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Native Growth Inhibitors from Citrus Shoots. Partition, Bioassay, and Characterization[1, 2]

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Abstract. **A partition method has been devised to separate auxins, gibberellins, and their respective inhibitors in plant extracts. Inhibitors counteracting gibberellin activity have been detected by a modified barley endosperm bioassay. An inhibitor found in young citrus shoots counteracts both auxin and gibberellin activities and behaves during partition and chromatography like abscisin II.**

The importance of native growth inhibitors has been emphasized by the recent studies on the chemistry and physiology of abscisin II (17). Abscisin II proves to be an inhibitor of auxin and gibberellin mediated growth processes (3, 16) and also of other hormonal responses (1, 8, 11).

Inhibitors of auxin have often been studied in the past and can easily be bioassayed by coleoptile elongation techniques. Inhibitors of gibberellin were also described (6, 16) and it has been shown that they may seriously interfere with bioassay of gibberellin like substances (9).

The commonly used bioassays for gibberellins are not suitable for the estimation of inhibitors. The barley endosperm bioassay technique (4, 5) has been adapted by us for the bioassay of these inhibitors. Measured amounts of exogenous gibberellin are added to vials containing either tissue extracts or water (controls), a full expression of gibberellin effect is found in controls, while native inhibitors partially or fully counteract gibberellin in the extract vials.

We have also devised a solvent partition method allowing easy separation of gibberellins from their inhibitors. Using these and other conventional techniques we separated and characterized inhibitors from young flushing citrus shoots.

Materials and Methods

Defoliated flushing citrus branchlets were homogenized in 80 % methanol with an "Ultra-Turrax" followed by cloth filtration and centrifugation at 1500 × *g* for 10 minutes. The extract was evaporated in a Buchi "rotavapor" at 45° to yield a concentrated water residue which was further partitioned as described in figure 1. Each fraction was concentrated and streaked on No. 3 MM Whatman paper strips. Ascending chromatography in individual cylinders was usually carried out by isopropanol, ammonia, water (80:0.1:19.9).

Auxins and their inhibitors were estimated by a modified wheat coleoptile section test (13).

Gibberellins and their inhibitors were tested as follows.

The method of Coombe *et al.* (4) for husked varieties was used with some modifications. Two seed halves were incubated for 32 hours at 25° in the darkness in vials 2.5 ml in volume, containing 1 ml of gibberellin solution or water and a 3 × 2 cm paper chromatogram section; sterilized water was used throughout. During incubation, vials were horizontally rotated at 1 rpm. The rotation ensures better aeration, thus solving the problems of solution depth discussed by Coombe *et al.* (4), and seems to provide for a better elution of chromatogram sections. The released reducing sugars were determined by the method of Noelting *et al.* (14) which is sufficiently accurate and much simpler than the method proposed by Paleg (15).

Results and Discussion

The partition method described in figure 1 has been developed in order to separate auxins, gibberellins and their inhibitors.

Typical results are shown in figure 2 depicting the biological activity of all the fractions of an extract from young lemon shoots bioassayed for auxins, gibberellins and their inhibitors. Each horizontal row in this figure shows the results of 1 bioassay technique with all the fractions; the lowest row shows the results of a separate experiment which will be discussed later. Each vertical column represents the activities of a determined fraction.

From the figure it is evident that the auxins partition into the etheric fractions I and II. Two main zones of auxin inhibition appear: the first

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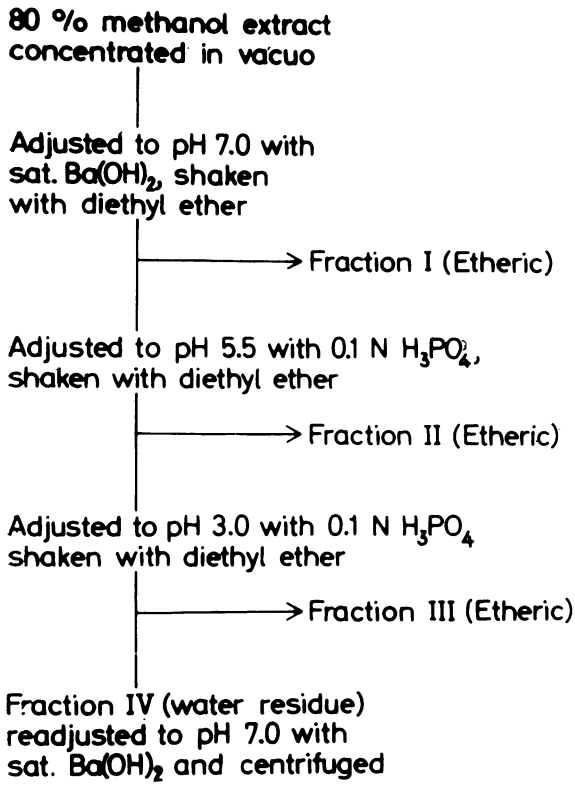

FIG. 1. Partition technique for natural growth regulators.

(R_F 0.7–1.0) is most strongly felt in fraction I while smaller amounts pass to the other fractions. The second inhibition zone appears only in the water residue (IV), at R_F values 0.0 to 0.3.

Gibberellin-like substances appear only in fraction III. The "activity" (5) found in fraction IV corresponds to the amount of native sugars present in the extract. The etheric fractions I-III are free of native sugars.

Gibberellin inhibitors appear mainly at R_F values 0.7 to 1.0 of fractions I and II: the former seems to contain more activity than the latter. The addition of exogenous gibberellin reveals the presence of these inhibitors which cannot otherwise be detected. The gibberellin like substances of fraction III and the native sugars of fraction IV appear here as in the row above, protruding from the "GA-control" line representing the sugar release induced by 1 ml of 10^{-8} M GA_3. The fluctuations around this control line are rather marked: therefore the use of this technique is recommended only for the sake of testing gibberellin inhibitors.

Different classes of growth promotors and inhibitors could therefore be separated into different fractions and R_F zones, as shown in the former paragraphs. The fact that auxin (and inhibitors) partition into the ether phase at neutral or mildly acidic pH values, might cause some surprise, since these compounds are usually found in the acidic fraction obtained at pH 3. In order to clarify these points, the following experiment was conducted: 7.5 μg of both IAA and abscisin II were dissolved in 80 % methanol, partitioned, chromatographed and bioassayed by the coleoptile test as described above for plant extracts. The results of this experiment are depicted in the lowest row of figure 2. IAA behaved like our native auxin, moving into the same fractions and R_F zones. Activity is also seen at the higher R_F values of fraction IV, probably caused by degradation products of IAA. Abscisin II behaved as our auxin and gibberellin inhibitor, appearing mostly in the neutral etheric fraction I at R_F zone 0.7 to 1.0. According to Larsen (10) the optimum pH range for ether partition of IAA is between 5.5 and 2.5, but his data show that partition is possible also at higher pH values. Kohler and Lang (9) succeeded in extracting their gibberellin inhibitors at pH ranges 7 to 5. It follows that our partition procedure, based on a gradual decrease in pH, brings into use some partition properties of growth regula-

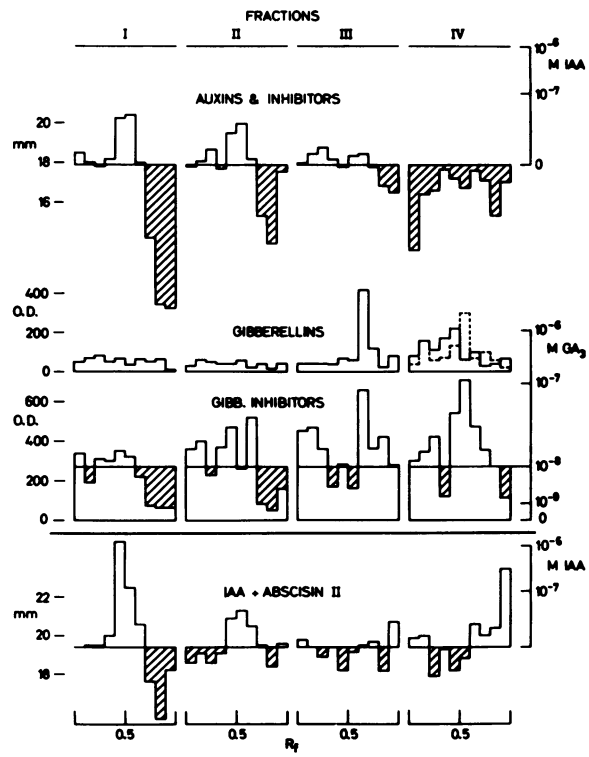

FIG. 2. Bio-assay histograms of an extract from lemon shoots, divided by partition into 4 fractions (I-IV) and tested for auxins and their inhibitors (upper row), gibberellins (second row) and gibberellin inhibitors (third row). The lowest row contains a control experiment, in which a mixture of IAA and abscisin II has been partitioned and bioassayed. The dotted line (gibberellins, fraction IV) represents the amount of native sugars on the chromatogram.

tors which had not been fully utilized previously. The qualitative differences between fractions I and II, however, seemed to be rather small and we decided in later experiments, to modify our partition procedure so that 1 mildly acidic fraction at pH 6 would substitute the 2 fractions at pH 7.0 and 5.5.

The inhibitor we found at R_F values 0.7 to 1.0 has already been shown to have inhibiting properties both with regard to auxin effects and to gibberellin effects. Its R_F values also correspond to those of the so-called β-inhibitor (2) which has been recently identified, at least in part, with abscisin II (12). We have also seen that abscisin partitions and chromatographs in a way similar to our inhibitor. Additional evidence in this direction is presented in figure 3.

The R_F zone 0.75 to 0.95 was cut from chromatograms of an etheric fraction obtained at pH 6.0, eluted with methanol overnight and rechromatographed contemporaneously with strips spotted with abscisin II in 4 different solvent systems. Results obtained by the wheat coleoptile assay are shown in sections A, B, and C of figure 3. They show that our inhibitor behaved chroma-

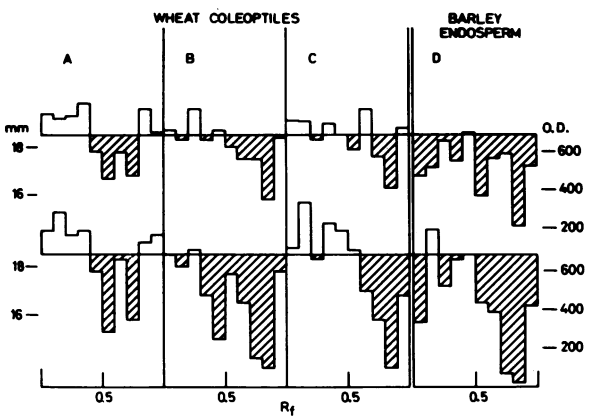

FIG. 3. A comparison between the chromatographic behavior of an inhibitor from citrus shoots (upper row) and abscisin II (lower row); conducted with the wheat coleoptile bioassay (A, B, C) and barley endosperm system (D). The following developing solvents were used: A) Diethyl-ether:Methanol:Acetic acid (50:50:0.1). B) Methanol:Butanol:Water:Acetic acid (50:20:30:0.1). C) Isopropyl-ether:Methanol: Water:Acetic acid (55:35:10:0.1). D) Isopropanol: Ammonia:Water (80:0.1:19.9).

tographically like abscisin II in each of the solvent systems tested. Section D of the same figure shows a similar comparison carried out with our test for gibberellin inhibitors; here again the properties of our inhibitor closely resemble those of abscisin II.

Abscisin II has already been identified in citrus fruits (7, 12). We suggest that it may be present also in young growing branchlets of citrus trees. The inhibitor seems to be present in considerable amounts in shoot tissues since 20 mg fresh weight equivalents still showed inhibiting effects when tested with our barley endosperm inhibitor bioassay. The physiological role of such inhibitors in young growing branchlets awaits further elucidation.

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