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# In Planta Production of Indole-3-Acetic Acid by *Colletotrichum gloeosporioides* f. sp. *aeschynomene*

Rudy Maor, Sefi Haskin, Hagit Levi-Kedmi, and Amir Sharon*

*Department of Plant Sciences, Tel Aviv University, Ramat-Aviv, Tel Aviv 69978, Israel*

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**The plant pathogenic fungus *Colletotrichum gloeosporioides* f. sp. *aeschynomene* utilizes external tryptophan to produce indole-3-acetic acid (IAA) through the intermediate indole-3-acetamide (IAM). We studied the effects of tryptophan, IAA, and IAM on IAA biosynthesis in fungal axenic cultures and on in planta IAA production by the fungus. IAA biosynthesis was strictly dependent on external tryptophan and was enhanced by tryptophan and IAM. The fungus produced IAM and IAA in planta during the biotrophic and necrotrophic phases of infection. The amounts of IAA produced per fungal biomass were highest during the biotrophic phase. IAA production by this plant pathogen might be important during early stages of plant colonization.**

Many fungi can produce auxins in axenic cultures (2, 5). Most species use tryptophan to produce indole-3-acetic acid (IAA), mainly through the indole-3-pyruvic acid and tryptamine pathways (18). The physiological role of auxins in fungi is not well understood. In most studies, auxin was added to the culture medium, and the effects on fungal growth and development were determined. It is not known whether exogenous IAA and endogenous IAA cause the same phenotype. One of the roles suggested for fungus-produced IAA is to mediate fungal-plant interaction. High concentrations of IAA can inhibit the hypersensitive response (7, 12) and may suppress expression of plant defense genes (16, 20). Furthermore, the disease symptoms caused by some fungal pathogens are similar to the symptoms caused by high IAA concentrations and include epinasty, tumor formation, and plant organ deformation (18). However, direct evidence for the involvement of IAA in plant diseases is available only for plant pathogenic bacteria (9, 10, 20). Fungal production of IAA in planta has never been shown.

Most plant pathogenic bacteria produce IAA through indole-3-acetamide (IAM). In this pathway, tryptophan is converted to IAM by tryptophan-2-monooxigenase (*iaaM*), and IAM is metabolized to IAA by IAM-hydrolase (*iaaH*). The capacity to produce IAA through the IAM pathway is associated with bacterial virulence and with gall formation (8, 9, 15, 19). Cohen et al. (4) transformed two strains of *Fusarium* pathogenic to *Orobanche* by using the bacterial *iaaM* and *iaaH* genes and showed that the transgenic isolates produced increased levels of IAA in axenic cultures. The transgenic strains also were more virulent on *Orobanche*, but whether the increased virulence was due to higher levels of IAA is not known, since IAA production in planta was not measured.

*C. gloeosporioides* f. sp. *aeschynomene* produces large amounts of IAA in axenic cultures through the bacterial IAM pathway (13). Whether the fungus produces IAA during plant colonization is unknown. Our objectives in the current work were to study the effect of tryptophan, IAA, and IAM on IAA biosynthesis and to determine whether *C. gloeosporioides* f. sp. *aeschynomene* produces IAA in planta.

**Fungi and plants.** We used *C. gloeosporioides* f. sp. *aeschynomene* isolate 3.1.3. The fungus was maintained on Emerson's YpSs (EMS) medium as previously described (14). *Aeschynomene virginica* (northern jointvetch) plants were used for plant assays. Plants were grown in 10-cm-diameter pots in a greenhouse at 28°C. Daylight was supplemented by fluorescent tubes with a 16-h light photoperiod.

**Plant inoculation.** Conidia were obtained from 5-day-old cultures grown on EMS medium as previously described (14). *A. virginica* plants 10 to 14 days old were inoculated by spraying to runoff with 5 ml of conidial suspension. After inoculation, the plants were placed in closed plastic cylinders with 100% humidity for the first 24 h, the cover was then removed, and the plants were maintained until the end of the experiment in the greenhouse at 28°C. Normally, small necrotic lesions appear after 3 days on the infected leaves, and plant mortality occurs after 5 to 10 days (14, 17).

**Effect of tryptophan, IAA, and IAM on production of IAM and IAA by the fungus.** *C. gloeosporioides* f. sp. *aeschynomene* conidia were germinated in pea extract as previously described (14). After 4 h, the germinated conidia were centrifuged (5,000 $\times g$ for 10 min), washed with sterile water, and resuspended in 4 ml of Czapek-Dox (CD) medium (14). One milliliter of the suspension was dispensed into each of four 250-ml flasks containing 50 ml of CD medium, and the flasks were incubated on an orbital shaker (190 rpm) at 28°C for 4 days. Tryptophan, IAM, or IAA was added to a final concentration of 2 mM, and the cultures were grown for another 4 h. The control treatment contained an equivalent volume of ethanol. After 4 h of growth in the presence of the metabolites, the mycelium was harvested by filtration and ground in liquid $N_2$.

Production of IAM and IAA by protein extracts was performed according to Clark et al. (3). The ground mycelium was suspended in 11.8 ml of protein extraction buffer: 10 ml of Tricine buffer (pH 8.3), 13.5 mM cetyltrimethylammonium bromide (CTAB), and 200 μl of protease inhibitor cocktail (Sigma, St. Louis, Mo.). The tubes were incubated for 10 min at 28°C with shaking at 150 rpm and centrifuged (7,600 $\times g$ for 10 min), the supernatant was filtered, and total protein was

* Corresponding author. Mailing address: Department of Plant Sciences, Tel Aviv University, Tel Aviv 69978, Israel. Phone: 972 3 640 6741. Fax: 972 3 6405498. E-mail: amirsh@tauex.tau.ac.il.

determined (1). The extract was separated into two tubes, tryptophan or IAM (1 mM) was added, and the tubes were incubated for 16 h at 28°C with shaking at 70 rpm in the dark.

**Indole extraction and analysis.** Protein extracts were acidified to pH 3.0 with 2.5 ml of 1 M HCl. For extraction of plant indoles, whole plants (upper part without roots) were ground in 10 ml of 1 mM HCl. The extracts were transferred to 50-ml tubes, ethyl acetate (10 ml) was added, the mixture was vortexed and centrifuged (7,600 $\times$ $g$ for 10 min), and the tubes were placed at $-20$°C overnight. The ethyl acetate upper layer was filtered, evaporated, and redissolved in 0.5 ml of methanol. For plant extracts, the concentrated filtrate was loaded on silica gel 60, $F_{254}$ thin-layer chromatography (TLC) plates (Merck, Whitehouse Station, N.J.), and chromatograms were obtained with chloroform-methanol-water (84:15:1). Indoles were identified on plates illuminated by 312-nm UV light, and bands with an $R_f$ similar to those produced by IAM and IAA standards were marked and scraped off the plate and extracted with 1 ml of ethyl acetate for 1 min. The mixture was centrifuged at 14,000 $\times$ $g$ for 10 min, and the supernatant was transferred to a new tube. The solvent was dried under vacuum, and the dry extract was redissolved in 50 μl of methanol. This procedure recovered 95% of the indoles present in plant extract before TLC separation.

Indole levels were determined by high-performance liquid chromatography (HPLC). Samples were injected with a Waters 717 Plus auto sampler (Waters, Millipore, Billerica, Mass.) and loaded onto a $C_{18}$ reverse-phase column (Inertsil ODS3, 5 μM; 150 by 4.6; MetaChem Technologies Inc, Torrance, Calif.) equipped with a 7.6- by 4.6-mm guard column (ALT96041; Alltech, Deerfield, Ill.). Indoles were separated on a 3 to 100% (vol/vol) methanol-water gradient containing 1% acetic acid over 25 min with a flow rate of 1 ml/min. Under these conditions, the retention times of IAM and IAA were 11.6 and 15.5 min, respectively. All experiments included three replications and were repeated several times.

**Production of IAA in axenic cultures.** We tested the effects of tryptophan, IAM, and IAA on IAA accumulation in axenic cultures. Exogenous tryptophan and IAM caused 5- and 4.5-fold increases in IAM production, respectively (Table 1). Tryptophan also increased IAA biosynthesis 2.7-fold, while exogenous IAM did not significantly affect IAA production. No IAM or IAA was produced in a buffer solution containing either tryptophan or IAM without the protein extract. The significant increase in IAA and IAM production rates induced by tryptophan and IAM indicates that IAA biosynthesis is enhanced by substrate availability. IAA did not cause any changes in indole production rate. These results contrast with those from IAM-producing bacteria, in which both IAM and IAA inhibit the enzymes involved in IAA biosynthesis (6). Our findings show that a relatively high basal level of the IAA-synthesizing enzymes is maintained in the fungus even in the absence of tryptophan, but endogenous tryptophan does not support IAA production. The enzymatic activity is enhanced and IAA can be produced when external tryptophan becomes available to the fungus. These results imply that for in planta production of IAA, the fungus must be able to utilize plant tryptophan.

**In planta production of IAM and IAA by *C. gloeosporioides* f. sp. *aeschynomene*.** Protein and indoles were extracted from *C. gloeosporioides* f. sp. *aeschynomene*-infected plants 48 and 96 h postinoculation. Production of IAM and IAA by the protein extracts and the levels of IAM and IAA in the infected and mock-inoculated plants were determined. Activities of both tryptophan monooxygenase and IAM hydrolase were detected in the protein extracts from infected plants at 48 and 96 h (Table 2). Much more IAA and IAM were produced by protein extracts from 96-h-infected plants than those from 48-h-infected plants. Neither IAM nor IAA was produced by the protein extracts from the mock-inoculated plants. Indole extracts from the infected plants contained IAM and IAA at both time points (Table 2). The highest levels of IAM were detected in plants 96 h after inoculation, while similar levels of IAA were measured at 48 and 96 h postinoculation. The ratio between IAM and IAA was higher at 96 h (2.6:1) than at 48 h (1.3:1). Neither IAA nor IAM was detected in mock-inoculated plants at either of the time points.

These results show that *C. gloeosporioides* f. sp. *aeschynomene*-infected plants contain elevated levels of IAA compared to the levels in uninfected plants. The detection of tryptophan monooxygenase and IAM hydrolase enzymatic activities as well as the detection of IAM in infected plants showed that *C. gloeosporioides* f. sp. *aeschynomene* not only expresses the IAA biosynthesis genes but also produces IAM and IAA in planta, since *A. virginica* plants do not possess these enzymatic activities and are unable to produce IAM.

TABLE 1. Effect of IAA, IAM, and tryptophan on the production rates of IAA and IAM by fungal protein extracts

| Inducing compound (2 mM)[a] | Production (nmol/mg of protein/h)[b] | | |
|---|---|---|---|
| | Tryptophan[c] | | IAM[d] |
| | IAM | IAA | |
| Control | 0.33a | 0.31a | 0.88a |
| Tryptophan | 1.68b | 0.66b | 2.38b |
| IAM | 1.50b | 1.00c | 1.25a |
| IAA | 0.43a | 0.31a | 0.69a |

[a] Fungal cultures were grown for 4 days, 2 mM the inducing compound was added, and proteins were extracted after 4 h. Enzymatic activities of tryptophan monooxygenase and IAM hydrolase were determined by 16 h of incubation with either tryptophan or IAM.
[b] Data are the average of three replications. Treatments in the same column that are labeled with different letters are statistically different ($P < 0.05$) according to the Tukey multiple comparison test.
[c] The levels of both IAM and IAA (in nanomoles per milligram of protein per hour) were determined using tryptophan as a precursor.
[d] Only the level of IAA was measured, using IAM as a precursor.

TABLE 2. Production rates of IAA by infected-plant protein extracts and accumulation of IAM and IAA in *C. gloeosporioides* f. sp. *aeschynomene*-infected plants

| Time (h) | Amt (nmol/plant) of[a,b]: | | IAA production by protein extracts (nmol/mg of protein/h)[b,c] |
|---|---|---|---|
| | IAM | IAA | |
| 48 | 300a | 230a | 1.4a |
| 96 | 530a | 205a | 13.8b |

[a] Indoles were extracted from whole plants 48 and 96 h postinoculation, and the amounts of IAM and IAA per plant were determined.
[b] Treatments in the same column that are labeled with different letters are statistically different ($P < 0.05$) according to the Tukey multiple comparison test.
[c] Proteins were extracted from infected plants, and the IAA production rates were determined after a 16-h incubation with IAM.

Only small amounts of mycelium invade a few host cells at 48 h, as part of the biotrophic infection stage (11, 17). Much more mycelium is produced after 96 h, at which time, the pathogen invades the plant cells and causes massive tissue damage. The increase in IAM hydrolase activity at 96 h probably reflects the larger fungal biomass. Despite the significant increase in fungal biomass at 96 h, IAM and IAA levels were similar at both time points. Thus, much more IAA was produced per unit of fungal biomass at the biotrophic phase. These results are consistent with the hypothesis that the limiting factor in IAA biosynthesis is the amount of available tryptophan. As shown earlier, IAA biosynthesis requires external tryptophan. Therefore, tryptophan must be exported from the plant into the fungus during the biotrophic stage to support IAA biosynthesis, since the fungus does not invade the host cells at this early stage and does not have direct access to the plant cellular metabolic pool. Whether tryptophan is actively transported by, for example, general or tryptophan-specific transporters or by simple diffusion needs to be further investigated.

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