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Acquired resistance to pimaricin in Cladosporium cucumerinum and Fusarium oxysporum f.sp. narcissi associated with decreased virulence

J. DEKKER and A. J. GIELINK

Laboratory of Phytopathology, Agricultural University, Wageningen

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Abstract

In laboratory experiments strains of *Cladosporium cucumerinum* and *Fusarium oxysporum* f.sp. *narcissi* were selected, which showed resistance to the fungicidal antibiotic pimaricin. Increased resistance appeared associated with decreased fitness in vitro (radial growth and sporulation on agar media) and in vivo (pathogenicity). The physiological background of a link between sensitivity to the fungicide and pathogenicity is discussed.

Introduction

Pimaricin belongs to a large group of polyene macrolide antibiotics which all are almost exclusively antifungal (Dekker, 1969). It is used against certain fungal pathogens in man, and to prevent moulding of freshly made cheese. Development of resistance to antibiotics in micro-organisms is a well known phenomenon, especially in bacteria, and to a lesser extent in fungi. In laboratory experiments with yeasts, resistance was obtained to polyene macrolide antibiotics, e.g. to nystatin (Athar and Winner, 1971), nystatin and amphotericin B (Littman et al., 1958) and candidin (Hebeka and Solotorovsky, 1965). Van Tuyl (1977) obtained pimaricin resistant strains of *Aspergillus nidulans*. No problems with development of pimaricin resistance, however, have yet been encountered in practice, i.e. in the medical field and in cheese production.

Pimaricin is also active against many fungal plant pathogens and it shows systemic action in seeds and plants (Dekker, 1957; Oort and Dekker, 1960). Application of this antibiotic for control of plant diseases, however, has been very limited for various reasons, among others instability (Dekker and Ark, 1959; Dekker, 1963). Recently it has been admitted in the Netherlands for control of bulb rot of narcissus (Langerak, 1977). As development of resistance in fungi to some systemic fungicides has caused many problems in practice, it seemed of interest to investigate whether strains of plant pathogens could be obtained with resistance to pimaricin and whether resistance problems might be expected to arise, when the antibiotic is used for control of plant diseases.

Materials and methods

Plants. One week old cucumber seedlings, cv. Lange gele tros were used for the pathogenicity tests. Narcissus bulbs, cv. Carlton were obtained from the Bulb Research Laboratory at Lisse, the Netherlands.

Pathogens. The in vitro and in vivo experiments were carried out with *Cladosporium cucumerinum* Ellis & Arth., kept in stock at the Laboratory of Phytopathology, Wageningen, and with *Fusarium oxysporum* Schlecht. f.sp. *narcissi* (Cooke & Massee) Snyder & Hansen, isolated from diseased bulbs.

The fungicide. The antibiotic pimaricin was kindly provided by Gist-Brocades N.V., Delft, the Netherlands. A formulation was used containing 0.3% pimaricin (Delvolan).

Spore germination test. Conidial suspensions were obtained by flooding a sporulating culture of the pathogens with 5 ml of an aqueous nutrient solution, containing 0.5% KH_2PO_4, 0.1% $(NH_4)_2SO_4$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% NaCl, 2% glucose and 0.2% yeast extract, removing the conidia from the agar surface by gentle scratching with a scalpel and filtering the suspensions through one layer of Monyl gauze. The suspensions were adjusted with nutrient solution to the required concentration. In the tests with *F. oxysporum* only microconidia were used. Double-concentrated fungicide solutions were diluted with equal volumes of conidial suspension, and 0.3 ml drops of the mixtures pipetted on glass slides. After 24 hours incubation at 20 °C and 100% r.h. percentages of germinated conidia were assessed by counting 100 conidia in each of three replicates; length of germ tubes of 100 conidia were measured. Using a range of fungicide concentrations allowed calculation of the respective ED_{50} values.

Radial growth and sporulation on agar medium. PDA plates were prepared containing pimaricin. Discs with mycelium, 5 mm in diameter, taken from the margin of a growing colony, were placed on the agar surface. The plates were incubated at 20 °C in the dark and the radial growth measured after approximately one week. Using a range of fungicide concentrations allowed calculation of $ED_5{}^0$ values.

To measure the degree of sporulation, a disc, 5 mm in diameter, was punched from a sporulating part of the colony, immersed in 1 ml of water in a test tube and placed on a whirlmix to liberate the conidia; the concentration was determined with a haemocytometer.

Assessment of pathogenicity. One-week old cucumber seedlings in the cotyledon stage with the first true leaf expanding were placed with their roots in 50 ml vials containing water, and inoculated with a conidial suspension of *C. cucumerinum* with the help of a De Vilbiss sprayer. After 7 days of incubation at 18 °C and 100% r.h., the disease was assessed, using a scale from 0 (healthy) to 6 (strongly diseased and dying).

In the outer layer of narcissus bulbs holes, 8 mm in diameter, were punched, in which pieces of agar with *F. oxysporum* f.sp. *narcissi* were placed and covered with cellotape. After 10 days of incubation at 18 °C, the disease was assessed by measuring the extension of the discolouration around the site of inoculum.

Isolation of pimaricin resistant strains. PDA plates were prepared containing concentrations of pimaricin normally lethal to the pathogen. Conidial suspensions of *C. cucumerinum*, used for inoculation of the plates, contained 10^8 conidia/ml, those of *F. oxysporum* f.sp. *narcissi*, about 10^7 conidia/ml. Conidial suspensions were distributed over the agar surface, 0.2 ml per petri dish, or mixed through the agar. After incubation in the dark at 20 °C for 5 days, the visible colonies were counted and their diameter assessed. A number of colonies were isolated for further study.

Results

A. Cladosporium cucumerinum. Conidia were plated out on PDA containing 4 ppm of pimaricin. The frequency of emergence of resistance appeared 1 in 10^7. From the resistant strains 18 were selected for further studies. Their sensitivity to pimaricin was determined in conidial germination tests, in which also length of germ tubes was measured. The ED_{50} values for germination and germ tube elongation were compared

Table 1. Resistance of *Cladosporium cucumerinum* to pimaricin (ED_{50} values of conidial germination and germ tube elongation) compared with fitness in vitro (radial growth and sporulation) and in vivo (pathogenicity).

	Resistance ED_{50} in ppm		Fitness (in absence of fungicide)		
	germination	germ tube elongation	radial growth[1]	sporulation[2]	pathogenicity[3]
RC 10	1.90	1.20	64	10	0.50
RC 2	1.82	1.53	64	19	0.10
RC 18	1.70	1.30	60	20	0.00
RC 17	1.70	1.00	64	11	0.65
RC 9	1.68	1.15	64	24	0.50
RC 4	1.68	1.21	64	29	0.00
RC 1	1.68	1.49	72	50	0.80
RC 3	1.63	1.35	68	43	1.10
RC 6	1.59	1.47	56	17	1.40
RC 7	1.48	1.62	60	27	0.90
RC 16	1.40	1.30	60	31	1.70
RC 5	0.86	1.08	60	11	0.95
RC 13	0.75	0.75	64	31	0.60
RC 12	0.60	1.55	64	20	0.10
RC 8	0.78	0.60	100	84	3.40
RC 19	0.40	0.37	100	75	4.90
RC 15	0.32	0.32	60	51	4.70
RC 20	0.32	0.42	100	51	4.70
S	0.22	0.21	100	100	5.7

[1] Average of 4 cultures, in % of sensitive strain.
[2] Average of 4 discs, in % of sensitive strain.
[3] Disease index, scale 0 (healthy) to 6 (dead or dying).
RC = resistant strains of *Cladosporium cucumerinum*; S = sensitive strain.

Tabel 1. Resistentie van Cladosporium cucumerinum voor pimaricine (ED_{50} waarden voor conidiënkieming en kiembuisgroei) vergeleken met vitaliteit in vitro (radiale groei en sporulering) en in vivo (pathogeniteit).

with the fitness of the resistant strains in vitro (mycelium growth and sporulation on fungicide-free agar) and pathogenicity (Table 1).

It appears that a significant increase in resistance is always accompanied by a strong reduction in pathogenicity. The following groups of resistant strains may be discerned:
1. Relatively high resistance, accompanied by very low pathogenicity, reduced growth and strongly reduced sporulation on agar (RC 10, 2, 18, 17, 9, 4, 1, 3, 6 and 7).
2. Moderate to low resistance, accompanied by low to moderate pathogenicity, reduced growth and strongly reduced sporulation on agar (RC 16, 5, 13 and 12).
3. Low resistance, accompanied by high to moderate pathogenicity, fairly normal growth on agar and moderately reduced sporulation (RC 8, 19, 15 and 20).

As even in the first group the level of pimaricin resistance is rather modest, it was investigated whether it could be increased by plating of conidia of resistant strains on agar, containing increasing levels of the antibiotic. In this way the ED_{50} of pimaricin for mycelium growth on agar could be increased from 2 μg/ml for the wild strain to about 8 μg/ml (Fig. 1). Higher levels of resistance were not obtained.

B. Fusarium oxysporum f.sp. *narcissi*. Conidia were plated on PDA, containing 4 ppm of pimaricin, or mixed through PDA, containing 0.5 ppm of this antibiotic. The frequency of emergence of resistance was 1 in 25×10^6. From the resistant colonies, 9 were selected for futher studies. Their sensitivity to pimaricin was determined in conidial germination and radial growth tests. Calculated ED_{50} values were compared with growth on agar in absence of the fungicide and with pathogenicity (Table 2).

It appears that the pimaricin-resistant strains may be divided in two groups, one with a low level of resistance (RF 8, 10, 11, 12 and 6) and one with a higher level of resistance (RF 2, 3, 4 and 5). The latter group possesses a reduced fitness in vitro (radial growth on agar in absence of the fungicide), the former one shows a fairly normal growth on agar. There is also a striking negative correlation between resistance, expressed as ED_{50} in the spore germination test, and pathogenicity.

Discussion

Polyene macrolide antibiotics complex with sterols, especially ergosterol and cholesterol. Fungicidal action is due to complexation with ergosterol, the principle sterol in

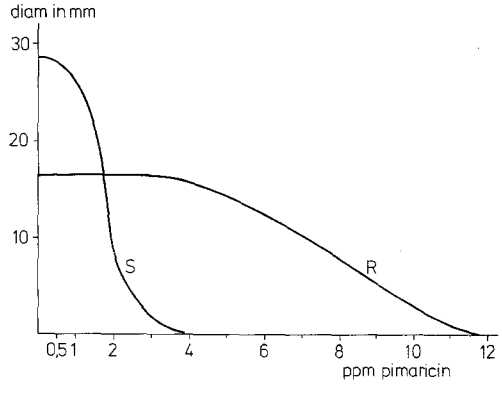

Fig. 1. Growth of *Cladosporium cucumerinum* on PDA, containing pimaricin, diameter in mm after 10 days. S = sensitive strain, R = resistant strain.

Fig. 1. Groei van Cladosporium cucumerinum op aardappel glucose agar met pimaricine, diameter in mm na 10 dagen. S = gevoelige stam, R = resistente stam.

Table 2. Resistance of *Fusarium oxysporum* f.sp. *narcissi* to pimaricin (ED_{50} values of conidial germination and mycelial growth) compared with fitness (radial growth on PDA and pathogenicity in absence of the fungicide).

	Resistance ED_{50} in ppm		Fitness (in absence of fungicide)	
	germination	radial growth	radial growth[1]	pathogenicity[2]
RF 5	0.39	2.70	82	59
RF 4	0.36	2.90	80	65
RF 3	0.35	2.70	81	68
RF 2	0.28	2.85	79	73
RF 12	0.19	2.00	93	86
RF 8	0.19	2.05	96	86
RF 11	0.18	2.30	91	89
Rf 10	0.17	1.35	93	92
RF 6	0.22	1.75	96	108
S - 1	0.12	1.90	99	112
S - 2	0.13	1.45	100	111

[1] In % of control S-2, average of 4 cultures.
[2] Discolouration around site of inoculation in mm, average of 4 treatments.
RF = resistant strains of *Fusarium oxysporum* f.sp. *narcissi*; S = sensitive strain.

Tabel 2. Resistentie van Fusarium oxysporum f.sp. narcissi voor primaricine (ED_{50} waarden voor conidiënkieming en myceliumgroei) vergeleken met vitaliteit (radiale groei op aardappel dextrose agar en pathogeniteit bij afwezigheid van het fungicide).

the fungal membrane, which may cause leakage and cell death (Hamilton-Miller, 1974). In studies with artificial membranes filipin, nystatin, etruscomycin and pimaricin were able to disrupt bimolecular lipid films containing lecithin and cholesterol in a 1 : 1 molar ratio (Van Zutphen et al., 1971).

It has been indicated that polyene resistance may be associated with an altered sterol pattern in fungi; this has been studied for nystatin in *Candida* spp. (Athar and Winner, 1971) and in *Saccharomyces cerevisiae* (Thompson et al., 1971; Woods, 1971; Molzahm and Woods, 1972). Lomb et al. (1975) showed that both lipid and sterol content changed in strains of *Torulopsis glabrata* resistant to nystatin and lucensomycin. The resistant strains possessed a strongly reduced amount of ergosterol and increased amounts of sterols which were biogenetically more primitive than ergosterol. In experiments with strains of *Candida albicans* and *C. utilis* resistant to nystatin and lucensomycin, Fryberg et al. (1976) found that the major sterols of successively more resistant cultures were successively more primitive biogenetic precursors of ergosterol.

It has been shown that the latter possess a lower affinity to polyene antibiotics, so that the fungal membrane contains fewer or weaker binding sites to the antibiotic, resulting in a decreased sensitivity to the fungicide. In agreement herewith, Johnson and Subden (1977) found that amphotericin B and filipin showed a stronger binding to ergosterol, which is the major sterol in strains of *Neurospora crassa* and *C. albicans* sensitive to these antibiotics, than to lichoesterol and eburicol, which were more important in the resistant mutants.

It has been reported that resistance to polyene antibiotics may be due to single gene mutation, as was shown for nystatin resistance in yeast by Woods (1971) and for

pimaricin resistance in *Aspergillus nidulans* by Van Tuyl (1977). It seems plausible that such mutations lead to alterations in sterol patterns, which are of crucial influence on sensitivity of the fungus to the antibiotics.

In experiments with *Candida* spp., Athar and Winner (1971) observed that isolates resistant to nystatin and amphotericin B showed a slower germ tube production and growth, a reduced ergosterol content and a diminished pathogenicity. The quantitative and qualitative changes in the sterols of the fungal membrane responsible for reduced affinity to polyene antibiotics, seem at the same time to lower the fitness of mutants which are resistant to these antibiotics. Also in our experiments with plant pathogens it appears that pimaricin resistance is associated with a decreased fitness in vitro as well as in vivo.

If the hypothesis of the existence of a link between increased resistance and decreased fitness proves to be correct, it would be important with respect to the fungicide-resistance problem in agriculture. It may be assumed that, even when mutation for resistance would occur frequently under field conditions, the build up of a resistant pathogen population would then be improbable or even impossible. This might hold promise for the development of systemic fungicides which are not or less prone to meet resistance problems in practice.

Samenvatting

Verworven resistentie tegen pimaricine bij Cladosporium cucumerinum en Fusarium oxysporum f.sp. narcissi gekoppeld aan een vermindering van de virulentie

In laboratoriumproeven werden stammen van *Cladosporium cucumerinum* en *Fusarium oxysporum* f.sp. *narcissi* verkregen, die resistent waren tegen het fungicide antibioticum pimaricine. Toegenomen resistentie bleek gepaard te gaan met een verminderde vitaliteit in vitro (radiale groei en sporulatie op een agar voedingsbodem) en in vivo (pathogeniteit) (Tabel 1 en 2). Het niveau van de resistentie tegen pimaricine bleek erg beperkt (Fig. 1). De fysiologische achtergrond van een koppeling tussen gevoeligheid voor het fungicide en pathogeniteit wordt besproken.

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Address

Vakgroep Fytopathologie, Postbus 8025, 6700 EE Wageningen, the Netherlands.

Book review

Neergaard, P.: Seed Pathology. MacMillan, London, Basingstoke, 1977. 2 vol., XXIV + 1187 pp., numerous figures and 5 colour plates, cloth-bound with cardboard casing. Price £ 60.

Paul Neergaard is an outstanding authority in seed pathology and director of the Government Institute of Seed Pathology for Developing Countries in Copenhagen. His exhaustive textbook is the fruit of his many years of teaching activity. He demonstrates that seed pathology is a major discipline in plant pathology of great economic significance and covers much more than just seed testing for the sake of quarantine with ensuing rejection, seed treatment or acceptance of a seed sample. It is particularly useful that glossary (38 pp.), references (142 pp.) and index including hosts, diseases and vectors (167 pp.) are separately bound in Vol. 2. The text (chapters indicated in parenthesis) is divided into five main sections: I. Pathogens – diseases – hosts; II. Mechanisms of seed transmission; III. Principles of control; IV. Seed health testing methods; and V. Assessment of seed-borne inoculum. It covers the anatomy of ovules and maturing seeds susceptible to infection (12) at various stages (10), depending on environmental conditions (11); thus quite different parts of the seed can be colonized (13). A different microflora develops during storage (7). Seed-borne organisms include mainly fungi (5), but also bacteria (4), viruses (3) and nematodes (6). They may affect germination and subsequent development of seedlings as well as consumptive value by deterioration and toxicity. Mycotoxin production is rather fully treated (8). After sowing, the seed-borne organisms compete with soil organisms and are transmitted to the seedling in various proportions, determined by the kind of pathogen, its position in the seed (14), environmental conditions (15, 16) and factors inherent to the pathogen (17) and host (18). The