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Altered Levels of Indoleacetic Acid and Cytokinin in Geranium Stems Infected with *Corynebacterium fascians*

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Tumor tissues of leafy galls of geranium (*Pelargonium zonale* cv. Irene) infected with *Corynebacterium fascians* contain decreased amount of indole-3-acetic acid (IAA) and exhibit increased cytokinin activity in the tissue culture bioassay compared with healthy stem tissues. Three active compounds, chromatographycally similar to zeatin, zeatinriboside and $N^6(\Delta^2$-isopentenyl)-adenine are present both in healthy and infected stems. Tumor tissues of leafy galls contain an additional cytokinin which is not present in traceable quantities in the extracts from healthy stems. The total cytokinin activity in extracts from leafy gall tissues was much greater than that in extracts prepared from healthy stem tissues.

The characteristic symptoms of the disease caused by *Corynebacterium fascians* are the shortening of internodes, leafy gall formation and high water content of the affected tissues. There are several reports which explain the growth abnormalities relative to hormonal imbalances (THIMANN and SACHS, 1966; KIRÁLY *et al.*, 1967; VAN ANDEL and FUCHS, 1972; SZIRÁKI *et al.*, 1975). AMES (1974) has shown that in the young seedlings of the tumor-prone amphiploid *Nicotiana suaveolens* X *Nicotiana langsdorffii* the IAA concentration decreased over 9 days. HAMILTON *et al.* (1974) established that no free IAA was present in tumor tissue of *Parthenocissus tricuspidata* and they separated several IAA metabolites by thin-layer chromatography. Two papers reported that *Corynebacterium fascians* can break down IAA (LACEY. 1948; KEMP and STEENSON, 1971). This phenomenon, namely decreasing of IAA level in the plant, may have a trigger effect in tumor formation (*cf.* AMES, 1974).

On the other hand, THIMANN and SACHS (1966) demonstrated that the diseased tissues of peas and the cultures of the infecting *Corynebacterium facians* contained chloroform-soluble cytokinins. Cytokinin measurements were based on retention of chlorophyll and on senescing of oat leaves. SEQUEIRA (1973) in his review expresses some doubt as to the nature of the presumed cytokinins because these above-mentioned bioassays are not sensitive and specific. KLÄMBT *et al.* (1966) isolated the cytokinins from cultures of *Corynebacterium fascians* and they were identified as N^6-(Δ^2-isopentenyl)-adenine, nicotinamide and 6-methylaminopurine by HELGESON and LEONARD (1966). These studies have attempted to interpret alterations in normal growth and development as the result of changes in cytokinin or in auxin levels of the infected plants.

In this paper we try to demonstrate that the extract of infected germanium stems shows altered cytokinin activity and altered level of IAA as compared to the extract of healthy stems. These hormonal changes may play an important role in leafy gall formation and development.

Materials and Methods

Plant material: Geranium (*Pelargonium zonale* (L.) Ait. cv. Irene) stems naturally infected by *Corynebacterium fasciens* (Tilford) Dowson, and uninfected healthy stems were harvested in greenhouse from several stocks. Infected stems had characteristic leafy tumors.

Cytokinin extraction and bioassay: Thirty grams of stems, healthy and in fected respectively, were washed and homogenized with 60 ml phosphate buffer pH 6.5 and extracted with 200 ml of 96% ethanol for 12 h at 4 °C. The extracts were filtered and centrifuged at 6000 *g* for 15 minutes and then the superantants were concentrated to the aqueous phase under vacuum at 35 °C. For extraction and purification of cytokinins the procedures of KIRÁLY *et al.* (1967), MILLER (1967) and VAN STADEN *et al.* (1972) were followed as we described previously (SZIRÁKI and GÁBORJÁNYI, 1974; SZIRÁKI *et al.*, 1975). Further purification of the cytokinins was carried out by paper chromatography on Whatman No. 1 paper. The solvent systems were: *t*-butanol : conc. NH_4OH : water (3 : 1 : 1, v/v/v) and 0.03 *M* borate buffer, pH 8.4. The samples to be chromatographed were dissolved in 90% ethanol and streaked onto chromatography papers. After drying, the chromatograms were divided into 10 equal stripes and tested for cytokinin activity in the soybean callus bioassay using the method of MILLER (1965) modified by KRASNUK *et al.* (1971). The migration of active compounds in stem extracts was compared with known cytokinins. The standards, namely zeatin (Z), zeatinriboside (ZR) and N^6-(Δ^2-isopentenyl)-adenine (2iP) were spotted as markers.

Twenty ml of tissue culture basal medium supplemented with agar and portions of chromatograms corresponding to specific R_F regions was added to each 50 ml Erlenmayer flask. After adjusting the pH to 5.8 with NaOH, the medium was sterilized by heating twice, at 100 °C for 45 min. A one-day-interval was allowed between heat treatments. Five callus explants were transferred to each flask. Cultures were maintained at 27 °C for 28 days and then weighed.

The experiments were repeated with similar results in five cases. Data of Tables show the results of one representative experiment. All the data are expressed on a dry weight basis, because the water content of infected stem is always higher than that of healthy stems. The dry material content of healthy stems was 19.2%, while that of the infected ones only 9.8%.

Extraction and assay of indole compounds: Healthy and infected stems were rinsed in tap water and blotted dry. Thirty grams fresh weight samples were extracted with 300 ml of 60% ethanol in a Waring Blendor for 10 min. As regards the extraction of indole compounds, we slightly modified the procedure of WIESE and

DE VAY (1970), (*cf.* SZIRÁKI *et al.*, 1975). For identification of the separated indole compounds authentic samples were spotted as markers. The markers were as follows: indole acetonitrile, indole propionic acid, indole-3-acetic acid, indole acetamide, 5-hidroxy-indole-3-yl acetic acid and tryptophane. The separated indole compounds on developed chromatograms were located and characterized by applying the SALKOWSKI's reagent and measured spectrophotometrically. The R_F regions from chromatograms corresponding to indole-3-acetic acid were eluted with ethanol and the adsorbance of eluates were measured at 280 nm using a UNICAM SP 800 spectrophotometer. The spectrophotometric measurements were carried out according to FLETCHER and ZALIK (1963). The experiments were repeated with similar results in five cases. Data are from one representative experiment, and expressed on a dry-weight-basis.

Results and Discussion

It was shown that geranium stems infected by *Corynebacterium fascians* contained lower level of indoleacetic acid than the healthy ones (Table 1). Other

Table 1

Spectrophotometric estimation of contents of indole-3-acetic acid in healthy and in *Corynebacterium fascians*-infected germanium stems on a dry weight basis*

Compound	μg/kg dry weight	
	healthy	infected
Indole-3-acetic acid	23±4	11±2

* Compounds were located by spraying a portion of the chromatographic paper (Whatman No. 1) with the SALKOWSKI's reagent. The corresponding R_F regions from unsprayed portion of the paper were eluted with ethanol and the absorbance of the eluate at 280 nm was measured spectrophotometrically and compared to known quantities of authentic indole-3-acetic acid.

authentic samples (mentioned in the Material and Methods) were also applied but we could not detected any of them. Only the IAA was located and characterized from plant extracts by applying the SALKOWSKI's reagent. The IAA content was estimated spectrophotometrically. The R_F regions from chromatograms corresponding to IAA were eluted with ethanol and the absorbance of eluates was measured at 280nm and compared to known quantities ofauthentic IAA. Decreased level of IAA in the affected tissues corresponds well with that shown by KEFFORD (1959) in the frenching disease of tobacco. Very recently AMES (1974) has shown that in a tumor-prone amphiploid hybrid tobacco the concentration of IAA decreased. This was confirmed by HAMILTON *et al.* (1974) in an other tumor

Table 2

Soybean callus bioassay of chromatograms of purified extracts of geranium stems, healthy and infected by *Corynebacterium fascians**

	Callus yield (mg/flask)									
	R_F 0.0–0.1	0.1–0.2	0.2–0.3	0.3–0.4	0.4–0.5	0.5–0.6	0.6–0.7	0.7–0.8	0.8–0.9	0.9–1.0
Healthy	166	326	390	184	144	353	391	211	234	403
Infected	977	263	421	396	255	163	318	411	180	357
Control (without extract)	220									

* Purified extracts were chromatographed on Whatman No. 1 paper in *t*-butanol–conc. NH_4OH–water (3 : 1 : 1, v/v/v). Cultures were grown on basal medium supplemented with portions of chromatograms corresponding to R_F regions. Each flask contained 5 callus explants. The experiments were repeated with similar results in five cases. Data of Table show the results of one representative experiment. The extracts were obtained from 10 g dry weight of stem/1000 ml medium.

Table 3

Soybean callus bioassay of rechromatographed extracts of chromatograms of geranium stems, healthy and infected by *Corynebacterium fascians**

	Callus yield (mg/flask)									
	R_F 0.0–0.1	0.1–0.2	0.2–0.3	0.3–0.4	0.4–0 5	0.5–0.6	0.6–0.7	0.7–0.8	0.8–0.9	0.9–1.0
Healthy	247	261	241	264	295	235	274	260	257	235
Infected	260	303	237	252	360	236	236	320	247	225
Control (without extract)	235									

* Samples were chromatographed on Whatman No. 1 paper in borate buffer (0.03 *M*, pH 8.4). Cultures were grown on basal medium supplemented with portions of chromatographed extracts in *t*-butanol–conc. NH_4OH–water (3 : 1 : 1, v/v/v) corresponding to 0.0–0.1; 0.2–0.3 and 0.6–0.8 R_F regions. The R_F regions from chromatograms were eluted with ethanol. Each flask contained 5 callus explants.

tissue. One can suppose that the decrease of IAA stems from the ability of *Corynebacterium fascians* to break down indoleacetic acid.

We found in this study that infected geranium stems contained increased level of cytokinin as compared to the healthy ones. The chromatographed extracts were tested in the soybean bioassay. The chromatograms were divided ten R_F regions and the individual regions were incorporated into the medium for bioassaying cytokinin activities. After a 28-day incubation period the fresh callus yields were weighed (Table 2). It is seen from the Table 2 that purified extracts represented a mixture of active materials. The extract purified from healthy stems shows high cytokinin activity at R_F 0.1 – 0.3; 0.5 – 0.7 and 0.9 – 1.0 regions. The extract purified from infected stem tissues exerts high cell division activity at 0.0 – 0.1; 0.2 – 0.4; 0.6 – 0.8 and 0.9 – 1.0 R_F regions. It is seen from these results that the extract of healthy plants contains active materials at three R_F regions and the extract of infected plants at four R_F regions. There is no active material in traceable quantities in the extracts of healthy stems at R_F 0.0 – 0.1. The peak migration of the active material was at R_F 0.9 – 1.0 and R_F 0.0 – 0.1 in healthy and infected stems, respectively. It is seen also from the data of Table 2 that the total cytokinin activity from infected tissues is higher than that of the healthy ones. The standards namely zeatin, zeatinriboside and isopentenyladenine were detected at R_F 0.81 – 0.86; 0.89 – 0.95 and 0.91 – 0.96, respectively. We suppose that the high cytokinin activity at R_F 0.9 – 1.0 both with healthy and infected stems originated from the isopentenyladenine content of plant material.

For characterization of the factors responsible for stimulating callus proliferation at the R_F regions 0.5 – 0.7 and 0.6 – 0.8 of healthy and infected samples respectively, and at the lower R_F regions, a second chromatographic separation was employed. The active materials from the given regions were eluted and rechromatographed in borate buffer (0.03 *M*, pH 8.4). It is seen from the Table 3 that both the extracts from healthy and infected tissues contain active material at 0.4 – 0.5 R_F region. It could be concluded that both extracts contain zeatin at these R_F regions, since the authentic zeatin was detected at R_F 0.48 – 0.53. The extracts of infected stems show high activity at R_F 0.1 – 0.2 and at 0.7 – 0.8 too. At these R_F regions the healthy stems show low, if any, activities. It is probable that the cell division activity located at R_F 0.7 – 0.8 is due to zeatinriboside (R_F 0.81 – 0.87).

One can conclude from these data that the infection of geranium by *Corynebacterium fascians* causes increasing cytokinin and decreasing auxin levels. This altered auxin–cytokinin rate could be the cause of development of leafy gall in geranium stems.

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