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FACTORS IN LEAVES OF *GINKGO BILOBA* L. THAT INFLUENCE FOLIAR ABSCISSION

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FACTORS IN LEAVES OF Ginkgo biloba L. THAT INFLUENCE FOLIAR ABSCISSION

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INTRODUCTION

Auxins, or growth promoting hormones, retard abscission of leaves when occurring naturally at a high level in the leaves, or of leaf petioles when applied experimentally to debladed petiole stumps. There is evidence of a seasonal decrease in auxin concentration directly associated with approaching plant senescence and as a result of shortening photoperiod. This phenomenon has been reported for many deciduous plants by Avery, Burkholder, and Creighton (1937), Wareing and Roberts (1956), Nitsch (1957), Hacskaylo and Goslin (1957a, b), and Kawase (1960). Shoji, Addicott, and Swets (1951) suggested that the important factor in abscission was the elimination of an auxin gradient normally maintained between the blade and the stem across the abscission zone when auxin production decreased in the blade as senescence advanced. Later studies by Guar and Leopold (1955) and Biggs and Leopold (1958) indicate that it is the quantity of auxin in the abscission zone rather than presence of a gradient that regulates abscission; a high concentration preventing, and a low concentration promoting abscission. Rubinstein and Leopold (1963) suggested that there are two separate stages of leaf abscission. One stage, an induction period of about six hours or more which is inhibited by auxin and a second, which is stimulated by auxin.

Sacher (1957) has suggested that auxin maintains normal selective permeability of cell membrances. In the narrow abscission zone where cells may be very sensitive to a change in auxin concentration, a drop in auxin level with senescence could cause a release of pectinases which would result in a disintegration of the middle lamella.

More recently a new group of naturally occurring factors which accelerate abscission of fruits and leaves has been reported. Addicott, Lyon, and Smith (1962) suggested that these factors represent a new class of plant hormones. Osborne (1955) obtained by diffusion into agar from senescent leaf pulvinoids of kidney beans and several species of trees, a factor which accelerated abscission of debladed petioles of bean plants, whether applied proximally or distally to the abscission zone. Similar factors were extracted from whole bean leaves by Biggs

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(1957) and by Rubinstein and Leopold (1962); from *Coleus* leaves by Jacobs, Shield, and Osborne (1962); and from cotton leaves by Hall, Herrero, and Katterman (1961). Several of these factors tested in the standard coleoptile bioassays counteracted the growth acceleration of the common auxin, indoleacetic acid. Osborne (1955) suggested that abscission of leaves could be controlled, not only by a change in the auxin balance, but also by a seasonal increase in this unidentified abscission factor.

Most progress in the study of abscission promotional factors has been made with cotton bolls from which two abscission factors have been isolated and purified. Carns, Hacskaylo and Embry (1955) found that a diffusate from the base of fresh cotton bolls inhibited growth response to indoleacetic acid in the standard *Avena* curvature test. The activity of the extract increased as bolls neared the time of abscission and decreased during the period of boll set. An abscission promoter, called abscisin I, was later extracted in petroleum ether from cotton burrs of mature fruit by Liu and Carns (1961). Another factor, named abscin II, was extracted from ovulary walls of young fruits by Ohkuma, *et al.* (unpublished). This purified substance in high concentrations inhibited growth of *Avena* coleoptiles in the presence of indoleacetic acid, but low concentrations stimulated growth above that of the controls. It is suggested that this activity could result from an interaction with an auxin. The two abscisins differ in chemical properties, but both of them accelerate abscission of debladed petioles of cotton explants when applied at 10^{-2} micro-grams per abscission zone.

Research is being conducted at the Ohio Agricultural Experiment Station, Wooster, Ohio, to isolate growth factors which influence foliar abscission that have been found in senescent leaves of *Ginkgo biloba* L. This species was chosen for study, because chlorosis develops rapidly and uniformly over the entire tree and subsequent abscission is rapid and complete. It is assumed that these distinct changes are accompanied by a rapid change in some growth factor or factors.

MATERIALS AND METHODS

Ginkgo leaves were collected from a 30 year old tree at weekly intervals from mid-August to late October. Leaves were selected from mid-portion of the crown at a middle position on the branches. Immediately following each collection, the leaves were placed in a polyethylene bag and stored in a deep freeze.

Sixty grams of frozen leaves, equivalent to 15 grams dry weight, were macerated in a Waring blendor with 50 ml. of petroleum ether. The plant material was divided evenly into three Soxhlet extracting

thimbles; each thimble was placed in a separate Soxhlet apparatus containing 150 ml. of petroleum ether. The solvent was refluxed through the plant material for a period of eight hours.

The petroleum ether extracts were extracted with 600 ml. of a solution of methanol and water (1:4-v/v). These fractions were separated and each was evaporated to a 15 ml. volume over a steam bath. Each ml. in this instance represented 1 gram dry weight of plant material.

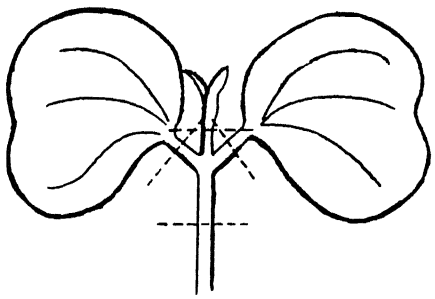
A series of three separate Soxhlet extractions with petroleum ether were performed on one sample of frozen plant material. The extracts were concentrated so that 1 ml. equalled 1 gram dry weight of the plant material. Two groups of soybean plants (ages 45 and 90 days) were sprayed with these extracts to test the efficiency of the extraction procedure and the effects of the extracts on plants of different ages.

A cotton explant bioassay was conducted to test small quantities of extracts or fractions of extracts for abscission promoting activity (Ohkuma, *et al.*, unpublished). Cotton seeds, *Acala* 4-42, were germinated in sand flats and grown for 14 days under a fifteen hour photoperiod. Uniform plants were selected and the apical meristem and leaf blades were removed, leaving five mm. long petiole stumps of the cotyledons (Fig. 1). The explants were immersed for 10 minutes in a 0.006 percent Roccal solution to kill bacteria, drained on absorbent paper, and transferred with sterile forceps to a stainless steel holder in a sterile petri dish. A layer of 1.5 percent bacterial agar in the bottom of each dish provided support and moisture for the explants.

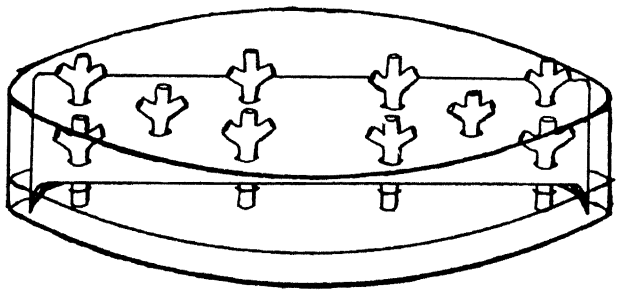
A frozen leaf sample of *Ginkgo* was oven dried at 80°F. for twenty-four hours and ground in a Wiley mill to a twenty mesh screen. Five gram dry weight samples were placed in Soxhlet thimbles for extraction as previously described The extract was concentrated to 15 ml.

One ml. of the petroleum ether extract was taken to dryness and 2 ml. of 2 percent agar was added. Five micro-liters of the mixture was applied to both petiole stumps of ten explants with a hypodermic syringe. The explants were kept in darkness at a constant temperature of 30°C. Abscission was tested daily by applying 5 grams of weight to the end of each petiole stump with an instrument made from wire and a bacteriological needle (Fig 1) in order to stimulate field conditions where the weight of the leaf blade applies mechanical pressure to the abscission zone. These procedures were performed under ultraviolet light.

The leaf blades of the cotyledons and apical meristem were removed from cotton plants kept in flats under greenhouse conditions. Abscission



FOURTEEN DAY COTTON SEEDLING



EXPLANTS IN PETRI DISH

TESTING ABSCISSION

Fig. 1.—Procedure for cotton explant bioassay.

was tested as described above with both the petroleum ether and alcohol-water phases of the extracts of frozen *Ginkgo* leaves.

A petroleum ether extract of oven dried *Ginkgo* leaves was applied with a brush to a terminal leaf of a black locust seedling, *Robinia pseudo-acacia*. The response of the seedling to the extract was observed during a four-week period.

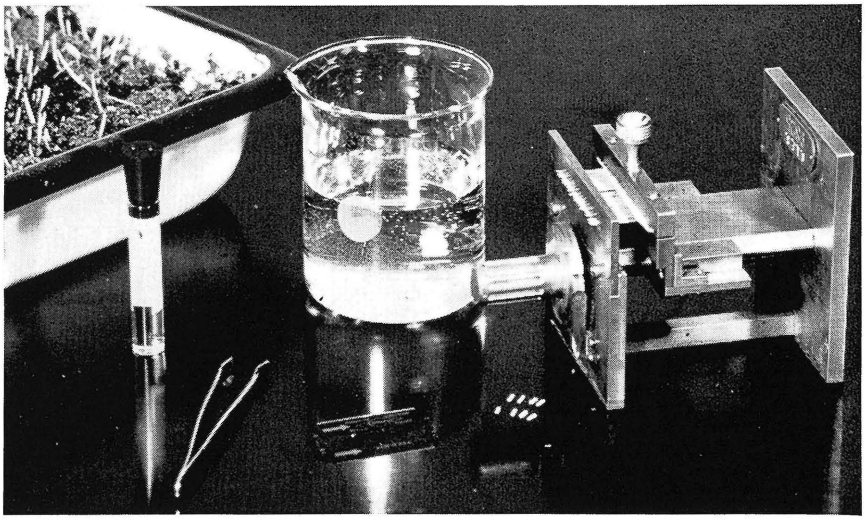


Fig. 2.—Germinating wheat seeds, shell vial, and Van de Weij coleoptile microtome for the wheat coleoptile straight-growth bioassay.

Petroleum ether and methanol-water fractions of the petroleum ether extracts of frozen *Ginkgo* leaves were separated by ascending paper chromatography. Each solution was applied in narrow bands to 1.5 inch wide strips of Whatman No. 1 chromatographic paper in aliquots of 1/20 ml., 1/2 ml. and 1 ml. The chromatograms were developed for 12 hours, using a solvent mixture of pentanol, butanol, ethanol, and water, 1:1:2.5:4 (v/v); then dried, examined for fluorescing bands, and cut into pieces for bioassay (Hacskaylo and Carns, 1955).

A straight-growth bioassay of wheat coleoptile sections was used to test the growth activity of the chromatograms (Hendershott, 1959). Approximately 1000 wheat seeds of the Atlas 66 variety were soaked for two hours in tap water prior to sowing and germinated in wet sawdust, under darkness, at room temperature, and at a high humidity. After three days the etiolated coleoptiles were broken off at the base and placed in a Van de Weij coleoptile microtome for sectioning (Fig. 2). Two mm. of the tips were discarded to remove the influence of naturally occurring auxins. Then 4 mm. sections were cut and placed in distilled water for a period of 3 hrs. Four coleoptile sections were placed in each shell vial (12 x 60 mm) wih one ml. of phosphate-citrate buffer solution, adjusted to a pH of 5.0, and a section of the chromatogram to be tested. These vials were stoppered and attached to a bicycle wheel which rotated slowly to reduce curvature of the coleoptiles caused

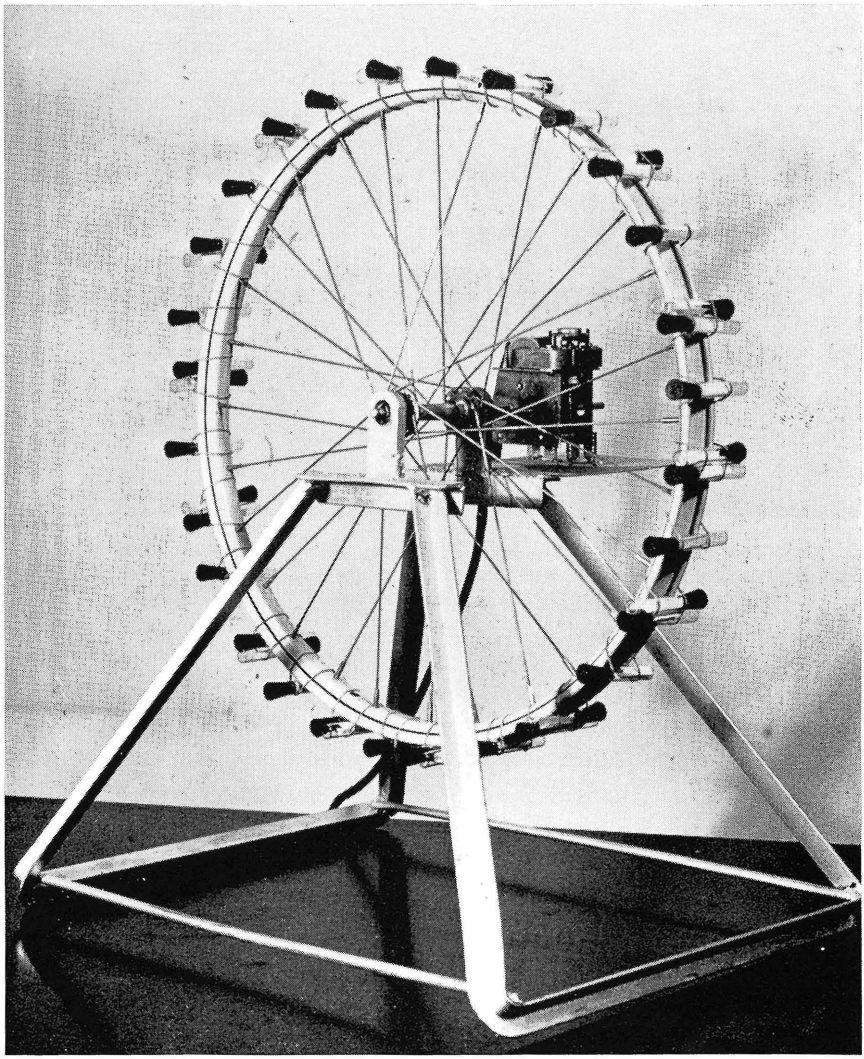


Fig. 3.—Shell vials containing wheat coleoptiles rotated to reduce the influence of gravity.

by gravity (Fig. 3). Twenty-four hours later, the coleoptile sections were measured under a calibrated binocular microscope to observe growth responses of the sections.

A series of Soxhlet extractions with petroleum ether were made from frozen *Ginkgo* leaves that were collected August 14, October 2,

and October 22, 1963. Paper chromatograms of petroleum ether and alcohol-water phases were bioassayed using a straight-growth test to determine any seasonal changes in naturally occurring growth factors.

RESULTS

Nearly all of the petroleum ether extractable growth factor or factors were removed from fresh *Ginkgo* leaf tissue in the first 8 hour period of extraction. There was a marked decrease of activity in the second period and relatively none in the last period. This was indicated by the response of 45- and 90-day soybean plants. Ninety percent of the leaves of the 90-day old plants sprayed with the extract of the first 8 hour period, abscised within 5 days (Fig. 4). Plants sprayed with the extract of the 2nd and 3rd period lost only one or two of the old, lower leaves. Forty-five day old plants lost 10 percent of the lower leaves after being sprayed with the extract of the first 8-hour period, and lost no leaves after being sprayed with the 2nd and 3rd 8-hour period extracts. Prior to abscission, leaves became chlorotic; abscission occurred at the base of the plant and progressed upward. The top two or three leaves did not abscise. Plants sprayed with pure petroleum ether were not affected.

When soybean plants in the initial flowering stages were sprayed with the petroleum ether extract, abscission occurred only in the lower, older leaves. There was no apparent effect upon the upper portions of the plant.

A petroleum ether extract of oven dried leaves applied to cotton explants promoted petiole abscission (Fig. 5). When the extract was separated and applied to debladed petioles of greenhouse cotton plants, the methanol and water phase promoted abscission of the petioles, while the petroleum ether phase did not.

A petroleum ether extract of oven dried senescent *Ginkgo* leaves was applied to a leaflet of a terminal leaf of a black-locust plant. The extract caused wilting of leaves within two days (Fig. 6.). Within one week, a few leaves had abscised; within three weeks, 90 percent of the leaves had abscised; and within four weeks, the plant died. Abscission began in the lower leaves. No abscission occurred in control plants treated with petroleum ether.

When the methanol-water phase of petroleum ether extracts of frozen *Ginkgo* leaves was chromatogrammed, 5 areas of fluorescence were observed under ultra violet light. The areas and colors of fluorescence were: R.f. 0.05, blue; R.f. 0.15, yellow; R.f. 0.25, violet; R.f. 0.60, blue; and R.f. 0.80, blue.

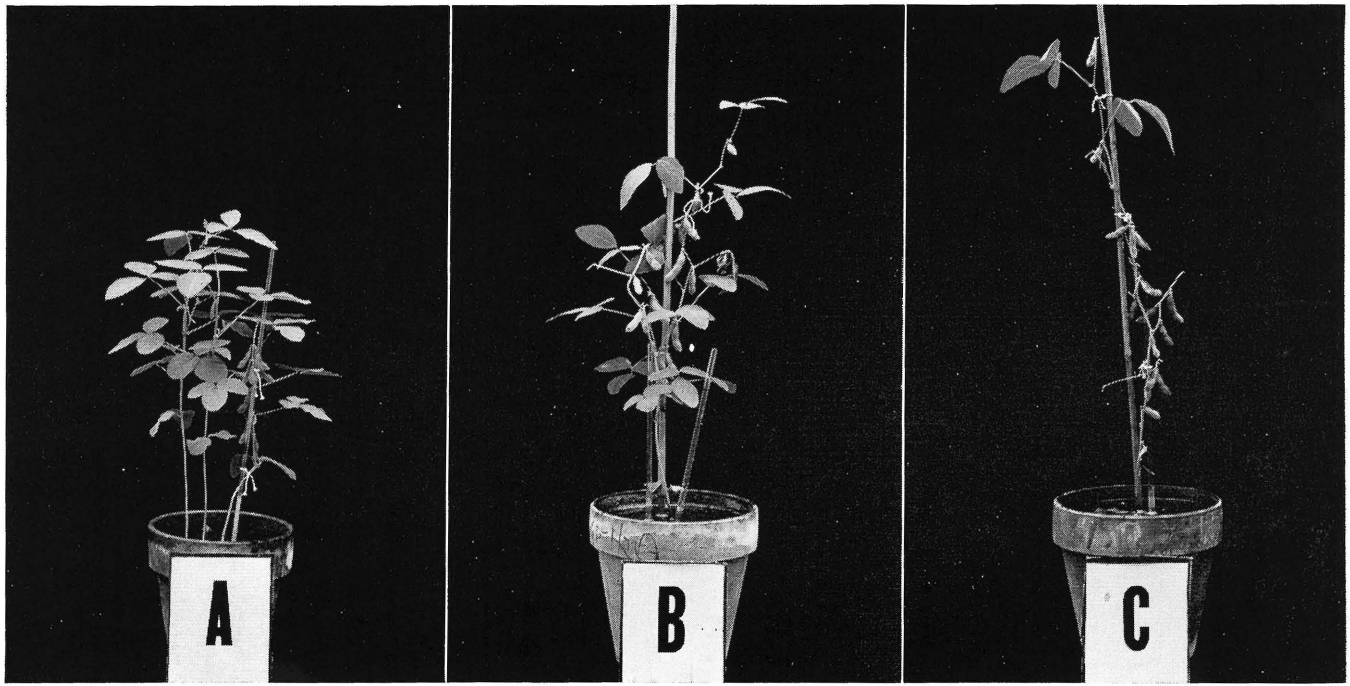


Fig. 4.—Appearance of soybean plants three months old, ten days after the application of a petroleum ether extract of senescent Ginkgo **leaves. A – control unsprayed; B – control sprayed with petroleum ether; C – sprayed with petroleum ether extract of** Ginkgo **leaves.**

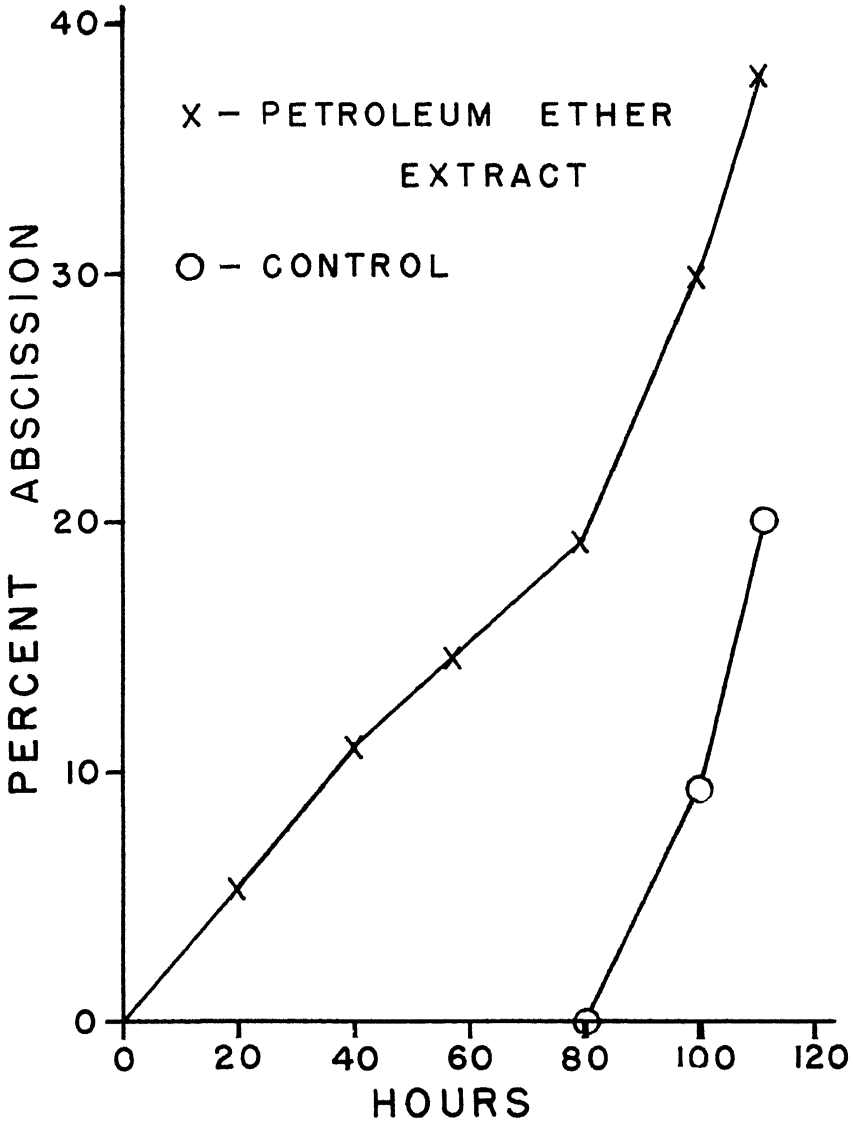
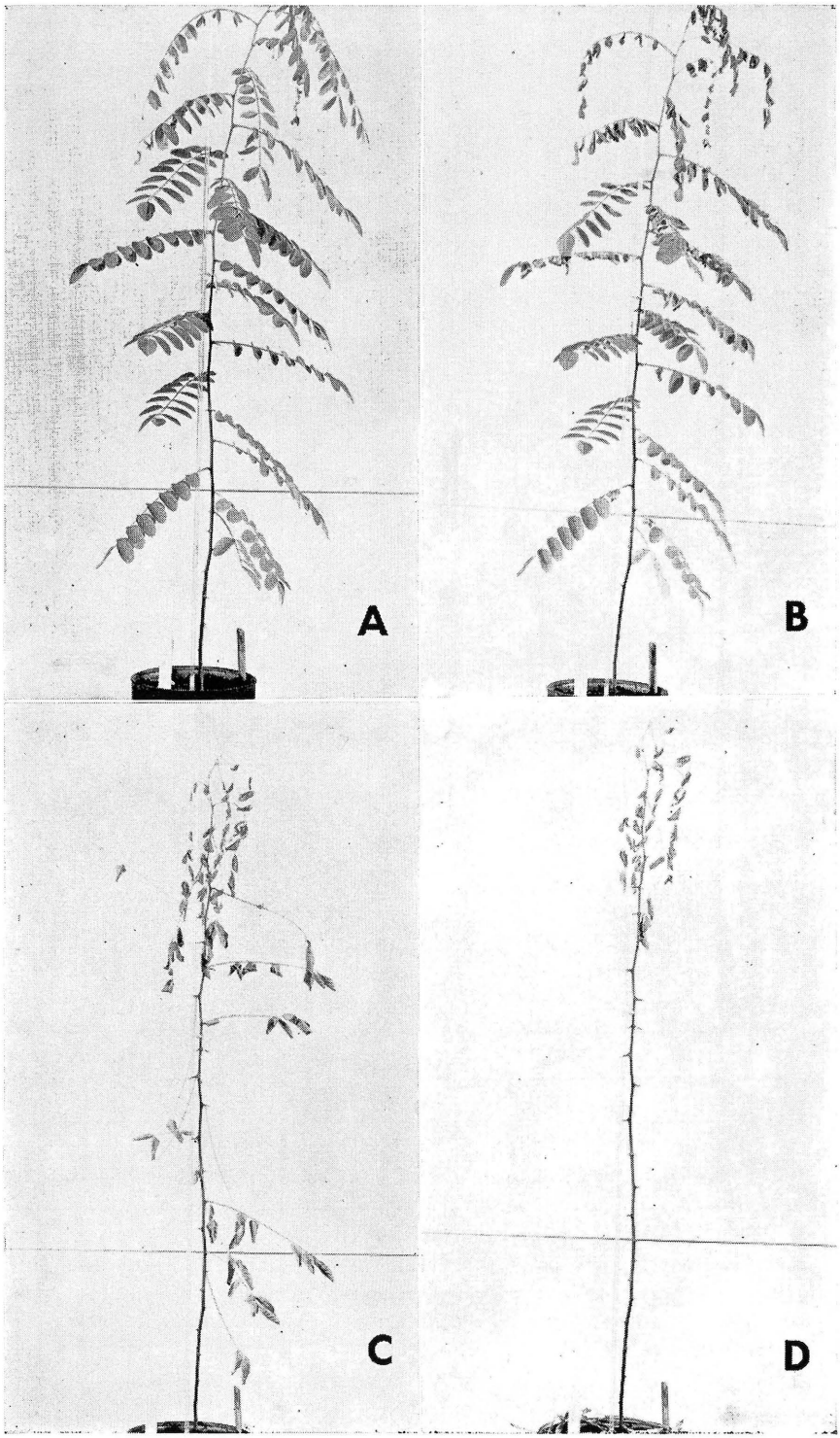


Fig. 5.—Rate of abscission of cotton explants treated with petroleum ether extracts.


A
B
C
D

Areas of growth promoting activity were observed at R.f. values of 0.10, 0.25, 0.60 and 0.75 on chromatograms when bioassayed with the straight-growth of wheat coleoptile sections. A significant decrease in the growth promoting activity was observed at R.f. 0.10 and 0.25 which correlated directly with the age of the leaves, collected on August 14, October 2 and October 22 (Table I and Fig. 7 and 8). As the age of leaves increased, growth-promoting activity of the extract decreased. It appears that there are at least two growth factors present in *Ginkgo* leaves which influence the elongation of wheat coleoptiles, and decrease in activity as the season progresses in late summer and early fall. It is interesting to note that during the same period of time, leaves of *Ginkgo* trees become chlorotic and abscise abruptly.

TABLE 1.—Average elongation in mm. of the coleoptile sections as related to age of Ginkgo **leaves of the chromatogrammed methanol phase at R.f. 0.10 and 0.25.**

	R.f. 0.10		
Ml. of Extract or Dry Wt.	Age of Leaves*		
	I	II	III
0.00	4.7544 mm.	4.6157 mm.	4.7450 mm.
0.05	6.2350 mm.	5.7000 mm.	4.2450 mm.
0.50	6.0875 mm.	6.7275 mm.	4.3200 mm.
1.00	6.0250 mm.	5.8575 mm.	4.5525 mm.
	L.S.D. 0.47 at 5% level		
	R.f. 0.25		
0.00	4.7544 mm	4.6157 mm.	4.7450 mm.
0.05	6.2800 mm.	5.2350 mm.	4.4225 mm.
0.50	6.0175 mm	5.9225 mm.	4.3800 mm.
1.00	5.4750 mm.	5.6525 mm.	4.4275 mm.
	L.S.D. 0.49 at 5% level		

*Date leaves were collected: I - August 14, 1963; II - October 2, 1963; III - October 22, 1963.

Fig. 6. (Left)—Appearance of black locust seedling four months old after the application of a petroleum ether extract of senescent Ginkgo **leaves. A – appearance of seedling after two days; B – appearance of seedling after one week; C – appearance of seedling after two weeks; D – appearance of seedling after three weeks.**

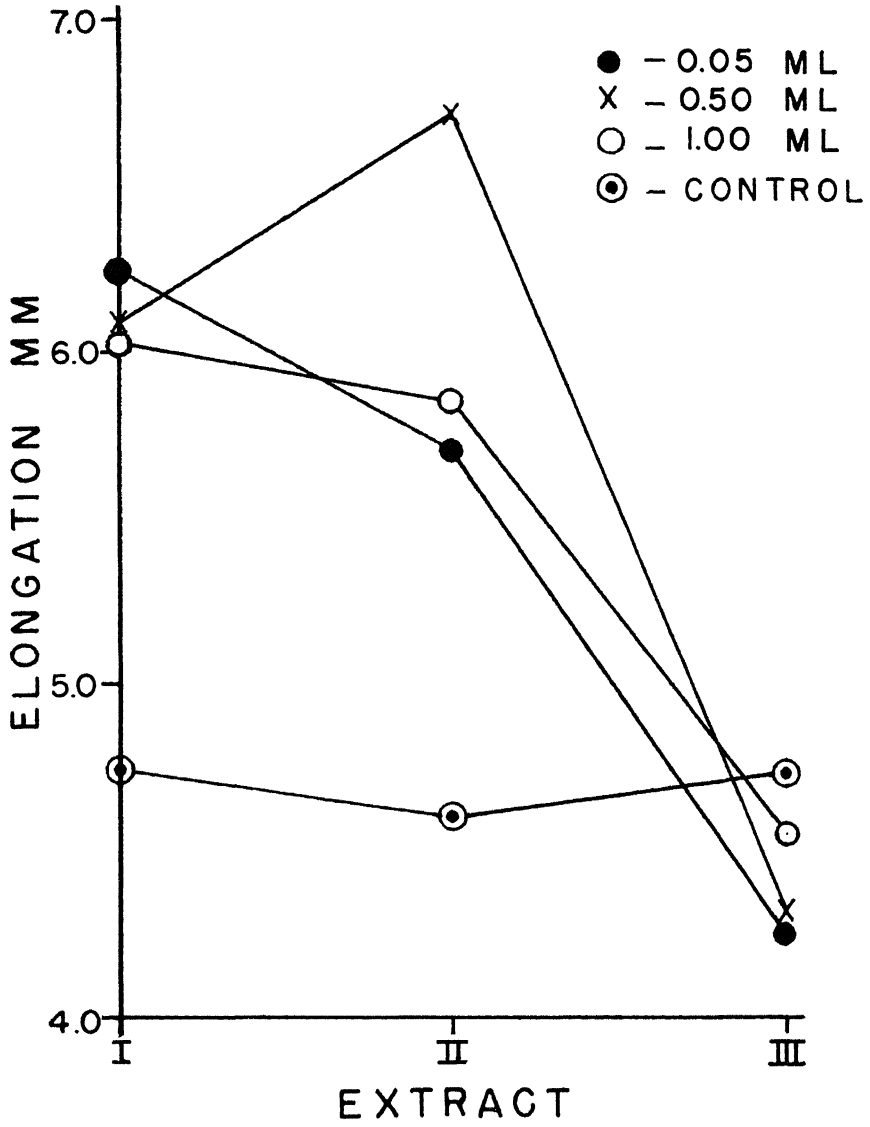


Fig. 7.—Elongation response of coleoptiles to a factor at R.f. 0.10. Date leaves were collected: I – August 14, 1963; II – October 2, 1963; III – October 22, 1963.

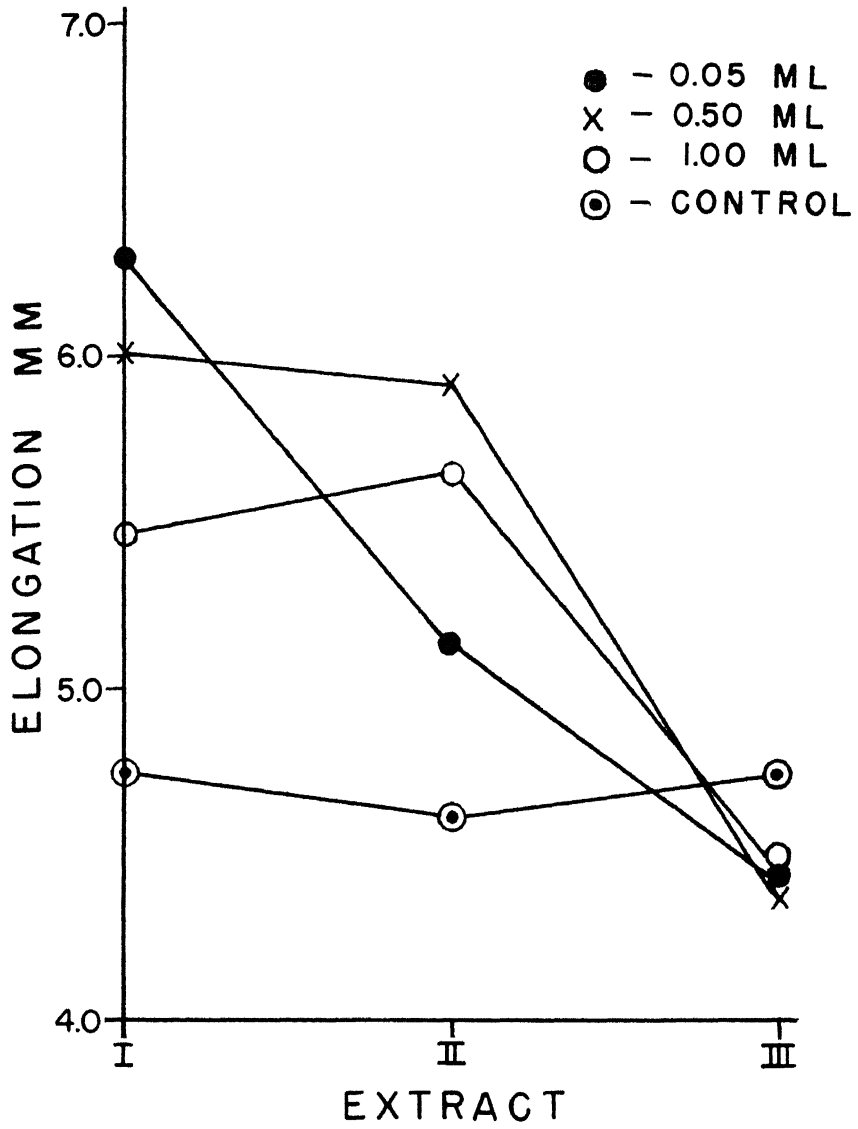


Fig. 8.—Elongation response of coleoptiles to a factor at R.f. 0.25. Date leaves were collected: I – August 14, 1963; II – October 2, 1963; III – October 22, 1963.

It is suggested that these two growth promoting factors are directly related to foliar abscission in *Ginkgo* trees. In young or less mature leaves, concentrations of the growth promoters are relatively high. As leaves grow older, concentrations decrease to near zero immediately prior to the time of leaf fall. Guar and Leopold (1955) found a quantitative relationship between auxin in the abscission zone and leaf fall. High concentrations of auxin retard abscission; low concentrations promote abscission.

Upon evaluating effects of the petroleum ether extract of senescent *Ginkgo* leaves on cotton explants and intact black locust and soybean plants, it was found that foliar abscission was induced; and in the case of black locust, the plants died. Treatment of young flowering soybean plants resulted in little effect upon foliar abscission or upon the plant as a whole. Foliar abscission was induced; in older fruiting plants, however, fruit abscission did not occur.

Combined observations suggest that the factor or factors which induce abscission are present in the leaf at all times; but the factor or factors which promote the elongation of the wheat coleoptiles and may prevent or retard abscission are relatively high in concentration in young, immature leaves, and decrease in concentration as the leaves become mature. Thus the factor or factors inducing abscission are inhibited in the young growing portions of the plant by growth promoters, and these inhibiting factors decrease as leaves and fruit become mature or senescent. At this stage, the abscission factor becomes active and abscission occurs.

SUMMARY

1. Leaves of *Ginkgo biloba* L. were collected in 1963 at weekly intervals from mid-August to late October (time of abscission).

2. Frozen and oven dried leaves of a *Ginkgo* tree were extracted with petroleum-ether for a period of eight hours; the petroleum ether solution was extracted with a methanol-water (1:4) solution and separated into fractions. Black locust and soybean plants and cotton explants were tested for abscission with the original petroleum ether extract. The petroleum ether and the methanol-water fractions were bioassayed using debladed cotton plants.

3. Paper chromatography was used in conjunction with the straight-growth test of wheat coleoptile sections. Two areas of the chromatograms of the methanol-water phase, R.f. 0.10 and 0.25, promoted growth of the wheat coleoptile sections. Concentration or activity of promoting factors decreased as the age of the leaf increased.

4. The rate of abscission was accelerated in cotton explants and in soybean and black locust plants when the petroleum ether extract was applied. There was little or no foliar abscission in young flowering soybean plants as compared to fruiting plants, where all but the young terminal leaves abscised. The fruit of senescent soybean plants did not abscise.

5. It is postulated that two biologically active growth factors occurring in the leaves of *Ginkgo biloba* L. which influence elongation of wheat coleoptile sections may also be involved in foliar abscission.

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