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STUDIES ON PHLOEM EXUDATION FROM YUCCA FLACCIDA HAW. XIII. EVIDENCE FOR THE OCCURRENCE OF A CYTOKININ NUCLEOTIDE IN THE EXUDATE

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SUMMARY

Phloem exudate of *Yucca* was analysed for cytokinin activity. After partition in a water- and butanol-1 fraction, almost no cytokinin activity was found in the butanol phase (cytokinin base and nucleosides). Application of alkaline phosphatase to the water layer gave rise to a strong positive cytokinin response with the soy-bean callus test, an amount of approximately 520 μg zeatin equivalent per litre being found in the exudate after treatment with alkaline phosphatase. The results indicate that a cytokinin, presumably zeatin, is translocated as a nucleotide.

1. INTRODUCTION

The occurrence of cytokinin in xylem sap has been well established: REID & BURROWS (1968), BURROWS & CARR (1969), CARR & BURROWS (1966).

Less is known of the occurrence of cytokinins in phloem sap. Recently, however, PHILIPS & CLELAND (1972) described the presence of cytokinins, which co-chromatographed with zeatin or zeatin ribonucleoside, in the honeydew of the aphis *Dactynotus ambrosiae*, feeding on *Xanthium strumarium*. They also showed that the honeydew from flowering plants contained more cytokinins than the honeydew from vegetative plants. HOAD (1973) also found cytokinin activity in phloem exudate of *Ricinus communis*.

Phloem exudate from the young inflorescence of *Yucca* can be collected in sufficient quantities to allow an analysis for cytokinins (TAMMES & VAN DIE 1964).

The aim of this study was to determine whether cytokinins were also present in phloem exudate of *Yucca*.

2. MATERIALS AND METHODS

2.1. Collection of sieve tube exudate

In June 1973, phloem exudate from the young inflorescence stalk of *Yucca* was tapped while the stalk was cooled. This process has been described earlier (TAMMES, VONK & VAN DIE 1969). The exudate was transported at 0 °C and stored at minus 20 °C until analysis.

2.2. Purification of the active compounds by column chromatography

The exudate was diluted with water and partitioned against water-saturated butanol-1. Cytokinin-like active compounds in the remaining water-layer were purified by a modified procedure of LETHAM (1966), a.e. by means of a De Acidite FF Column as shown in *fig. 1*. Column washings were continued with deionised water till sucrose free, tested by the anthrone method (MORRIS 1948).

2.3. Paper- and thin layer chromatography

Paper chromatography of the fractions A, B, C, D_1, and D_2 (*fig. 1.*) was carried out in: butanol-1: Conc. NH_4OH (4:1 v/v) on Whatman No. 3 filter paper (System 1). The chromatograms were divided into equal strips and eluted with fifty per cent ethanol: water (v/v).

When the soy-bean callus test was used (2.4), the fractions corresponding with zeatin and zeatin ribonucleoside from System 1 were rechromatographed in: butanol-1: acetic aced: water (4:1:1 v/v) as System 2. These chromatograms were cut into ten equal pieces after being sprayed with water and intensively dried, in accordance with HEWETT & WAREING (1973), to avoid toxic residues in the callus test. They were then eluted with fifty per cent ethanol-water (v/v) and tested in the soy-bean callus bioassay.

Thin layer chromatography was carried out with desalted samples (Chromatodesalter, Pleuger) in ethanol: water (8:2 v/v) on Polygram SiL. N-HR/U.V. 254 plates (Machery-Nagel). Zeatin and zeatin ribonucleoside co-chromatographed on all chromatograms.

2.4. Bioassays

After evaporation of the different fractions in vacuo at 30 °C, the *Amaranthus* bioassay was used (BIDDINGTON & THOMAS 1973). Instead of the concentration L-Tyrosine recommended by these authors, 5 mg/ml phosphate buffer and a total amount of twenty explants per petridish were used.

As a second bioassay, the soy-bean callus tissue test (MILLER 1963) was used. Zeatin was used as a standard for both bioassays.

2.5. Treatment with alkaline phosphatase

The water-layer remaining after elution of the De Acidite FF column with formic acid and partition against butanol (*fig. 1*) was concentrated to ten ml in vacuo, and an equal volume of 0.2 M Tris (hydroxymethyl) amino methane buffer 0.02 M in $MgCl_2$, pH 8.2 was added. After addition of alkaline phosphatase (free of phosphodiesterase, Boehringer) 2 mg/ml, the solution was kept for 24 h at 37 °C (*fig. 1*, D_2). As a control, another ten ml exudate was treated in the same way but without enzyme (*fig. 1*, D_1). In order to test the possible active degradation products of alkaline phosphatase, twenty-five ml of an eighteen per cent sucrose solution in 0.04 M phosphate buffer, pH 8.2, was treated as described for the water-layer.

Ten ml exudate pH 8.2 diluted with 10 ml dionised water partitioned against water-saturated Butanol-1.

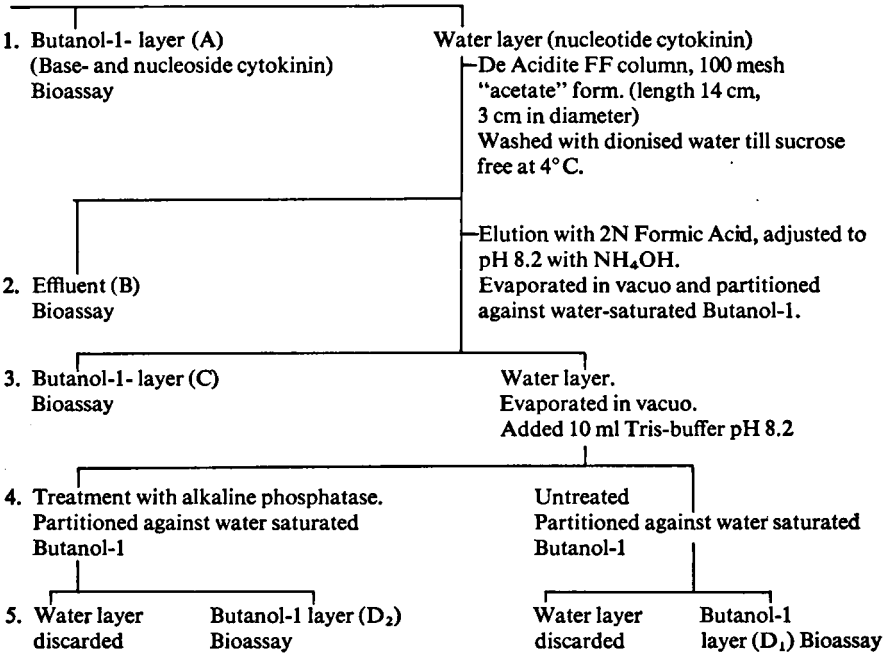

Fig. 1. Flow diagram for the extraction of cytokinin-like substances from bleeding sap of *Yucca*.

3. RESULTS

The extraction procedure as developed by LETHAM (1966) is based on a separation of the free base cytokinins and nucleoside cytokinins from the nucleotide cytokinins. Cytokinin-free base and nucleoside cytokinins can be extracted from aqueous solutions by partition against water-saturated butanol at pH 8, whereas the nucleotide cytokinins remain in the water layer (*fig. 1*, Step 1).

The large amount of sucrose in the phloem exudate does not allow a direct measurement of cytokinin activity by the *Amaranthus* bioassay, probably due to plasmolysis of the *Amaranthus* explants. For this reason the water-layer containing nucleotide and sucrose was run on a negatively charged column (*fig. 1*, Step 1). The nucleotide cytokinins are retained by the negatively charged resin, whereas the sucrose is eluted from the resin by water.

Surprisingly, the butanol layer, which usually contains the free base and nucleoside cytokinins, showed hardly any activity in the *Amaranthus* bioassay (*fig. 2*, Fraction A).

Since the nucleotide cytokinins are almost inactive in the callus bioassay (ENGELBRECHT 1971, VAN STADEN & WAREING 1972) and nothing is known

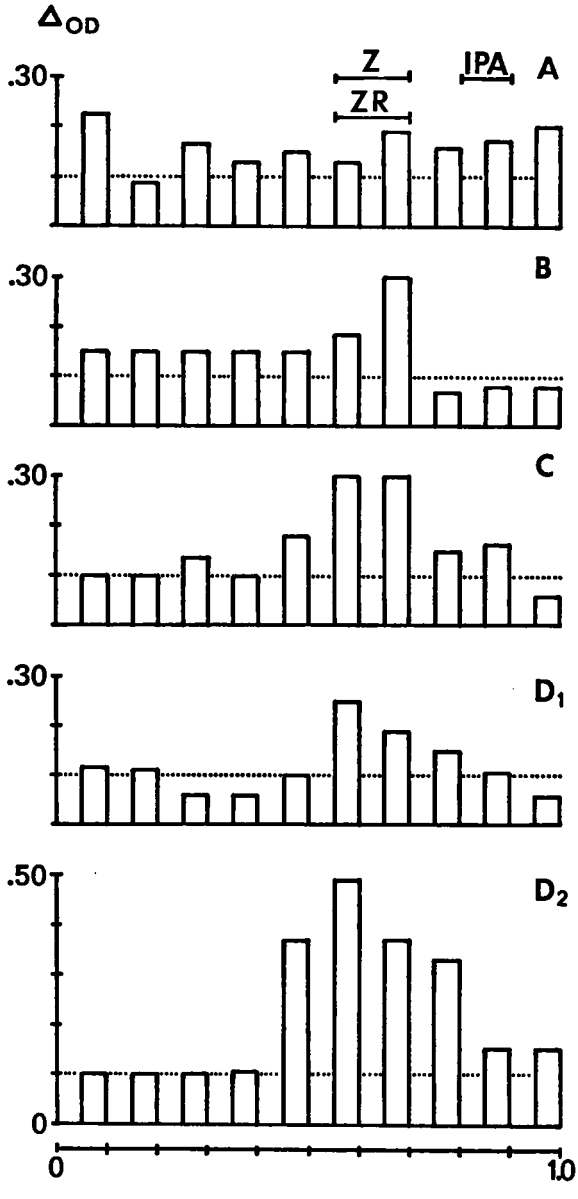

Fig. 2. Histogram of the *Amaranthus* bioassay from ten ml exudate, treated as described in extraction flow diagram *(fig. 1)*. Ordinate: △ O.D. 542–620 nm. Absciss: Rf value in butanol-1: conc. NH_4OH (4:1 v/v). Cytokinin standard:
z = zeatin zr = zeatin ribonucleoside. IPA = N^6-(Δ^2- isopentenyladenosine). Dotted line represents control (zero value).

about their activity in the *Amaranthus* bioassay, the nucleotide cytokinins were first converted into nucleoside cytokinins by alkaline phosphatase treatment (*fig. 1*, Step 4). After partitioning with water-saturated butanol, the cytokinin activity passed into the butanol layer (*fig. 2*, Fraction D_2). The control experiments, without addition of alkaline phosphatase, showed only very little cytokinin activity (*fig. 2*, Fraction D_1). The small amounts of cytokinin activity in Fractions B and C (*fig. 2*) may be due to a slight breakdown of the cytokinin nucleotide on the resin, or to breakdown during evaporation of the formic acid fraction.

A similar experiment was carried out with 50 ml phloem exudate. The exudate was partitioned three times with water-saturated butanol. The remaining water layer was treated directly with alkaline phosphatase and not passed through the negatively charged resin. The fraction finally obtained (D_2), was assayed in the soy-bean callus bioassay instead of in the *Amaranthus* bioassay. Again Fraction D_2 was the only active fraction (*fig. 3*, A and B).

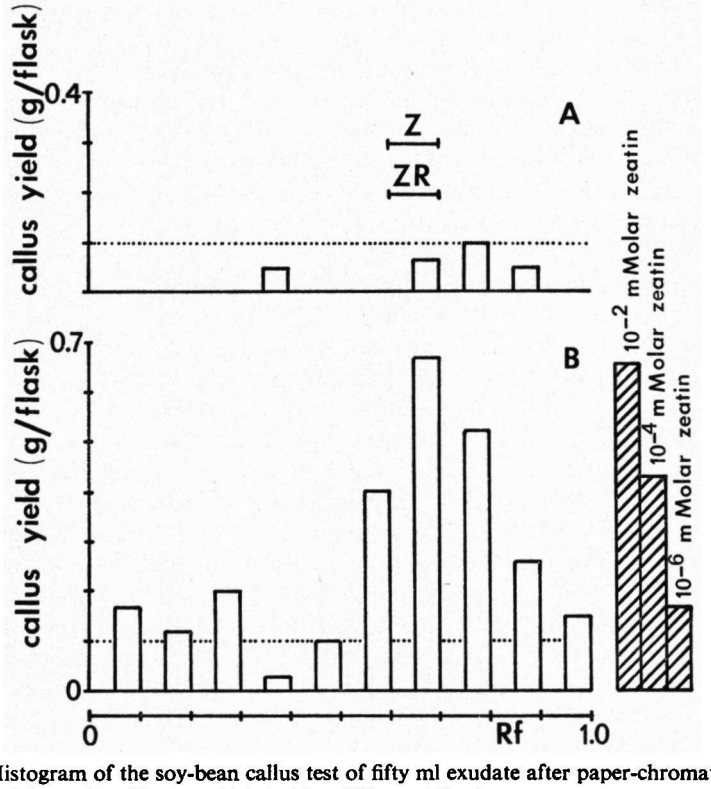

Fig. 3. Histogram of the soy-bean callus test of fifty ml exudate after paper-chromatography in: butanol-1 : acetic acid : water (4:1:1 v/v) at different Rf values.
A: butanol-1 layer(fraction A). B: water-layer, after treatment with alkaline phosphatase (fraction D_1). Cytokinin standard: z = zeatin zr = zeatin ribonucleoside. Dotted line represents control(zero value).

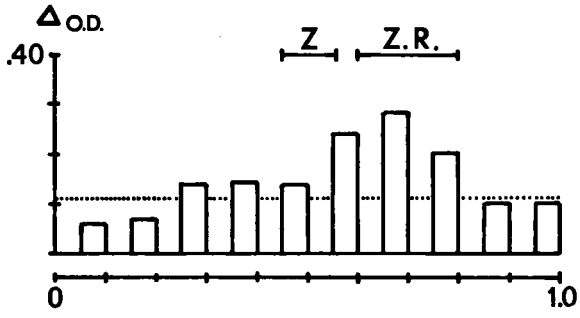

Fig. 4. Histogram of the *Amaranthus* bioassay from five ml exudate, treated as described in extraction flow diagrams (*fig. 1*) after T.L.C. on polygram plates. Ordinate: Δ O.D. 542–620 nm. Absciss: Rf value in ethanol: water (8:2 v/v). Cytokinin standard: z = zeatin zr = zeatin ribonucleoside. Dotted line represents control (zero value).

The results of both experiments clearly indicated the presence of a nucleotide cytokinin in the phloem exudate. No clear evidence could be obtained of the occurrence of free base- or nucleoside cytokinins.

The exact chemical nature of the nucleotide is not known with certainty, but based on Rf values in different solvent systems, the presence of isopentenyl adenosine (IPA) could be ruled out (*fig.* 2). Solvent systems as used in the experiments depicted in *figs. 2* and *3* do not discriminate between zeatin and zeatin ribonucleoside. However, these two compounds can be separated from each other by using ethanol:water (8:2 v/v) as a solvent on a silica gel thin layer chromatogram. The results shown in *fig. 4* made it highly likely that during alkaline phosphatase treatment the nucleotide cytokinin from the phloem exudate is converted into zeatin ribonucleoside.

From the results obtained by the callus bioassay, a concentration of about 520 μg zeatin equivalents/l was calculated. Based on a total amount of eighteen per cent dry matter for *Yucca* exudate (TAMMES & VAN DIE 1964), a quantity of approximately 3 μg zeatin equivalent/g dry weight could be calculated.

Nucleic acids are known to occur in phloem exudate of *Robinia pseudacacia* (ZIEGLER & KLUGE 1962), of *Salix triandra*, and of *Yucca flaccida* Haw. (KLUGE, BECKER & ZIEGLER 1970). Since cytokinins also occur in nucleic acids, it appeared necessary to check whether nucleic acids could form a source for cytokinins if treated with alkaline phosphatase only. For this reason ribonucleic acid (RNA) from yeast and t-ribonucleic acid (t-RNA) from brewer's yeast (Boehringer, 5 mg each) were treated as described (*fig. 1*) and tested in the *Amaranthus* bioassay. Neither the butanol layer, nor the other fractions showed any positive response for cytokinins (data not given), ruling out the possible occurrence of nucleotide cytokinins as breakdown products of nucleic acids in the phloem exudate. These observations support the finding that cytokinins can only be detected in nucleic acids if RNA or t-RNA are first hydrolysed by KOH or phosphodiesterase before treatment with alkaline phosphatase (DEKHUIJZEN & STAPLES 1968).

4. DISCUSSION

Strong evidence has been presented for the view that a nucleotide cytokinin, presumably of zeatin, occurs in the water-soluble fraction of the phloem exudate of the inflorescent stalk of *Yucca*. No butanol soluble cytokinins could be detected in phloem exudate.

Data on the occurrence of cytokinins in phloem exudate are very limited. PHILIPS & CLELAND (1972) detected cytokinin activity in the butanol-soluble fraction of the excretory products from aphides feeding on *Xanthium* plants, but did not analyse the activity of the water-soluble cytokinins in the phloem sap. Therefore the possibility that nucleotide cytokinins are converted into nucleoside cytokinins in the intestine of the aphides cannot be excluded.

The observation that cytokinin activity is due to a nucleotide is not in accordance with the conclusion of ZIEGLER & KLUGE (1962). They found no nucleotides or nucleosides in the phloem exudate of *Robinia*. It is of interest, however, that these authors described the occurrence of a U.V. absorbing compound with a maximum of 264 nm, a shoulder at 268–270 nm, and a minimum of 236 nm. In the light of the present results, it may be suggested that a cytokinin (zeatin, zeatin ribonucleoside, 2IP and IPA, U.V. absorption in 50% ethanol: water, maximum 268–270 nm; minimum 230–240 nm) was present in the sap of *Robinia*.

Far less is known about the biological activity of nucleotide cytokinins than of free base and nucleoside cytokinins in different bioassays. In general, the following order of decreasing sensitivity in the callus bioassay is found: purine base, nucleoside, nucleotide (ENGELBRECHT 1971, VAN STADEN & WAREING 1972). There is a possibility that, as has been suggested by VAN STADEN & WAREING (1972), the nucleotide is the major form in which cytokinins are translocated and/or stored within the plant.

The present finding of high amounts of nucleotide cytokinins in the inflorescent stalk before the opening of the flower buds suggests that the nucleotide or its conversion products, zeatin and zeatin ribonucleoside, play and important role in the flowering of *Yucca*. The importance of cytokinins for the flowering process has been suggested earlier by MAE & VONK (1974), who showed that bud blast symptoms on *Iris* could be partly overcome by the injection of cytokinins into the flower buds. PHILIPS & CLELAND (1972) also showed that in flowering *Xanthium* the butanol soluble cytokinin concentration in the sap was about seven times higher than in the phloem of vegetative plants.

The results of this study confirm the results of PHILIPS & CLELAND (1973) and HOAD (1972) on the occurrence of cytokinin-like substances in phloem. Evidence has been presented for the view that cytokinin nucleotide, presumably of zeatin, is translocated in the inflorescent stalk of *Yucca*

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