APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1985, p. 660–663
0099-2240/85/030660-04$02.00/0

Vol. 49, No. 3

Role of Penicillic Acid in the Phytotoxicity of *Penicillium cyclopium* and *Penicillium canescens* to the Germination of Corn Seeds

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Received 19 July 1984/Accepted 19 December 1984

***Penicillium cyclopium* and *Penicillium canescens* cultures inhibited the germination of corn. The phytotoxic compound was isolated by solvent extraction and thin-layer chromatography on silica gel. The phytotoxin was identified as penicillic acid by mass spectrometry, nuclear magnetic resonance, and infrared spectroscopy. Gas-liquid chromatography on a capillary glass column separated the two epimeric forms of penicillic acid. The maximum production of penicillic acid was obtained with *P. cyclopium* cultures grown at 25°C. The phytotoxicity of penicillic acid was manifested by its ability to alter the germination of corn. The percent inhibition of germination was directly proportional to the logarithm of the penicillic acid concentration. Growth of the main root was reduced 50% by concentrations of 500 μg/ml.**

Penicillium cyclopium and *Penicillium canescens* are the main contaminant fungi detected in seeds harvested in the northwest of France. Such contaminants are fearsome, since they affect the seeds before harvest time and may find optimal developing conditions when the seeds are stored, leading to alteration of the germination quality of these seeds.

We have investigated here whether the altered germinative qualities of the seeds were solely due to the growth of contaminating fungi or to the related action of phytotoxic metabolites.

P. cyclopium may produce more than 20 toxic metabolites, among which were identified ochratoxin A (5), penicillic acid (2), cyclopiazonic acid (11), and penitrems A and B (12). *P. canescens* was less widely studied but produced citrinin (8) and canescin (3). Of these mycotoxins, penicillic acid was reported to inhibit the growth of young plant roots of rice (25) and of oat seedlings by lessening their respiration (1). It was also reported to inhibit urease (23) and RNase (26) activity.

We demonstrated the phytotoxic effect of the culture filtrates of *P. cyclopium* and *P. canescens* on the germination of corn seeds and isolated and identified penicillic acid as a responsible agent. Such inhibitory properties of penicillic acid have not previously been reported.

The temperature for optimal production of penicillic acid was determined, and sound corn seeds were infected with penicillic acid-producing and non-producing *P. cyclopium* strains. Detection and dosage evidence for its major involvement in the alteration of germinating potencies.

(The work reported here is contained in the thesis of J. Keromnes in the University of Brest [1982] in partial fulfillment of the requirements for the degree of Doctorat de Spécialité.)

MATERIALS AND METHODS

Reagents. The silylation reagents, *N,O*-bis trimethylsilyltrifluoracetamide and trimethylchlorosilane were obtained from Merck, Darmstadt, Federal Republic of Germany. Stationary-phase SE 30 was obtained from Supelco, Rungis, France. Penicillic acid was a gift from Le Bars (INRA, Toulouse, France). Thin-layer chromatography (250-nm) silica gel plates were used for purification (Merck GF 254) and for fluorodensitometric assays (Merck G 60). All solvents were reagent grade.

Biological materials. The corn seeds (Limagrain LG 9 variety) which germinated with a 97 to 99% yield were surface sterilized with diluted sodium hypochlorite (22). *P. cyclopium* (strains Pc.11 and Pc.4) and *P. canescens* were isolated from spoiled seeds and maintained on a nutritive medium (malt extract, 20 g/liter; agar, 15 g/liter).

Phytotoxin production. A corn extract was prepared by grinding 150 g of corn for 30 s in a Waring blender, boiling it for 1 h in 1 liter of water, and filtering it through cloth. The filtrate was made up to 2 liters, poured into Roux bottles (100 ml), and sterilized at 120°C for 20 min. The sterile liquid medium was inoculated with one fragment of 7-day-old mycelium and incubated at 25°C in the dark.

Phytotoxins were also produced on a solid substrate by inoculating 1 kg of hypochlorite-cleaned corn seeds (22) with one of the isolated strains (pulverization of 10^7 spores in 1 ml of sterile water) and incubating them for 45 days in a closed chamber at 18°C with a relative humidity of 85% (4).

Preparation of culture extracts. Filtrates (100 ml) of liquid cultures were adjusted to the pH (5.4) of the control, sterilized by membrane filtration (Millipore HAWP04700; pore size, 0.22 μm), and extracted three times with 100 ml of ethyl acetate. The combined solvent fractions were dried with anhydrous $MgSO_4$ and concentrated in a rotary vacuum still.

Purification of extracts. The dried extract was dissolved into 5 ml of ethyl acetate. One 500-μl portion was streaked on a thin-layer chromatography plate which was developed in chloroform-methanol (95:5, vol/vol). UV-absorbing bands (254 and 366 nm) were eluted with ethyl acetate and dried before testing for phytotoxicity in a germination assay. Rechromatography of the topic eluate with chloroform-ethyl acetate-formic acid (60:40:1, vol/vol/vol) allowed further purification before crystallization in ethyl acetate-hexane.

Chemical identification procedures. Trimethylsilylether derivatives (TMS) were prepared by adding 100 μl of an *N,O*-bis-trimethylsilyltrifluoracetamide-trimethylchlorosilane (99:1, vol/vol) mixture to 100 μg of the dried compound and incubating the solution for 3 h at room temperature. Injection of solid trimethylsilylether derivatives into a gas chromatograph (Girdel, Paris, France) was done with a Ros

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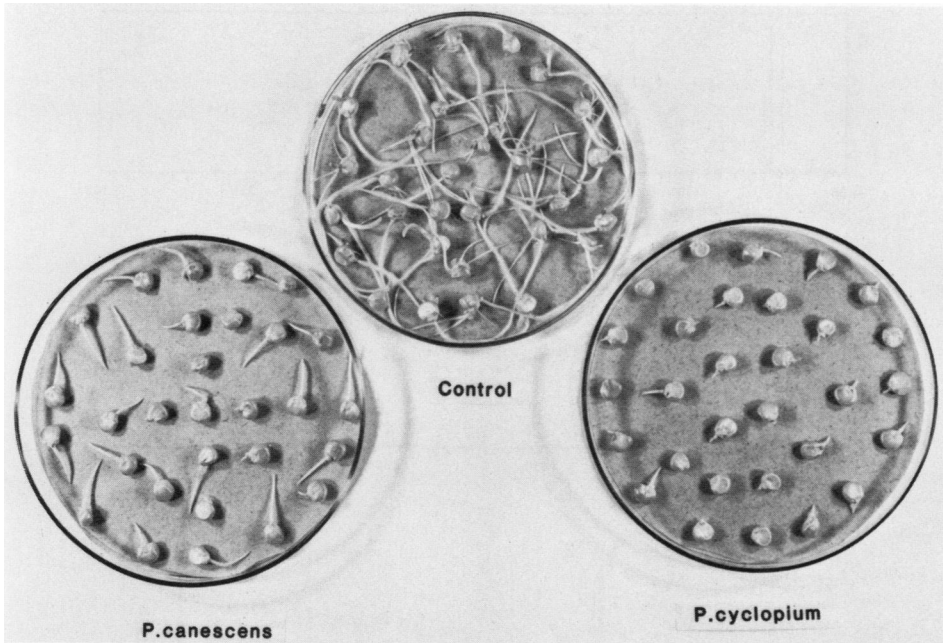

FIG. 1. Phytotoxicity in filtrates from 11-day-old cultures of *P. cyclopium* and *P. canescens* incubated at 25°C: effect on the germination of the corn seeds.

solid injector (24). The glass capillary column (47 m, 0.28 mm internal diameter) coated with an SE 30 phase by the static method was directly coupled with a quadrupole mass spectrometer (Ribermag Nermag, Rueil, France). The carrier gas was helium (1 ml/min), and the oven temperature was 135°C. The energy of the electrons in the mass spectrometer source was set at 70 eV.

Proton nuclear magnetic resonance spectra were taken in deuterated chloroform with a JEOL X100 nuclear magnetic resonance spectrometer and with tetramethylsilane as internal reference standard.

Infrared spectra (Pye Unicam SP 2000 IR spectroscope) of the compound were made up from a suspension in Nujol.

Assay of penicillic acid. A solution of penicillic acid in ethanol (0.8 mg/ml) was standardized spectrophotometrically (ε_{224} = 10,445 in ethanol [13]).

Thin-layer chromatography plates containing the unknown and standards were exposed for 10 min to 20% NH_4OH fumes, and the fluorescent derivative was measured by fluorodensitometry (Vernon fluorodensitometer equipped with a 366-nm filter for excitation and an emission filter transparent to blue). The penicillic acid quantities were computed in micrograms per milliliter of culture or micrograms per gram of corn seeds.

Germination tests. Germination tests were used for the measurements of phytotoxicity. Crude or purified dried extracts were dissolved in distilled water and sterilized by filtration. Hypochlorite-cleaned seeds (22) were placed on sterile blotting paper wetted by the rest solutions in petri dishes.

For culture filtrates, 25 seeds and 25 ml of filtrate were placed in the petri dish, whereas for extracts, 10 seeds and 10 ml of the sterilized extract were placed in the petri dish. After 4 days of incubation at 25°C, germination occurred. Inhibition of root growth, defined as the decrease of root length relative to that of the controls, was expressed as a percentage.

Seeds experimentally contaminated with the fungi (strains Pc.11 and Pc.4) were incubated for 45 days and then subjected to a "cold test germination" (100 seeds were incubated for 10 days at 8°C and then for 4 days at 23 to 24°C in sand wetted to 70% of its full water retention). The germination potencies were the percentage of germinated seeds growing into normal young plants.

RESULTS

Identification of penicillic acid in *P. canescens* and *P. cyclopium* cultures. Filtrates from 2-week-old cultures of *P. canescens* and *P. cyclopium* inhibited the germination of corn seeds, but *P. cyclopium* filtrates were much more toxic than those from *P. canescens* (Fig. 1). Both crude and purified extracts from the cultures were inhibitory.

The unknown toxic compound was separated on GF 254 plates and was located under 254-nm UV light as a dark spot. The R_f values in chloroform-methanol (95:5, vol/vol) and in chloroform-ethyl acetate-formic acid (60:40:1, vol/vol/vol) were 0.37 and 0.35, respectively. One single phytotoxic molecule was isolated from cultures and crystallized as white needles from mixtures of ethyl acetate and hexane. Both species yielded the same compound, which had the same melting point (82°C) and color reaction with ammonia as authentic penicillic acid. When the trimethylsilylether derivative was analyzed by gas chromatography on capillary glass columns, two peaks were obtained at retention times of 11.6 and 11.8 min (Fig. 2), which corresponded with those obtained with authentic penicillic acid which contained the two epimeric forms. The molecular ion of the epimeric forms measured by mass spectrometry was at the 242 mass (Fig. 2). Furthermore, spectra (OH and CH at 3,330 cm^{-1}, COOH at 1,740 cm^{-1}, CO at 1,645 cm^{-1}) and nuclear magnetic resonance spectra (2 H at 5.58 ppm, 2 H at 5.2 and 5.1 ppm, 3 H at 3.90 ppm, and 3 H at 1.75 ppm) were identical with those of authentic penicillic acid. These results agree with reported data on mass spectrometry (15), nuclear magnetic resonance (13), and infrared spectra (7, 14) of penicillic acid.

Dose-related effects of penicillic acid. Cultures of *P. cyclopium* grown in liquid medium at 25°C contained larger quantities of penicillic acid and showed more intense phyto-

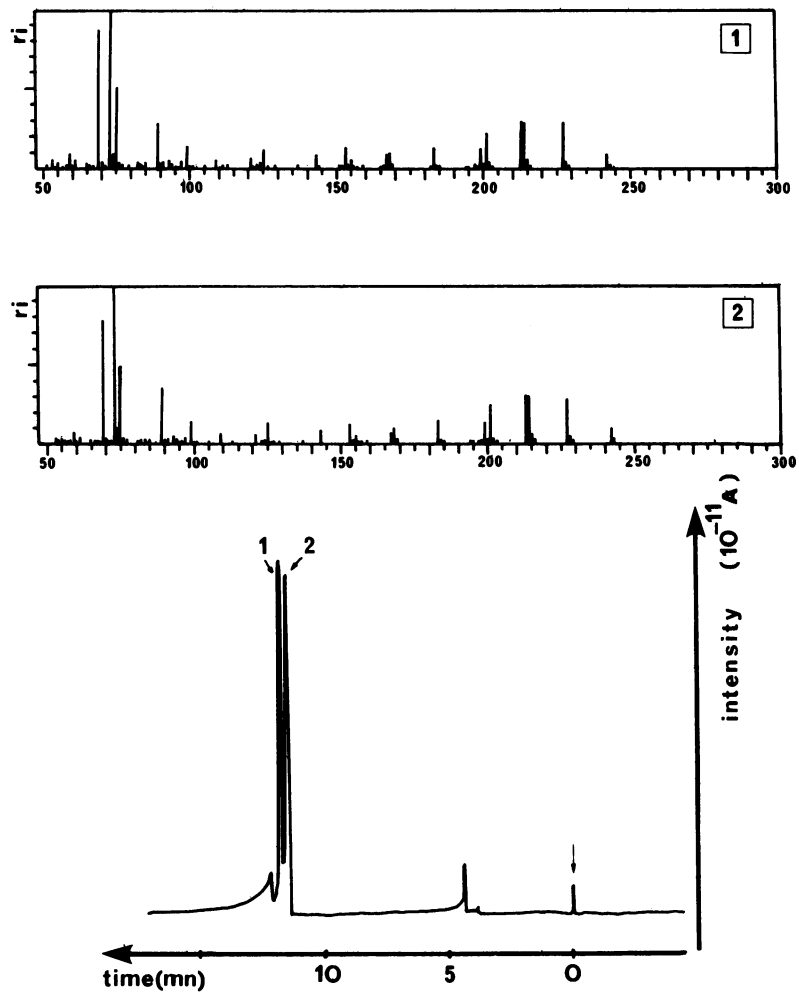

FIG. 2. Gas chromatogram and mass spectra of the epimeric forms (1) and (2) of the penicillic acid trimethylsilylether derivative.

toxic effects than cultures grown at 20 or 27°C. The maximal toxic effect was observed at day 11 of culture and resulted in roots of young plants 70% shorter than those of the controls. In that case, the penicillic acid concentration in the culture medium was 1,050 μg/ml. Within the range of concentrations used here, the percent inhibition of root growth was directly proportional to the logarithm of the penicillic acid concentration (Fig. 3). The root growth was decreased 50% by a solution containing 500 μg of penicillic acid per ml. A concentration of 115 μg/ml was required to detect a significant ($P < 0.05$) inhibition. In the case of culture filtrates, similar results were observed.

***P. cyclopium* and germination of stored corn seeds.** A penicillic acid-producing strain (Pc.11) and a non-producing strain (Pc.4) were distributed on fresh normal seeds and stored for 45 days. The cold test germination gave 50 and 68% normal young plants with Pc.11 and Pc.4, respectively. The control sample gave 96% normal young plants. The concentration of penicillic acid was measured and found to be 1.18 μg/g in the lot infected with the Pc.11 strain. No measurable quantity of penicillic acid (detection limit, 0.05 μg/g) was found in the Pc.4-infected lot or in the control lot.

DISCUSSION

Penicillic acid was identified in cultures of *P. cyclopium* and *P. canescens* isolated from infected corn seeds. Two tautomeric forms have been described previously (2), the cyclic form occurring in acidic or neutral medium and the linear form occurring after the opening of the ring in alkaline medium. The in vivo pH range favors the cyclic form (Fig. 4). The existence of epimeric OH groups has not been shown for the cyclic form, although such epimers would be expected to form during the cyclization of the molecule. The

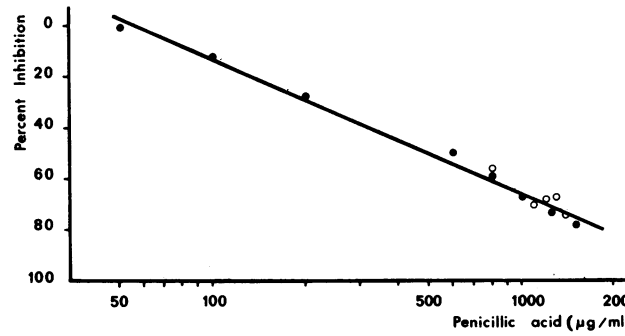

FIG. 3. Inhibitory effect of standard penicillic acid (●) and culture filtrates containing penicillic acid (○) on the growth of the main root of corn seeds.

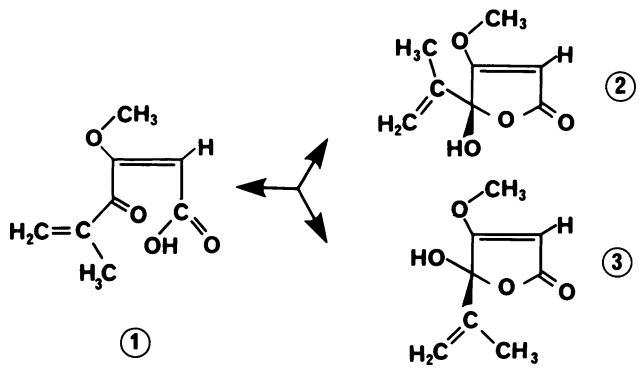

FIG. 4. Formulae of the isomeric forms of penicillic acid: linear structure (1) and cyclic epimeric forms (2, 3).

small difference in polarity expected between the epimers explains why they were not detected in authentic penicillic acid until high-resolution gas chromatography with capillary glass columns (95,000 theoretical plates). Support for this hypothesis was given by the identical areas of the chromatographic peaks and the identical mass spectra of the two peaks. Direct chemical proof awaits their separation by a preparative technique. The occurrence of such epimers calls for possible differences in their biological activity and this determination also awaits isolation of larger quantities.

Penicillic acid is mostly known for its toxicity to animals (1, 10, 19, 21). Our work showed that penicillic acid is also toxic to corn seeds during germination. Penicillic acid should now be considered as a phytotoxin as are aflatoxin B1, rubratoxin B, and zearalenone (4). The toxicity of penicillic acid is about 5% that of aflatoxin B1, which gave 50% inhibition of seed germination at the dose of 25 μg/ml, whereas 500 μg of penicillic acid per ml were necessary for a 50% decrease of the main root length. Nevertheless, penicillic acid occurs in such high quantities (up to 2% dry weight) in infected corn (15) that it must be considered seriously. Several other factors increased the seriousness of penicillic acid as phytotoxin. About 50% of *P. cyclopium* strains produce penicillic acid (16), and its biosynthesis is higher on broken kernels (17). Penicillic acid is stable in substrates with low water content (18) and accumulates at the low temperatures (6) of typical storage conditions.

P. cyclopium and *P. canescens* strains produced phytotoxic penicillic acid in culture and in corn seeds, but some evidence for other phytotoxic metabolites was gathered. Specifically, strains Pc.4 of *P. cyclopium* which did not produce penicillic acid caused an appreciable decrease in seed germination. It would seem that the phytotoxicity of mycotoxin producing organisms warrants further investigation.

ACKNOWLEDGMENTS

We thank J. Pelhate (Laboratoire d'Agrobiologie, Faculté des Sciences, Brest, France) for the *Penicillium* strains and J. Le Bars (Laboratoire de Pharmacologie et Toxicologie, INRA, Toulouse, France) for the sample of penicillic acid and help in its quantitation.

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