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Evidence for Cytokinin in Bacterial Leaf Nodules of *Psychotria punctata* (Rubiaceae)

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ABSTRACT

Cytokinin activity based on two bioassays was at least 100-fold higher in *Psychotria punctata* leaf discs with bacterial nodules than in discs without them. Nodulated discs from young leaves yielded 0.4 to 6 μg of cytokinin (zeatin equivalents) per g fresh weight of leaf tissue, whereas non-nodulated discs from the same leaves yielded 0 to 0.003 μg per g fresh weight. These estimates probably include free-base cytokinins and, if present, any nucleoside cytokinins precipitable by acidic silver nitrate. Cytokinin concentrations in *Psychotria* leaf nodules appear to be higher than normally found in green leaves of other plants. In 1-butanol-acetic acid-water (12:3:5, v/v), the one peak of activity chromatographed with an R_F similar to zeatin's, but both number and identity of the active substance(s) remain unknown. These findings suggest that a cytokinin is produced by bacteria in leaf nodules of *P. punctata* and that it is involved in the symbiosis.

Miehe (10) first proposed the idea that host plants require a growth-stimulating substance produced by their leaf nodule bacteria. Later, Humm (4) first used the term, hormone, for this hypothetical substance. Becking (1) provided evidence for cytokinin in leaf nodules of *Psychotria mucronata*, reporting that isolated nodules placed in contact with young oat leaves caused Chl retention in the contact area. He also reported that senescing host leaves remain green in the vicinity of each nodule after other portions of the leaves have become yellow. Such "green islands" are suggestive of cytokinin localization in the nodules (12). Pereira *et al.* (13) found that bacteria isolated from two leaf-nodulated species of *Ardisia* produce cytokinin(s) when grown *in vitro*.

In studies of other bacteria-angiosperm symbioses, Rodriguez-Barrueco and Bermudez De Castro (16) have detected cytokinins in the root nodules of alder, and Phillips and Torrey (14, 15) have found them in a medium in which pure cultures of *Rhizobium* had grown.

The purposes of this investigation were to determine whether leaf nodules of *Psychotria punctata* contain cytokinin(s), to measure their concentrations, and to compare these concentrations with those in other portions of the same leaves.

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MATERIALS AND METHODS

Extraction and Partial Purification of Cytokinins. Using a syringe equipped with a tooled hypodermic needle, leaf discs about 1.5 mm in diameter were punched from leaves of greenhouse-grown plants of *Psychotria punctata* Vatke (*P. bacteriophila* Val.). Equal numbers of discs with and without nodules were punched from each leaf. All discs were removed from young leaves about 2 cm in length. Only young, expanding leaves were sampled because Whitmoyer and Horner (20) had shown earlier that bacteria in these leaves are metabolically active, whereas in fully expanded leaves the bacteria are degenerating. Discs with and without nodules were collected in separate batches of 1500 each (about 0.75 g fresh weight/batch). Discs were punched out, then immediately frozen in twice distilled, absolute ethanol at about −35 C. Each batch was homogenized in a glass homogenizer for 10 min. Subsequent extraction and purification steps are summarized schematically in Figure 1. Details are as described by Short and Torrey (17, 18). Nucleotide cytokinins, if present, were probably excluded by this procedure. They are expected to remain in the aqueous phase upon partition with butanol. This phase was discarded (Fig. 1). Nucleoside cytokinins, if present, may have been excluded upon silver precipitation, but this is not fully clear. Some nucleosides may precipitate under the acidic conditions used (18). Nucleosides not excluded may have been hydrolyzed to free bases upon hot acid extraction of the silver precipitate (9). Our assays probably estimate free-base cytokinins and any nucleoside cytokinins precipitable by acidic silver nitrate.

Paper chromatograms were dried in air for 12 hr, then cut into 10 strips, each of 0.1 R_F unit width, for bioassay. In each experiment, an extraction solvent blank was processed through all steps of purification and bioassay; results were used in correcting assay values plotted in Figures 2 and 4. Each zeatin calibration value (Figs. 2 and 4) was derived by subtracting from it the result obtained on control medium without zeatin.

Radish Cotyledon Bioassay. The bioassay developed by Letham (7, 8) was used in two experiments to measure cytokinin activity on paper chromatogram strips. Thirteen cultivars of radish (*Raphanus sativa* L.) were tested on six kinetin concentrations and basal medium without kinetin. White Icicle and New Champion (Earl May Seed and Nursery Co., Shenandoah, Iowa) were slightly more responsive to kinetin than the other cultivars. When these two were tested on zeatin, White Icicle was more responsive and its response to logarithm of concentration more nearly linear than New Champion. White Icicle was used in all other work reported here.

Seeds were germinated on blotter paper (Rapaco blotting paper, Rochester Paper Co., Rochester, Mich.). To reduce variation, only seeds held by a sieve of 2 mm pore diameter and passing through a sieve of 2.6 mm pore diameter were germinated in

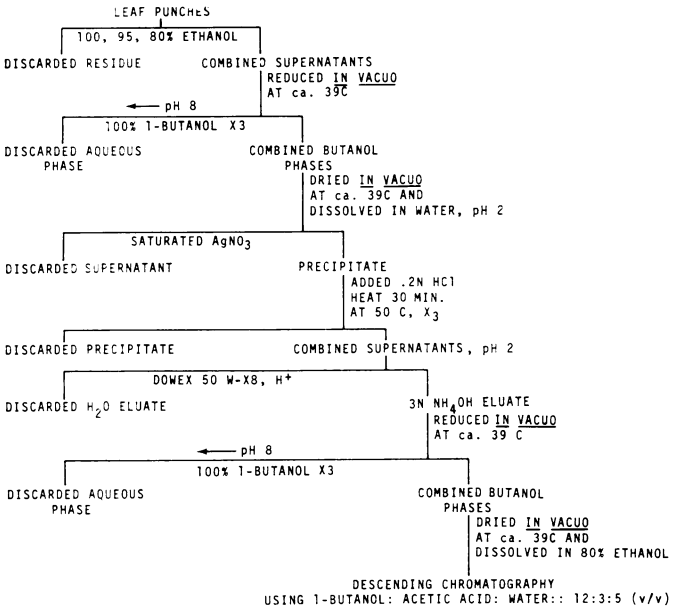

FIG. 1. Procedure of Short and Torrey (17, 18) used to extract and partially purify cytokinin from leaf discs of *Psychotria punctata*.

darkness at 24 ± 1 C for 3 days. The inner cotyledon and a hypocotyl section of 2 mm length (the latter for detecting gibberellin activity) were excised from selected seedlings and 10 of each were incubated together in one 9-cm Petri dish containing 3 ml of 2 mM potassium phosphate at pH 5.9 (7) in which was included either the chromatogram strip to be assayed or a plain strip and one of six calibrating concentrations of zeatin. Cotyledons were placed with their inner (adaxial) surfaces down against the wetted paper. Aseptic conditions were not maintained but visible contamination was not evident. Four test dishes were enclosed in a clear plastic crisper lined with wet paper. Each crisper was enclosed in a polyethylene bag. After incubation for 3 days under weak fluorescent light (40–45 ft-c) at 24 ± 1 C cotyledons were blotted and weighed. Hypocotyl section lengths were measured with a ruler.

Soybean Callus Bioassay. Seeds of soybean (*Glycine max* [L.] Merrill, cv. Acme) were surface-sterilized with a half-strength solution of commercial bleach (Miracle bleach; 5.25% [w/v] sodium hypochlorite) for 10 min. Seeds were imbibed for 15 hr in sterile distilled H_2O, then cotyledons were removed, sliced into sections, and cultured on the medium of Miller (11), which contains 5 mg/l IAA and 0.5 mg/l kinetin. After about 5 weeks, callus proliferating from the cotyledon sections was excised and cultured. Callus was subcultured at 3-week intervals and used in bioassays after each had aged 6 weeks so as to reduce cytokinin carry-over. The bioassay medium was identical except that it contained no kinetin.

Each chromatogram strip was placed in a 500-ml flask and extracted with 10 ml of 80% (v/v) ethanol. The extract was dried at room temperature. Two hundred ml of medium were prepared in the same flask containing the dried residue. After heating to dissolve the agar, 50-ml aliquots were distributed to four flasks and autoclaved. Three pieces of soybean callus inoculum were transferred to each flask. The inoculum had a mean fresh weight of 32 mg per piece with a coefficient of variation of 26%. Cultures were maintained in darkness at 24 ± 1 C. Callus fresh weights were measured after 6 weeks of culture on test medium or on medium with one of six calibrating concentrations of zeatin.

RESULTS

Radish cotyledon bioassay results (Fig. 2) show that leaf discs with nodules yield more cytokinin activity than discs without nodules. Results almost identical to those of Figure 2 were obtained in a second experiment using the same bioassay; both amount and R_F distribution of activity were similar. Based on calibration values obtained with known concentrations of zeatin (Fig. 2), discs with nodules yielded 0.5 and 0.4 μg of cytokinin per g fresh weight in the first and second experiment, respectively; discs without nodules yielded 0.003 and 0.002 μg/g fresh weight. In a third experiment (Fig. 4), the soybean callus bioassay was used. Discs with nodules yielded 6 μg of zeatin equivalents/g fresh weight; discs without nodules yielded no detectable activity (Fig. 4). Soybean calluses cultured on chromatographed material from leaf discs with nodules are shown in Figure 3.

In both experiments using radish cotyledons for cytokinin bioassay, activity was located between R_F 0.6 and 0.8 (in the portions represented by R_F 0.65 and 0.75 in Fig. 2) whether from discs with or without nodules. When crystalline zeatin was chromatographed, most of its activity was located between R_F 0.7 and 0.9,

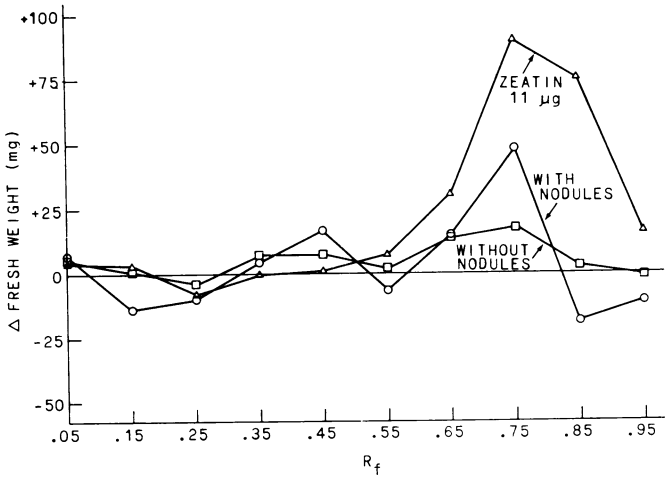

FIG. 2. Chromatographic distribution of cytokinin activity from *Psychotria* leaf discs with and without bacterial nodules after processing as in Fig. 1. The radish cotyledon bioassay was used. Each value along the ordinate is per 10 cotyledons and is the difference between the assay value for the actual extracted material and that for the extraction solvent blank. A leaf disc fresh weight of 0.25 g was represented in each ml of medium for bioassay. Calibration values for 1 nm, 10 nm, 0.1 μM, 1 μM, and 10 μM zeatin were respectively 13, 26, 38, 51, and 117 mg/10 cotyledons.

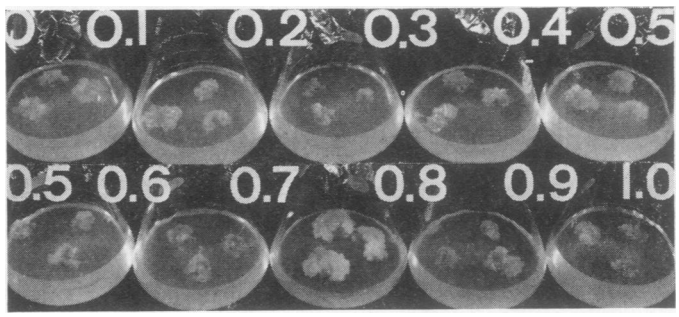

FIG. 3. Soybean callus bioassay results as a function of R_F when material extracted from *Psychotria* leaf discs with nodules was partially purified (Fig. 1). One representative culture of the four for each chromatogram strip is shown after 6 weeks' growth.

but small amounts extended to the adjacent portions with lower and higher R_F (R_F 0.65 and 0.95 in Fig. 2). In the experiment using soybean callus (Fig. 4), activity was confined between R_F 0.7 and 0.8. Zeatin's chromatographic distribution (Fig. 4) was similar to that described earlier (Fig. 2).

Because radish cotyledon expansion is promoted by high gibberellin levels as well as by low cytokinin levels (7, 8), radish hypocotyl sections, which elongate in response to low gibberellin levels, were incubated with cotyledons to check on specificity of the cotyledon response. In neither experiment was hypocotyl elongation promoted by that portion of the chromatogram (R_F 0.6–0.8) which promoted cotyledon expansion. In both experiments, hypocotyl elongation was promoted instead at R_F 0.3 to 0.5 and 0.8 to 1.0. At R_F 0.3 to 0.5 leaf discs with nodules yielded activity equivalent to 0.2 μg GA_3/g fresh weight; discs without nodules yielded activity equivalent to 2 μg GA_3/g fresh weight. At R_F 0.8 to 1 activity was equivalent to 1 μg GA_3/g fresh weight whether derived from discs with or without nodules.

Radish hypocotyl elongation was inhibited by material at R_F 0 to 0.3 whether from discs with or without nodules. In both cases, the strongest inhibition was observed at R_F 0.1 to 0.2; discs without nodules (41% inhibition) were as effective as discs with nodules (35% inhibition). Hypocotyl elongation was also inhibited at R_F 0.5 to 0.8 but only by extracts from discs with nodules. These resulted in 40, 27, and 41% inhibition at R_F 0.5 to 0.6, 0.6 to 0.7, and 0.7 to 0.8, respectively.

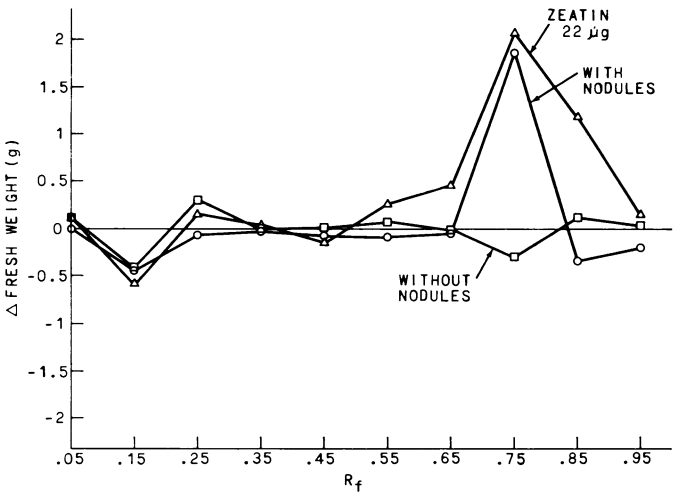

FIG. 4. Same as Fig. 2, except soybean callus bioassay results are plotted. Results are on a per culture basis; each culture consisted of three calluses. A leaf disc fresh weight of 0.375 g was represented in each 100 ml of medium for bioassay. Calibration values for 1 nM, 10 nM, 0.1 μM, 1 μM, and 10 μM zeatin were respectively 1.08, 1.46, 1.85, 2.21, and 2.84 g/culture.

DISCUSSION

Psychotria leaf discs with bacterial nodules yield more cytokinin activity than discs without nodules when extraction and partial purification (Fig. 1) are followed by either of two cytokinin bioassays (Figs. 2–4). One peak of activity, unusually narrow, was consistently observed when chromatograms were subdivided and the strips assayed. This activity, like zeatin's, was maximal in the 0.7 to 0.8 R_F strip (Figs. 2 and 4). Unlike zeatin, it appeared not to extend into the 0.8 to 0.9 R_F strip; only inhibition was observed there when material from discs with nodules was chromatographed (Figs. 2 and 4). Masking by inhibitor at this R_F may account for the narrow cytokinin profile observed (Fig. 4). The differences in chromatographic behavior between zeatin and the unknown(s) when developed in 1-butanol-acetic acid-water (12:3:5, v/v) may be, therefore, more apparent than real. No other solvent systems were tried because of the difficulty of obtaining sufficient nodulated discs. The number and identity of cytokinins remains unknown.

The possiblity that the lower level of apparent cytokinin activity from discs without nodules is due to a higher level of masking inhibitor(s) from them at the same R_F cannot be completely excluded by our findings. However, this seems unlikely based on results obtained with radish hypocotyl sections. Only material from discs with nodules was inhibitory between R_F 0.6 and 0.8.

The concentration of cytokinin in nodules of young *Psychotria* leaves appears to be high compared to that in other parts of the same leaves and in leaves of other species (3, 5, 19). In *Psychotria* nodules it appears to be at least 2 orders of magnitude higher than in young leaves of *Xanthium* (19), for example.

Whether the nodule cytokinin is produced by nodule bacteria is not known. The ability of two bacterial cultures, each derived from a different leaf-nodulated species of *Ardisia*, to produce cytokinin when grown *in vitro* (12) makes it seem likely that they synthesize cytokinin *in situ* also. However, other interpretations of our findings and those of Becking (1) merit consideration. First, nodule bacteria may induce cytokinin accumulation or synthesis by host cells within or near the nodules. If so, then the question becomes a different, but similar one: what substance or substances are the bacteria secreting to induce leaf cells to accumulate or synthesize cytokinin? Second, nodule bacteria may simply play no role in the accumulation or synthesis of cytokinin in the nodule. Based on intuition and the earlier findings discussed below, we favor the view that cytokinin found in leaf nodules is produced by nodule bacteria.

To establish that cytokinin is directly involved in a leaf nodule symbiosis, a requirement for it by host plants as well as its production by the symbionts must be shown. The only evidence for such a requirement was obtained in a tissue culture study reported from this laboratory (6). *Psychotria punctata* stem callus fails completely to grow in the absence of exogenous cytokinin and requires an unusually high cytokinin concentration for optimal growth *in vitro*. Its optimum for callus growth (about 10 mg of BA or kinetin/l) is the highest yet reported for a tissue culture. The cytokinin requirement is paralleled by a high cytokinin tolerance. Both callus growth and root formation persist on a saturated solution of BA or kinetin (6). These findings suggest that *Psychotria* tissue is unusually active in cytokinin degradation or inactivation. Excessive degradation or inactivation could result in a dependence on cytokinin-producing bacteria and lead to symbiosis. Therefore, we, like Pereira *et al.* (13) and Becking (1), find the hypothesis of a cytokinin-mediated symbiosis to be an attractive explanation of the facts known to date about leaf nodule bacteria and their host plants.

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