only these were stimulated by adenine and coconut milk. The formation of roots was not stimulated by adenine. In view of these data, Howell and Skoog state that "it is unlikely that adenine is 'identical' with 'caulocaline'" as has been suggested by Galston and Hand. It is noted here, however, that Galston and Hand (21) state that "adenine may not be the only substance actually functioning as the caline." Al-Talib and Torrey (1) have

shown adenine sulfate (20 to 60 mg/l), in the presence of 10^{-6}M IAA, to be inhibitory to isolated buds of Pseudotsuga taxifolia. Auxin alone leads to reduced leaf expansion and unorganized cellular pro- liferations at the base of the buds. Callus development is accentu- ated by addition of kinetin.

In the recent review by Humphries and Wheeler (28) on the physi- ology of leaf growth, literature is cited which shows a stimulation or non-stimulation of leaf disk expansion by adenine, depending on the species. Other references are cited where kinetin has shown a posi- tive effect in leaf disk expansion in the dark, but a negative effect in the light. Humphries and Wheeler make the point that the concept of phyllocaline is partially fulfilled by the gibberellins but that probably several hormones are concerned in leaf growth.

Jensen (29) has presented the information that isotopically labelled adenine is incorporated into onion root tip cells. Where the amount per cell of nucleic acid or protein is increasing (meri- stematic tip), the incorporation is constant. This indicates synthe- sis with little turnover. Where the amount per cell of nucleic acid or protein is constant (region of elongation), incorporation in- creases tremendously, indicating a great increase in turnover. The differentiating vascular tissue shows a significantly higher rate of incorporation of adenine, particularly by the nuclear and nucleolar fractions than the other tissue. While this was an abstract and no additional discussion was presented, it seems that these data fit the observations presented by Torrey (63) concerning lateral root

initiation on pea root sections. Better quoted than paraphrased,
Torrey states,

> Taken all together one might visualize a gradient of
> decreasing concentration of effective endogenous auxin and
> adenine, from root tip toward root base. The high effective
> concentration of adenine in the tips is confirmed by the lack
> of response of tips to added adenine sulfate and their rela-
> tive insensitivity to kinetin inhibition.

Danckwardt-Lillieström (12) has shown that buds may easily be
initiated at the cut end of isolated root tips of _Isatis tinctoria_
when grown _in vitro_. The amounts of shoots formed, however, decrease
with successive subculture. No shoots were formed after the third
transfer. Kinetin, at 60.90 ug per liter, induced shoots at the cut
end of root tips in their sixteenth transfer. At higher rates of
kinetin, shoots were induced throughout the length of the roots. The
assumption is made that kinetin is a natural hormone in the root of
this plant and, in the first transfer, it is in the right concentra-
tion to induce shoots but decreases in concentration thereafter.
Torrey (60), working with an isolated root clone of _Convolvulus
arvensis_ L., has shown that kinetin at 0.1 mg per liter markedly
stimulates bud formation, mainly on the distal end (root end) of the
10-mm root segment. Light augments the induction of buds due to
kinetin treatment. Kinetin at 0.1 mg per liter stimulates the growth
of laterals in the dark but inhibits them in the light. Light is es-
sential for the development of normal shoots on untreated segments.
Buds on these segments are truly endogenous and originate in pericy-
cle, usually opposite the protoxylem poles. The kinetin induced struc-
tures appear to be incompletely organized buds, but no histological
comparison between them and normal endogenous buds was made at that
time.

Anatomy of Subterranean Parts

The literature review of this section will be presented in chronological order and discussed with respect to four areas: (1) horizontal underground parts, (2) transition zones, (3) origin of primordia, and (4) tissue patterns.

Horizontal underground parts. In a review of taxonomic treatment of E. esula vs. E. virgata, Bakke (3) made reference to several descriptions of the two species. Among these, A. deCandolle referred to the presence of "smooth rhizomes many branched . . . " and C. V. Morton described the species E. esula as having " . . . rhizomes always present." Bakke (3), in collecting underground material, obtained "surface feeding roots and rhizomes . . . " indicating by diagrams the structures referred to as rhizomes. Esau (18), collectively citing several workers, presents this description:

> The taproot is often called the primary root; branches of the first order, the secondary roots; and branches of the secondary roots, the tertiary roots. Some plants may have root branches of fourth and even fifth orders. In perennial species the taproots and the older laterals undergo secondary growth. At this stage of development they serve as conductors of food and water, and as storage and anchorage organs. Absorption, on the other hand, is carried on mainly by the ultimate branchings which are in primary state of growth. The fine absorptive branches—the feeder roots—remain short and are often fragile and short-lived.

Coupland and Alex (8) attributed the persistence of underground parts to the presence of buds on rhizomes and roots and found that (9) "the number of vegetative buds per unit length of root (or rhizome) tended to be greatest below the soil surface." Selleck et al. (53) found

the roots to be perennial, woody and tough, with numerous, spreading rhizomes near the surface. In the fall, when stems deteriorated, buds from these rhizomes and roots enlarged. Two-inch sections of "rhizomes," brought into the greenhouse in October, regenerated readily. Raju, Steeves, and Coupland (49) referred to these rhizomes of previous authors as lateral long roots " . . . which grow horizontally away from the parent root, then turn obliquely down ultimately becoming vertical."

Transition zone. Hanson and Rudd (26) stated that "stems may extend a foot or more before transition is reached." Coupland and Alex (8) reported horizontal structures transitional between stem and root arising from vertical underground stems and from main roots as deeply as 20 inches. Unpublished results cited by Coupland and Alex stated that "eventually these horizontal structures turn downward to become vertical tap roots for shoots originating near the bend." It was also indicated by Coupland and Alex (9) that stem and root-stem transition tissue comprise a large proportion of the total weight of the plant in uppermost soil layers. Wicks (66) stated that if buds were located irregularly on an underground stem, "it might be assumed that this was root since it was underground. At the most it may be considered a stem-root transition zone." Bakshi and Coupland (4) state that "root-to-stem transition has been observed in the region 15 to 22.5 cm below the soil surface." They postulated that maybe Hanson and Rudd (26) found transition areas 30 cm below the soil surface because a lateral bud from a main root had outgrown the original

stem. Selleck et al. (53), citing Bakshi and Coupland, state that the transition zone "was observed to occur between 3.7 and 5.5 in." below the soil surface.

Origin of primordia. Priestley and Swingle (46) found hypocotyledonary buds in Linaria and Anagallis to be epidermal in origin and, thus, exogenous. However, there was a centripetal differentiation of meristem through cortex, endodermis, and pericycle resulting in a vascular connection with the main stele. In Convolvulus arvensis L., buds on the hypocotyl are endogenous. In the root of Euphorbia cyperissias L., buds form from pericyclic phellogen. These are endogenous although often misconceived in the literature as exogenous, since the phellogen is near the periphery and centripetal differentiation of procambial strands is from internal meristematic parenchyma. Bakshi and Coupland (4) saw no symmetrical arrangement of lateral organ primordia in young roots as in older roots where they originated opposite protoxylem points. Lateral primordia could not be designated as root or shoot before their emergence from the parent root. They also observed lateral organs of underground stem internodes to be endogenous while at nodes the lateral organs were exogenous.

Tissue patterns. Hanson and Rudd (26) noted that, although triarch roots were most common in leafy spurge, tetrarch, pentarch, and polyarch patterns also occurred. They reported periderm arising from a well-developed cortex. Bakke (3) also described a heavy cortical layer covered by cork surrounding the xylem. He also reported concentric rings of storage parenchyma within a ring of vessels forming

annual rings. Bakshi and Coupland (4) did not observe these concentric
rings. They did, however, find that the cortex and epidermis die and
peel off making way for developing pericycle.

PROCEDURES AND RESULTS

Techniques for Procurement of Plant Material

Early attempts to conduct experiments on leafy spurge in sterile
nutrient culture were thwarted by inability to obtain adequate numbers
of contamination-free seedlings. Seed germination was extremely slow
or non-existent. Since the aseptic technique precluded the use of
vast numbers of seeds, tests were conducted to determine which seeds
and under what conditions the most uniform seedlings could be obtained.

Seed Germination

Due to the extreme genetic and developmental variability that
exists in leafy spurge, all seeds used in germination tests, growth
studies, and bud initiation and development studies were harvested at
the same time, at the same place, and hand sorted by the same person.

Leafy spurge seeds, collected in September of 1962, were sorted
into three color classes: mottled, grey, and purple. Purple seeds
correspond closely to those classed by Wicks and Derscheid (67) as
reddish-brown. Pictures of these three classes are shown in figure 1.
Seeds were subjected to various conditions of light and temperature.
Temperature was alternated from 20 C to 30 C every 12 hours, unless
otherwise stated. Each treatment was replicated three times with 100
seeds per treatment being planted on distilled water-saturated blot-
ters in plastic germinating dishes. Light (when used) was from an
18-inch florescent tube placed 1 foot above the seeds.
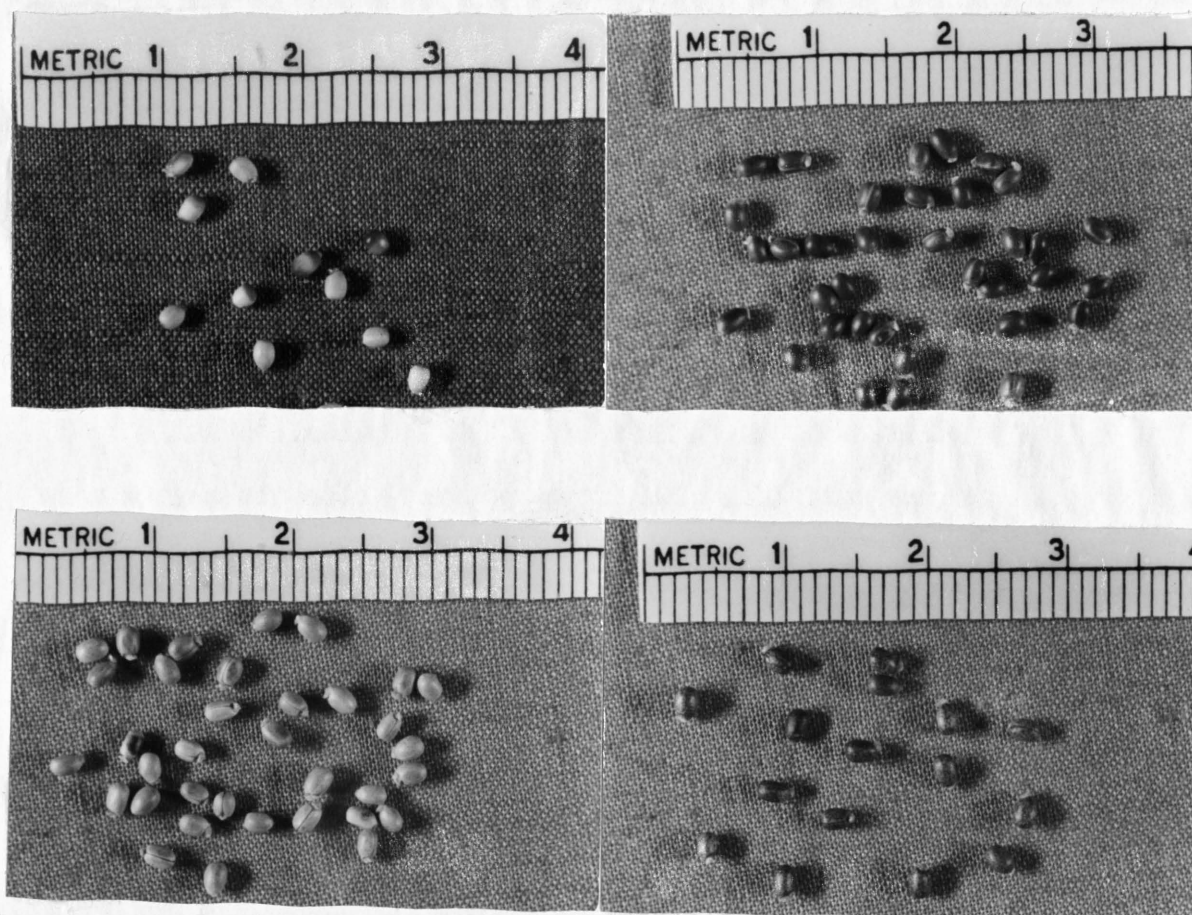
Fig. 1. Leafy spurge seeds, separated as to color and maturity.
Upper left—yellow and yellow with brown bands, non-viable.
Upper right—purple, least mature. Lower left—grey,
next in maturity. Lower right—mottled,
most mature

Figure 2 shows the effect of constant light on the germination of mottled seeds, as compared to constant darkness. Both treatments were subjected to alternating temperature conditions. Light reduced both the percent and rate of germination. Figure 3 shows the effects of light and temperature on the germination of mottled seeds. One treatment was constant light and alternating temperatures for 6 days followed with constant darkness at 20 C. The second treatment consisted of only one alternation of temperature (20 C to 30 C to constant 20 C) in constant light, until the sixth day when it was placed in constant darkness at 20 C. The third set of seeds received no alternation of temperature but remained at 20 C in constant light, until the sixth day when it was placed in constant darkness, also at 20 C. Germination percentage increased after placing the seeds in the dark. Increasing the number of temperature alternations increased the germination rate and percentage.

Germination of all three classes of seeds, mottled, grey, and purple, when subjected to 18 days of alternating temperatures, in constant darkness, is shown in figure 4. Mottled seeds had the highest rate and percentage of germination, followed by grey seeds.

The effect of 1 alternation and 2 alternations in constant darkness, on all three color classes of seeds, is shown in figure 5. Germination was lower for all three classes when temperature alternated only once. The difference was greater for more mature seeds.
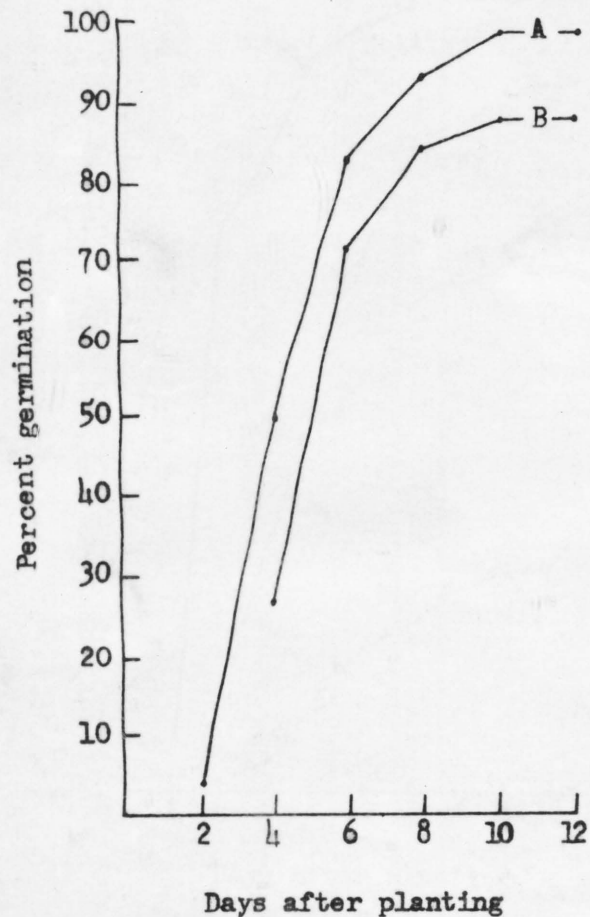
Fig. 2. Germination of mottled seeds of
leafy spurge under light and dark con-
ditions. (A) Alternating 12-hour
periods, 20 C-30 C, constant
darkness (B) Alternating 12-
hour periods, 20 C-30 C,
constant light
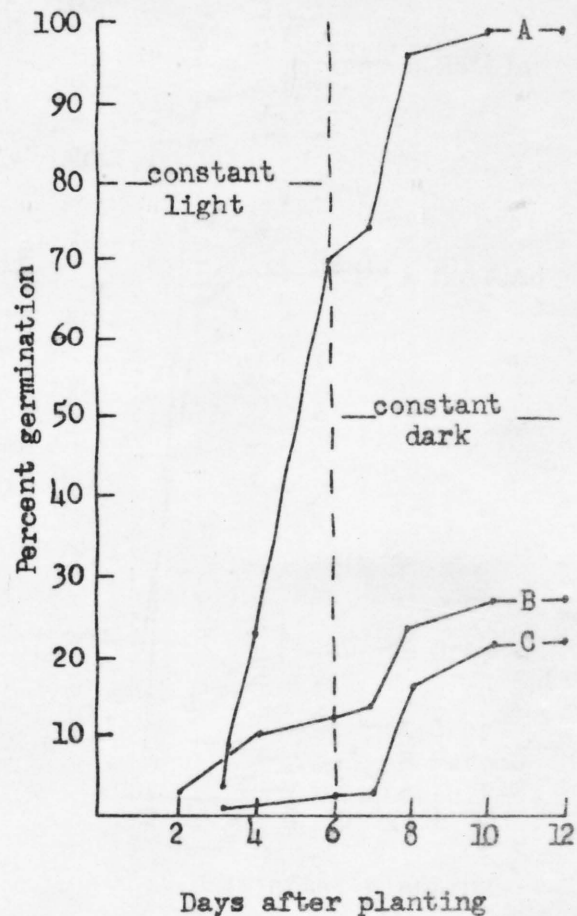
Fig. 3. Germination of mottled seeds of
leafy spurge under different light and
temperature conditions. (A) Six days
of alternating 12-hour periods, 20 C-
30 C (B) One day of alternation
as in (A), then constant 20 C
(C) Constant 20 C
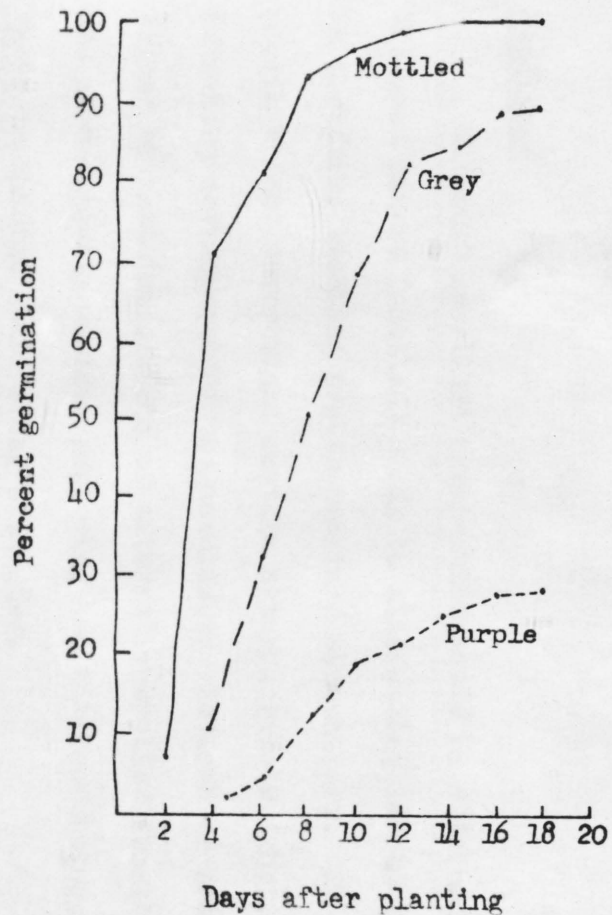
Fig. 4. Germination of leafy spurge seeds
according to color. All seeds,
alternating 12-hour periods,
20 C-30 C, constant darkness
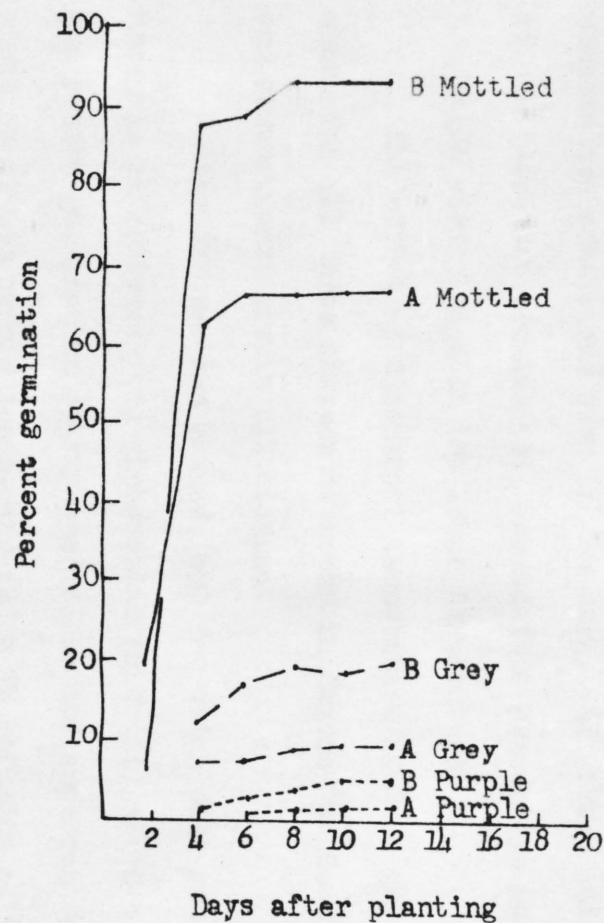
Fig. 5. Germination of leafy spurge seeds
under different temperature conditions.
(A) One alternation, 20 C-30 C-constant
20 C (B) Two alternations 20 C-30 C-20
C-30 C-constant 20 C. Alternating
periods were 12 hours each,
constant darkness

Figure 6 shows the effect of a constant temperature of 30 C until the twelfth day when seeds were placed in constant 20 C. Both temperature conditions were in the dark. All three color classes received these two treatments. Germination percentage was low until the seeds were placed in the lower temperature.

The effect of a constant temperature of 20 C in darkness is shown for all three classes of seeds in figure 7. Germination rate and percent were low in all classes.

Other information gained, but for which data are not present here, is as follows: (1) gibberellic acid (GA) at 10 mg per liter did not retard germination of any seed class and appeared to increase germination of the purple seeds, and (2) a 1% solution of H_2O_2 also appeared to increase the germination percent of purple seeds. Root growth was seriously inhibited on seedlings from all seed classes when treated with H_2O_2.

Seedlings

Although excellent germination could be obtained by subjecting the most mature (mottled) seeds to alternating temperature conditions in the dark, seedling growth remained non-uniform. It was considered possible that seeds might contain natural growth substances which were affecting development of the seedlings. Several experiments were designed to test the effects of externally applied growth substances and seed extracts without employing the time consuming, sterile, in vitro techniques still being attempted.
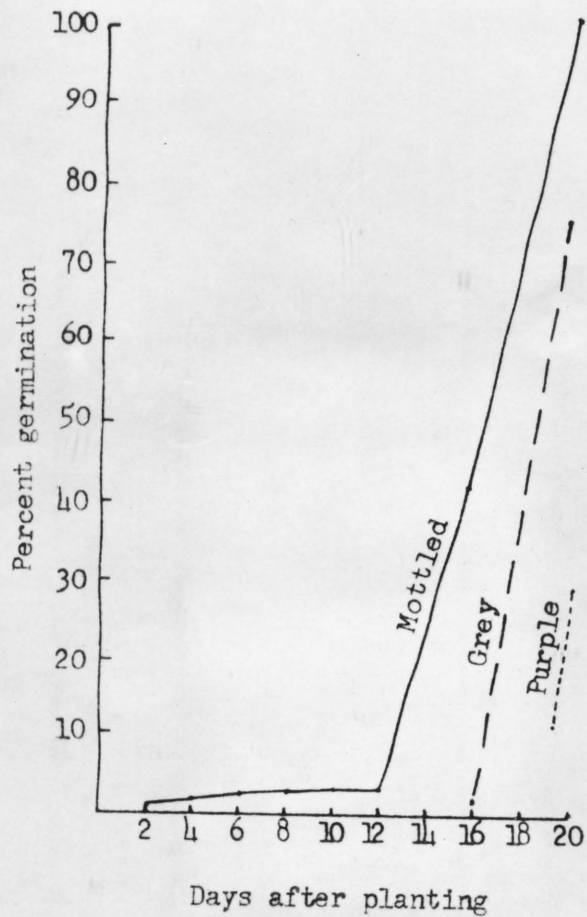
Fig. 6. Germination of leafy spurge seeds
in the dark, at constant 30 C for 14
days, followed by constant 20 C
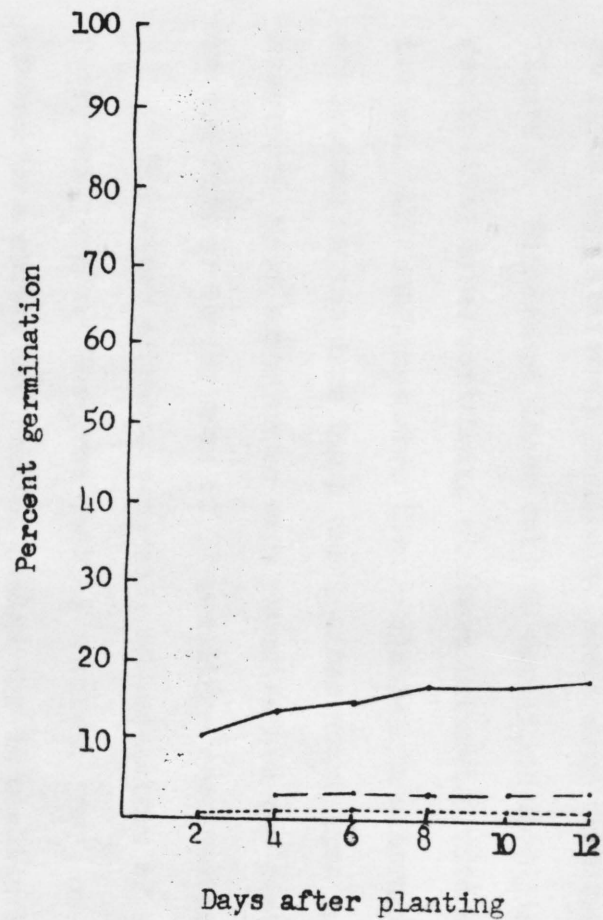
Fig. 7. Germination of leafy spurge seeds
at 20 C.   Mottled ——————
Grey — — — Purple -----------

Effects of growth substances and seed extracts. In the fol-
lowing series of experiments, mottled seeds were germinated under
alternating temperature conditions in complete darkness. On the fourth
or fifth day, uniformly germinated seeds were selected as indicated in
figure 8. Sixteen of these uniform seedlings were placed in plastic
germinating boxes containing blotters saturated with the solution to be
tested. All seedlings were then subjected to alternating temperature
conditions in the dark for 6 days, after which time root length was
measured. Most experiments were repeated and the root growth expressed
in the figures is the mean of 32 seedlings per treatment.

Hot water extracts were made of each class of seeds--mottled,
grey, and purple. One gram each of mottled, grey, or purple seeds was
ground in a mortar and pestle, boiled for 10 minutes in 100 ml of
water, the residue removed by filtration, and the supernatant cooled.
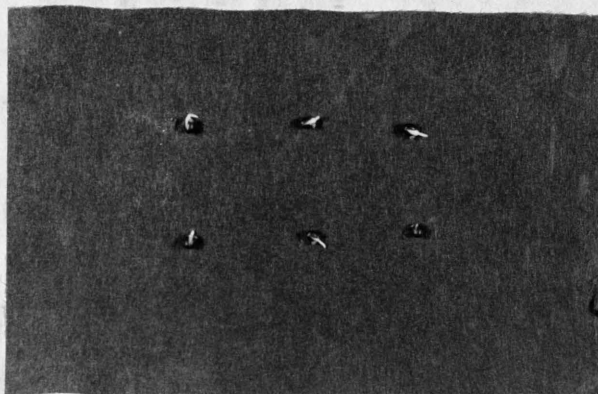
Fig. 8. Uniformly germinated seeds when placed on
blotters saturated with the test solutions

Seed extracts and one-half dilutions of the extracts were
tested against seedling growth, and the results shown in figures 9
and 10. There was less growth from seedlings treated with extracts
from less mature seeds and less growth from seedlings treated with
the higher concentration of seed extract.

The effects of adenine sulfate, kinetin, and indoleacetic acid
(IAA), alone and in certain combinations, are shown in figure 11.
There was less growth of seedlings treated with either kinetin or IAA
than with adenine sulfate or water and less growth with kinetin plus
adenine sulfate or kinetin plus IAA than with any of these chemicals
alone.

The purine antagonist, 8-azaguanine, at 50 and 25 mg per liter,
was tested against growth of leafy spurge seedlings, and the results
shown in figure 12. Less growth occurred with 50 mg per liter
8-azaguanine than with 25 mg per liter. Growth was increased at both
rates when adenine sulfate was included with the purine antagonist.

Growth in nutrient culture. The basal nutrient solution used
was the standard White's media as modified by Machlis and Torrey (36);
however, the molarity of sucrose used was 5.8×10^{-2} rather than
6.0×10^{-3}. One additional modification was made in that iron chelate
was used in place of $Fe_2(SO_4)_3$. An iron-ethylenediaminetetraacetate
(Fe-EDTA) chelate stock was prepared as follows: Na_2EDTA, 800 mg/l;
$Fe_2(SO_4)_3$, 381 mg/l. Three ml of the iron chelate stock were used
per liter of final media. Klein and Manos (33) found the above con-
centration of Fe-EDTA to be optimum for cultures of Daucus carota.

Solution   Root Growth in 6 Days

Water

Mottled seed extract

1/2 Dilution mottled seed extract

Grey seed extract

1/2 Dilution grey seed extract

Purple seed extract

1/2 Dilution purple seed extract

4   8   12   16   20   24   28   32 mm
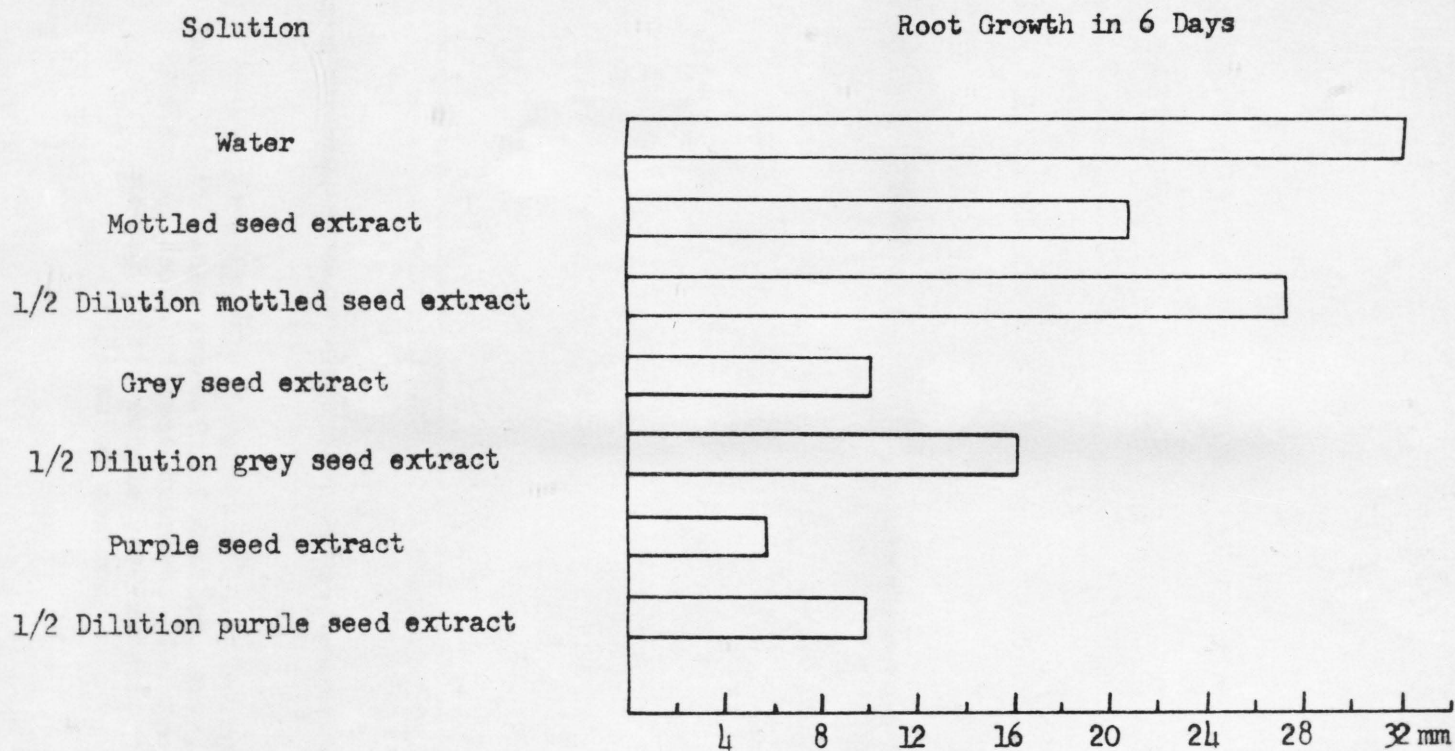
Fig. 9. Root growth in mm of leafy spurge seedlings when uniformly germinated seeds
were placed on blotters saturated with various solutions. Root growth is
the mean of 32 seedlings per treatment.
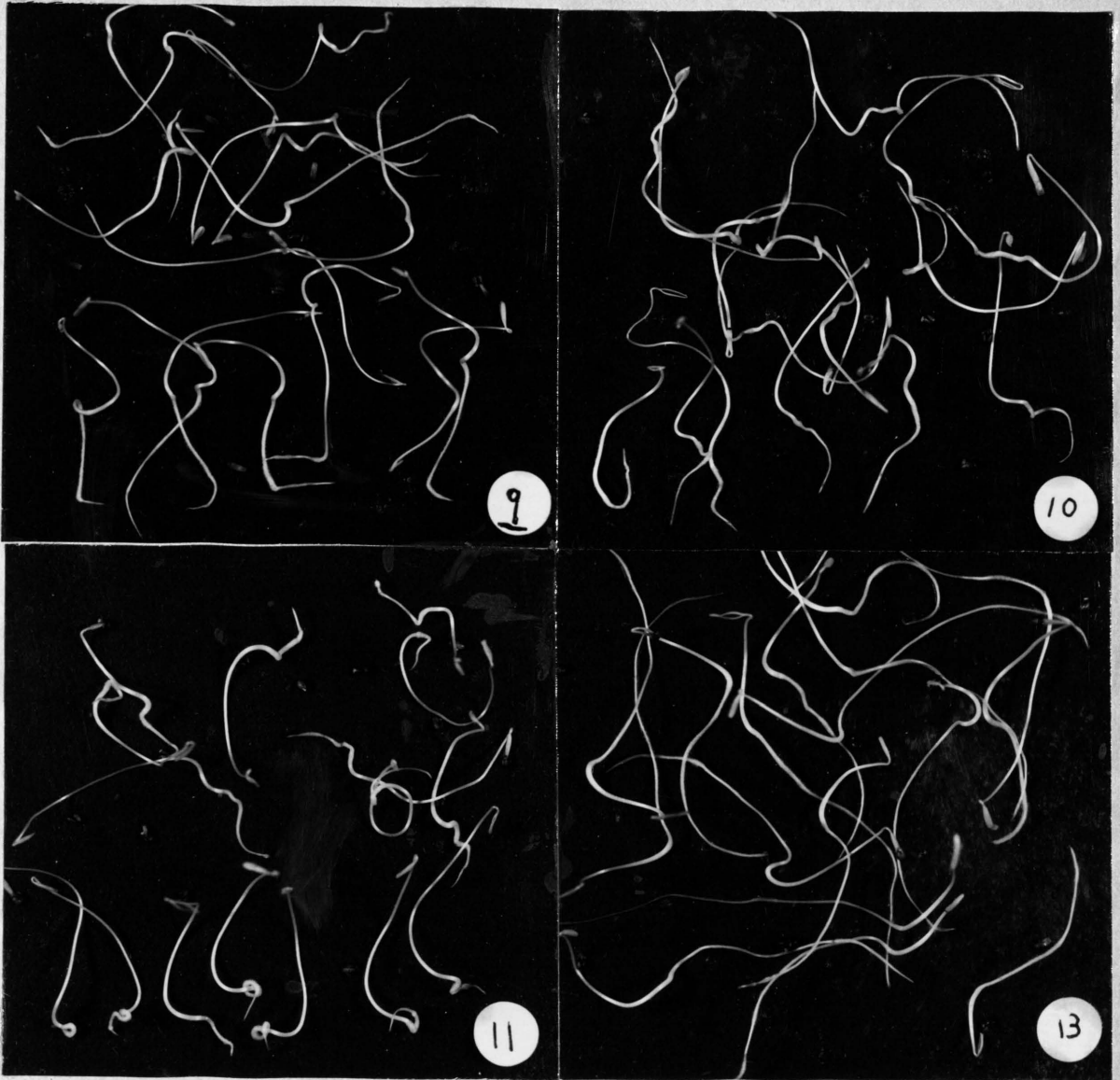
Fig. 10. Growth of uniformly germinated leafy spurge seedlings after
6 days in hot water extracts of mottled, grey, and purple
seeds. (9) mottled seed extract, (10) grey seed
extract, (11) purple seed extract,
(13) water control
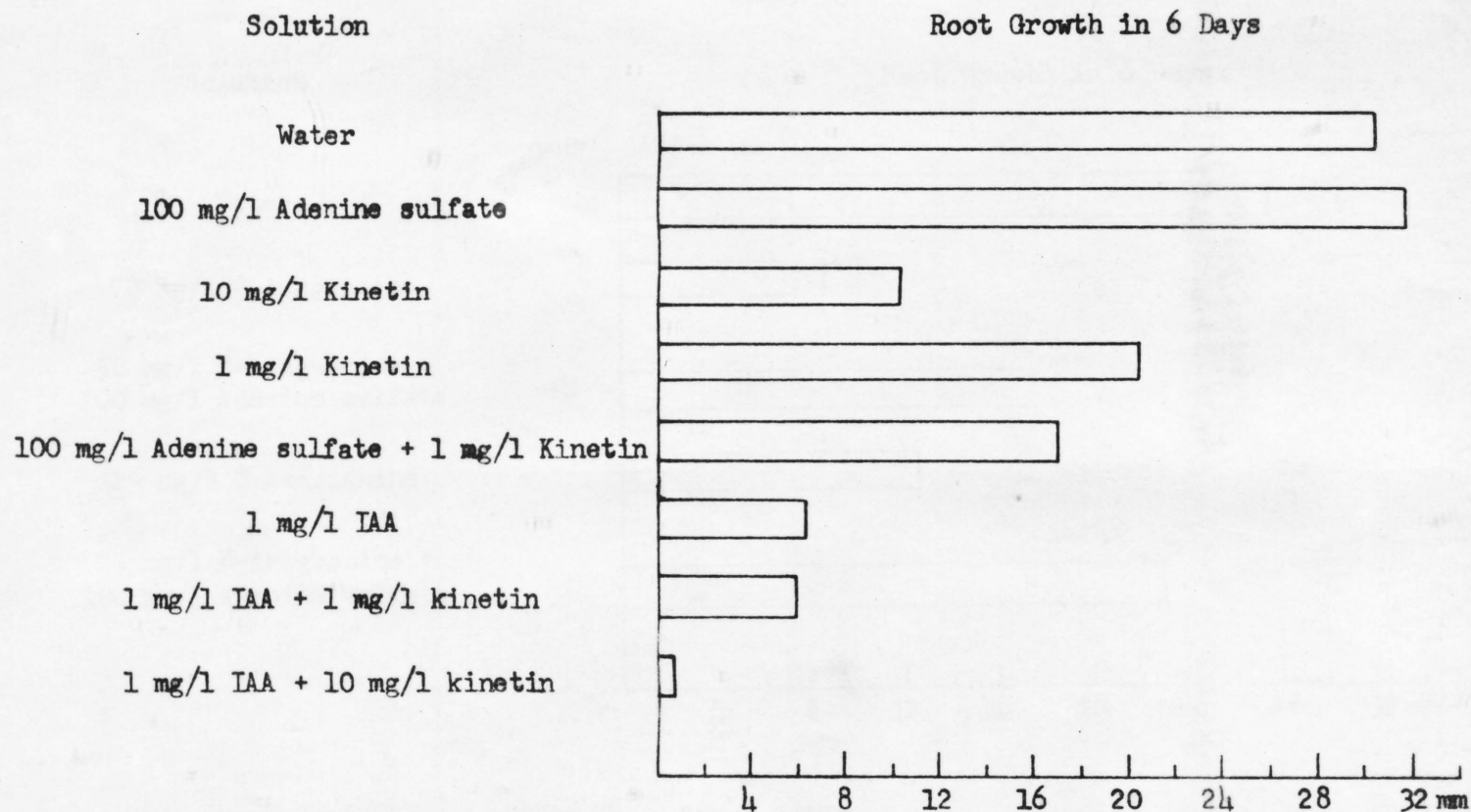
Solution   Root Growth in 6 Days

Water

100 mg/l Adenine sulfate

10 mg/l Kinetin

1 mg/l Kinetin

100 mg/l Adenine sulfate + 1 mg/l Kinetin

1 mg/l IAA

1 mg/l IAA + 1 mg/l kinetin

1 mg/l IAA + 10 mg/l kinetin

```
          4    8    12   16   20   24   28   32 mm
```

Fig. 11. Root growth in mm of leafy spurge seedlings when uniformly germinated seeds
were placed on blotters saturated with several solutions. Root
growth is the mean of 32 seedlings per treatment.

Solution   Root Growth in 6 Days

Water

50 mg/l 8-Azaguanine

50 mg/l 8-Azaguanine +
100 mg/l Adenine sulfate

25 mg/l 8-Azaguanine

25 mg/l 8-Azaguanine +
100 mg/l Adenine sulfate

          4   8   12   16   20   24   28   32 mm
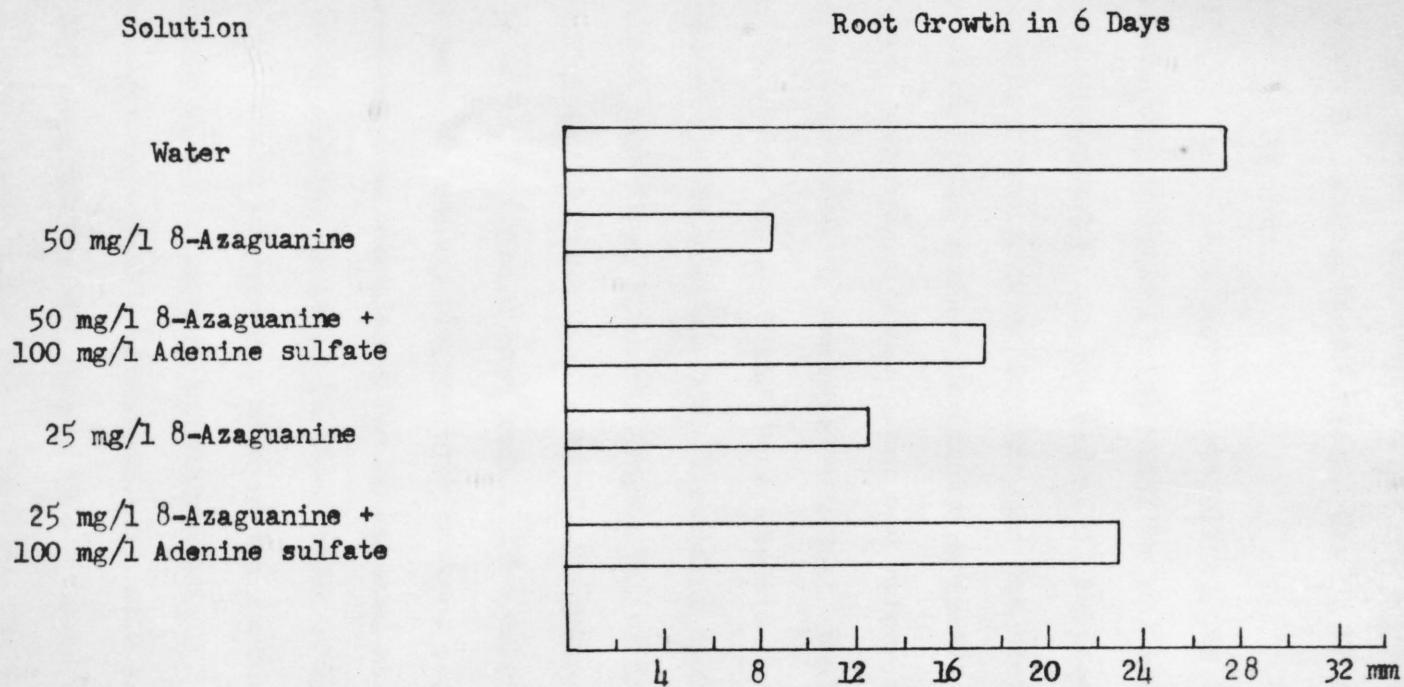
Fig. 12. Root growth in mm of leafy spurge seedlings when uniformly germinated seeds
          were placed on blotters saturated with several solutions. Root
          growth is the mean of 16 seedlings per treatment.

The water used in preparation of stock solutions and final media was deionized, glass distilled with potassium permanganate, and redistilled, using an all-glass apparatus. The pH of all media after autoclaving was 4.9 to 5.0.

Mottled seeds were surface-sterilized for 12 minutes in 200 ml of a 50% solution of Hilex (5.25% NaOCl by wt) containing one drop of Tween-20 (a surfactant). At the end of 12 minutes, the seeds and sterilant were poured into a strainer and the seeds washed with sterile distilled water. The seeds were placed in previously autoclaved, 9-cm Petri dishes, containing filter paper and water, and subjected to alternating temperatures in complete darkness. While germination appeared to be slower in sterilized than unsterilized seeds, most seeds had germinated by the seventh day. Depending on the experiment to be run, isolated root tips, hypocotyl segments, or intact seedlings were transferred from the Petri dishes to 125-ml Erlenmeyer flasks containing 50 ml of the liquid test media. The culture flasks containing the media were previously plugged with cotton, capped with small, non-waxed paper cups and autoclaved for 20 minutes at 15 pounds pressure. All growth substances employed in these studies were added prior to autoclaving. Where glucose was used as the carbohydrate source, it was equimolar with the sucrose normally used.

In order to reduce contamination as much as possible, all transferring and subculturing was done inside a transfer box equipped with an ultra-violet, germicidal lamp. The lamp was turned on at least one-half hour prior to each use of the transfer box. To reduce dust

particles and air-borne contamination, the small room where the work was performed was sprayed before each use with propylene glycol. These techniques reduced contamination to a minimum.

Experiments with intact and decotyledonized seedlings were conducted, but presentation of data must await further experimentation and refinement of technique. Some consistent and very marked effects were noted, however. (1) Lateral roots were initiated after decapitation of the root tip, and subsequent removal of the vegetative tip arrested the development of the newly initiated lateral roots; (2) no epicotyl growth was obtained in decotyledonized plants in 18-day experiments, while all control plants developed epicotyls (average of 55 mm) in the same length of time; (3) less root growth was obtained in glucose-adenine sulfate treatments than in sucrose-adenine sulfate treatments. Almost no growth was obtained on seedlings in 100 and 500 mg per liter adenine sulfate regardless of the carbohydrate source.

The extreme physiological variability existing in seedling plants of leafy spurge is shown in figures 13 and 14. Two seedlings of the same age were grown in basal media supplemented with 50 mg per liter adenine sulfate in the dark, at 25 C for 5 months, without transfer. Figure 13 shows a seedling plant which developed a stem of 127 cm and a main root of 109.2 cm, a total of 236.2 cm, with numerous lateral roots on the stem. The coiled portion of the seedling, in the center of the photograph, is root. The lateral roots are quite long as compared to the many short laterals formed on the root of the plant in figure 14. The seedling shown in figure 14 developed a stem
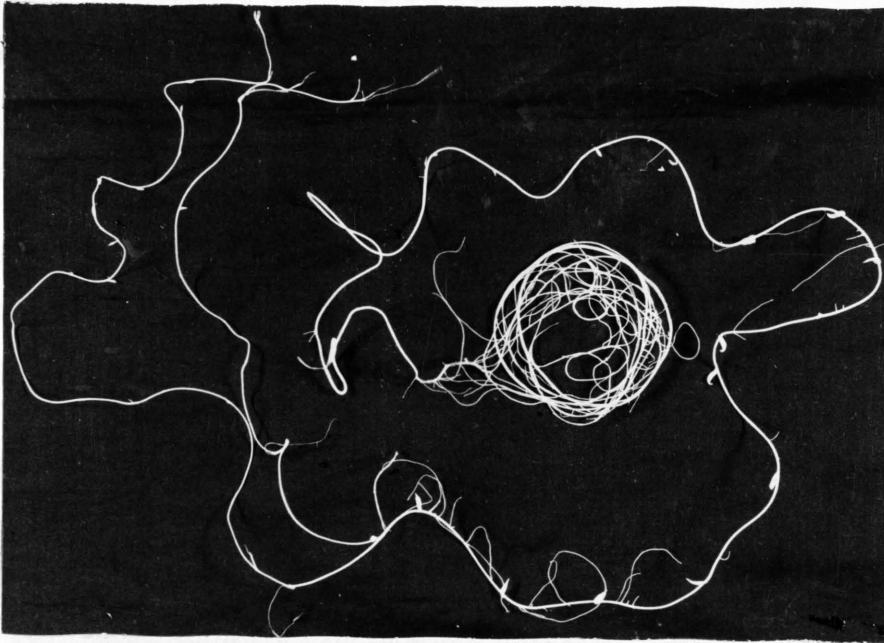
Fig. 13. A leafy spurge seedling, grown for 5 months in culture, at
25 C, in the dark, on basal medium supplemented with 50 mg/l
adenine sulfate, and demonstrating a high shoot/root ratio
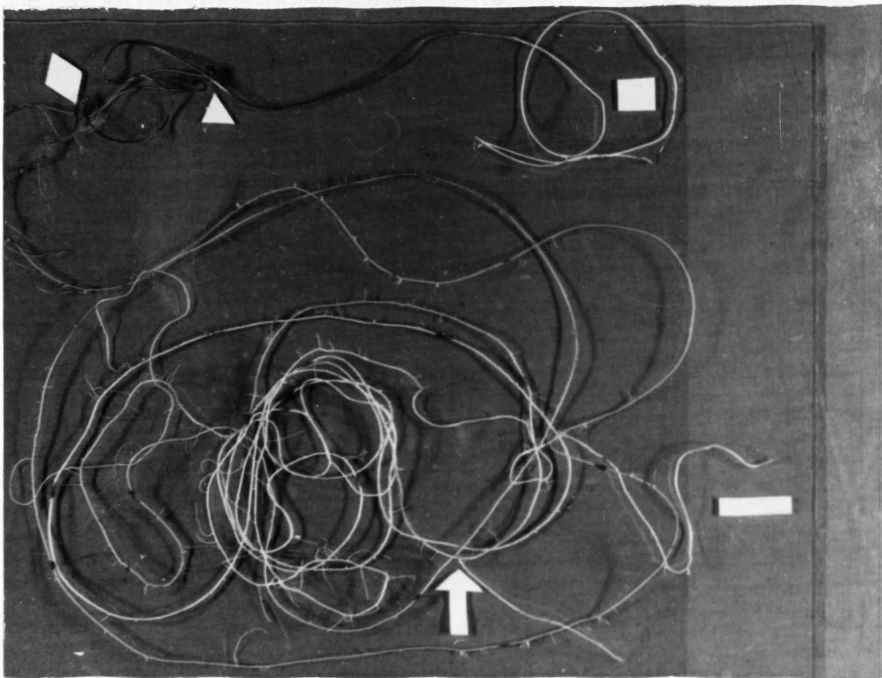
Fig. 14. A leafy spurge seedling, grown for 5 months in culture at
25 C, in the dark, on basal medium supplemented with 50 mg/l
adenine sulfate, and demonstrating a low shoot/root ratio.
□ = stem, △ = cotyledons, ◇ = union of hypocotyl
and root, ⇨ = shoot, ▭ = main root tip

of only 50.8 cm and a main root of 193.0 cm, a total of 243.8 cm.
Figure 15 demonstrates lateral root initiation and subsequent growth
of the laterals when the main root tip was removed. The root tip was
removed when the seedling was 2.5 cm long. Total growth period was
5 months.
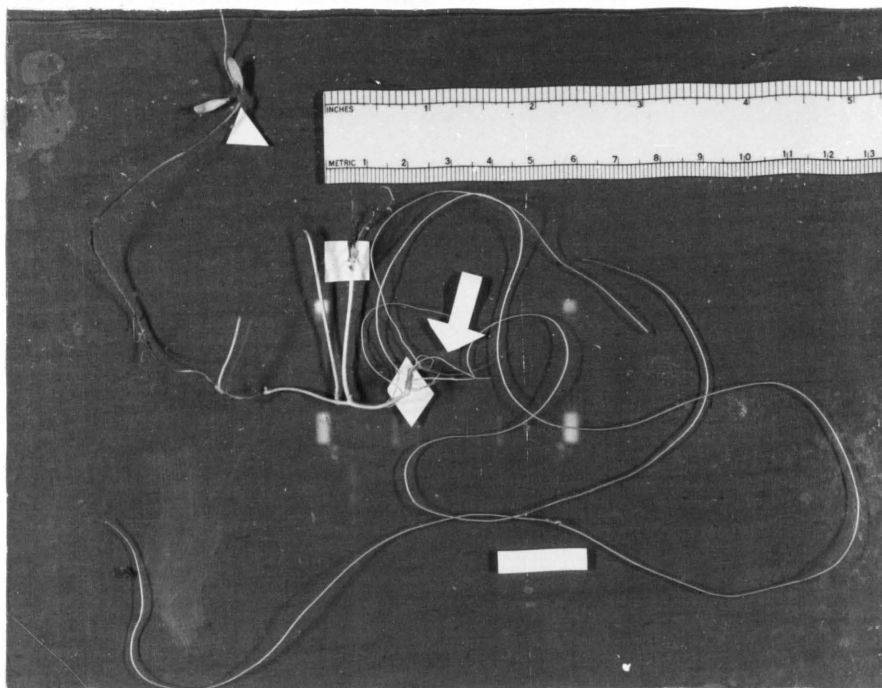
Fig. 15. A leafy spurge seedling, grown for 5 months in culture at 25 C,
in the dark, on basal medium, and demonstrating the initiation and
subsequent growth of lateral roots after removal of the main
root tip. □ = a crown of buds formed on a shoot that had
its origin in the hypocotyl, ◇ = union of root with
hypocotyl, ⇨ = lateral roots, ▭ = buds formed on
two of the developing lateral roots,
△ = cotyledons

As shown in the preceding photographs, considerable seedling growth was obtained when 50 mg per liter adenine sulfate was incorporated in the basal media. Very little growth was obtained, however, on seedlings placed in media containing both 50 mg per liter adenine sulfate and 50 mg per liter 2,6-diaminopurine. The adenine antagonist was not tested alone.

Numerous attempts were made to culture intact leafy spurge seedlings on solid, nutrient, agar media, in the dark, but growth was very limited. Even for periods up to two months, root growth never exceeded 120 mm for any seedling.

Isolated Plant Parts

Considerable difficulty was encountered in obtaining uniform growth of isolated roots. Attempts to establish growth of isolated roots which failed were: serial dilutions of IAA from 1 to 10^{-8} mg per liter; kinetin at 10, 1, 10^{-1}, and 10^{-4} mg per liter; GA at 10, 1, and 10^{-2} mg per liter (and combinations of the above); coconut milk at 0.2, 1, 2, 4, and 6% of the media; casein hydrolysate; and yeast extract. Despite the substances added, liquid media seemed to allow more growth (however slight) than solid or semi-solid agar. No growth was obtained when 3-mm root tips were used, but some root tips grew if they were 10 or 15 mm in length when placed in the test solutions. Consequently, all remaining experiments were conducted using 10-mm root tips and liquid media.

Since adenine was known to stimulate the growth of certain plant organs and since alternation of temperature provided maximum

germination of leafy spurge seeds, several experiments were designed to determine the effect of the purine on root growth at constant and alternating temperatures. Since it was also known that some plants utilize glucose more readily than sucrose, the former was tested with and without adenine sulfate and at constant and alternating temperatures.

All treatments did not always contain the same number of root tips due to occasional contamination, damage to the tissue in transfer, or lack of uniform seedlings. The treatments and results of all experiments are shown in table I and the analyses of variance given in table II. Orthogonal comparisons were made to test for significant differences among treatments. In experiment I, the mean root growth in 18 days was significantly greater in sucrose (102 mm) than in glucose media (51 mm) over all treatments. Root growth was significantly greater in the sucrose plus adenine sulfate at 20 mg per liter treatment (experiment I and II) than in the sucrose alone, but root length was not significantly increased by the addition of 50 mg per liter adenine sulfate (experiment III). When adenine sulfate was incorporated with glucose, however, a significant reduction in root length was observed. Experiment I showed significance at the 10% level and experiment II at the 1% level. In sucrose-based media, root growth was significantly greater under alternating temperature conditions than under constant temperature, regardless of the adenine sulfate level (experiment I and IV). Root growth was significantly less when grown in glucose under alternating temperatures, but the difference was primarily due to the lack of growth when adenine sulfate was added

Table I. Increase in Root Length, in 18 Days, From 10-mm Root Tips, When Subjected to Various Treatments

Treatments	Experiments				Mean
	I	II	III	IV	
Sucrose, (20 C)	64	50	96	140	
	42	47	45	16	
	23	35	42	14	
	21	18	41	12	
	8	5	24	6	
			21		
			17		
Mean	31.6	31.0	40.9	37.6	35.8
Sucrose, adenine sulfate - 20 mg/l (20 C)	166	107	113		
	64	103	103		
	61	103	74		
	53	35	52		
	5	8	45		
			28		
			17		
Mean	69.8	71.2	61.7 *		70.5
Mean of 20 C	50.7				
Sucrose, (20 C - 30 C)				232	
				230	
				205	
				175	
				105	
Mean				189.4	189.4
Sucrose, adenine sulfate - 20 mg/l (20 C - 30 C)	222				
	218				
	213				
	187				
	180				
Mean	204.0				204.0
Mean of sucrose	101.8	51.1	51.3	113.5	

* Sucrose, adenine sulfate - 50 mg/l (20 C) - not included in the mean for Sucrose, adenine sulfate - 20 mg/l (20 C).

Table I. (continued)

Treatments	Experiments				Mean
	I	II	III	IV	
Glucose, (20 C)	114	153	161		
	121	102	157		
	84	66	51		
	34	61	45		
	37	34	34		
			34		
			27		
Mean	78.0	83.2	72.7		78.0
Glucose, adenine sulfate - 20 mg/l (20 C)	127	83			
	76	77			
	22	41			
	17	18			
	7	8			
Mean	49.8	45.4			47.6
Mean of 20 C	63.9				
Glucose, (20 C - 30 C)	124				
	102				
	57				
	27				
	11				
Mean	64.2				64.2
Glucose, adenine sulfate - 20 mg/l (20 C - 30 C)	20				
	17				
	7				
	7				
	5				
Mean	11.2				11.2
Mean of 20 C - 30 C	37.7				
Mean of glucose	50.8	64.3	72.7		

Table II. Analysis of Variance of the Increase in Length, in 18 Days,
From 10-mm Root Tips When Subjected to Various Treatments

Source	Experiment I		Experiment II		Experiment III		Experiment IV	
	df	MS	df	MS	df	MS	df	MS
Segments	4	7807.6	4	4801.1	6	4021.8	4	4563.3
Treatments#	6	19453.6**	3	2827.8**	2	1832.7	1	57608.1**
Sucrose vs Glucose								
s,sa,SA vs g,G,ga,GA	1	22294.3**						
s,sa vs g,ga			1	871.2				
s,sa vs g						2142.9		
SA vs s, sa	1	78336.3**						
sa vs s	1	3648.1*	1	4040.1**		1522.6		
S vs s								57608.1**
g,ga vs G,GA	1	3432.2*						
G vs GA	1	7022.5**						
g vs ga	1	1988.1	1	3572.1**				
Error	24	523.5	12	324.9	12	783.1	4	1486.85
Total	34		19		20		9	

* Significant at 5% level.   ** Significant at 1% level.
# Sucrose, glucose, adenine sulfate at 20 C, abbreviated s,g,a; and at 20 C - 30 C, abbreviated S,G,
A, respectively. Adenine sulfate was used at 20 mg/l in Exp. I & II and at 50 mg/l in Exp. III.

(experiment I). As shown in table I and figure 16, considerable
variability existed within some treatments; however, a composite plot
of these data presents the general response to the various treatments
previously described. The composite plot is presented in figure 17.
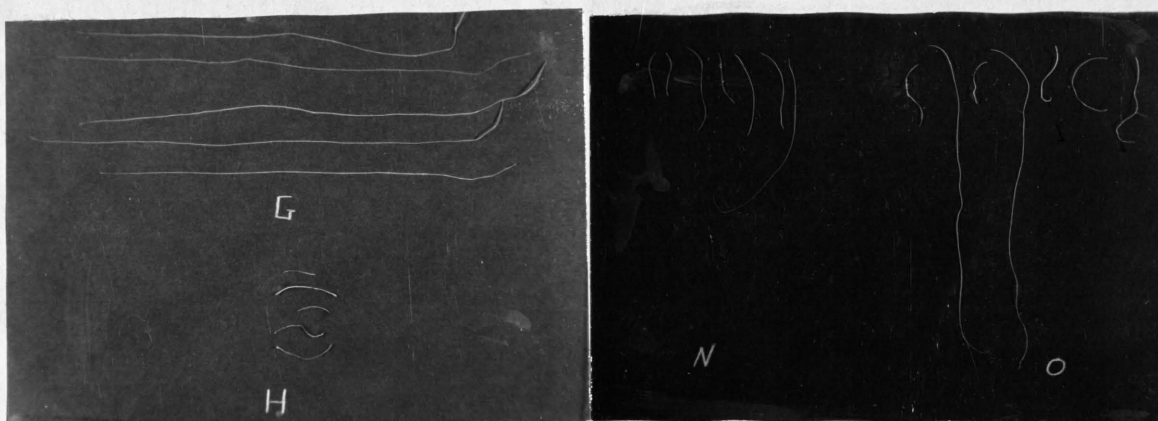
Fig. 16. Growth of isolated root tips of leafy spurge grown in various
media for 18 days at 20 C. (G) sucrose, 20 mg/l adenine sulfate
& alternating temperatures, (H) glucose 20 mg/l adenine
sulfate & alternating temperatures, (N) sucrose,
(O) glucose

Isolated lateral root tips excised from older plants showed the
same response as root tips from seedlings when grown in glucose or
glucose-adenine sulfate media, with and without alternation of
temperatures.

An experiment was conducted to determine the effects of glucose,
sucrose, and sucrose with 50 mg per liter adenine sulfate upon the
growth of hypocotyls at 20 C in the dark. Seedlings were obtained by
the same aseptic technique as described in the previous section. The
point of union of root with the hypocotyl was plainly visible, and
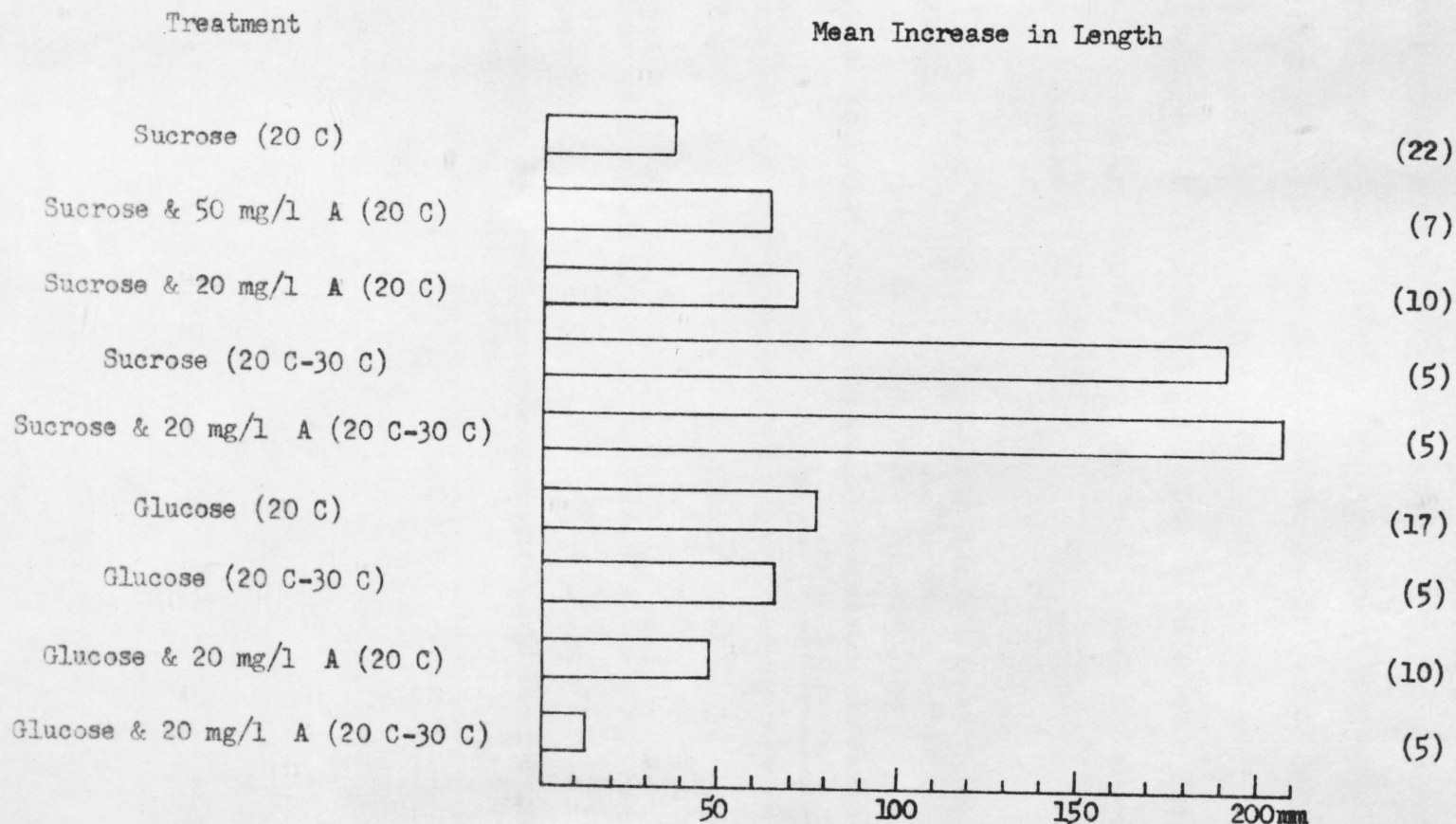
Fig. 17. Mean increase in length of isolated 10-mm root tips in 18 days when subjected to various media and growth conditions. The number in parentheses indicates the number of root tips tested. Adenine sulfate is abbreviated, A.

that part of the seedling above this junction was removed and subjected to the various treatments listed in table III. At the end of 18 days, hypocotyl and epicotyl lengths were recorded. Results are shown in table III and figure 18. Growth of hypocotyl and epicotyl was greater in the sucrose-adenine sulfate treatment than for sucrose alone. The hypocotyls in both treatments containing sucrose were much longer than those in the glucose medium. No epicotyl growth was observed in the glucose treatment.

Table III. Mean Increase in Length of Leafy Spurge Hypocotyls and Epicotyls (in 18 days, at 20 C, in the dark), after the Entire Root was Removed from the Seedling

	Mean increase in mm	
Treatment	hypocotyl	epicotyl
Sucrose (20 C)	103.14 - (7)*	24.5
Sucrose & 50 mg/l adenine sulfate (20 C)	120.12 - (8)	55.7
Glucose (20 C)	32.38 - (8)	0

* Number of segments tested.

—
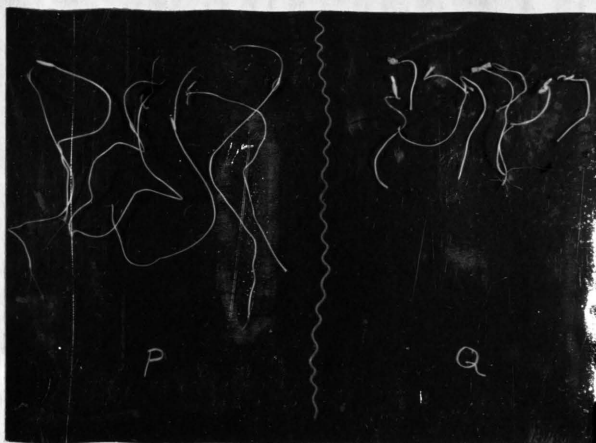
Fig. 18. Growth of leafy spurge hypocotyls and epicotyls (in 18 days, at 20 C, in the dark), after the entire root was removed from the seedling. (P) sucrose, (Q) glucose

Bud Formation and Development

Anatomy of Subterranean Parts

The underground parts of mature plants of leafy spurge were collected from Oakwood Lakes State Park, 10 miles northwest of Brookings, South Dakota, and from a roadside area on the northeast edge of South Dakota State College campus. Plants from both sites were collected in September. All plants were carefully exhumed and measured to determine the exact location of the various plant parts in the soil. Segments of the various plant parts were taken to the laboratory and prepared for microscopic examination. The tissues collected were killed and fixed in formalin-acetic acid-alcohol (FAA), softened with 50-50 ethyl alcohol-hydrofluoric acid for 2 weeks, embedded in celloidin, and sectioned with the sliding microtome. Sections were made 15 microns thick and stained with safranin and fast green.

Seedling tissue was collected from month-old plants grown in the greenhouse. The young tissues were killed and fixed in FAA, dehydrated in the ethyl-butyl alcohol series, impregnated with and embedded in paraffin, and sectioned on the rotary microtome. Seedling tissues were sectioned 10 microns thick and stained, using Triarch's quadruple staining technique.

All sections were cleared in xylol and mounted in Harleco mounting medium.

The drawing in figure 19 represents a clone and includes the main plant axis with its primary tap root, older lateral roots, an oblique root, numerous feeder roots, and several stems arising from the underground horizontal roots.

Horizontal underground parts. All horizontal and oblique structures examined proved to be roots, with both triarch and tetrarch protoxylem being found on the same plant. A section taken from a triarch horizontal root and containing an adventitious endogenous bud (marked as 3 in figure 19) is shown in figure 20. A section illustrating a horizontal tetrarch root (marked as 5 in figure 19) is shown in figure 21. Numerous pink buds were present on the horizontal and oblique roots.

Transition zone. The transition zone of leafy spurge was macroscopically visible within the first 2 days after germination, especially when the seedling was exposed to an even slight amount of light. The collet (lower hypocotylary swelling) was pink in color; the root below was white, and root hairs were visible as shown in figure 22.
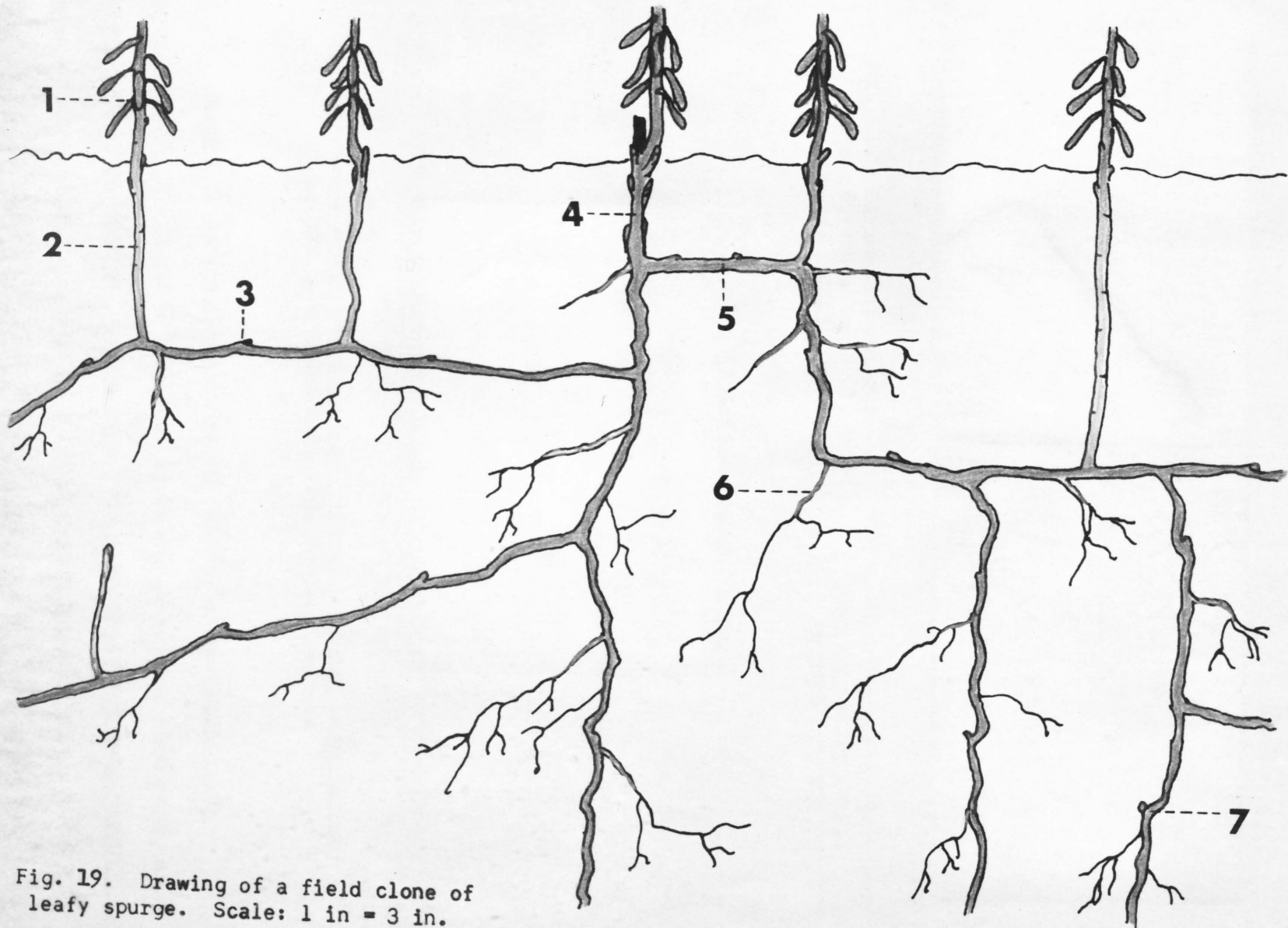
Fig. 19. Drawing of a field clone of leafy spurge. Scale: 1 in = 3 in.
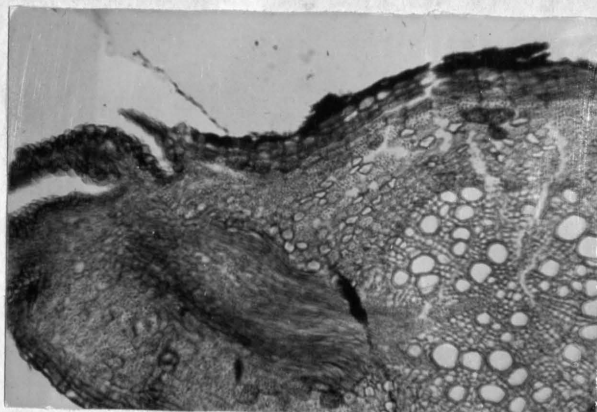
Fig. 20. Horizontal triarch
root of mature leafy
spurge. (80X)
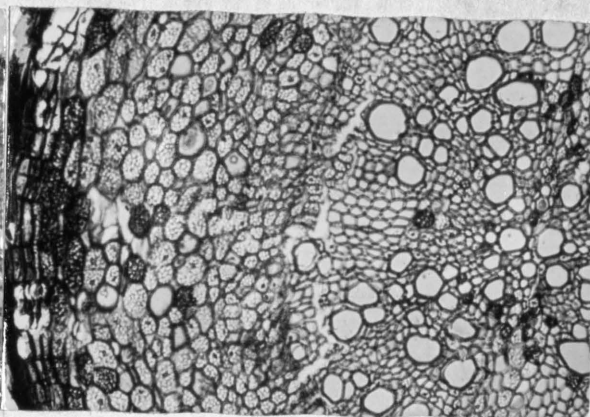
Fig. 21. Horizontal tetrarch
root of mature leafy
spurge. (125X)

Fig. 22. Germinating seeds of leafy spurge, showing pigmentation
above and root hairs below the transition zone

Figure 23 is a scale drawing of part of a month-old leafy

spurge seedling. Numbers have been inserted at various locations from

which 10-micron sections were taken. These sections are shown in fig-

ures 24 through 29. The distal ends of the main root axis or lateral
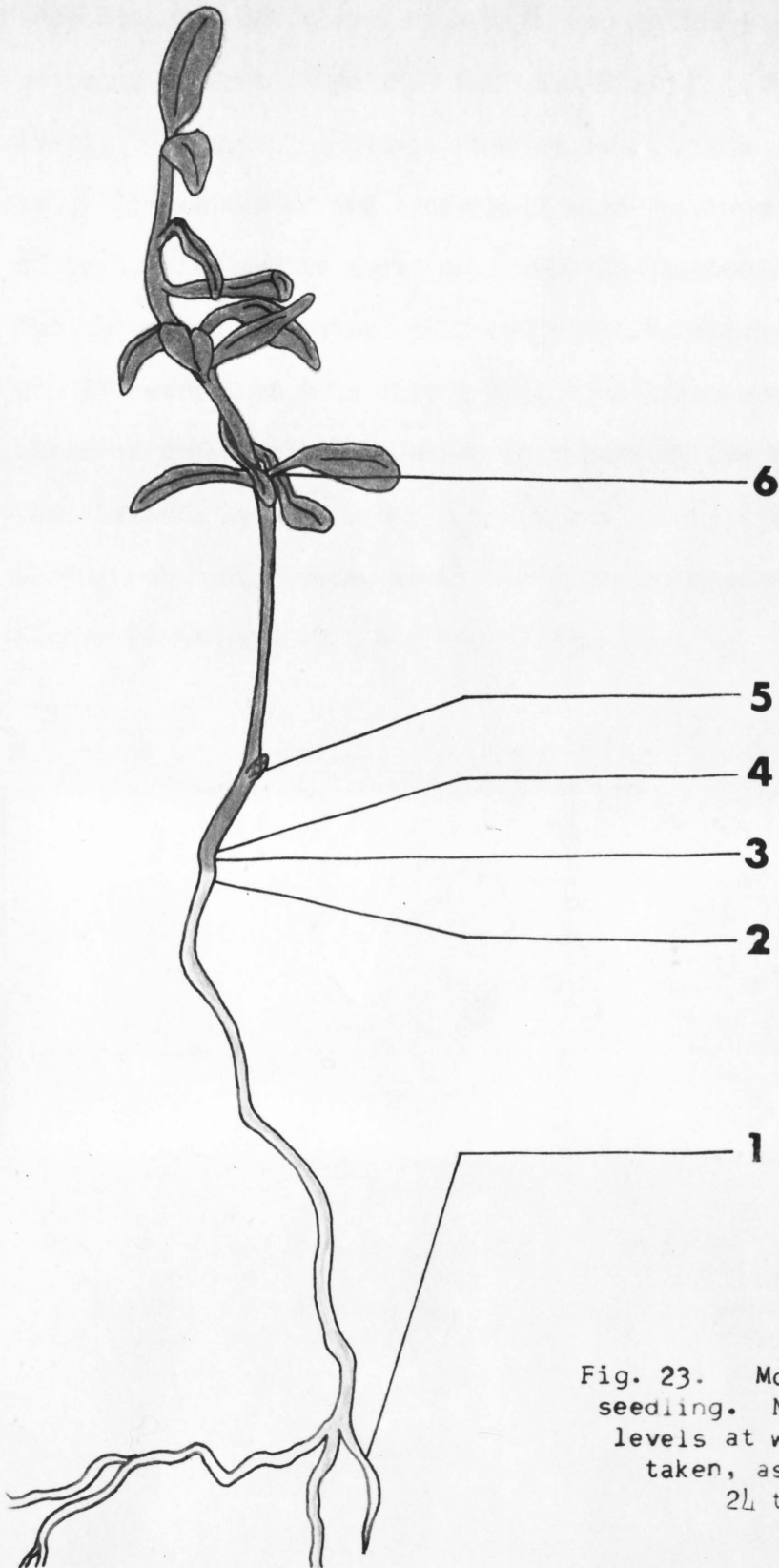
Fig. 23.   Month-old leafy spurge
seedling.   Numbers 1-6 indicate
levels at which sections were
taken, as shown in figures
24 through 29.

root branches had diarch xylem (figure 24 and marked as 1 in figure 23)
becoming triarch (figure 25 and marked as 2 in figure 23) at a higher
level. The triarch pattern was maintained into the base of the col-
let. The center of the transition zone appeared not to have a pattern
of protoxylem and is shown in figure 26 (marked as 3 in figure 23).
The beginning of central pith and endarch protoxylem is shown in fig-
ure 27 (marked as 4 in figure 23). The tetra-endarch collateral
stele of upper collet is shown in figure 28 (marked as 5 in figure 23).
The distance between 2 and 4 in figure 23 was 2540 microns. A section
through the cotyledons, lower stem, and cotyledonary buds is shown in
figure 29 (marked as 6 in figure 23).
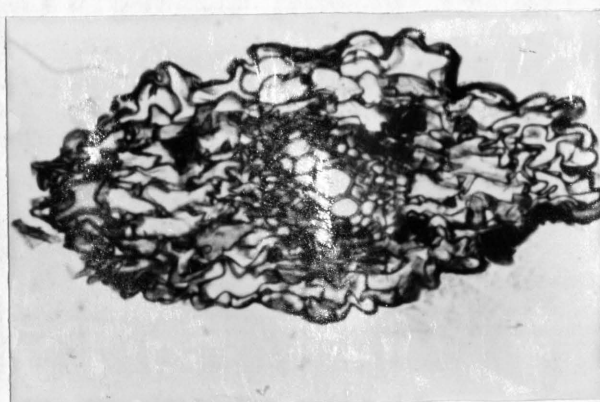
Fig. 24. A section from month-
old leafy spurge seedling,
taken 1.5 cm behind the
root tip. (400X)
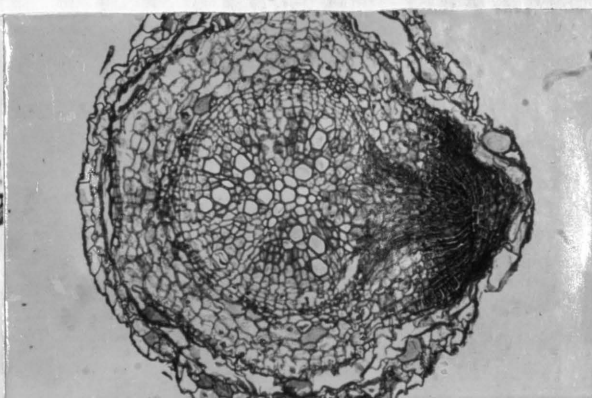
Fig. 25. A section from month-
old leafy spurge seedling,
taken just below the
transition zone.
(125X)
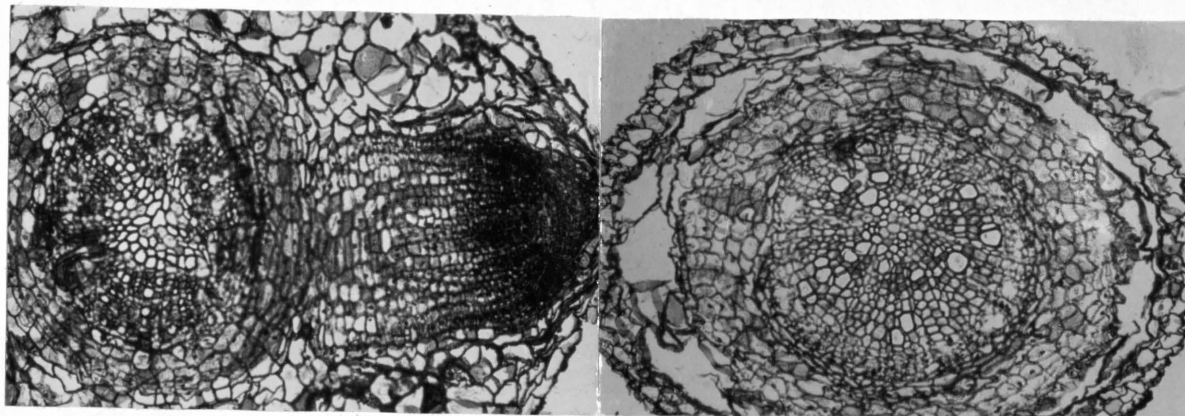
Fig. 26. A section from month-
old leafy spurge seedling,
taken in the center of
the transition zone.
(125X)

Fig. 27. A section from month-
old leafy spurge seedling,
taken in the upper
transition zone.
(125X)
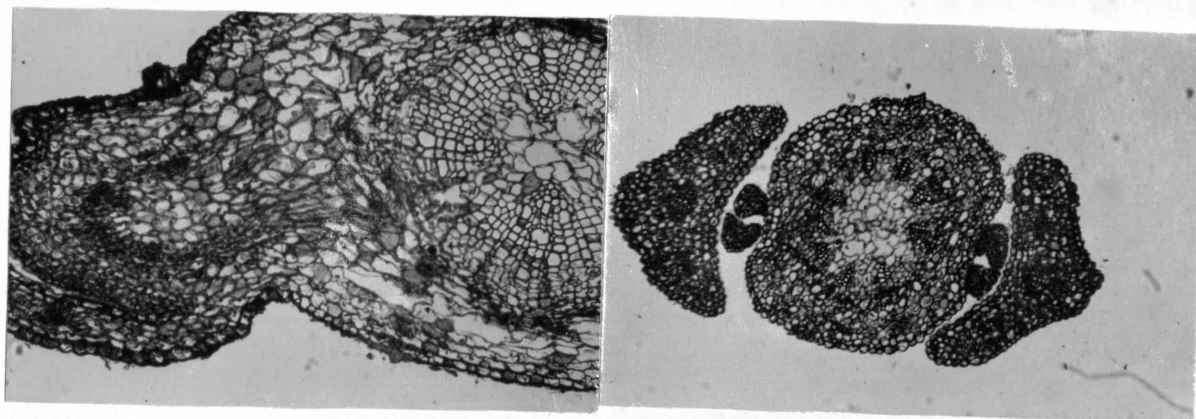
Fig. 28. A section from month-old
leafy spurge seedling, taken
in the lower hypocotyl.
(125X)

Fig. 29. A section from month-
old leafy spurge seedling,
taken through cotyledons,
lower stem, and cotyle-
donary buds. (80X)

All shoots of a mature clone were similar in structure except for one. This exceptional shoot (of the original seedling) contained a "crown" of adventitious buds on the collet, with a triarch or tetrarch tap root (shown in figure 30 and marked as 4 in figure 19) directly below ground. The others had a crown of buds originating in stem tissue, these stems (shown in figure 31 and marked as 2 in figure 19) extending to the horizontal or oblique roots from which they originated as endogenous buds. Feeder roots have little or no secondary growth, are diarch at the distal end, and usually triarch through the rest of their axes (shown in figure 32 and marked as 6 in figure 19).
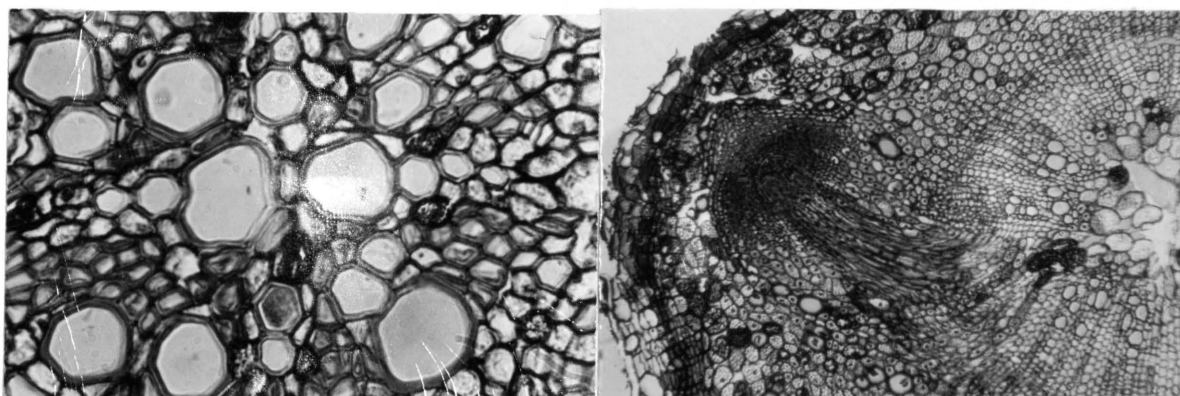
Fig. 30. Tetrarch tap root of mature leafy spurge, sectioned just below the transition zone. (400X)

Fig. 31. Underground stem of mature leafy spurge arising from horizontal root. (80X)
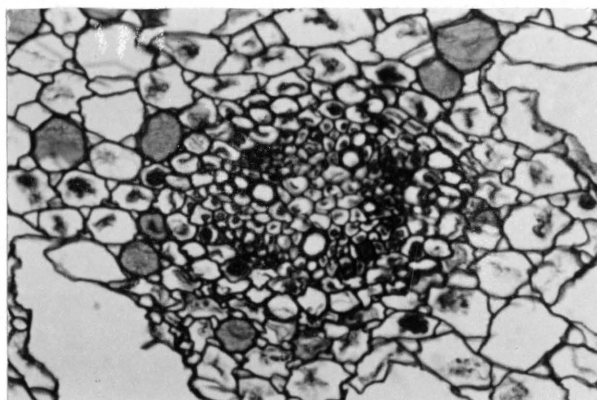
Fig. 32. Cross section of feeder root of leafy spurge and showing
three distinct primary poles and one xylem vessel located in the
normal fourth position. (400X)

Figure 33 illustrates that the crowns formed at the soil sur-
face, on stems originating from horizontal or oblique roots, were
similar to those crowns found at the transition zone. The difference
was readily determined by excision and examining for the presence or
absence of pith directly below the crown.

Origin of primordia. All root primordia, whether adventitious
from shoots (figure 31), lower hypocotyl as shown in figure 34, or
from roots (figure 25 and also shown in figure 35), had endogenous
origin from pericycle or pericyclic tissue.

Bud primordia arose exogenously from nodes of underground
stems. Buds on upper hypocotyl appeared to be exogenous (figure 28)
because of the continuous epidermis on both bud and main axis. How-
ever, the procambial development as deep as the vascular strands of
hypocotyl was indicative of an endogenous origin from a pericyclic
tissue. A bud was found in the lowermost part of the collet arising
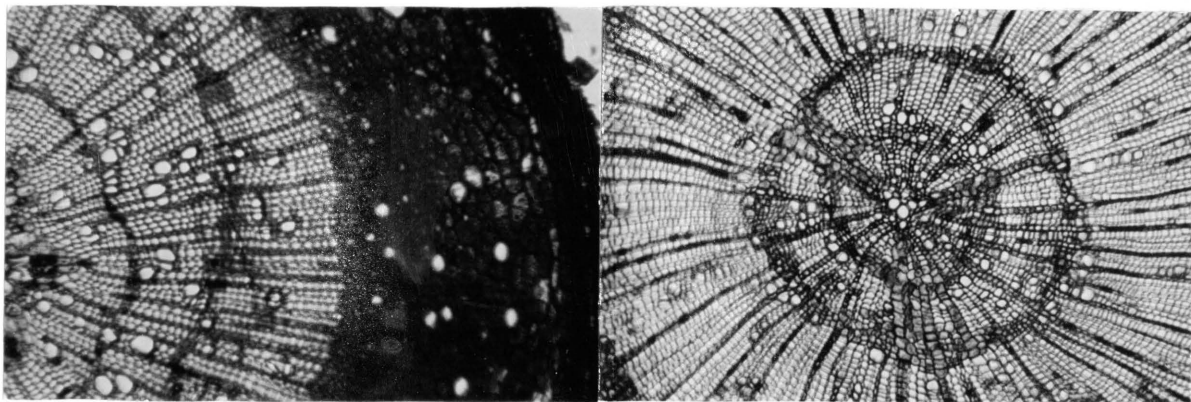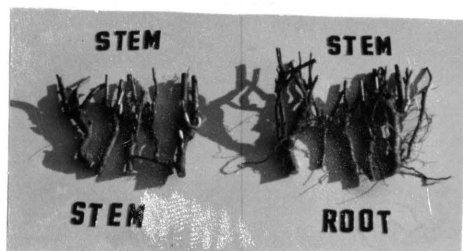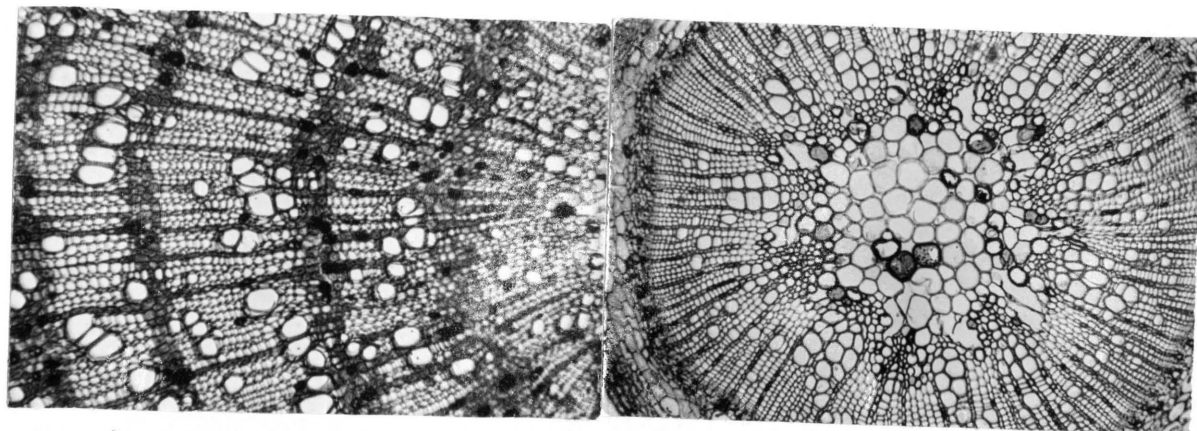
Fig. 33. Crown formed on stem tissue (center-left) and at the tran-
sition zone (center-right) of mature plants of leafy spurge.
Upper left--stem above crown showing concentric rings of
parenchyma (80X). Upper right--young stem above crown
without parenchyma rings (marked as 1 in figure 19)
(125X). Lower left--stem below crown showing
concentric rings of parenchyma (80X).
Lower right--root showing
concentric rings and
parenchyma rays
(125X)
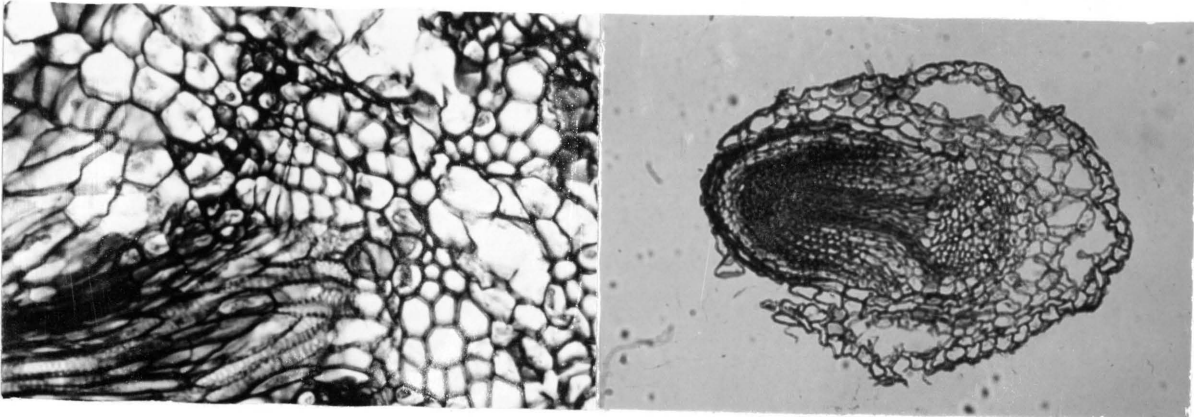
Fig. 34. Section through lower
hypocotyl of leafy spurge
seedling, showing endoge-
nous lateral root
primordium.
(400X)

Fig. 35. Section through young
triarch root, showing lateral
root primordium originating
from the flank of a xylem
wing. (125X)

endogenously from clearly rootlike tissue as shown in figure 36. In

young roots, the origin of buds was endogenous from the original

pericycle layer. In more mature roots, whether horizontal (figure 20)

or vertical as shown in figure 37 (marked as 7 in figure 19), bud ori-

gin appeared to be exogenous from outermost layers of pericycle. But

here, as in the lower hypocotyl of figure 28, the procambial differ-

entiation extended to the vascular system of the main root.
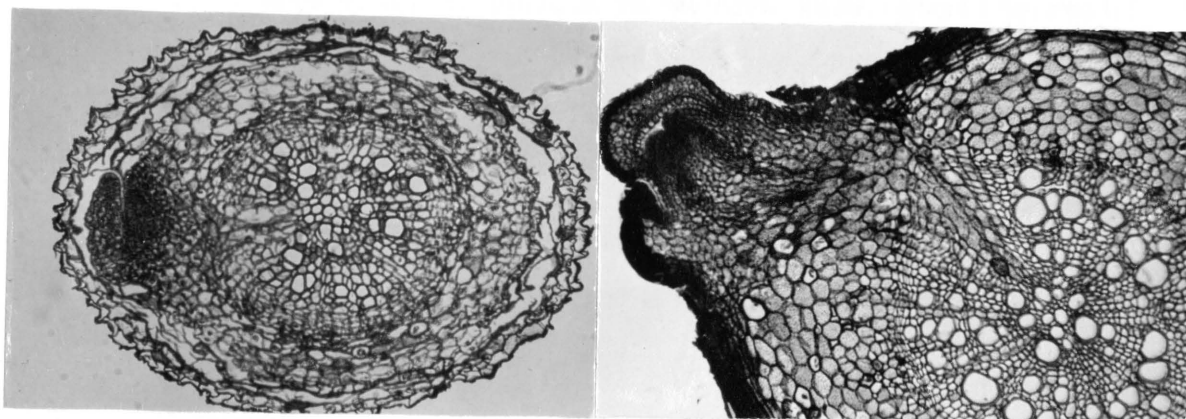
Fig. 36. Section from month-old leafy spurge seedling, taken in the lower part of the collet and showing endogenous bud. (125X)

Fig. 37. Section through bud on vertical root of mature leafy spurge. (125X)

Tissue patterns. Without exception, diarch xylem patterns were observed on distal ends of young roots, and triarch or tetrarch xylem prevailed throughout the remainder of underground root tissue. No organs were observed with more than four xylem wings.

Cortex and epidermis were sloughed off quite early in root development. Pericycle, therefore, was the outermost tissue in all except the feeder roots and the very young lateral roots. The cork, whenever present, either on underground stem (figure 31) or on roots (figure 37), originated from a pericyclic phellogen.

On large vertical roots, -arch patterns were determined in the field observing the transversely cut surface just below the crown. The broadening parenchyma rays opposite the protoxylem points appeared somewhat lighter in color than the surrounding xylem.

Concentric rings of storage parenchyma were often observed above the crown in stem tissue, and below the crown in stem tissue, or in root tissue (all shown in figure 33).

Hypocotyl Segments

Mottled leafy spurge seeds were germinated under aseptic conditions. The seedling plants were severed just above the union of root and hypocotyl and the upper portions placed in basal media in the dark at 25 C. The hypocotyls were allowed to extend for 1 month, attaining a length of about 150 mm. Some epicotyl extension also occurred during this time. Three cm at both extremes of the hypocotyl were discarded, the remainder being sectioned into 1-cm lengths. These hypocotyl segments were selected at random and placed in flasks containing 50 ml of basal media, supplemented with kinetin at 5, 1, 10^{-2}, and 0 mg per liter; and IAA at 1, 10^{-2}, 10^{-4}, and 0 mg per liter, in all possible combinations. Each flask contained 2 segments, with 3 flasks for each treatment. All flasks were placed in light at constant 25 C for 3 weeks. An identical set of treatments was placed in the dark at constant 25 C. Segments placed in the dark produced no buds in the 3-week period; however, considerable callus was formed on the segments in all treatments containing IAA at 1 and 10^{-2} mg per liter. The results of bud formation on the light grown segments are shown in figures 38, 39, and 40. Maximum bud formation was obtained with 10^{-2} mg per liter kinetin. Fewer buds were formed in the treatment containing kinetin at 1 mg per liter and the treatment without kinetin or IAA. While the buds were formed in 1 to 2 weeks, normal

Mg per liter IAA

Mg per liter kinetin	1*	10^{-2}**	10^{-4}	0
5	Contam‑ination	cc c c c cccccc	b c ccc c c	cc ccc cccc cc c c
1			Contam‑ination c s	bbbb bb b
10^{-2}	s s	s s s sss	ssss s s ss	bbbbb bb b bbb bbbb b
0	ss ssss	rs ss Contam‑ination	cc r sss	b b b bb

Fig. 38. Buds (b), roots (r), unidentified swellings (s), and iso‑lated patches of callus (c), emerged from 1‑cm hypocotyl segments of leafy spurge, cultured for 3 weeks, in the light, at 25 C, in kinetin and IAA, alone, and in combinations. ____ = a hypocotyl segment, * = very heavy callus on both ends and along the length of the segments, ** = heavy callus on both ends of the segment
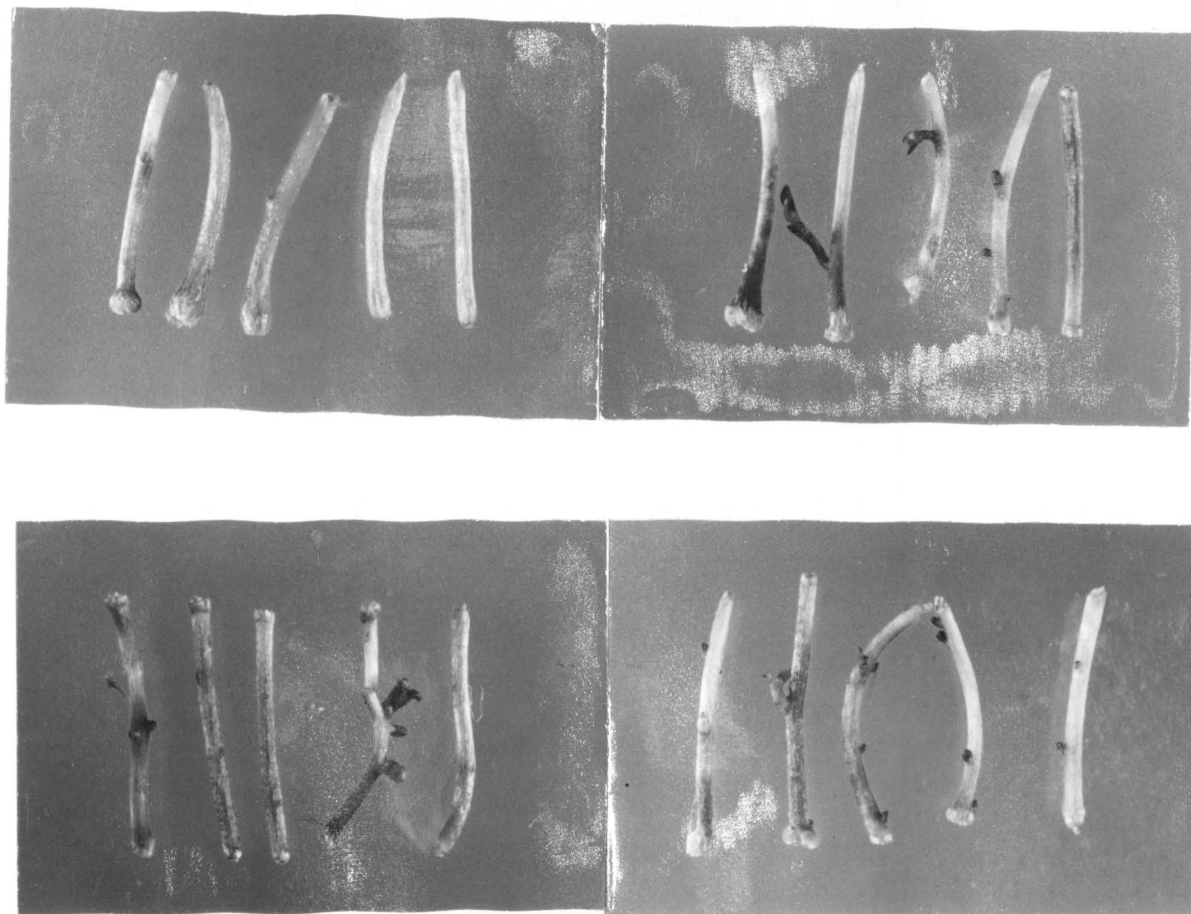
Fig. 39. Bud emergence on leafy spurge hypocotyl segments, cultured
for 3 weeks, at 25 C, in the light, in various concentrations of
kinetin and IAA. Upper left--kinetin 10^{-2} and IAA 10^{-4} mg/l.
Upper right--no kinetin, no IAA. Lower left--kinetin,
1 mg/l, no IAA. Lower right--kinetin 10^{-2} mg/l,
no IAA
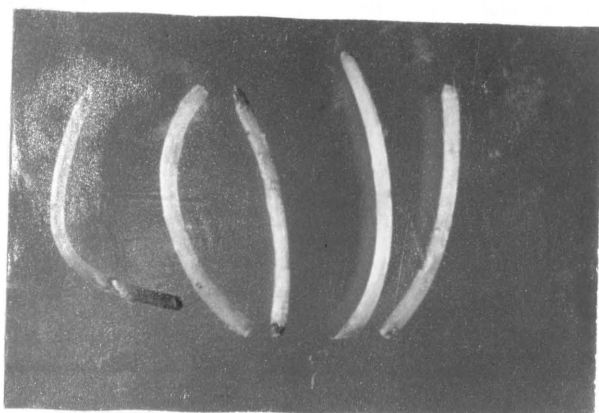
Fig. 40. Small patches of undifferentiated meristems formed on leafy
spurge hypocotyl segments, cultured for 3 weeks, at 25 C, in the
light, in 5 mg/l kinetin

extension of the shoot was lacking. Organs were not designated as
buds unless leaves were evident. As indicated in figure 38, many
swellings were apparent which could not be visually identified as buds
or roots. These swellings were most prevalent in the treatments con-
taining 10^{-2} mg per liter kinetin. Small patches of undifferentiated
meristem were observed in the treatments containing the highest rate
of kinetin (5 mg/l). In treatments containing IAA at 1 mg per liter,
heavy callus was generally observed over the entire segment. In treat-
ments containing IAA at 10^{-2} mg per liter, heavy callus was observed
at both ends of the segment, but callus was restricted to the basi-
petal ends in the treatments 10^{-4} mg per liter and no IAA. In the
latter two treatments, the amount of end callus decreased as the con-
centration of kinetin increased.

The epidermal splitting and sloughing found on a hypocotyl
segment treated with IAA, in the dark, is shown in figure 41. When
the hypocotyl segments treated with IAA were cultured in the light,
the callus was more typically bulbous and resembled that found on
hypocotyl segments treated with coconut milk in the dark.
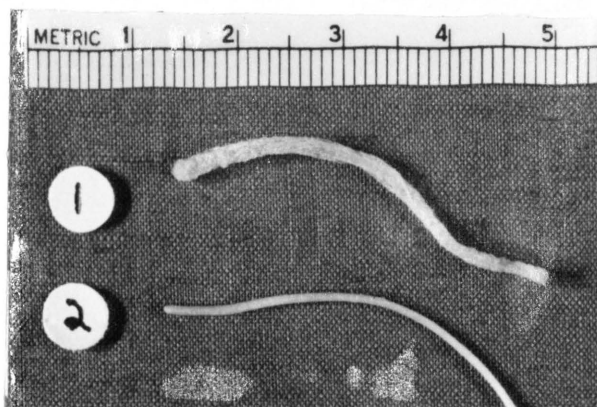
Fig. 41. Callus formed on hypocotyl segment of leafy spurge when
placed in media supplemented with IAA and cultured in the dark.
(1) 1 mg/l IAA, (2) no IAA

Buds were formed on hypocotyls of leafy spurge seedlings after
removal of the growing point. It was generally observed, however,
that more buds were formed on the hypocotyl when the organ was se-
verely wounded, but not severed, below the cotyledons. Buds formed on
hypocotyl and roots formed at the distal end of the hypocotyl are
shown in figure 42. Although buds were formed in 2 to 3 weeks after
removal of the growing point, very little growth of the new organ
occurred unless the seedling root was left intact.
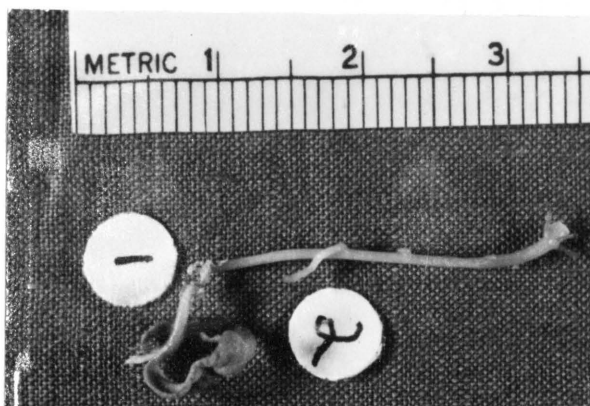
Fig. 42. Bud and root formation on leafy spurge hypocotyl, in 47 days after wounding below cotyledons. (1) the wound, (2) a bud

Root Segments

Seedling plants were grown for 83 days, in the dark, at 20 C on (1) a basal medium using sucrose as the carbohydrate source and (2) a sucrose based medium but supplemented with 50 mg per liter adenine sulfate. The plant in each treatment which appeared to have the best growth was selected for bud formation tests. Fifty mm of the root tip and about 100 mm of the upper part of the root of each plant were removed. The remainder of the main root axis, now quite free of lateral roots and devoid of visible buds, was cut into 1-cm segments and the segments placed in a Petri dish containing sterile distilled water. Root segments, selected at random, were divided among 25 flasks with 5 flasks per treatment. The treatments were: kinetin at 1, 10^{-1}, 10^{-2}, and 0 mg per liter and IAA at 1 mg per liter. A total of 115 segments were available from the plant grown in the medium not supplemented with adenine sulfate. They were subcultured for 7 weeks at

22 C in the light. Bud and lateral root initiation is shown in figures 43 and 44. The number of buds formed in 10^{-1} mg per liter kinetin was double that formed in 10^{-2} mg per liter kinetin, and the number formed in the latter concentration of kinetin was double that in the control. Nineteen times as many lateral roots were formed in treatments containing 1 mg per liter IAA as in the basal medium alone.

From a total of 90 segments taken from the plant grown in a sucrose basal medium, but supplemented with 50 mg per liter adenine sulfate, only 5 buds were formed. These 5 buds were found as follows: 0, 2, 2, and 1 buds in treatments containing the 1, 10^{-1}, 10^{-2}, and 0 mg per liter kinetin, respectively. No lateral roots were formed and the visually undetermined swellings noted on the segments from the plant grown in the sucrose-based medium were absent. However, callus induction was observed on the segments from this second plant treated with IAA as shown in figure 44.

	mg/1 kinetin			mg/1 IAA
1	10⁻¹	10⁻²	0	1

1	10^{-1}	10^{-2}	0	1
s	bsss	rr	s	rrrs
s	bb	br	s	rrr
s	b	s	b	rr
bs	bb	bb	ss	rrs
ss	b	s	br	rr
r	bbs	b	b	rrr
s	b	b	b	ss
ss	bss	b	r	r
bs	s	r	r	rrr
ss	b	b		rrrr
	bs	bs		rrr
	b	bs		rrr
	ss			rrs
	b			rr
	bs			rrss
	b			rrrrr
	ss			rrr
	b			rrs
				rrrs
				rrr
				rr
				rss
				rrr
2 b	18 b	9 b	4 b	0 b
12 s	13 s	4 s	4 s	12 s
1 r	0 r	4 r	3 r	57 r

Fig. 43. Buds (b), roots (r), and unidentified swellings (s), emerged from 1-cm root segments, subcultured for 7 weeks, at 22 C, in the light, in basal media, supplemented with kinetin and IAA, at various concentrations. The original plant had been grown for 83 days on basal media.
———————— = a root segment
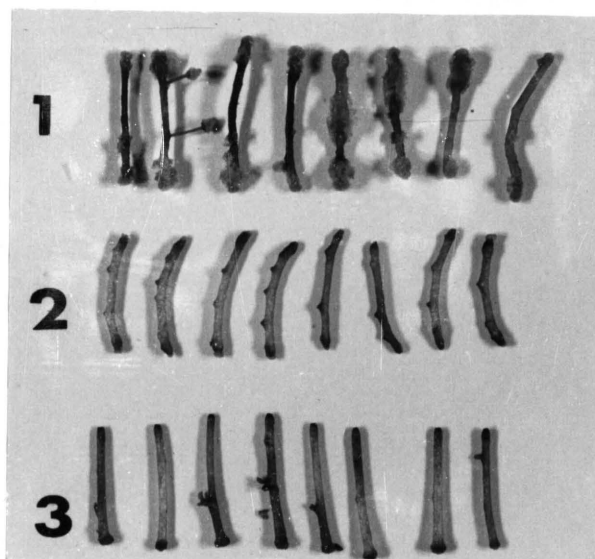
Fig. 44. Organ emergence on isolated, 1-cm, root segments of leafy
spurge. (1) segments from a seedling plant which had been grown
for 83 days in a sucrose basal medium supplemented with 50 mg/l
adenine sulfate, the segments then placed in media containing
1 mg/l IAA; (2) segments from a seedling plant which had
been grown for 83 days in a sucrose basal medium not
containing adenine sulfate, the segments then
placed in media containing 1 mg/l IAA;
(3) segments from the plant as in (2)
but containing no IAA. Subculture
of all segments was for 7 weeks
in the light at 22 C

During the course of this study, microscopic examinations were

made on both intact seedlings and on isolated root segments. These

examinations were made in an effort to determine whether bud or root

primordia were the cause of the so-called "unidentified swellings."

All primordia observed were endogenous in origin, arising deep within

the pericycle, and usually found opposite protoxylem poles. In some

cases, what appeared to be leaf primordia were observed prior to organ

emergence. Transections of certain other organs revealed a central core of vascular tissue, plainly differentiated prior to emergence. Examples of these differentiated organs are shown in figures 45 and 46. Due to the fact, however, that some patches of meristematic cells could not be identified positively as root or bud, the original notation of undetermined swellings, made under examination with a dissecting microscope, was retained. Buds on root segments, like those on hypocotyl segments, were formed under several types of treatments but failed to develop normally. What appeared superficially to be scale leaves with axillary buds were, in reality, adventitious buds with extended first leaves. An apparently dormant bud with extended leaves is shown in figure 47. Quite frequently, leaves of the young bud exhibited distinct folds on the outer epidermal layer, as shown in figure 48.
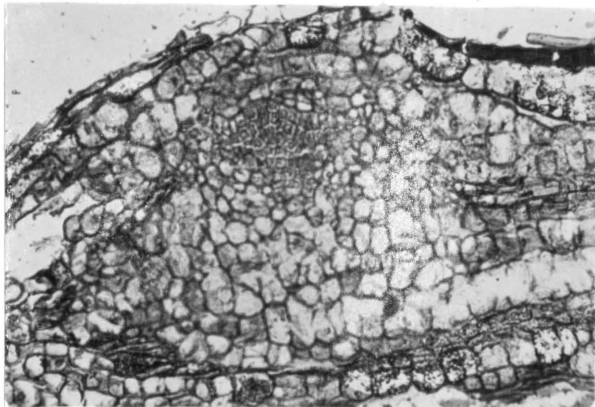
Fig. 45. Longitudinal section
of leafy spurge root segment,
cultured in vitro, showing
vegetative bud with
beginning leaf
primordia.
(125X)
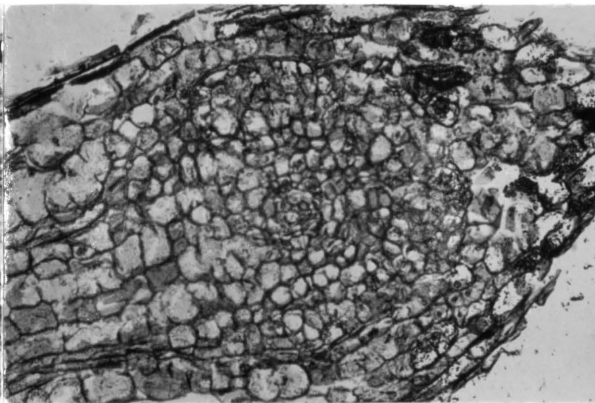
Fig. 46. Longitudinal section of
root segment with transverse sec-
tion through a lateral root,
showing a central core of
procambium formed prior
to organ emergence from
the main root axis.
(125X)
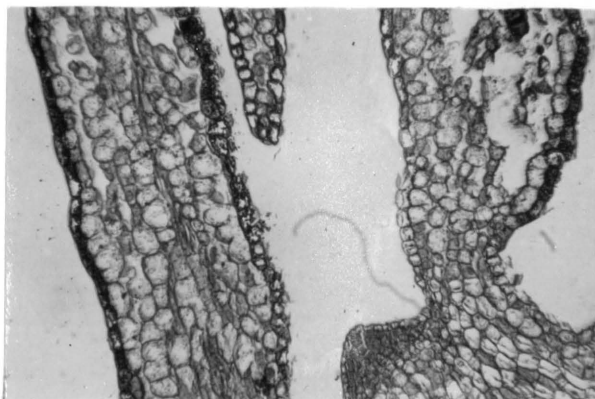
Fig. 47. Transverse section of
a vegetative bud from isolated
root segment of leafy spurge,
showing dormant apex, but
extended first leaves.
(80X)
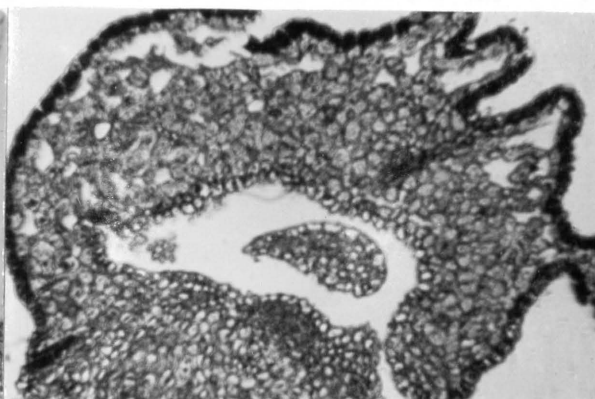
Fig. 48. Transverse section through
leafy spurge root segment with
vegetative bud, showing dis-
tinct folds on the outer
epidermal layer of the
extended first leaf.
(80X)

Root and hypocotyl segments, placed in nutrient media supplemented with 10% coconut milk, produced callus at the cut ends within 2 weeks. Callus continued to form until entire segments were covered. These effects are shown in figure 49. Upon microscopic examination of coconut milk-treated root segments, numerous patches of meristematic cells were observed on what appeared to be the main root axis underlying the callus. External callus and meristematic clusters are shown in figure 50.
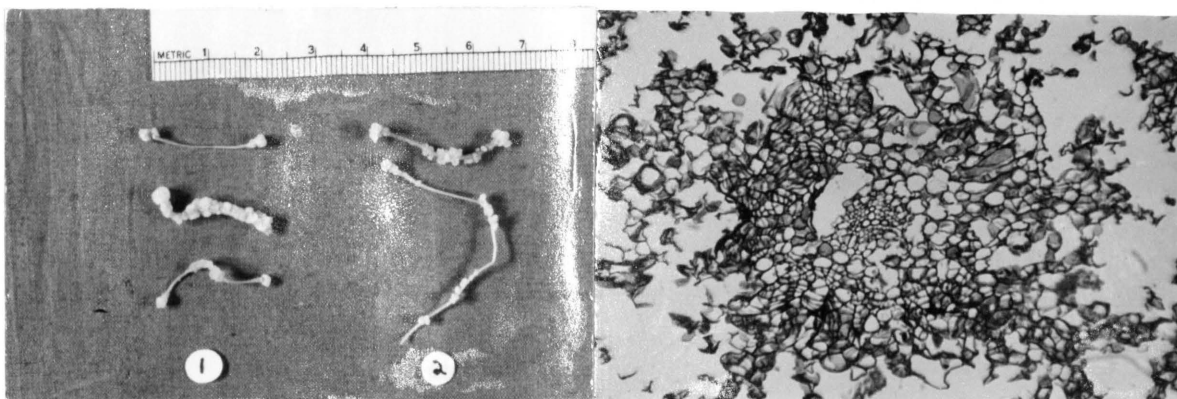
Fig. 49. Callus formed on root and hypocotyl segments of leafy spurge, when placed in media supplemented with 10% coconut milk, in the dark. (1) root segments, (2) hypocotyl segments

Fig. 50. Cross section through leafy spurge root segment cultured in media containing 10% coconut milk, showing external callus and numerous meristematic clusters. (80X)

Intact seedlings of leafy spurge grown in culture exhibited the same marked apical dominance as was observed on field or greenhouse plants; however, removal of the growing tip resulted in axillary bud development only if the root of the seedling plant were left intact. In addition, epicotyls failed to develop when separated from the seedling. The same situation was found concerning adventive buds on roots. Buds which emerged from growing isolated roots developed slowly at first (1 to 2 weeks) and then more rapidly, until extension of shoots reached the rate of about 1 cm per day as shown in figure 51. Removal of the root tip on similar plants during the course of the shoot's elongation, however, arrested their development. The bud shown in figure 51 was visible 17 days after placing the isolated 10-mm root tip section into the basal medium. In 33 days, the shoot had extended to 2 cm. In 93 days the shoot was 26.5 cm in length, in contrast to the 1- to 2-mm buds on isolated root segments shown in figure 44.
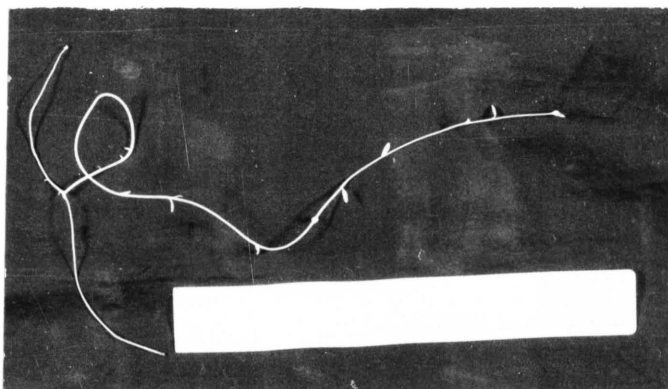
Fig. 51. Active shoot from an isolated root of leafy spurge, grown in vitro at 20 C, in the dark, for 93 days

Preformed buds, on isolated root segments, failed to elongate in 10, 5, 1, or 10^{-2} mg per liter GA, in the dark or light at 20 C or at room temperature in the light. Neither did buds develop when subjected to alternating temperatures. A few buds elongated slightly in some treatments after 2 to 3 months, but consistent results were lacking and no data are presented.

DISCUSSION

Germination and Seedling Growth

Mottled seeds of leafy spurge were found to have the most rapid
and highest germination percentage, followed by grey and purple seeds.
These data agree with those of Selleck et al. (53). Wicks and
Derscheid (67) have shown that mottled seeds are most mature but ob-
served that grey seeds germinated more completely. The difference was
not great and, in one case, Wicks (66) found that germination of mot-
tled seeds was slightly higher than grey. The durations of the ger-
mination tests conducted by Wicks and Derscheid (67) were 29 and 33
days compared to the 12-, 18-, or 20-day tests presented in this paper.
Increasing the number of days of the tests, under continued alterna-
tion of temperature, would probably have increased the germination
percentage of the grey seeds to approximately that of the mottled.
Light decreased both the percentage and rapidity of germination of
mottled seeds. This is in agreement with Selleck et al. (53). Other
seeds were not tested in this manner.

It was observed that increasing the number of temperature al-
ternations increased the germination percentage of all classes of
seeds. It was clear that certain seeds were induced to germinate at
only one alternation of temperature, others required two alternations,
and others required still more. As seeds increased in maturity,
(purple, grey, and mottled), fewer alternations were required for maxi-
mum germination. Mottled seeds approached maximum germination with
only two alternations. The requirement for thermoperiodicity did not

disappear after removal of the seed coat, as suggested by Koller
et al. (35). Germination was in fact lower after removal of the seed
coat, probably due to internal damage or increased activity of micro-
organisms. Lower germination after seed-coat removal was also ob-
served by Selleck et al. (53). Hanson and Rudd (26), Brown and Porter
(6), and Selleck et al. (53) found that leafy spurge seeds germinated
better at 30 C than at 20 C. The reverse of this was found in these
studies, although germination was low in both cases. However, when
seeds were exposed to 14 days of constant 30 C, followed by constant
20 C, germination increased to that provided by continued alternations
of temperature. It appears, then, that some changes within the seed
were brought about by the higher temperature which were necessary for
germination at the lower temperature. These data support at least one
of the theories proposed by Koller et al. (35) that there is a pos-
sible creation of a balance of the intermediate materials of respira-
tion during the high temperature part of the cycle, which would induce
germination during the lower temperature cycles. From the data at
hand, another explanation might be that the alternation of temperature
or the prolonged period of 30 C might result in the destruction of an
inhibitor.

While data are limiting, indications are that both GA and H_2O_2
slightly increased germination of the purple (least mature) seeds.
If, upon further tests, this were proved to be the case, it might be
explained on the basis that GA releases the dormancy induced by cer-
tain blastocholines. For example, coumarin is listed by Audus (2) as

a blastocholine, and Koller et al. (35) state that GA has been known
to break the dormancy induced by coumarin. Also, H_2O_2 might oxidize
the germination inhibitors, as proposed by Ching (7). These sugges-
tions would appear to have some merit when it is recalled that the
least mature seeds of leafy spurge (purple) contained the most potent
growth inhibitors. The possibility is not overlooked, however, that
the concentration of the inhibitor might remain constant but that
seeds of increasing maturity contain increasing amounts of an anti-
inhibitor or growth promoting substance similar to those found by
Wareing and Villiers (65), Purves et al. (48), and Ricard and
Nitsch (50).

Seed coats are known to contain germination and growth inhibi-
tors but, as mentioned, removal of the seed coat of leafy spurge seeds
did not improve germination or subsequent growth of the seedlings. In-
hibitors might still reside in the endosperm, cotyledons, or embryo.
Despite the seat of the natural inhibitor(s), it seems likely that it
(they) may contribute to poor seedling development, as has been shown
with alfalfa and clover (64). A suggestive idea is that the inhibi-
tor(s) may be responsible, at least in part, for the high mortality
rate of field seedlings of leafy spurge.

Kinetin and its hydrolysis product, adenine, appear to play no
role in the germination of leafy spurge seeds, although the following
discussion emphasizes their importance in the subsequent growth and
development of the seedlings.

Root growth decreased when adenine sulfate was added with the already inhibitory kinetin. Synergistic inhibition also resulted when kinetin and IAA were added together. The inhibition caused by the purine antagonist, 8-azaguanine, was partially removed by adenine sulfate. Concentrations of adenine sulfate greater than 500 mg per liter or kinetin greater than 10^{-1} mg per liter were required to appreciably prevent seedling growth. When these two substances were incorporated with sucrose or glucose (in vitro experiments), adenine at 100 mg per liter or kinetin at 10^{-4} mg per liter was sufficient to prevent growth. An analogous situation, involving another type of growth substance, is seen in the work of Purves and Galston (47). The inhibition of pea epicotyl sections caused by relatively high rates of IAA (10^{-4} mg per liter) was only apparent in the presence of the carbohydrate sucrose.

As shown by analysis (table II), isolated root growth was significantly reduced by glucose-adenine sulfate (20 mg per liter) but significantly increased by sucrose-adenine sulfate (20 mg per liter) when compared to root growth in these carbohydrates alone. However, hypocotyl and epicotyl growth was severely restricted in glucose as compared to sucrose. While glucose and adenine were not tested together on these tissues, it may still be implied that adenine is not totally responsible for all reduction in growth in glucose media. That there is a differential toxicity of certain sugars has been presented in the review of literature (24).

The most striking results obtained from the isolated root tip experiments were those showing maximum growth due to alternating temperatures. The question arises, as in the case of seed germination, whether this response was due to the breakdown of an inhibitor or the formation of growth promoting substances, or possibly even both. It would prove interesting to see if a prolonged period of 30 C, subsequently followed by constant 20 C, would allow the same amount of growth as did alternating temperatures. A similar situation is found in Musa balbisinia Colla in that seeds are induced to germinate in 5 to 6 days of alternating temperatures, but additional temperature changes are required for normal seedling development (57).

Adenine has been shown to play a definite role in the initiation and growth of lateral roots upon epicotyl and root sections of Pisum (21, 63). Galston and Hand (21) suggest that adenine fulfills the requirement of rhizocaline. Torrey (63) has described its interactions with kinetin and IAA in lateral root initiation. An increase in the number of lateral roots was observed on seedlings of leafy spurge when grown in adenine sulfate-supplemented media. Lateral root induction, after root tip removal, also supports the theory presented by Torrey. Explanations as to why adenine sulfate provided a significant increase in isolated root tip growth must, however, be purely conjectural at this time. A suggestive idea is that an inhibitor (possibly a kinin) is present in the root tip and antagonized by the natural metabolite, adenine. It would also appear that adenine or at least its precursors may be derived from the non-root portion of the

seedling. Torrey (63) has shown that cambium activity requires some factor or precursors derived from cotyledons and cites that adenine and mixtures of amino acids promoted growth of decotyledonized pea seedlings. Support of these ideas is seen by the fact that 2,6-diaminopurine (an adenine antagonist) effectively removed the stimulation of root growth due to adenine. Additional experimentation is desirable, however, since the antagonist was not tested alone. The increase in root length brought about by the alternation of temperatures is accommodated by the idea that the level of inhibitor is reduced and the relative concentration of effective, endogenous adenine is increased and, therefore, no external adenine is required. These substances must be, however, only a fraction of those involved in the multifactor process of growth.

Knowles and Zalik (34) found that removal of the cotyledons of Viburnum trilobum Marsh. resulted in the breaking of epicotyl dormancy. The exact opposite of this response was observed with leafy spurge; however, a difference existed as to procedure. Knowles and Zalik tested seedlings grown in vermiculite with no mineral nutrients or carbohydrate source added. For leafy spurge, the experiments were conducted in culture. Experiments by Brown and Gifford (5), on pine embryos, demonstrated that sucrose, when supplied through the cotyledons, exerted a much greater beneficial effect on the rate and duration of root growth than when supplied to the embryo directly by way of the root. The suggestion is offered that this might explain why seedlings of leafy spurge did not develop when placed on agar media,

since the cotyledons were not in contact with the nutritive substances.

It is suspected that the high or low shoot/root ratios exhibited by seedling plants of leafy spurge is due to the imbalance of natural metabolites and their antagonists. In addition, it seems likely that this physiological variability is expressed by the seedling but that the naturally occurring growth substances affected were present during and retained after germination. The release of growth inhibition was somehow triggered by the alternation of temperature.

The experiments discussed above are vulnerable to the criticism that (1) the number of replications was inadequate, and (2) the variability within some treatments was so great as to invalidate any attempted interpretations. It is felt, however, that despite these objections, the experiments have served their purpose, that of acquiring a technique and establishing a procedure for the culture of leafy spurge in vitro. It is also felt that some general conclusions must be drawn in order to provide some working hypotheses for further tests. Direct proof must, in many cases, await additional experimentation and refinement of technique.

Bud Formation and Development

Chemical Factors

In addition to undifferentiated callus formation, many definite patches of meristematic cells were observed in coconut milk-treated root segments. Neither the callus nor meristems seem out of the ordinary in light of the many works concerned with the growth regulatory effects of this liquid endosperm as cited by Audus (2) and Miller (40).

Maximum bud formation on hypocotyl segments of leafy spurge was obtained with 10^{-2} mg per liter kinetin, in the light. Fewer buds were obtained on segments treated with 1 and 0 mg per liter kinetin. IAA removed the effect of bud formation at the above rates of kinetin (figures 38, 39, and 40). Wickson and Thimann (69) have shown that the concentration of applied, radioactive IAA in excised pea stem sections was reduced by exposure to light, simultaneous treatment with kinetin, and by decapitation of the plants some days before the sections were cut. These same conditions also favored bud growth. On leafy spurge hypocotyl segments, the first two conditions stimulated bud formation. The third condition was not established.

Callus formation on these hypocotyl segments also seems to demonstrate the interaction of purine and auxin. Kinetin, at 5 mg per liter, induced patches of undifferentiated meristem. IAA at 10^{-2} mg per liter induced heavy callus at both ends of the segments, but callus was restricted to the basipetal ends when treated with 10^{-4} and 0 mg per liter. At the latter two rates of IAA, the amount of end callus decreased as the concentration of kinetin increased. Skoog and Miller (56) and Miller (40) have described the same phenomenon; namely, that untreated tobacco stem segments produced considerable callus at the basipetal end with this effect being removed by 0.86 mg per liter kinetin. The large amount of basipetal end callus in the absence of added IAA or high rates of kinetin is felt to be due to the polar distribution of endogenous auxin. That this polar movement actually occurs in isolated stem segments of tobacco and pea and in bean

hypocotyl segments has been well demonstrated by Keitt and Skoog (30) and Wickson and Thimann (69).

The effect of kinetin and IAA on organ formation in isolated root segments was studied, but interactions between these substances were not tested. The obvious difference between these tests and those on hypocotyl segments is that 10^{-1} mg per liter kinetin stimulated maximum bud formation on root segments (figure 43), and 10^{-2} mg per liter stimulated maximum bud formation on hypocotyl segments. It would appear that the concentration of natural purine is lower in the root than in the hypocotyl. This conclusion may be substantiated by the fact that stem and hypocotyl have apparent photosynthetic mechanisms whereas the root does not and the postulation that kinin is synthesized in stem tissue in the light (68). Galston and Warburg (20) have also postulated the formation of a "third factor" in green tissue, which would appear to satisfy the requirement of kinin. It is recalled at this point that very few buds were formed on hypocotyl segments in the dark. It should also be mentioned, however, that light did accelerate bud formation on root segments of Convolvulus (60).

IAA is known to cause the formation of lateral roots at concentrations well beyond those thought to be physiologically active. This response was definitely shown (figures 43 and 44) on isolated root segments taken from a clone of leafy spurge. It is interesting to observe that in all cases the lateral root primordia were formed on the convex side of the root segment. The root of the parent plant had

coiled around the inside of the culture flask and the root segments retained this slight bend after they were cut and during their sub-culture. This would appear to substantiate the existence of "a transverse polar gradient in a root forming hormone" as discussed by Sinnott (55). Basipetal end callus was apparent on untreated root segments, but callus formation was slight or non-existent on the root segments treated with 1 mg per liter IAA. The latter case is in direct contrast to the large amount of callus induced by 1 mg per liter IAA on hypocotyl segments. Of interest here is the fact that IAA-treated root segments, (from a plant grown on media supplemented with 50 mg per liter adenine sulfate), produced much callus but no lateral roots. It seems logical to assume that the level of purine necessary for callus formation, due to high IAA level, was limiting on root segments from plants not grown on adenine sulfate-supplemented media, but optimal for lateral root formation. However, in root segments from adenine sulfate-grown plants, the purine level was supraoptimal for lateral root formation. Rapid incorporation of adenine into onion root tip cells has been shown by Jensen (29). It is assumed that this metabolite, adenine, was also readily incorporated in leafy spurge roots, especially in light of the typical, purine response observed in the root segments.

Buds that had emerged from isolated 1-cm segments of root or hypocotyl failed to elongate. GA or alternating temperatures did not serve to release the dormancy of the buds. Buds formed on isolated roots (tips included) or buds on hypocotyl or root of shoot-decapitated seedlings did produce excellent growth in culture.

Apparently, some factor(s) or at least precursor(s) is(are) essential for active bud growth.

The anatomical studies conducted on leafy spurge root segments, cultured _in vitro_, have shown that both buds and roots are endogenous and have their origin in pericycle, usually opposite protoxylem poles. These studies have also shown that primordia may quite often be distinguished as roots or shoots prior to their emergence through the epidermis. Since, however, some few primordia could not be identified, and both root and shoot primordia were found, all visible swellings on the segments could not be counted as either buds or roots. It appears significant that more of the "unidentified swellings" were noted at the concentration of kinetin which induced maximum bud formation and that many of these swellings appeared as buds when examined microscopically.

Although buds or roots may sometimes be identified prior to their emergence from the parent organ, these studies do not preclude the possibility of an indifferent meristem as has been suggested (4, 60). If one does exist, the technical question may judiciously be raised as to the use of the words initiation, formation, and emergence as employed in this writing. If an initial is a cell that remains within the meristem indefinitely by combining self-perpetuation with the additions of cells to the plant body (Esau (18)), then the word initiate may very well be used to describe the first division of the initial which eventually leads to the formation of a primordium. Formation, then, is proposed to be the organization of a meristem into either root or shoot. This in fact would be differentiation.

Emergence is, therefore, the first macroscopically visible indication
of developmental growth. Although data were collected by observing
the number of emerged lateral organs, it is felt that the word forma-
tion has been legitimately used. Since, in the hypocotyl tests, the
primordia quite often remained microscopically undifferentiated when
10^{-4} mg per liter IAA and 10^{-2} mg per liter kinetin were used together,
yet buds actually emerged when the IAA was omitted, the effect of
kinetin must be that of formation, as described above. That kinetin
was also instrumental in stimulating the initiation of visible buds
and the unidentifiable swellings may be assumed since kinetin, in com-
bination with IAA, induces mitoses followed by cytokinesis, "leading
to patches of small meristematic cells between undivided cells (13)."
A high kinetin/IAA ratio has been reported to induce bud formation and
a low ratio to induce root formation (23, 56, 58). While this funda-
mental relationship was first observed in tests on tobacco pith and
stem tissue, the literature has shown that it exists in other plants
and tissue, as cited by Miller (40). That buds formed on isolated hy-
pocotyl and root segments not treated with kinetin or IAA are due to
the proper endogenous concentrations of natural kinin and IAA may be
substantiated by the words of Skoog and Miller (56).

> We do know that both kinins, of as yet unknown chemical
> nature, and auxins are present in leaf and stem tissues. It
> may be logically assumed that they participate in meristematic
> activity, differentiation and organ development in the intact
> plant in the same manner as in tissue cultures.

That initiation, as defined above, had not taken place prior to sub-
jecting the segments of leafy spurge to the various treatments may be

logically assumed since sections taken from intact organs of similar age possessed no meristematic patches or organized primordia when examined microscopically.

Anatomy of Subterranean Parts

Horizontal underground parts. Rhizomes are horizontal underground stems. All underground stem tissue examined in leafy spurge was vertical and a product of adventitious bud development from horizontal, vertical, and oblique roots.

Transition zone. Connection between primary vascular systems of the shoot and root involves spatial adjustments between systems of differently oriented parts and different directions of differentiation in the horizontal plane. This necessarily shows some features, intermediate or transitional, between shoot and root. The region of the seedling where structural details change between root and shoot systems is called the transition region (18) or transition zone.

Only one transition zone per clone was present in leafy spurge. This was found in the collet (lower hypocotylary swelling). Examination of leafy spurge seedlings revealed that transition from tri-exarch radial stele of the root to tetra-endarch collateral stele of the upper collet was completed in approximately 2500 microns. Dichotomizing of 2 of the 4 bundles of the upper collet resulted in 6 bundles entering the cotyledons. The juncture of stem and root at the place where the stem originated as an adventitious bud was a mere extrusion of stem with endarch collateral stele from within the root

with exarch radial stele. As defined above, this cannot be called a transition zone.

In undisturbed mature clones, it was not difficult to distinguish between the original plant axis and the stems that arose as buds from lateral roots. If a transverse cut directly above a crown of buds revealed stem tissue with a prominent pith and an equally prominent pith was also evidenced below the crown, this axis was a stem that had developed from an adventitious bud. If, however, root tissue was found directly below the crown, the axis was that of the original seedling and the crown, therefore, was the collet containing the transition zone. Based on the above method, it is estimated that, in established patches of leafy spurge, less than 10% of the above-ground structures have arisen from the original plant axis.

Origin of primordia. An exogenous lateral primordium originates from primary superficial initials. An endogenous primordium originates from deep-seated tissue, usually pericycle.

All root primordia had endogenous origin from pericycle or pericyclic tissue. Buds on roots were also endogenously formed. In the case of buds on young roots, differentiation was centrifugal from original pericycle layers located at protoxylem poles. In the case of older roots, their origin was from active pericycle (located toward the periphery of the organ). Centripetal differentiation of procambium, however, established their contiguity with xylem and phloem of the main axis via the pericyclic tissue. All buds, on the region of the hypocotyl with endarch protoxylem, were exogenous since the epidermis of bud and main axis was a continuous layer. Centripetal

differentiation of procambium to the vascular tissue of the main axis made the primordia appear quite similar to the buds arising from active pericycle of roots.

Tissue patterns. Diarch xylem patterns were observed in tips of all roots examined. In the seedlings studied, triarch xylem prevailed throughout the remainder of the root. Although a tetrarch condition was never found in the seedlings studied, it doubtless exists since tetrarch patterns were observed just below the crown in many original, mature axes. Feeder roots examined had, in general, triarch xylem at the proximal end, becoming diarch toward the tip. A feeder root was examined with a triarch condition established and a fourth pole less distinct and not continuous with the main vascular core. That no -arch pattern greater than tetrarch was observed does not rule out the possibility of their existence.

Phloem was very poorly developed, possibly because of the supplementary conducting system in leafy spurge, the laticiferous ducts.

Since cortex and epidermis were sloughed off early, pericycle became the outermost tissue protected by cork.

SUMMARY AND CONCLUSIONS

Leafy spurge seeds were sorted into three color classes:
mottled, grey, and purple. Seeds were subjected to various condi-
tions of light and temperature. Alternations of temperature were 12
hours each, 20 C and 30 C. Light (when used) was from an 18-inch
florescent tube placed 1 foot above the seeds.

Uniformly germinated mottled seeds were subjected to various
growth substances and seed extracts. Aseptically germinated mottled
seeds provided seedlings or isolated plant parts for numerous tests
conducted in vitro. Adenine sulfate was tested against the growth of
intact or parts of plants in both sucrose and glucose media. Root
segments from plants grown under sucrose and sucrose-adenine condi-
tions were subjected to treatments with kinetin and IAA. Hypocotyl
segments were subjected to various rates of kinetin and IAA, alone
and in combinations. Anatomical studies were conducted on isolated
root segments and seedling plants in culture and on seedlings and
mature plants grown in the greenhouse and in the field.

The following conclusions are drawn:

1. Rate of germination and germination percentage are highest
for mottled seeds followed, in order, by grey and purple seeds. Light
decreases both the rapidity and percent germination. Mottled seeds
approach maximum germination with only two alternations of temperature,
but seeds of decreasing maturity (grey and purple) require more. The
germination percentage of seeds exposed to 14 days of constant 30 C,
followed by constant 20 C, equals that provided by continuous alter-
nations of temperature. Seed extracts inhibit seedling growth.

Inhibition is less from extracts of more mature seeds than from less mature seeds.

2. Vegetative parts fail to develop normally unless the root tip is left intact. Glucose markedly reduces hypocotyl and epicotyl extension after the root is removed from the seedling, as compared to sucrose. Maximum growth of isolated root tips is obtained when the roots are subjected to the same alternating temperature conditions as are the parent seeds and seedlings from which they are removed. Epicotyl growth is delayed in decotyledonized seedlings.

3. Adenine sulfate at concentrations up to 800 mg per liter does not affect seed germination, but concentrations above 500 mg per liter do retard growth of the seedlings. Adenine sulfate further decreases root growth when added with the already inhibitory kinetin. Adenine sulfate at 20 mg per liter increases the growth of isolated root tips when grown in a sucrose-based media; however, growth is variable. Root segments from a plant grown on sucrose-based media are induced to maximum lateral root initiation by 1 mg per liter IAA. On root segments from a plant grown on sucrose-based media supplemented with adenine sulfate, IAA only induced callus.

4. Kinetin at 10 mg per liter has very little, if any, effect upon germination but seriously inhibits the growth of the seedlings. Synergistic inhibition is seen when IAA and kinetin are added together. Rates of kinetin as low as 10^{-4} mg per liter inhibit root growth in vitro. Maximum stimulation of bud formation on hypocotyl segments occurs at 10^{-2} mg per liter kinetin, and this effect is removed by 10^{-4} mg per liter IAA. The end callus forming on untreated or segments treated with 10^{-4} mg per liter IAA may be reduced by the

addition of kinetin. On root segments, maximum stimulation of bud formation is at 10^{-1} mg per liter kinetin. Stimulation to bud initiation is reduced or non-existent if the plant from which the segments are taken is grown on adenine sulfate-supplemented media.

5. The main axis of a mature plant of leafy spurge is composed of aboveground stem(s), a hypocotyl region varying from a few millimeters to a few centimeters, a transition zone, and the primary root. This vertical, primary root produces lateral roots, feeder roots, and adventitious buds. The lateral roots, and roots of other orders, produce additional lateral roots, feeder roots, and adventitious buds. Adventitious buds may extend forming vertical, underground and aboveground shoots. In established patches of leafy spurge, about 90% of the aboveground shoots arise from adventitious buds on lateral roots. Crowns of buds, at and just below the soil surface, are formed both from vertical stems and from the collet or hypocotyl region of the main axis.

6. The transition zone of leafy spurge is found in the collet (lower hypocotylary swelling). The transition from exarch radial stele of the root to endarch collateral stele of the upper collet is complete in approximately 2500 microns. All root primordia have endogenous origin from pericycle and pericyclic tissues. Buds arise endogenously in roots but exogenously in the region of the hypocotyl with endarch protoxylem. In many cases, primordia can be identified as roots or shoots prior to their emergence from the main axis.

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