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Endogenous Cytokinins in *Bougainvillea* 'San Diego Red'

I. OCCURRENCE OF CYTOKININ GLUCOSIDES IN THE ROOT SAP[1]

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ABSTRACT

Using chromatographic, chemical, and enzymic techniques, 11 compounds capable of stimulating the division of soybean callus cells were tentatively identified in the root sap of *Bougainvillea* 'San Diego Red.' These cytokinin-like compounds included phosphorylated and glucosylated forms of zeatin, ribosylzeatin, and their dihydro derivatives. In addition, isopentenyl adenosine and isopentenyl adenine were apparently also present. The occurrence of glucosylated derivatives in the root sap of plants has not been substantiated previously.

Although there is an extensive body of literature pertaining to the presence of cytokinin-like compounds in the xylem sap of plants, only limited information is available on the nature of the compounds concerned (12). TLC and paper chromatographic techniques, in conjunction with bioassays, have resulted in the detection of two peaks of cytokinin activity in most sap analyzed. Most activity was usually associated with the nonpolar peak that co-chromatographed with zeatin and ribosylzeatin. More thorough investigation of this peak resulted in the identification of zeatin, ribosylzeatin, and dihydrozeatin in the xylem sap of a number of plants (4, 7, 14). The polar peak, although not chemically characterized, could be hydrolyzed partially by phosphatase enzymes to yield an active compound that co-chromatographed with ribosylzeatin. This phosphorylated compound is considered to represent zeatin ribotide, regarded widely as a bound form of cytokinin (12). However, inasmuch as: (*a*) the polar peak is frequently incompletely hydrolyzed by alkaline phosphatase (14); (*b*) cytokinin glucosides are present in plant roots (13, 16); and (*c*) small amounts of a compound that co-chromatographed with zeatin glucoside were detected in the xylem sap of white lupins (2), we postulated that glucosylated compounds are present in the root exudate of plants (12). This paper provides evidence for the occurrence of glucosylated cytokinins in the root sap of *Bougainvillea*.

MATERIALS AND METHODS

Rooted cuttings of *Bougainvillea* 'San Diego Red' were cultured in the greenhouse under long day conditions to the stage of 15 mature leaves as described previously (9). Root exudate was collected for 24 h by decapitating the plants 2 cm above soil level and attaching rubber tubes to the cut stumps. After collection, the sap was stored as 100-ml batches in plastic bottles at −20 C until required.

Cytokinin activity in the crude root sap was determined by thawing a required aliquot and taking it to dryness *in vacuo* at 35 C. The residue was resuspended in 35% ethanol, filtered through Whatman No. 1 paper and then fractionated on a Sephadex LH-20 column (2.5 × 90 cm). The constituents in the extract were separated by eluting the column with 35% ethanol at a flow rate of 20 ml h^{-1}. The eluates collected from the column, as 40-ml fractions, were placed in 50-ml flasks and dried on a hotplate at 40 C in a stream of air. Twenty ml culture medium was then added to the flasks and the cytokinin activity determined by the soybean callus bioassay as outlined previously (5). Three peaks of cytokinin-like activity were detected in the crude root sap extract (Fig. 1). Two of these peaks co-chromatographed with zeatin and ribosylzeatin, respectively. The third peak, which was more polar, eluted between 120 and 240 ml on the Sephadex LH-20 column.

More information regarding the nature of the three peaks reported in Figure 1 was obtained by fractionating a larger aliquot of crude sap on Sephadex LH-20 and collecting fractions A, B, and C separately as indicated. Further chromatographic, chemical, and enzymic techniques were used to establish tentatively the identity of the cytokinin-like compounds present in these individual fractions.

An aliquot of fraction A treated with β-glucosidase (almond emulsin, Calbiochem) (11) and then refractionated on the Sephadex LH-20 column resulted in the disappearance of most of the activity from its original elution volume (120–240 ml) and the shifting of this activity to those elution volumes where ribosylzeatin and zeatin elute normally (Fig. 2A). This infers that the *O*-glucosides, where the glucose moiety is attached to the side chain, of both ribosylzeatin and zeatin formed part of the polar peak detected in the crude root sap. When the β-glucosidase hydrolyzed extract from fraction A was treated with $KMnO_4$ (5, 8) before being fractionated, the residual activity associated with the polar peak (Fig. 2A) at elution volume 160–240 ml disappeared com-

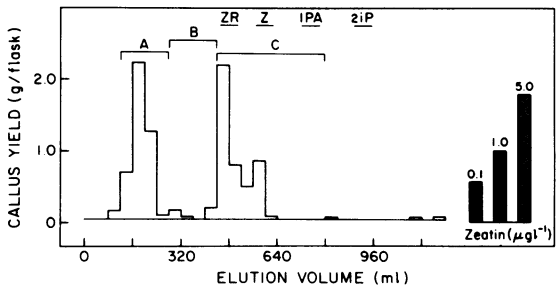

FIG. 1. Soybean bioassay after fractionation of equivalent of 100 ml crude root sap on a Sephadex LH-20 column with 35% ethanol. ZR: ribosylzeatin; Z: zeatin; IPA: isopentenyl adenosine; 2iP: isopentenyl adenine. A, B, and C represent fractions collected for subsequent analysis.

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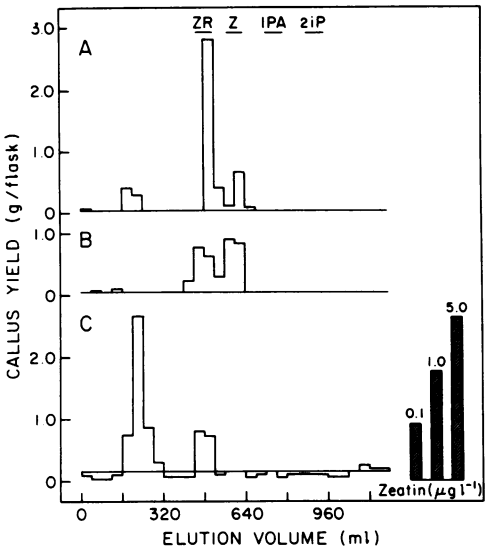

FIG. 2. Soybean bioassays of fractions collected as indicated in Figure 1, treated in various ways, and again fractionated on Sephadex LH-20 with 35% ethanol. Each histogram represents activity from equivalent of 100 ml root sap. A: fraction A treated with β-glucosidase; B: fraction B treated with β-glucosidase; C: fraction A treated with alkaline phosphatase. ZR: ribosylzeatin; Z: zeatin; IPA: isopentenyl adenosine; 2iP: isopentenyl adenine.

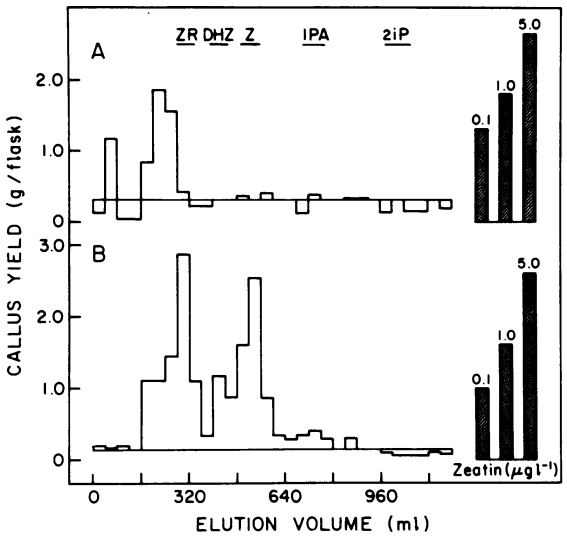

FIG. 3. Soybean bioassays of fractions collected as indicated in Figure 1 and refractionated on a Sephadex G-10 column with 5% ethanol. Each histogram represents the activity from the equivalent of 100 ml root sap. A: fraction A; B: fraction C. ZR: ribosylzeatin; DHZ: dihydrozeatin; Z: zeatin; IPA: isopentenyl adenosine; 2iP: isopentenyl adenine.

pletely. The activity that coeluted with ribosylzeatin was reduced by 21%, whereas the peak that coeluted with zeatin showed a 10% loss in activity. As $KMnO_4$ treatment breaks the double bond in the side chain of compounds such as zeatin (5), it seems that most of the activity associated with the *O*-glucosides of ribosylzeatin and zeatin was attributable to their dihydro derivatives which move with the parent compounds.

Fraction B, which originally showed no detectable activity in Figure 1, probably due to the presence of impurities, yielded two peaks of activity following treatment with β-glucosidase. These peaks co-chromatographed with ribosylzeatin and zeatin, respectively (Fig. 2B). Additional treatment with $KMnO_4$ reduced the activity of these two peaks by the same magnitude as found for fraction A.

When fraction A was treated with alkaline phosphatase (chicken intestine, Sigma) (15) and refractionated on Sephadex LH-20, most of the cytokinin activity remained at the original elution volume. A small peak of activity could, however, be detected in the region where ribosylzeatin elutes normally (Fig. 2C). Additional evidence that the polar peak detected in the crude root sap (Fig. 1) consisted predominantly of compounds other than phosphorylated compounds was obtained by separating an aliquot of fraction A on a column (1.5 × 90 cm) packed with Sephadex G-10 swollen in 5% ethanol. By eluting the column with 5% ethanol at a flow rate of 20 ml h^{-1}, the cytokinin-like compounds in fraction A could be separated into two peaks (Fig. 3A). Only the smaller peak (elution volume 40–80 ml) could be hydrolyzed with alkaline phosphatase. These data suggest that at least five compounds that are active in the soybean callus bioassay are present in the combined fractions A and B collected from crude root sap. These compounds are tentatively identified as dihydrozeatin ribotide, and the glucosides of ribosylzeatin, zeatin, and their dihydro derivatives.

Separation of fraction C (Fig. 1) on a Sephadex G-10 column eluted with 5% ethanol, resulted in the detection of three peaks of cytokinin-like activity (Fig. 3B). These peaks coeluted with ribosylzeatin, dihydrozeatin, and zeatin, respectively. The peak that eluted most rapidly off the column was spread over a large volume (160–360 ml) and eluted only partly with ribosylzeatin, suggesting that it contained compounds other than ribosylzeatin alone. Treatment with $KMnO_4$ did in fact result in the elimination of that part of the peak that coeluted with ribosylzeatin. The rest of the activity other than that which coeluted with dihydrozeatin also disappeared following this treatment.

The use of exchange resins confirmed the above results showing that the cytokinin complement of *Bougainvillea* root sap is more complex than was indicated in Figure 1. By adjusting the pH of the crude root sap to 2.5 and following the normal Dowex 50W-X8 cation exchange purification as described previously (11), six peaks of activity could be detected after Sephadex LH-20 fractionation (Fig. 4A). Two of these (elution volume 160–280 and 320–360 ml) were more polar than the cytokinin markers. The other four coeluted with ribosylzeatin, zeatin, isopentenyl adenosine, and isopentenyl adenine, respectively. The effluent from the Dowex 50 column yielded three peaks of cytokinin-like activity. One was polar and eluted between 160–240 ml whereas the other two smaller peaks coeluted with the isopentenyl derivatives (Fig. 4B). These three peaks of activity were also detected (Fig. 4C) in an extract that was obtained by adjusting the pH of the crude root sap to 8.4, passing it through Dowex 1, and then removing the adsorbed cytokinins with 2 N HCOOH (5). This latter column is used for the retention of cytokinin nucleotides, which are apparently not retained by a cation exchange resin such as Dowex 50 (5).

DISCUSSION

By using chromatographic, chemical, and enzymic techniques, in conjunction with the soybean callus bioassay, 11 compounds capable of stimulating cell division could be detected in the root sap of *Bougainvillea* plants growing under long day conditions. As was the case in earlier studies where high performance liquid chromatography techniques were used for the analysis of tomato root exudate (1), and where the spring sap of sycamore trees was investigated by means of combined GC-MS (7), it could be demonstrated that the cytokinin complement of *Bougainvillea* root sap is complex.

Phosphorylated cytokinins frequently referred to as bound forms of the hormone (12), contributed only about 20% of the

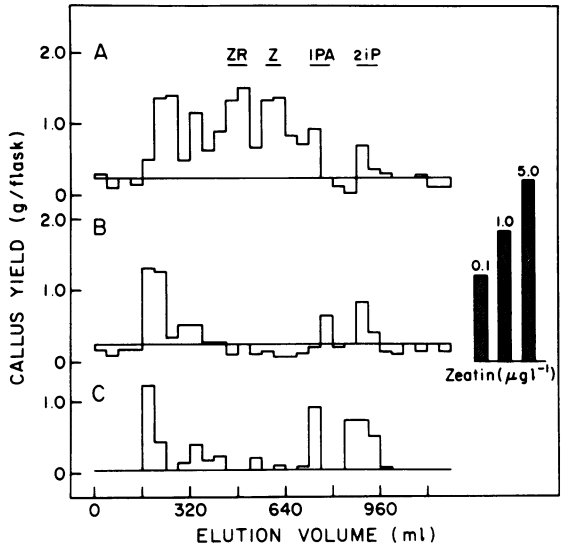

FIG. 4. Soybean bioassays after the fractionation of the equivalent of 100 ml purified root sap on a Sephadex LH-20 column with 35% ethanol. A: compounds retained on a Dowex 50 cation exchange resin; B: compounds in the effluent from Dowex 50; C: compounds retained on a Dowex 1 anion exchange resin. ZR: ribosylzeatin; Z: zeatin; IPA: isopentenyl adenosine; 2iP: isopentenyl adenine.

activity of the polar peak (elution volume 120–240 ml) detected after fractionating crude root sap on a Sephadex LH-20 column eluted with 35% ethanol. The bulk of the activity in the polar peak represented compounds that could be hydrolyzed with β-glucosidase. Subsequent treatment of the hydrolyzed compounds with $KMnO_4$ revealed that most of the glucosylated cytokinins were resistant to oxidation. Their chromatographic behavior infers that these compounds are the dihydro derivatives of zeatin and ribosylzeatin. The presence of these glucosylated cytokinins in root sap has not been reported previously. Since cytokinin glucosides are present in plant roots (13, 16), and have been detected in phloem sap (3, 10), the possibility that their presence in root sap was due to phloem transport can not be disregarded.

The phosphorylated and glucosylated cytokinins are regarded as storage forms of these hormones (6, 12) and, consequently, may serve as a translocatable source from which the active cytokinins are derived. The presence of cytokinin derivatives with a saturated side chain may confer a degree of stability to these compounds that is necessary for the specific control of metabolic processes in plants. The precise function of the individual cytokinins remains to be elucidated.

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