Neth. J. Pl. Path. 81 (1975) 38-41

Sensitivity of Ustilago maydis to pyrazophos and one of its conversion products, and failure to induce resistance with UV-treatment

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Accepted 15 August 1974

Abstract

2-Hydroxy-5-methyl-6-ethoxycarbonylpyrazolo(1,5-a)pyrimidine (PP), a conversion product of pyrazophos, shows considerable toxicity to *Ustilago maydis*, when administered to this fungus in a solution at pH < 5. Evidence was obtained that *U. maydis* may convert pyrazophos into PP, and that the latter compound is the toxic principle responsible for the action of pyrazophos.

By UV-irradiation of sporidia no PP-resistant mutants were obtained. This does not support the hypothesis that this toxicant acts by specific inhibition of one enzyme system.

Introduction

The organophosphorus fungicide pyrazophos, 0,0-diethyl 0-(6-ethoxycarbonyl-5-methylpyrazolo(1,5-a)pyrimid-2-yl)phosphorothioate, has shown systemic, preventive and curative action against a number of powdery mildew fungi. Recent studies by De Waard (1974) demonstrated also sensitivity of *Piricularia oryzae* and *Colletotrichum lindemuthianum*, whereas all other fungi tested were found rather insensitive. This author showed that in sensitive fungi, but not in insensitive fungi, pyrazophos is metabolically converted into two fungitoxic products, namely the phosphate analogue of pyrazophos (PO-pyrazophos) and 2-hydroxy-5-methyl-6-ethoxycarbonylpyrazolo (1,5-a)pyrimidine (PP). The latter compound, which is believed to be the fungitoxic principle, inhibits growth of *P. oryzae* at a pH < 5. The selective action of pyrazophos seems to depend on ability for uptake of this compound and conversion into PP. As this compound inhibits not only respiration but also protein- and nucleic acid synthesis, no definite conclusion was reached by De Waard (1974) whether the toxicant acts specifically on some respiratory process or by non-specific interaction with cell constituents.

The purpose of our work was to investigate whether, after UV-irradiation, resistance to PP might develop in fungi. Because sensitivity to site-specific fungicides can usually be drastically modified by single gene mutations (Dekker, 1969, review 1971) this might provide evidence in support of or against site-specific action by PP. For these experiments *Ustilago maydis* was chosen, as this organism is very suitable for

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genetic analysis, and some information on its respiratory system and on mutations affecting sensitivity to respiratory inhibitors was available (Georgopoulos and Sisler, 1970; Georgopoulos et al., 1972). As smut fungi had not been included in the tests of De Waard, it was necessary to study first the possible toxicity of pyrazophos and of PP to *U. maydis*.

Materials and methods

A strain of *U. maydis*, known to carry no other mutations except for methionine and pantothenic acid requirements, was taken from the collection of the first author.

Samples of pyrazophos and PP, kindly provided by Dr M. A. de Waard (Laboratory of Phytopathology, Agricultural University, Wageningen), were originally obtained from Farbwerke Hoechst A. G., Frankfurt, W-Germany. A medium, described by Georgopoulos et al. (1972), was used for toxicity tests; this medium was buffered at pH 4.5 and appropriately supplemented. The chemicals were added to the media from stock solutions in acetone and the concentration of the solvent adjusted to 1.0% (v/v) in treated and control samples.

Inoculum, approximately 0.1 mg of dry weight per ml of liquid medium, was taken from mid- to late log-phase liquid cultures. Toxicity was expressed as the percentage reduction of colony formation on agar plates or the percentage inhibition of dry weight increase in liquid cultures, as determined by measuring the optical densities, and converting these to mg of dry weight per ml by reference to a standard curve.

In order to obtain fungicide-resistant mutants, sporidia were irradiated with UV until 70% of them were killed, and plated out on a fungicide containing medium.

Fungitoxicity

In vitro. Pyrazophos at concentrations up to 10^{-3} M did not reduce the number of colonies obtained from sporidia of *U. maydis* on agar medium. The size of the colonies, however, was significantly reduced even at a concentration of 10^{-5} M. At concentrations above 4×10^{-5} M precipitation of pyrazophos was obvious in the agar medium but as colonies developed clear zones were observed, indicating that the fungicide was either taken up by the sporidia or converted to a more soluble form. In similar experiments PP significantly reduced the number of colonies at a concentration of 10^{-4} M and completely prevented colony formation at 5×10^{-4} M. Transferring agar blocks, 0.5 mm diam., from medium, containing 10^{-3} M PP to fungicide free plates, 40 hours after the sporidia were plated, did not result in the formation of colonies. This indicates that most cells were killed by this treatment.

In liquid cultures, pyrazophos did not inhibit initial growth at 3.2×10^{-5} M (maximum solubility). After a 16 h incubation period a 32% inhibition of dry weight increase was caused. PP, however, caused a growth inhibition of *U. maydis* directly from the beginning of the experiment (Fig. 1). From these data the ED_{50} and ED_{95} values of PP for growth during the first 16 hours of incubation were calculated at 1.3×10^{-4} and 4×10^{-4} M, respectively.

In vivo. An experiment on control of corn smut by PP was carried out according to the method described by Georgopoulos et al. (1974). Seedlings were inoculated with

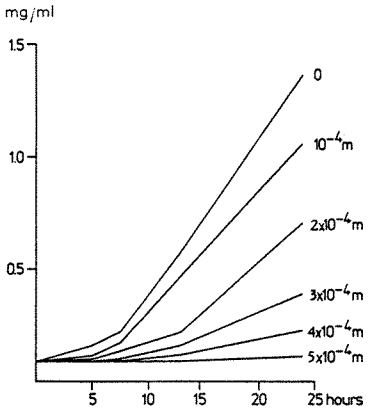

Fig. 1. Effect of PP on dry weight increase of *Ustilago maydis* in liquid culture.

Fig. 1. Effect van PP op de toename van het drooggewicht van Ustilago maydis in vloeibaar medium.

sporidia of compatible strains, suspended in a 4×10^{-4} M solution of pyrazophos or PP with 1% acetone. For comparison carboxin was included, 200 mg active material being applied per kg soil; in another treatment pyrazophos, 750 mg active material per kg soil, was applied in the same way. Contrary to carboxin, which gave 100% control, no disease reduction was obtained with pyrazophos or PP.

Tolerance to PP

Approximately 1.5×10^8 survivors of UV-irradiation were plated out on medium containing 10^{-3} M of PP. Although this is only twice the minimal concentration preventing colony formation of untreated cells, no growth was observed during 3 weeks, which indicates that no resistant mutants resulted from the UV-irradiation. When survivors of UV-irradiation were plated out on medium containing 6×10^{-4} PP, which is only slightly more than the minimal inhibitory concentration, colonies started to become visible after one week, and their numbers increased with prolonged incubation. It was found, however, that such late formation of colonies on this concentration of PP was sometimes possible even with non-irradiated cells, particularly when some cell aggregates were present in the suspension to be plated.

Toxicity tests in liquid cultures with a number of colonies, developing after UV-irradiation on medium containing 6×10^{-4} PP, did not reveal the presence of strains resistant to PP. It is worth noting that during these tests *U. maydis* showed a profound ability to be 'trained' to grow in media containing higher PP concentrations. After a few transfers to fungicide free medium, however, this PP-tolerance disappeared again. De Waard (pers. comm.) has a similar experience with *P. oryzae*. This phenomenon suggests that some inducible system might become operative when the fungus is grown in the presence of sublethal concentrations of PP.

Discussion

In liquid medium pyrazophos inhibits dry weight increase of *U. maydis* after a prolonged incubation period, but, in contrast to PP, it did not affect the initial growth rate of *U. maydis*. This indicates, in agreement with the hypothesis of De Waard (1974),

that also *U. maydis* converts pyrazophos into PP and that PP is the toxic principle. Also the observation that pyrazophos does not affect colony formation, but reduces colony size, points towards this conclusion.

In spite of the fungitoxicity of pyrazophos and PP in vitro, no control of maize smut was obtained. Although the reason of this failure has not been investigated, it should be stated that in our experiment conditions were highly favourable for disease development and that the inoculation was very severe, a situation which is probably seldom encountered in practice.

The failure of UV-treatment to induce mutations resulting in PP resistance does not support the hypothesis that the toxicant acts by specific inhibition of one enzyme system.

Samenvatting

Gevoeligheid van Ustilago maydis voor pyrazofos en een van zijn omzettingsprodukten, en het uitblijven van resistentie na UV-bestraling

2-Hydroxy-5-methyl-6-ethoxycarbonylpyrazolo(1,5-a)pyrimidine (PP), een omzettingsprodukt van pyrazofos, was in aanzienlijke mate toxisch voor *Ustilago maydis*, wanneer toegediend in een oplossing die een pH < 5 had. Aanwijzingen werden verkregen dat ook *U. maydis* in staat is pyrazofos om te zetten in PP en dat de laatste verbinding verantwoordelijk is voor de werking van pyrazofos.

Na UV-bestraling van sporidiën werden geen mutanten verkregen, die resistent waren tegen PP. De hypothese dat de werking van PP zou berusten op de specifieke remming van één enzymsysteem, wordt hierdoor niet gesteund.

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