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Determination of the Cytokinin Complement in Healthy and Witchesbroom Malformed Proteas

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Abstract. Cytokinins from normal and witchesbroom malformed stems of proteas were determined by radioimmunoassay following sample resolution by high-performance liquid chromatography (HPLC). Material from the early stages of shoot malformation had increased cytokinin concentrations which, over time, declined to the concentrations found in normal-growing stems. The cytokinin complement of the malformed structures was different from that of normal stems. The high concentrations of isopentenyladenosine detected appear to be related to the loss of the correlative inhibition of lateral buds and the development of the witchesbroom structures and may result from localized changes in cytokinin biosynthesis and/or metabolism.

Proteas are an important family of the Fynbos Plant Kingdom and comprise approximately 1400 species (Rourke 1980). Numerous species are grown commercially and have economic importance in countries like Israel, Australia, South Africa, and the states of California and Hawaii (Ben-Jaacov 1986). Witchesbroom is a common and striking malformation and has been found in almost all species of Proteacae (Myburgh and Rust 1971) and can cause economic losses in commercial plantations as malformed structures never flower. In the case of *Protea cynaroides* certain axillary and very occasionally terminal buds of the plant appear to lose control of correlative bud inhibition and all buds sprout. As more and more buds sprout the malforming structures become smaller and more complex (Fig. 1). After about 2 months, bud inhibition is restored and the malformation enlarges resulting in classical witchesbroom structures.

The exact cause of Protea witchesbroom remains unclear (Knox-Davies et al. 1986). There is speculation that the malformation is caused by a toxin or mycoplasm injected into the plant by a microscopic mite (Myburgh and Rust 1971, Rust and Myburgh 1976), but this is not supported by any scientific evidence. Attempts to transmit witchesbroom, using *Aceria proteae* mites (the supposed vector), to healthy plants have not been successful (Dorrington 1983).

Plant growth regulators (PGR), and particularly cytokinins, have been implicated in witchesbroom malformation in higher plants (Greene 1980). The increased cytokinin concentrations in the malformed structures are usually associated with microorganisms such as *Corynebacterium fascians* in pea (Roussaux 1965) and geranium (Balàzs and Sziràki 1974), *Taphrina cerasi* in cherry (Barthe and Bulard 1974), and *Exobasidium uvae-ursi* in bearberry (Norberg 1968).

Witchesbroom in Protea differs from the typical symptoms described by Greene (1980) in that there is no thickening of the internodes, no reduction in the growth of the main shoot, and a specific microorganism has not been implicated. The possibility that cytokinins may be involved in Protea witchesbroom does not appear to have been investigated. In this study the concentrations of cytokinins in normal and witchesbroom malformed *P. cyanaroides* were determined during a 12-week growth flush.

Materials and Methods

Plant Material

Protea cyneroides from a commercial planting near Stellenbosch in the Fynbos region of South Africa was used for cytokinin analyses. Material was collected, rapidly frozen, lyophilized, milled, and stored at −80°C until analyzed. Material was collected 10, 30, and 70 days after the first harvest date and prepared in the same manner. The sample dates were according to developmental stages of the malformation (e.g., sample date 1 =

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Fig. 1. Typical witchesbroom malformation and normal growth in *Protea cynariodes* equivalent to the growth stage represented by sample 4.

the earliest stage that a clear distinction between the two types of material could be made and maximum loss of control of bud inhibition; 2 = restoration of control of bud inhibition and the start of rapid shoot growth; 3 = rapid shoot elongation; 4 = hardening off). After the last sampling the flush of shoot proliferation had completed growth and was hardening off. The equivalent normal tissue was from a growth flush which began at approximately the same time, was harvested on the same dates, and had ceased growing after about 70 days.

Cytokinin Extraction and Purification

One-gram samples from each collection date from normal and malformed shoots were extracted in 20 ml of 80% methanol in the dark for 24 h at 4°C with stirring. The samples were then centrifuged at 20,000 *g* for 10 min and 10,000 dpm [^{3}H]DHZ (38 Cu mmol^{-1} from Amersham International plc) added to the supernatant to determine recoveries. After being passed through a 0.45-μm PTFE filter, the filtrate was reduced to dryness in a Savant vacuum concentrator. The dried extract was dissolved in 10 ml of ammonium acetate buffer (0.05 M, pH 8.5). The pH of the extract was checked to ensure that it remained above 8.

A modification of the method of Sagee et al. (1986) was used for the purification of cytokinins. The extract was passed over a 6 ml polyvinylpyrrolidone (PVP Polyclar AT) column at 40 ml h^{-1}, a 6-ml Sephadex DEAE A-25 ion-exchange column, and a SepPak C_{18} cartridge connected in series. The columns and cartridges were conditioned and activated in advance. The columns were then washed with 40 ml of 0.01 M ammonium acetate (pH 8.5). The PVP column containing phenolic contaminants was discarded. The C_{18} cartridge (containing the cytokinins) was removed, flushed with 10 ml distilled water, and the cytokinins eluted with 5 ml methanol. The sample was then reduced to dryness using a Savant vacuum concentrator.

High-Performance Liquid Chromatography (HPLC) Isolation of Cytokinins

The partially purified dried extracts (1 g equivalent) were dissolved in 1 ml of 90% HPLC grade methanol and filtered through a 0.45-μm millipore HV filter and 800 μl injected into the HPLC (the remaining 200 μl was used to calculate recoveries). Separations were achieved on a Waters gradient HPLC fitted with a 10 × 300 mm μBondapack semiprep C_{18} column and a U6K variable volume injector. Retention times of zeatin (Z), zeatin riboside (ZR), dihydrozeatin (DHZ), dihydrozeatin riboside (DHZR), isopentenyladenine (2iP), and isopentenyladenosine (IPA) were determined using authentic standards. The column was eluted with a gradient of 10% methanol in 0.1 N acetic acid (with the pH adjusted to 3.5 with triethylamine) to 50% methanol over 70 min at a flow rate of 1 ml min^{-1}. The gradient was then held constant at 50% methanol for 10 min and then taken to 100% methanol over 10 min and held at 100% methanol for a further 20 min. The immunohistograms were obtained from 1-min fractions collected from 20–100 min and dried in a Savant concentrator. Fractions of 3–4 min were collected corresponding to the total individual elution times obtained from both the authentic cytokinins and the immunohistograms, dried in a Savant concentrator, and used for the determination of cytokinin concentration.

Quantitation of Cytokinins

HPLC fractions were dissolved in 1.6 ml of 100% methanol and subjected to radioimmunoassay for Z, DHZ, and isopentenyl-type cytokinins (Cutting et al. 1983, Hofman et al. 1986). Serial dilution tests were carried out on all fractions showing immunological activity. All radioimmunoassay quantitations were done in triplicate. Raw data was analyzed using an on-line computer and the Securia data reduction radioimmunoassay package (Packard Instrument Company, 1986 publication no. 169-3016). Data was then corrected for cross-reactivity and recoveries. There were two HPLC runs per individual sample per harvest date, and all quantitative results are the means of the two runs.

Results

HPLC separation of the partially purified extract from both normal and malformed Protea stems yielded three peaks with immunological activity co-chromatographing with IPA, ZR, and DHZ (Figs. 2 and 3). Serial dilutions showed the extracts to be free of interfering contaminants (Fig. 4). The duplicate results from the HPLC runs were very similar but this is expected in a method where the only error is machine or operator.

On the first sample date, the concentrations of ZR, DHZ, and IPA were as much as five times higher in witchesbroom malformed stems than in normal-flushing stem material. The concentrations then declined rapidly and after 70 days were similar to the concentrations in normal tissue (Fig. 5). Of particular interest was the very high concentrations of IPA at the first sample date.

Discussion

Although quantitative differences between the cy-

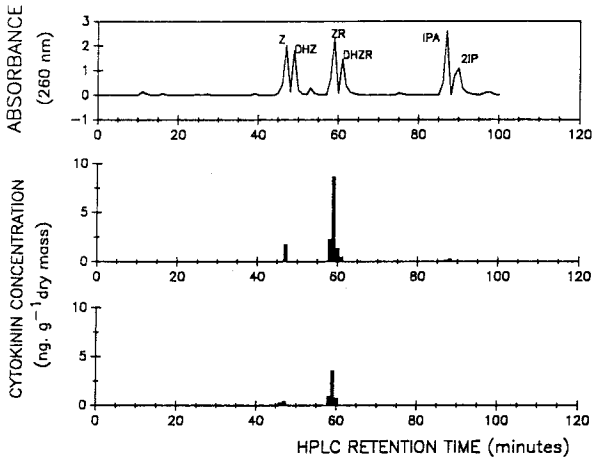

Fig. 2. HPLC immunohistograms from witchesbroom malformed and normal protea stem extracts from growth stage 1 using an anti-ZR serum. The UV trace using authentic cytokinin standards showing HPLC retention times is presented at the top of the figure.

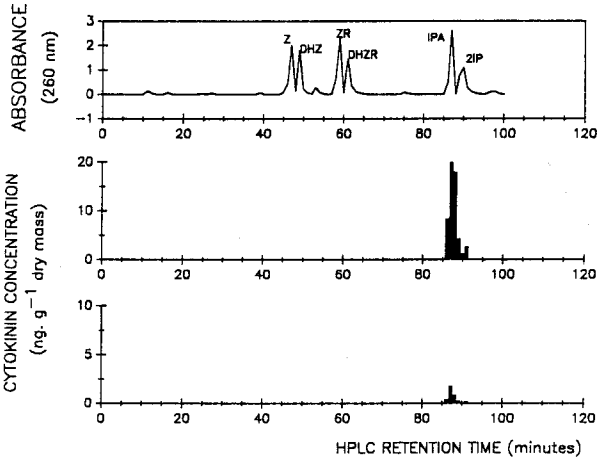

Fig. 3. HPLC immunohistograms from witchesbroom malformed and normal protea stem extracts from growth stage 1 using an anti-IPA serum. The UV trace using authentic cytokinin standards showing HPLC retention times is presented at the top of the figure.

tokinin complement of normal and malformed stems were observed, it should not be overlooked that there could be additional differences, due to the presence of endogenous cytokinins which do not cross-react with the three antisera types used in this study. It would be unrealistic to believe that the differences found in this study were due to supply from the roots, as the witchesbroom malformation is usually part of a normal stem. Rather, it appears that there is a localized loss of correlative inhibition

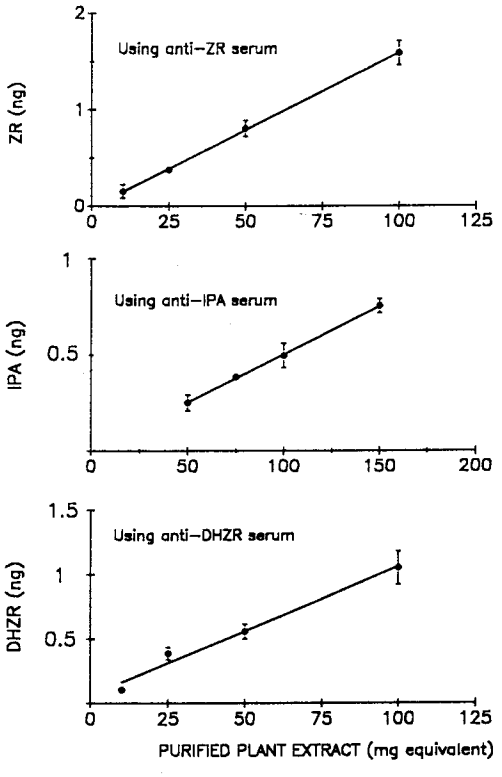

Fig. 4. Determination of possible nonspecific interference in the radioimmunoassays for the different cytokinins using serially diluted purified extracts (bars indicate mean ± SD, N = 3).

of axillary shoot growth associated with a large increase of cytokinin concentration during witchesbroom formation. Although there is still limited understanding of how the process of correlative inhibition is controlled (Hillman 1984), PGRs are thought to be involved (Martin 1987).

There are many examples of exogenous cytokinin application causing temporary release of lateral buds from inhibition in intact plants (Rubinstein and Nagao 1976, Sachs and Thimann 1967), but exogenous application of gibberellin is usually necessary for normal lateral shoot development (Ali and Fletcher 1970). In our study endogenous cytokinin concentrations were higher during the period of loss of correlative lateral bud inhibition and the restoration of apical dominance was paralleled by a decline in cytokinin concentration. Most importantly, localized application of benzyladenine (a synthetic cytokinin) causes the formation of witchesbroom structures in other species of *Proteaceae* (de Swardt 1989).

The higher concentrations of cytokinins in malformed structures could be due to a number of causes: increased export from roots, reduced localized utilization, or localized synthesis. The first

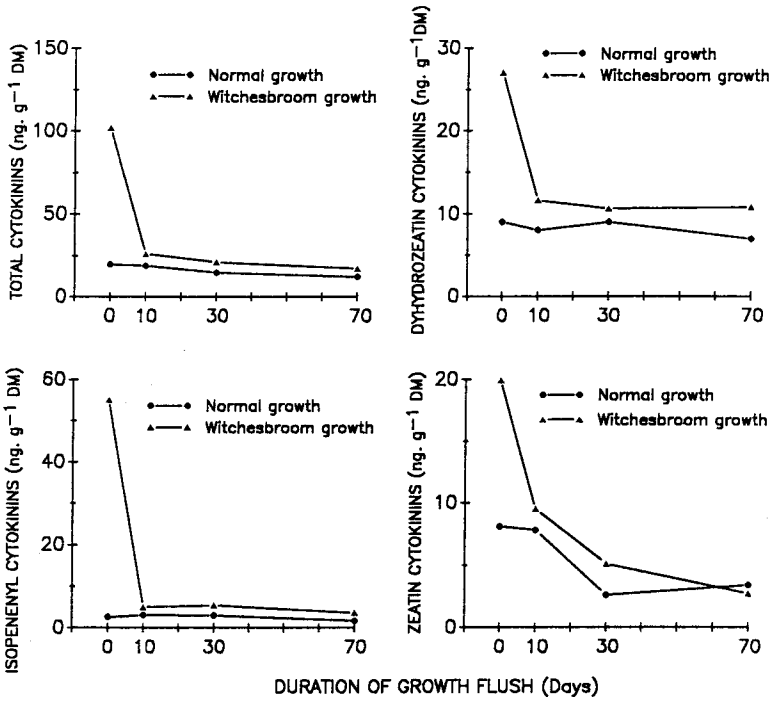

Fig. 5. Cytokinin concentrations in witchesbroom malformed and normal protea stem extracts from the four sample times spanning one growth flush. Results presented are the means of the duplicate samples after HPLC separation and have been corrected for cross-reactivity and recoveries.

possibility has little merit, since all structures on the plant should behave similarly. Clearly, this was not the case as normal growth and witchesbroom occurred on the same stem (Fig. 1). The other two options are difficult to assess as there is little knowledge of cytokinin utilization and synthesis in shoots. Increased synthesis is a possibility considering the high concentrations of particularly IPA, which could be a precursor of other cytokinins (Letham and Palni 1983). If this is the case witchesbroom would make an excellent model to study both this process and correlative inhibition.

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