Isolation, Purification, and Biological Activity of an Inhibitor from *Septoria tritici*

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ABSTRACT

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Methyl-3-indole carboxylate (3-indole carboxylic acid methyl ester [ICA.Me]) was identified in liquid cultures of *Septoria tritici*. For physiological studies, ICA.Me was synthesized from commercial 3-indole carboxylic acid (ICA) by methylation with diazomethane. Inhibition of growth of the melanin-producing isolate ISR398 by ICA.Me on thin-layer chromatography (TLC) plates was recorded at a concentration of 0.02 mg/ml. Inhibition by 3-indole acetic acid (IAA) was recorded at a concentration of 25-fold, whereas no inhibition was recorded for ICA at a concentration range of 0.02–0.5 mg/ml. Growth of *S. tritici* on liquid media was differentially affected by different concentrations of ICA.Me. Complete inhibition of *S. tritici* isolates ISR398 and ISR8036 was recorded at 0.08 mg/ml of ICA.Me, with only partial inhibition of ISR7901 at that concentration. The regulatory effects of these indole compounds were tested on wheat coleoptile and cucumber hypocotyl segments. IAA markedly increased growth of both coleoptiles and hypocotyls at 0.5–0.7 μg/ml. Application of ICA and ICA.Me caused a slight decrease in hypocotyl growth and a slight increase in coleoptile growth at concentrations ranging from 0.4 to 20.0 μg/ml. It is possible that ICA.Me may be involved in regulating the pathogen's population on the phylloplane and within wheat tissue.

Interactions between organisms are mediated in many instances by secreted inhibitory products of diverse chemical nature (22). Some of the inhibitory compounds are involved in antibiosis, namely, in which a metabolite produced by one organism has an harmful effect on the other (21). There are numerous examples for antibiosis in fungi for both nonvolatile and volatile compounds (3,4,5,19,23). In addition, fungi may produce compounds that are self-inhibitors (autoinhibition), mainly involved in suppressing germination and production of appressoria (1). In *Colletotrichum gloeosporioides*, the self-inhibitor was chemically defined as dihydro-5-hydroxy-5(8-pentyl-2-oxocanyl)-acetyl-2(3H)-furanone (gloeosporone) (16). In *Dictyostelium discoideum*, the self-inhibitor was identified as 2-dimethylamine-6-oxypurineriboside (2).

Inhibitory substances are specific to species from which they have been identified and lack chemical similarities (1), with the exception of different cinnamic acid derivatives found in several rust fungi (1,9,12,18,20). Methyl 3,4-dimethoxycinnamate secreted from urediospores of *Puccinia graminis* was reported to self-inhibit their germination (18). The same compound was involved in self-inhibition of *Puccinia arachidis* (9). As is the case with antibiosis, the chemical natures of the identified self-inhibitors are rather diverse (1).

In some cases, chemicals secreted by fungi have synergistic effects on germination and growth, as reported in the rusts for 6-methyl-5-hepten-2-one and n-nonanal (10,11). Stimulating substances in high concentration might cause self-inhibition of germination. N-nonanal was found as the most effective substance in these cases (10). It also has been suggested that substances exhibiting inhibitory effects are involved in regulating population structure (16).

The purpose of this study was to elucidate the nature of chemical product(s) secreted by *Septoria tritici* in vitro and its possible involvement in regulating fungal growth.

MATERIALS AND METHODS

Organic extraction. *S. tritici* cultures were grown for 4–5 days in liquid medium consisting of malt (Difco Laboratories, Detroit, MI) (4%), yeast extract (Institut Pasteur, France) (1%), and sucrose (4%). Cultures consisting mainly of conidia were centrifuged for 15 min at 5,860 *g*. The supernatant was extracted three times in ethyl acetate (200 ml of solvent per 100 ml of culture filtrate). The fractions were evaporated to dryness under vacuum and weighed. Extractions were made from 10–30 L of growth medium in which conidia concentration was about 10^7 spores per milliliter.

Rapid extraction. *S. tritici* isolates were grown for 4–5 days in shake liquid malt media, after which cultures were passed through Whatman No. 1 filter paper. One liter of culture filtrate, free of conidia, was adjusted to pH 2.5 with HCl and was passed through a 1.0- × 0.5-cm packed C_{18} reverse-phase column (Sep-Pak C_{18} cartridge, Millipore Waters Associates, Milford, MA) that fitted to a syringe. The column was rinsed with distilled water followed by 10 ml of ethyl acetate. After the passage of the culture filtrate, the column was washed with 30 ml of distilled water and with 30 ml of ethyl acetate. The water and ethyl acetate fractions were evaporated in a vacuum and placed on silica gel thin-layer chromatography (TLC) plates (Kiesel-gel $60F_{254}$, E. Merck, Germany). The TLC plates contained 1:2 (v/v) petroleum ether/ethyl acetate mixture. The plates were dried, placed under an 254- or 366-nm ultraviolat lamp, and fluorescing spots were marked. The plates were then stained with vanillin (2% vanillin, 10% H_2SO_4, 70% methanol in H_2O). Comparable plates not treated with vanillin were used for biological assays.

Biological assay using TLC. The silica gel TLC plates were sprayed with a 4-day-old suspension of 1×10^7 conidia per milliliter of the melanin-producing isolate ISR398 (ATCC148507) of *S. tritici* (17). The plates were incubated in a moist chamber under light for 48 h at 25 C.

Purification of antifungal fractions. Fractions exhibiting fungal growth inhibition were scrapped from the vanillin-treated TLC plates, suspended in ethyl acetate, and shaken for 24 h. These fractions were applied on TLC plates and tested for growth inhibition of isolate ISR398. Weighed fractions were applied to a 6- × 2-cm silica gel column (Kiesel-gel 60H, E. Merck) connected to a vacuum system. The column was washed with double its volume of petroleum ether (40–60 C, BDH, U.K.). The adsorbed fractions were eluted with changing ratios of solvent mixture of

increasing polarity of ethyl acetate in petroleum ether, whereas at the end it was washed with methanol. Fractions expressing biological activity were repeatedly passed through fresh Kieselgel 60H columns and tested for purity under ultraviolet spectrums of 254 and 366 nm and vanillin staining. Parallel-unstained TLC plates were tested for biological activity after each purification. Purified fractions showing inhibition of *S. tritici* were analyzed by nuclear magnetic resonance (NMR) and mass spectra techniques. ^{1}H NMR spectra were recorded on a Bruker AM-360 spectrometer (Bruker, Karlsrough, Germany) operating at 360 MHz, and a Fenningan 4921 mass spectrometer (Quardopole-type Instrument) (Fenningan-Mat, San Jose, CA) was employed.

Methylation of indole-3-carboxylic acid (ICA). A methyl group was added to ICA to yield indole-3-carboxylic acid methyl ester (ICA.Me) or methyl-3-indole carboyxlate. The methylation was conducted as follows: solution A, 5 ml of water in which 1 g of KOH was dissolved was added to 16 ml of cold diethylene glycol monomethyl ether; solution B, in a separate Erlenmeyer flask, 3 g of diazald (N-methyl-N-nitroso-p-toluenesulfonamide, Aldrich, Milwaukee, WI) was dissolved in 25 ml of diethyl ether and was added to solution A. The upper fraction in solution B was moved to a distillation apparatus from which diazomethane and purified ether were collected. One gram of commercial ICA (Sigma, St. Louis, MO) was dissolved separately in 10 ml of 50% methanol and 50% diethyl ether to which the distilled diazomethane was added until the solution attained a yellow color. The organic solvents were evaporated, and fractions were analyzed on TLC (compared to *S. tritici* product) and by NMR.

Effect of indole compounds on fungal growth. *S. tritici* isolates, ISR398 (isolated from the bread wheat cv. Hazera 84, Israel), ISR7901 (from cv. Shafir, Israel), and ISR8036 (from cv. Shafir, Israel) were grown in liquid malt medium in Erlenmeyer flasks fitted with side-attached test tubes. Concentrations of 0.04, 0.06, and 0.08 mg/ml of indole-3-acetic acid (IAA) (Sigma), ICA, and ICA.Me were added separately to the medium after autoclaving. The inoculated preparations were shaken at 20 C on a rotary shaker. The growth of *S. tritici* was assessed daily by measuring turbidity with a Klett-Summerson photoelectric colorimeter (Klett Mfg., NY) with a red filter.

Effect of indole compounds on cucumber and wheat. Seedlings of cucumber (*Cucumis sativus* L.) cv. Delila (Hazera Seed Co., Israel) and of wheat (*Triticum aestivum* L.) cv. Shafir (Son64A/Tzpp//Nai60/3/FA) (Hazera Seed Co.) were grown at 25 C in the dark for 5 days. Washed and weighed 1-cm-long cucumber hypocotyl segments were placed in test tubes (10 segments per test tube with three tubes per treatment) in a solution consisting of 3 ml of phosphate buffer (pH 7.0), 0.1 mM $CaCl_2$, 2 mM KCl, and 10 mg/L of chloramphenicol succinate. Concentrations of 0.5, 1.0, 4.0, 7.0, 10.0, and 20.0 μg/ml of IAA, ICA, and ICA.Me were added to test tubes shaken for 5 h in the dark at 25 C. At the end of the incubation, the hypocotyl segments were dried and weighed. Wheat coleoptile segments pruned to a length of 1.2 cm were treated similarly to the cucumber hypocotyl segments. The effect of IAA, ICA, and ICA.Me on growth of cucumber hypocotyl and wheat coleoptile segments was calculated as follows: percentage of growth = (Δweight/weight at t_0) $\times$ 100, in which t_0 = weight at time zero.

RESULTS

Purification of inhibitory fraction(s). Inhibition of growth of the melanin-producing isolate ISR398 was observed in spots on TLC plates onto which culture filtrates of ISR398 and ISR8036 were applied. Clear zones were observed on the TLC plates on which growth was inhibited, in contrast to the dark conidial-mycelial mat on which no inhibition occurred. Consecutive purification of the extract through preparative chromatography on a silica gel chromotography column with petroleum ether and ethyl acetate yielded a fraction (no. 8) at 22.5% ethyl acetate in petroleum ether that inhibited ISR398 on TLC plates. The inhibitory compound was identified as ICA.Me. The ^{1}H NMR spectrum of the purified compound in fraction no. 8 identified by the 360-MHz Bruker is as follows:

$\delta(CDCl_3$; TMS = 0):
8.20 br (NH,1H), 7.86 m (1H), 7.74 d (J = 8.5 Hz, 1H), 7.35 m (1H), 7.20 m (2H), 3.54s($0CH_3$) ppm.

The mass spectrum (m/e EIMS) is as follows: 175 (87%, M^+), 144 (100%, M-0Me), 116 (14%, M-CO_2Me), 89 (5%). The mass and NMR spectra of the extracted purified compound were identical to the synthetic ICA.Me synthesized from ICA according to the methylation procedure described in the Materials and Methods section. The extracted and synthetic fractions were also identical on TLC plates and differed from IAA and from ICA (Fig. 1). *S. tritici* isolates ISR398, ISR7901, and ISR8036 all yielded ICA.Me after rapid extraction directly from the medium through a C_{18} reverse-phase column (Sep-Pak) and thereafter were chromatographed on TLC silica gel plates and analyzed by NMR.

Biological activity. The organic extraction of purified ICA.Me yielded 0.04 μg/ml of medium on which ISR398 grew. At high concentrations, the inhibitor was not soluble in water and not diffusible in aqeous solution. ICA.Me inhibited *S. tritici* growth on TLC plates (soluble in ethyl acetate or methanol) at a concentration of 0.02 mg/ml, whereas IAA inhibited growth at 0.5 mg/ml. No inhibition was recorded for ICA at a range of 0.02–0.5 mg/ml. IAA and ICA had no inhibitory effect on growth of ISR398 in liquid culture at concentrations ranging from 0.04 to 0.08 mg/ml for 96 h, whereas ICA.Me expressed inhibitory effects already at 0.04 mg/ml, with complete inhibition of growth at 0.07 mg/ml (Fig. 2). Similar inhibitory effects were recorded for isolate ISR8036. The same concentrations of ICA.Me had less inhibitory effects on isolate ISR7901. The magnitude of growth of the three isolates at the range of 0–0.04 mg/ml of the indole compounds was rather different. Application of IAA, ICA, and ICA.Me to wheat seedling leaves at 1, 2, and 5 mg/ml did not result in necrosis or chlorosis for a 10-day period. Leaves treated with dimethyl sulfoxide showed a chlorotic response, whereas methanol had no visible effect.

Growth of cucumber hypocotyls and wheat coleoptiles was affected by IAA at concentrations of 0.5–0.7 μg/ml (Fig. 3). A decrease in growth was recorded at concentrations greater than

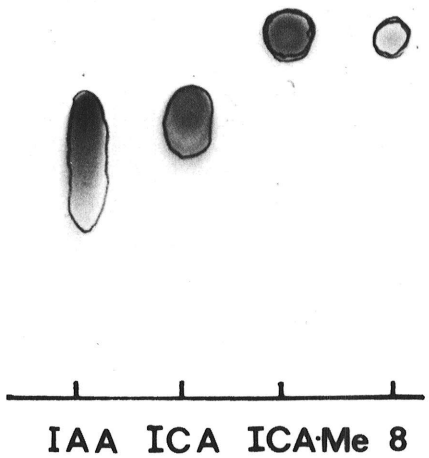

Fig. 1. A comparative chromatography on silica gel thin-layer chromatography (TLC) plates between synthetic 3-indole carboxylic acid methyl ester (ICA.Me), 3-indole carboxylic acid (ICA), and 3-indole acetic acid (IAA), and a purified fraction (no. 8) produced by *Septoria tritici* in liquid medium.

10.0 μg/ml. Both ICA and ICA.Me expressed no increase in cucumber hypocotyl growth and a slight decrease in wheat coleoptile growth. The concurrent addition of both IAA and ICA.Me (10 μg/ml each) to cucumber hypocotyls and wheat coleoptiles resulted in a 50% increase in growth from that of IAA alone.

DISCUSSION

A compound inhibiting growth of *S. tritici* in vitro was extracted by organic extraction (ethyl acetate) and by direct extraction (Sep-Pak column) from culture medium on which *S. tritici* isolates grew. The compound identified as ICA.Me, has not been reported previously in fungi or in plants of higher taxonomic order. ICA has been identified in higher plant extracts on the basis of physicochemical evidence (6,7,21). Products of the oxidative degradation of IAA containing indole and methyl groups (3,3-bisindolylmethane [3IM] and 3-methyleneoxindole [MeOx]) were identified in *Puccinia graminis* f. sp. *tritici* and were capable of stimulating mycelial growth and transition from germ tube to mycelial growth. The transition phenomenon was antagonized by IAA and indole-3-aldehyde (13). Allen (1) attributed the inhibitory activity of the bean rust inhibitor methyl 3,4-dimethoxy-cinnamate to the ester group position and the 3-methoxy group. It was suggested that IAA and related indole compounds including ICA have a regulatory effect on fungal development on the plant (13). ICA was reported to be a metabolite of tryptophol in lower and higher plants in the metabolic pathway of IAA from tryptophan (15). In the *Lupinus luteus-Rhizobium lupini* system, ICA is produced in young roots by enzymatic catabolism of IAA (6). Iskric (14) claimed that in autotrophic plants ICA is a product of IAA catabolism; however, parasitic plants (e.g., *Orobanche*) are capable of producing ICA and related compounds not via IAA catabolism. The enzymic oxidative degradation of IAA by peroxidase constitutes an important regulatory mechanism in the control of auxin concentration (7).

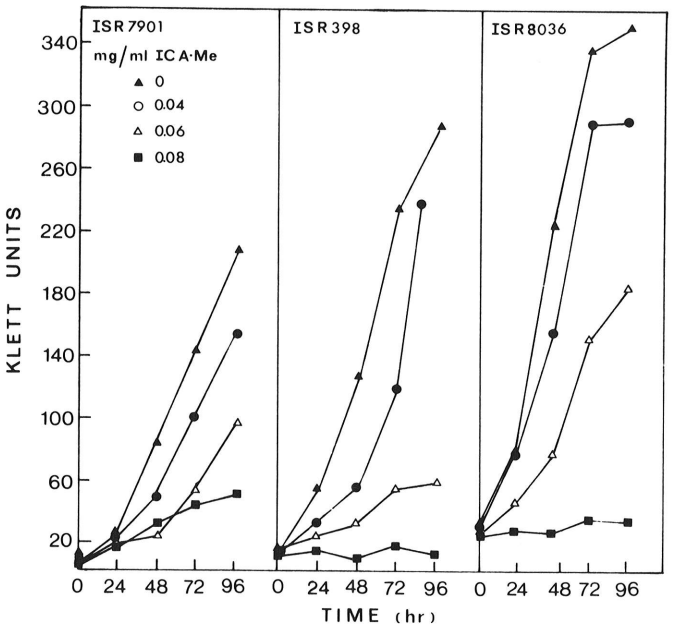

Fig. 2. The effect of 3-indole carboxylic acid methyl ester (ICA.Me) concentration on growth (absorbance units) over time of three Israeli *Septoria tritici* isolates in liquid malt medium.

The indolic compounds are active in balancing the IAA level in the host-parasite systems. The indolic compounds may compete for receptor sites of IAA and thus may be involved in regulating IAA level in plant tissue (24). The metabolic pathway(s) by which ICA.Me is produced in *S. tritici* cultures is not yet clear. Both IAA and ICA were not inhibitory to *S. tritici* in the same concentrations as ICA.Me, yet, whether IAA or ICA were also excreted by *S. tritici* was not ascertained because the bioassay was capable of detecting only inhibitory compounds. Inhibition of growth of *S. tritici* on TLC plates by ICA.Me occurred at 0.02 mg/ml, which is many folds greater than the amount produced by the fungus in liquid medium. It is likely that both the TLC bioassay and the detection of inhibition of growth by absorbance are not sensitive enough to associate the in vitro inhibition to that possibly occuring on the phylloplane and in the wheat tissue. Yet, regulatory effects on growth of cucumber hypocotyl and wheat coleoptile segments by IAA, ICA, and ICA.Me were recorded at concentrations less than 1.0 μg/ml.

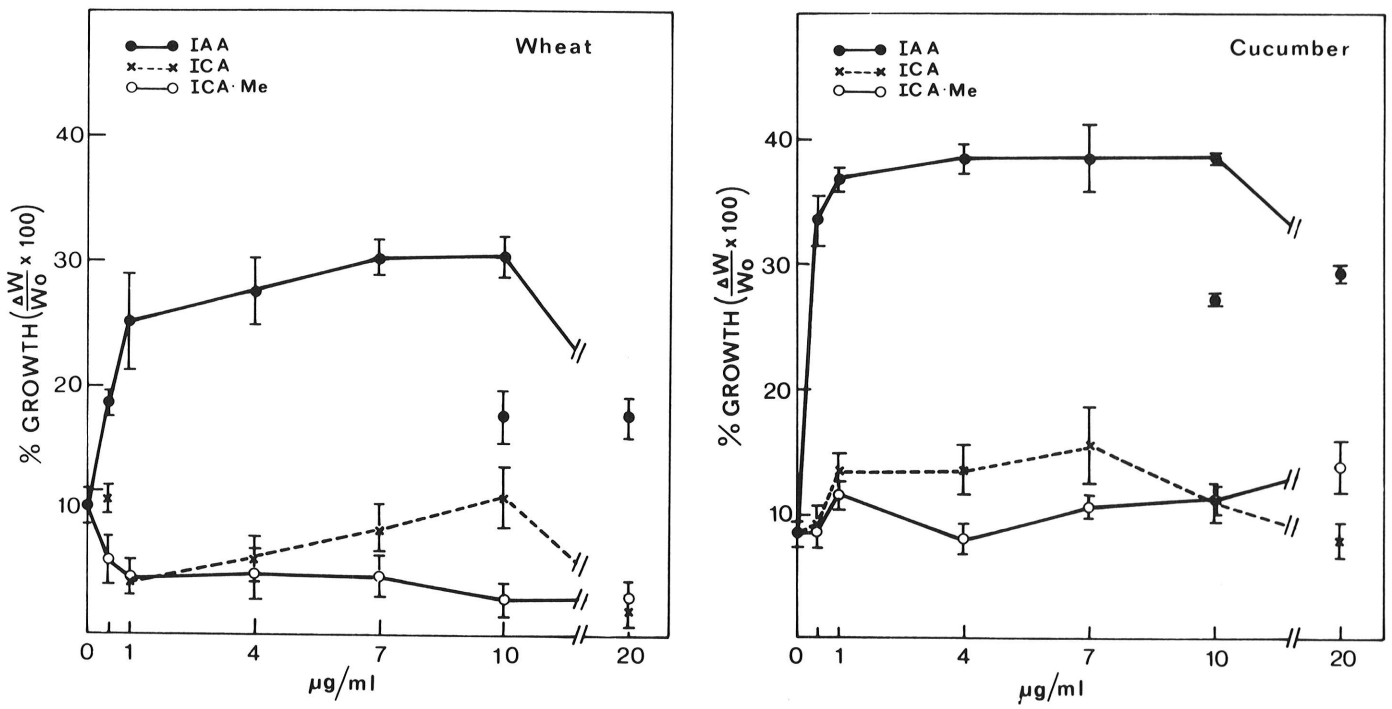

Fig. 3. The effect of different concentrations of 3-indole acetic acid (IAA), 3-indole carboxylic acid (ICA), and 3-indole carboxylic acid methyl ester (ICA.Me) on growth of cucumber hypocotyl and wheat coleoptile segments.

S. tritici isolates when mixed were reported to suppress pycnidia coverage on wheat leaf seedlings (25). It is possible that the production of inhibitors in vitro may be related to the pycnidia suppression phenomenon. The involvement of ICA.Me in inhibition of fungal development in vitro and in vivo can be ascertained by producing specific antibodies to ICA.Me. The differential concentration response (production/inhibition) is hypothesized to serve as a regulating mechanism (directly or via IAA metabolism) in the host and thus affects fungal development. Such mechanisms may contribute to the regulation of fungal population on the phylloplane.

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