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Department of Botany, University College of Wales, Aberystwyth, Cardiganshire, United Kingdom

GIBBERELLIN-LIKE ACTIVITY IN BLEEDING-SAP OF ROOT SYSTEMS OF *HELIANTHUS ANNUUS* DETECTED BY A NEW DWARF PEA EPICOTYL ASSAY AND OTHER METHODS

By

I. D. J. PHILLIPS and R. L. JONES

With 5 Figures in the Text

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Introduction

Little work has yet been done with a view to determining the site, or sites, of gibberellin synthesis in higher plants. With few exceptions extractable gibberellin-like substances have been found in greatest amounts in seeds, particularly immature seeds, smaller quantities being present in vegetative parts, such as leaves, stems, stolons and roots. There is some evidence (WHEELER, 1960) that young expanding leaves contain higher levels of gibberellin-like substances than older leaves. However, gibberellins are known to be translocated readily in the plant, moving both in the xylem and phloem (ZWEIG *et al.*, 1961). Thus, the occurrence of relatively high concentrations of gibberellins in particular plant parts does not necessarily indicate that they are synthesised there.

LOCKHART (1957) performed a series of experiments designed to identify the organ of gibberellin synthesis in *Pisum sativum* (var. "Alaska") seedlings, and concluded that gibberellins important in the regulation of epicotyl elongation are produced in the apical bud. He noted that excision of cotyledons or roots resulted in suppressed epicotyl elongation, and that the application of 1 μg gibberellic acid (GA_3) to the apex of de-rooted or decotyledonised seedlings did not replace the need for either of these organs with reference to epicotyl elongation growth. However, excision of the apical bud also resulted in depressed stem elongation, but in this case a similar GA_3 application to decapitated plants allowed epicotyl elongation to proceed at a rate equal to that seen in intact seedlings. Application of indole-3-acetic acid in lanolin to the cut surface was found not to compensate for removal of the apical bud, but rather at concentrations above 0.1 μg/g lanolin inhibited epicotyl elongation, either in the presence or absence of exogenous GA_3. A number of authors have subsequently accepted

LOCKHART's results as having demonstrated that the natural endogenous source of gibberellins in higher plants is the apical bud of the shoot. This is, perhaps, unfortunate as LOCKHART's experiments were subject to a number of limitations. Firstly, applications of GA_3 stimulate stem elongation in intact as well as decapitated „Alaska" pea seedlings (LOCKHART's own results show this). Thus it is possible that the stimulation of growth by exogenous GA_3 in decapitated seedlings was no more than the stimulation by GA_3 in intact plants. In other words, that internode elongation was limited by endogenous gibberellin levels equally in intact and decapitated plants. Secondly, it is likely that the situation in etiolated six day old seedlings, as used by LOCKHART, is very different from that pertaining in more mature light-grown plants. Lastly, it would seem naive to base conclusions on experimental results obtained with decapitated plants since removal of the apical bud inevitably creates an abnormal situation within the stem.

In contrast to LOCKHART's findings, there is considerable evidence that roots do produce growth-factors, perhaps hormonal in nature, which are important in shoot growth and development, some of which may perhaps be gibberellin-like in nature. WENT (1938, 1943, 1951) noted the inhibitory effect which root removal has upon stem growth, and ascribed this as being due to removal of the source of a stem growth hormone which he named "caulocaline". Similarly, CHAILAKHYAN (1960) has shown that removal of the roots from the long day plant *Rudbeckia bicolor* prevents the plant responding normally to long-day treatment, in so far as such de-rooted plants do not flower under long days, and also do not show the normal associated stem elongation (or "bolting") response. This suggests that the site of production of the long-day induced stem elongation factor resides in the root system. The well known activity of gibberellins in the induction of stem elongation in non-induced long day plants indicates the possibility that the stimulus coming from the roots in photoperiodically induced plants of *Rudbeckia bicolor* may be of a gibberellin-like nature. Other indirect evidence has previously led to the suggestion that roots serve as a source of gibberellin for the stem tissues in *Helianthus annuus*, a day-neutral plant, and that such root-synthesised gibberellins are translocated to the shoot system via the xylem (PHILLIPS, 1964a, 1964b). The presence of gibberellin-like substances in a long established clone of excised tomato roots growing in culture (BUTCHER, 1963) provides good evidence that root tissues are capable of synthesising these substances.

To investigate further the possibility that gibberellins are supplied to the shoot from the root system, an examination was made of the "bleeding-sap" from root systems of sunflower plants following the removal of the shoot system. Bleeding-sap collected from several plant

species have in the past been found to contain various substances such as amino acids, minerals, sugars, enzymes and growth-active substances (IVANOFF, 1963).

Materials and Methods

Bleeding-sap was collected from three month old plants grown in the field. The shoot system was removed from each plant at a point just above the cotyledonary node and a piece of rubber tubing, which led to a conical receiving flask, was slipped over the cut stump. The bleeding-sap was collected from the flask twice daily over a period of several days. The volume of each half-daily sample was noted, and the autonomic cycle of bleeding-sap production reported by GROSSENBACHER (1939) and SKOOG *et al.* (1938) was observed in these experiments (Fig. 1). The sap was finally bulked and stored at —15° C until used.

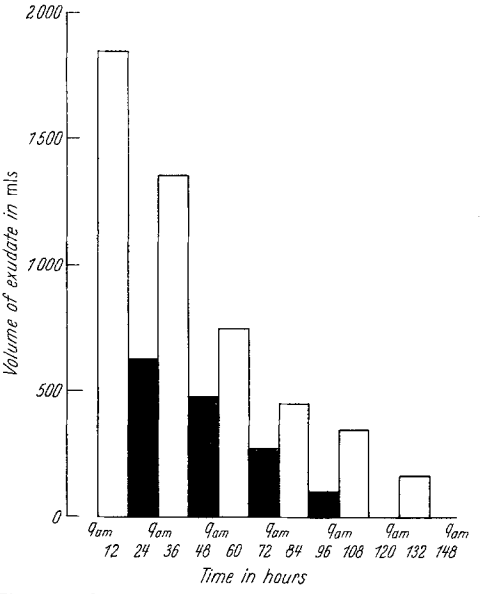

Fig. 1. Volumes of bleeding-sap (exudate) obtained from 52 three month old sunflower root systems growing under field conditions. Results expressed as volume in ml collected every 12 hours following removal of the shoot system

Bleeding-sap was assayed for gibberellin-like activity in 500 ml volumes. Each 500 ml aliquot was brought to pH 8.0 by addition of 5% sodium bicarbonate, extracted three times with equal volumes of redistilled ethyl acetate, brought to pH 2.5 with 5% hydrochloric acid and finally extracted three times as before with ethyl acetate. Each of the two ethyl acetate fractions so obtained was reduced to dryness *in vacuo* at 35° C and redissolved in a small volume of absolute redistilled methyl alcohol, prior to strip loading on to 10×35 cm strips of Whatman No. 3 chromatography paper. The chromatograms were developed in a descending manner in isopropanol/ammonia/water (10:1:1 v/v).

Chromatograms of bleeding-sap fractions have been bioassayed in various ways, the most frequent being to use a new dwarf pea assay.

Dwarf-pea epicotyl assay — The assay is based upon the elongation response of excised dwarf pea epicotyls to gibberellin.

Dwarf pea (var. "Meteor") seeds are soaked for 8—12 hours in tap water and then sown in seed boxes at a depth of 2.5—3″ in damp vermiculite. After four days growth at 25° C in *darkness*, the seedlings are at a stage of growth in which the first internode has virtually ceased elongation and the second internode is about to commence growth. At that time the epicotyls are severed from the cotyledons and roots, washed in distilled water and stood upright in small glass tubes containing 4 ml of test solution, or distilled water in the case of controls.

Ten excised etiolated epicotyls are placed in each tube, and left in an *illuminated* (fluorescent "daylight" tubes) incubator at 20° C for three days. At the end of this time the mean length of epicotyls above the first node is determined for each tube. The initial length of each epicotyl above the first node can be regarded as zero, so that the final length above the node is taken as measure of absolute growth over the three day incubation period.

The sensitivity of this test to gibberellin A_3 is shown in Fig. 2, and it can be seen that significant stimulation ($P<1\%$) of elongation occurs with GA_3 concentrations of 10^{-4} mg/l and above. Further, the system is insensitive to auxins such as indole-3-acetic acid (IAA), and there is no additive or synergistic interaction between auxin and gibberellin (Fig. 3); the only effect of IAA being to inhibit elongation at concentrations greater than 0.1 mg/l. Coumarin at concentrations up to 25 mg/l does not affect the responsiveness of the epicotyls to GA_3, β-inhibitor obtained from leaves of *Acer pseudoplatanus* (PHILLIPS and WAREING 1958) also did not influence the bioassay when used at concentrations which are highly active in the wheat and oat coleoptile section assays.

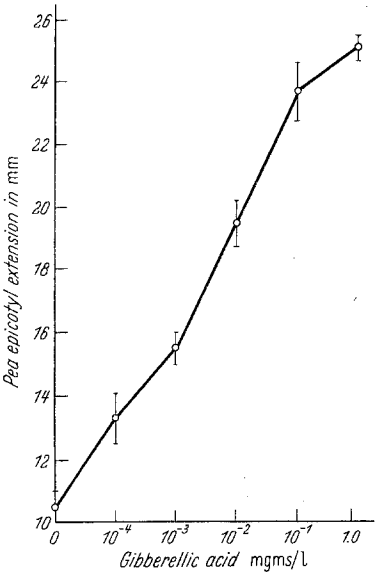

Fig. 2

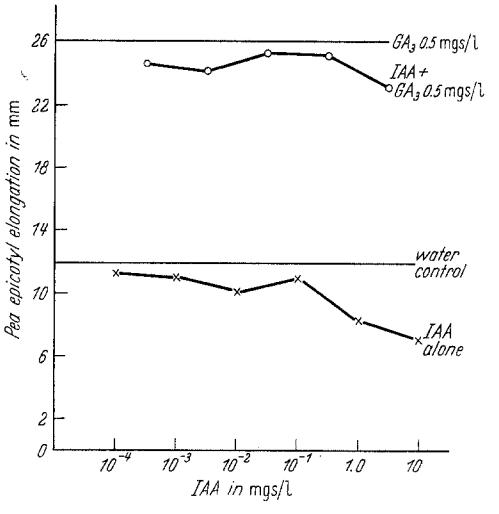

Fig. 3

Fig. 2. Relationship between gibberellic acid (GA_3) concentration and excised dwarf pea epicotyl elongation response over three days at 20° C in the light (epicotyls etiolated at start of treatment)

Fig. 3. Effect of indole-3-acetic acid (IAA), gibberellic acid (GA_3) and combinations of the two upon the elongation response of excised dwarf pea epicotyls

Where the assay is being used in conjunction with paper chromatography the paper is eluted with water prior to the incubation with pea epicotyls, and the paper not included in the tube during the incubation period. If thin-layer chromato-

graphy is used then the Kieselgur or silica-gel can be left in the tube with water and epicotyls.

Barley-endosperm assay — The technique used was as described by COOMBE and COHEN of the Waite Institute, Adelaide, S. A., Australia (see also NICHOLLS and PALEG 1963).

Results

A 500 ml sample of bleeding-sap obtained from the root systems of fifty-two field-grown plants was treated as above and bioassayed with dwarf pea epicotyls. The sample tested was taken from the bulked bleeding-sap obtained over the first twenty-four hours following removal of the shoot system. It can be seen (Fig. 4) that there was considerable gibberellin-like activity present in the acidic ethyl-acetate soluble fraction, and a smaller amount present in the basic-neutral ethyl-acetate fraction. In both cases only one peak of activity occurred under the chromatographic conditions used, with an R_f value of approximately 0.6—0.7. It is possible that the smaller amount of activity in the basic-neutral ethyl acetate soluble fraction is due to incomplete separation of acidic substances into the acidic fraction rather than to the existence of a separate basic gibberellin.

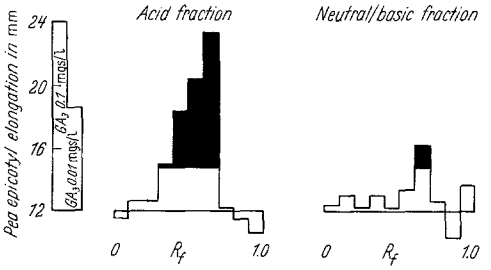

Fig. 4. Dwarf pea excised epicotyl assay of acidic and basic-neutral ethyl acetate soluble fractions of a 500 ml sample of root bleeding-sap taken from 2,460 ml obtained from 52 plants in 24 hours. Fractions were chromatographed on paper in isopropanol: ammonia: water (10:1:1 v/v). Darkened areas indicate growth significantly greater ($P < 1\%$) than controls. (Control elongation = 12.0 mm)

The presence in root bleeding-sap of substances active in the dwarf pea epicotyl assay has been fully confirmed in many other experiments. The amount of gibberellin-like activity present in equal volumes of bleeding-sap has been found to fall off after the first twenty-four hours following removal of the shoot system.

Gibberellin-like activity in bleeding-sap has also been detected by means of the barley endosperm assay which depends upon the measurable increase in reducing sugars which occurs in isolated slices of endosperm following the induction of amylase synthesis by gibberellins. The quantity of sugar present in each treatment was determined spectrophotometrically at 560 mμ in a Hilger and Watts Uvispeck photoelectric spectrophotometer. The instrument was zeroed with a reagent blank solution.

Two 500 ml volumes of first 24 hour bleeding-sap were each treated identically, and the acidic ethyl acetate soluble fraction was tested in the barley endosperm assay, following chromatography on Whatman No. 1 paper. Each chromatogram was divided up into ten regions between the starting line and solvent front, and each strip of paper so obtained was eluted with 1.5 ml of distilled water containing 500 μg streptomycin. Into each of the ten eluates from one of the two duplicate chromatograms were placed two slices of barley seed endosperm. Nothing

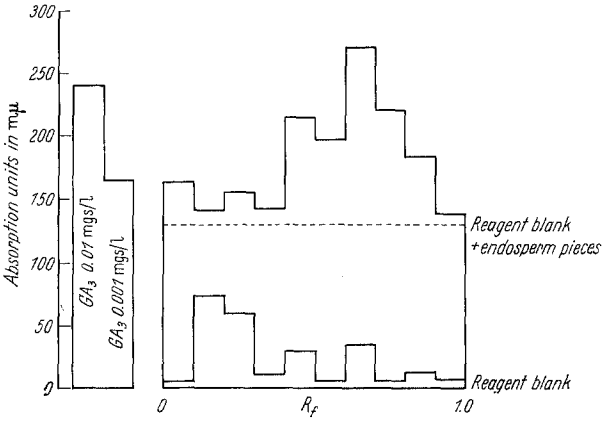

Fig. 5. Barley endosperm assay of acidic ethyl acetate soluble fraction of 500 ml sample of root bleeding-sap obtained and treated as in Fig. 4. Zero represents spectrophotometer zero with reagent solutions. Bottom histogram, chromatogram eluates after incubation without endosperm slices. Upper histogram, chromatogram eluates after incubation with endosperm slices. Horizontal dotted line, reading obtained with reagents to which endosperm slices had been added. GA_3 controls were incubated with endosperm slices. All treatments containing endosperm slices can be related to the horizontal dotted line

was added to the eluates of the second chromatogram. All eluates were incubated for 24 hours in darkness at 35° C. The sugar content of each tube was then measured, and the results expressed in spectrophotometer absorption units. Control tubes containing endosperm slices, streptomycin and GA_3 at 10^{-3} or 10^{-2} mg/l were included in the series of treatments. The result is shown in Fig. 5. There is evidence that sugars, or other substances raising the spectrophotometer reading, were present on the developed chromatogram, with most of these occurring at R_f 0.1—0.3. It can be seen that endosperm slices in the presence of distilled water and streptomycin but in the absence of GA_3 also cause a certain amount of sugar to be released into the water. Thus, the reading of 131.5 absorption units obtained with endosperm + streptomycin corresponds to a control level for the eluates in which endosperm was included and for the GA_3 control tubes each of which contained endosperm, and is thus indicated as a horizontal dotted line in Fig. 5. Maxi-

mum sugar increase by endosperm slices can be seen to have occurred in eluates obtained from the region of the chromatogram corresponding to R_f 0.4—0.9, with the peak of activity lying at R_f 0.6—0.7.

Discussion

The results presented above afford strong evidence that the root system in *H. annuus* is a site of gibberellin synthesis, and that root-synthesised gibberellins are translocated upwards to the shoot system. It would appear likely that the active substance or substances are conducted in the xylem, as it has been found in other experiments not reported here that gibberellin-like activity is present in bleeding-sap collected at a point above a steam-girdled region of the stem. The possibility that the root system of *H. annuus* is important as a source of gibberellin for the shoot, and that root-synthesised gibberellin is transported to the shoot in the xylem has been suggested previously on the basis of circumstantial evidence (PHILLIPS, 1964a).

The gibberellin-like activity in acidic ethyl-acetate fractions of bleeding-sap has also been demonstrated (unpublished) by means of the dwarf maize (*Zea mays*) leaf-base plus mesocotyl assay (FRANKLAND and WAREING 1962) using several dwarf mutant varieties.

The fact that bleeding-sap contained substances active in the induction of amylase synthesis in barley endosperm (Fig. 5) would suggest that the active material is a gibberellin rather than a gibberellin precursor. The basis for this suggestion is that it is unlikely that endosperm tissue would contain the spectrum of enzymes necessary for gibberellin synthesis.

The R_f value of the gibberellin-like activity was found to be similar in both dwarf pea epicotyl and barley endosperm assays, indicating that the same material is responsible for the responses in both tests (Figs. 4 and 5). Also, it can be seen from Fig. 5 that sugars present on the chromatogram do not run at the higher R_f value associated with the gibberellin activity, and it is unlikely therefore that contaminating substances are greatly influencing the response of barley endosperm to the eluate of region R_f 0.6—0.7.

Paper chromatography is known to have limitations in the separation of known gibberellins, and it is possible that what appeared to be one major zone of activity on chromatograms in these experiments was due to the presence of several gibberellins. Consequently, our current investigations of this problem are being conducted with the use of thin-layer chromatography on Kieselgur and silica-gel. Nevertheless, it appears that the active material is acidic in nature, and soluble in ethyl acetate. Bioassay of the aqueous residues following extraction with ethyl acetate has revealed no remaining gibberellin-like materials.

The quantity of gibberellin-like material appearing in the bleeding-sap can be expressed in terms of μg GA_3-equivalents. On this basis it can be calculated that a total level of activity equivalent to approximately 0.5 μg GA_3 was present on the chromatograms. This was obtained from 500 ml of bleeding-sap taken from the total 2,460 ml which was collected over the first 24 hour exudation period from fifty-two root systems. This means that almost 0.05 μg GA_3 – equivalents moved upwards from each root system over a twenty-four hour period following removal of the shoot system. If this amount of gibberellin activity is normally donated each day to the shoot it seems reasonable to assume that it is of considerable physiological importance in the shoot.

The relevance of the above findings to the hormonal control of normal growth and developmental processes of the sunflower shoot is being investigated further. The discovery of gibberellin-like activity in root bleeding-sap, and earlier findings that auxin levels in the shoot of *H. annuus* are partially regulated by the metabolic activities of the root system (PHILLIPS 1964a, b) lend credance to the suggestion made previously that shoot and root have important hormonal relationships with one another (PHILLIPS, *loc. cit.*) This view is supported by the detection of growth-inhibitors in bleeding-sap of several plant species (DAVISON 1963), and evidence for a root-synthesised kinin being important in leaf growth and senescence (MOTHES and ENGELBRECHT 1963; LOEFFLER and VAN OVERBEEK 1964).

Summary

Gibberellin-like activity was found to be present in bleeding-sap obtained from root systems of *Helianthus annuus*. The presence of activity was detected with a newly described simple and sensitive dwarf pea epicotyl assay, and by the barley endosperm and dwarf maize (*Zea mays*) tests.

The gibberellin-like substance, or substances, are acidic and ethyl acetate soluble, having an R_f value of 0.6—0.7 in paper chromatography with isopropyl alcohol: ammonia: water (10:1:1 v/v).

The quantity of gibberellin-like activity produced by the roots was estimated at approximately 0.05 μg GA_3 – equivalents/plant/day.

Other evidence for gibberellin synthesis in roots is discussed, together with the possibility that shoot and root have significant inter-relationships with regard to the production and utilisation of other types of growth hormone.

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Zusammenfassung

Am Blutungssaft des Wurzelsystems von *Helianthus annuus* wurde ein gibberellinartiges Wirkungsvermögen festgestellt. Das Vorhandensein dieser Aktivität wurde mittels eines neubeschriebenen einfachen und empfindlichen Tests an Zwergerbsen-Epikotylen sowie mit dem Gerstenendosperm- und dem Zwergmais-Test entdeckt.

Die gibberellinartigen Stoffe haben Säurecharakter und sind in Äthylacetat löslich. Ihr R_f-Wert wurde papierchromatographisch mit Isopropanol: Ammoniak: Wasser = 10:1:1 v/v als Laufmittel zu 0,6—0,7 ermittelt.

Das Maß der von der Wurzel produzierten gibberellinartigen Aktivität lag bei etwa 0,05 μ g GH_3-Äquivalenten pro Pflanze und Tag.

Andere Hinweise auf eine Gibberellinsynthese in Wurzeln werden besprochen und ferner die Möglichkeit, daß in Sproß und Wurzel eine signifikante Wechselbeziehung hinsichtlich der Produktion und Verwendung anderer Wuchsstoffarten besteht.

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Dr. I. D. J. PHILLIPS, and R. L. JONES,
Department of Botany, University College of Wales,
Aberystwyth, Cardiganshire (Great Britain)