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Communication

Cytokinin Secretion by *Frankia* sp. HFPArI3 in Defined Medium[1]

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ABSTRACT

***Frankia* sp. HFPArI3 (host plant *Alnus rubra* Bong.) was grown in defined medium and the culture solution was analyzed for the presence of various cytokinins and related compounds. N^6-(Δ^2-isopentenyl) adenosine was the only cytokinin detected by both high performance liquid chromatography and gas chromatography-mass spectrometry, at levels of approximately 1 ng/ml culture medium.**

Frankia sp. HFPArI3 is a branched, septate, filamentous actinomycete which forms nitrogen-fixing root nodules on the woody plant host *Alnus rubra* (1, 2). The *Frankia* nodule is a perennial structure formed by repeated branchings of modified lateral roots termed nodule lobes. Plant growth regulators such as cytokinins and indole acetic acid have been found to occur at high levels in nodules of *Alnus glutinosa* (10). *Alnus* nodules, harvested at the time of leaf senescence, contained large amounts of a zeatin glucoside-like cytokinin and smaller amounts of a zeatin or zeatin riboside-like compound (4).

The reported elevated levels of cytokinins are suspected of playing a role in *Alnus* root nodule development, but it has not been determined in nodule studies, where both host and endophyte are present, whether the ultimate source of the cytokinin is *Frankia* or host tissue. The present study examines the release of free cytokinin by the *Frankia* isolate HFPArI3 in defined culture medium.

MATERIALS AND METHODS

HPLC Studies. Homogenized, washed suspensions of *Frankia* sp. HFPArI3 (3) were transferred from yeast-glucose maintenance medium to standing cultures of 10 ml defined medium lacking nitrogen in 18 × 150 mm culture tubes. The defined medium was modified from Murry *et al.* (5) and consisted of (mM): 10 KH_2PO_4/K_2HPO_4 (pH 7.0); 0.1 $MgSO_4 \cdot 7H_2O$, 0.7 $CaCl_2 \cdot H_2O$, 0.02 FeNaEDTA, 5 sodium propionate; 1 ml/l of trace element stock containing (mM) 46.2 H_3BO_3, 11.5 $MnCl_2 \cdot H_2O$, 0.765 $ZnSO_4 \cdot 7H_2O$, 0.501 $CuSO_4$, 0.10 $Na_2MoO_4 \cdot 2H_2O$, 0.0036 $CoSO_4$; 1 ml/L vitamin stock consisting of (mM) 0.005 thiamin HCl, 0.025 nicotinic acid, 0.025 pyridoxine HCl, 0.005 folic acid, 0.005 Ca pantothenate, 0.110 biotin, 0.005 riboflavin. Cultures were incubated at 28°C.

Frankia cultures were processed for HPLC weekly from 2 to 8 weeks after transfer. Samples were agitated and then centrifuged at 1800 rpm for 10 min to separate the pellet from the supernatant. The supernatant was passed through Sep-Pak C18 cartridges (*Waters Assoc., Milford, MA*). Sep-Paks were eluted with 4 ml reagent methanol, and the eluate was evaporated to dryness under reduced pressure. The remaining solid was dissolved in 100 μl 20% reagent methanol/80% reagent H_2O, of which 40 μl was applied to a 4.6 × 250 mm, 5 μm particle size, reversed-phase C18 HPLC column (*Alltech Assoc., Deerfield, IL*). The HPLC used was a Hewlett-Packard model 1084 B with model 79785 A variable wavelength detector. The column was eluted with reagent methanol and 0.2 M acetic acid buffered with triethylamine to pH 3.3 in the following step gradient: 15 to 24% methanol in 6 min, 24% methanol for 12 min, 24% to 50% methanol in 1 min, 50 to 58% methanol in 9 min, 58 to 60% methanol in 2 min, and 60 to 100% methanol in 2 min. Column temperature was maintained at 38°C. Comparison of peak areas of standards at various wavelengths showed that the optimum UV absorbance for most cytokinin standards was at 272 nm on our system.

Sample chromatograms were compared with those of known standards of adenine, adenosine, t-Z,[2] d-Z, c-Z, t-ZR, d-ZR, c-ZR, 2iP, and iPA (*Sigma Chemical Co., St. Louis, MO*). Medium which was not inoculated with *Frankia* was also prepared and subjected to HPLC to determine whether media components co-eluted with cytokinin standards.

GC-MS. *Frankia* culture solution was analyzed by GC-MS with selected ion monitoring to confirm the presence of cytokinins identified by HPLC and to determine approximate amounts present in the culture solution. *Frankia* sp. HFPArI3 was grown as described above for 27 d. Supernatant from three culture tubes was combined before being passed through 1 Sep-Pak cartridge. Three replications of three tubes each were prepared and analyzed. The centrifuged pellet of *Frankia* mycelium from each sample culture tube was lyophilized to dryness and weighed. The weights of the three tubes per replication were combined. Data are presented as ng cytokinin (iPA) per mg *Frankia* dry weight and per ml culture solution. The culture solutions were eluted through Sep-Pak cartridges as described for HPLC. Methanol eluate was evaporated to total dryness under reduced pressure, and dissolved in 25 μl of derivatizing agent bis(trimethylsilyl)

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[2] Abbreviations: t-Z, *trans*-zeatin; d-z, dihydro-zeatin; c-Z, *cis*-zeatin; t-ZR, *trans*-zeatin riboside; d-ZR, dihydro-zeatin riboside; c-ZR, *cis*-zeatin riboside; 2iP, N^6-(Δ^2-isopentenyl) adenine; iPA, N^6-(Δ^2-isopentenyl) adenosine.

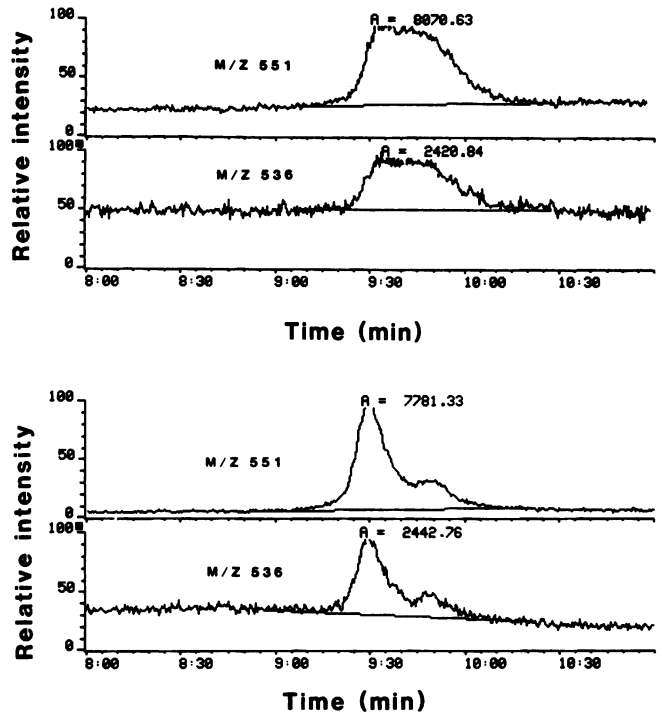

FIG. 1. GC-MS chromatogram of *Frankia* sample (upper) and 36 ng iPA standard (lower). Molecular ion ($M^{+\cdot}$) m/z 551 and $[M\text{-}CH_3]^+$ m/z 536. A = peak area.

trifluoracetamide (BSTFA) with 1% trimethylchlorsilane (TMCS) and 25 μl reagent acetonitrile. One μl of derivatized sample was applied by on-column injection to a 15 M DB-1 (methyl silicone) capillary GC column programmed from 220 to 260°C at 2°/min. Compounds were ionized by electron ionization (70 eV).

Cytokinins were detected by selected ion monitoring of the molecular ion $M^{+\cdot}$ and the $[M-15]^+$, corresponding to loss of methyl group. Quantitation was performed using external standards of 26, 20, and 36 ng for t-ZR, c-ZR, and iPA, respectively. The concentrations of the standards were based upon the estimated concentration of each of the cytokinins by HPLC. The limit of detection of the mass spectrometer was 10 pg/ml injected.

RESULTS AND DISCUSSION

HPLC chromatograms of *Frankia* culture solution showed peaks of UV absorbance at retention times corresponding only to t-ZR, c-ZR, and iPA. Samples of media alone had no interfering peaks in the windows of iPA or t-ZR, but a large peak was detected at the retention time corresponding to c-ZR. Alteration of the elution gradient by changing solvent pH or solvent ratio led to shifting of retention times of corresponding sample peaks in relation to t-ZR and c-ZR standards, although it did not completely separate c-ZR standard from the interfering medium compounds. Profile modification did not change the corresponding sample peak in relation to iPA standard. Peaks corresponding to iPA were detected by HPLC in samples from all dates tested (2- to 8-week-old cultures).

GC-MS detected 1.15 ng ± 0.09 (mean ± SE, n = 3) iPA per ml culture solution or 29.76 ng ± 2.75 (mean ± SE, n=3) iPA per mg dry weight of *Frankia* (Fig. 1). t-ZR and c-ZR were not detected at 16.7 pg/ml culture solution.

The results indicate that iPA is the single major cytokinin released into the environment by *Frankia* sp. HFPArI3, under the cultural conditions of this experiment, and as compared with standards available. The 1.15 ng per ml iPA produced by *Frankia* is similar to the 1.0 ng per ml total cytokinin production estimated for *Rhizobium* at mid-log phase (6).

Cytokinins in pea nodules were closely correlated with nodule development (8). Nodule cytokinin was highest during the period of high mitotic index and decreased during nodule senescence. During nodule development, much of the cytokinin activity was located in the white, meristematic region of the nodule, an uninfected zone, but moderately high cytokinin activities were also associated with infected tissue. Using GC-MS, Wang *et al.* (9) were unable to detect large amounts of cytokinins in pea nodules; however, they were also unable to detect cytokinins in nonnodulated roots under their experimental procedures.

We hypothesize that in the *Alnus–Frankia* symbiotic interaction, *Frankia* releases iPA into the host tissue. IPA could be converted by the host to zeatin riboside and/or zeatin. It is possible that *Frankia* alone will convert iPA to other cytokinins such as zeatin riboside under different cultural conditions or in the presence of a stimulus from *Alnus* roots. *Rhizobium* was shown to produce zeatin-like compounds in much greater quantities when exposed to *Phaseolus* root exudates (7).

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