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Benomyl-resistant Fusarium-isolates in ecological studies on the biological control of fusarium wilt in carnation

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Abstract

Ecological properties and stability of benomyl resistance of three benomyl-resistant mutants of nonpathogenic *Fusarium*-isolates antagonistic to fusarium wilt in carnation, and three benomyl-resistant mutants of a pathogenic isolate of *Fusarium oxysporum* f.sp. *dianthi* were evaluated *in vitro* and in glasshouse experiments. The benomyl resistance of the nonpathogenic mutants was stable under all conditions tested, also after a 1000-fold increase of the population in sterilized soil. Mutants of the pathogen were stable during all *in vitro* tests, but after proliferation in carnation stems only part of the population was benomyl resistant.

Compared to the wild type, mutants of the pathogen were less pathogenic, also if the *in vitro* properties were similar. Colonization of carnation by benomyl-resistant nonpathogenic *Fusarium* in the presence of the pathogen showed that the antagonistic effect correlated with the presence of the nonpathogenic isolates within the carnation stem. The wild types and two of the mutant nonpathogenic *Fusarium*-isolates controlled fusarium wilt in the susceptible cultivar Lena for 50% or more.

UV-induced benomyl resistance appeared to be a valuable marker to distinguish between different *Fusarium* isolates and to study the population dynamics, but intensive screening of the mutants is a prerequisite since alterations in antagonism and pathogenicity can occur.

Additional keywords: nonpathogenic *Fusarium*, *F. oxysporum* f.sp. *dianthi*, colonization.

Introduction

Fusarium wilts are soil-borne plant diseases which cause serious problems in several crops. The difficulties encountered in controlling fusarium wilt pathogens by conventional methods make control with biological agents an attractive and useful addition to the plant disease control measures already available. Nonpathogenic or less virulent *Fusarium* spp. were found to suppress several formae speciales of *F. oxysporum* Schlecht. in different crops (Alabouvette et al., 1987; Cugudda and Garibaldi, 1987; Mattusch, 1990). Wilt in carnation, caused by *F. oxysporum* f.sp. *dianthi* (Prill. & Delarc.) Snyder & Hansen race 2, can be suppressed by the nonpathogenic *Fusarium*-isolates 618-12, Fmon, and Fo47 (Lemanceau et al., 1992; Mattusch, 1990; Postma and Rattink, 1991, 1992; Rattink, 1987; Tramier et al., 1987).

Although nonpathogenic *Fusarium*-isolates are promising antagonists, repeated experiments under similar conditions have resulted in various degrees of disease suppression (Postma and Rattink, 1991). For the successful application of antagonists on a large scale, consistent results are essential. Detailed knowledge of colonization by the nonpathogenic isolate and information about the mechanisms of wilt suppression may help improve the reliability and the success of biological control. However, detection and enumeration of

nonpathogenic isolates in the presence of the pathogen is difficult, since they can not be distinguished from the pathogen by selective media or morphological characteristics. A way to overcome this problem, is the use of genetic markers like resistance towards the fungicide benomyl (Andrews, 1986).

Resistance to benomyl does not necessarily influence the fitness of the isolate (Dekker, 1984), and this resistance has already been proved a useful selective marker for several fungal species (Couteaudier and Alabouvette, 1990a, 1990b; Gordon et al., 1989; Papavizas and Lewis, 1983; Papavizas et al., 1982; Sivan and Chet, 1989). However, benomyl-resistant mutants have been found with improved antagonistic properties (Papavizas and Lewis, 1983; Papavizas et al., 1982; Sivan and Chet, 1989) or an altered virulence pattern (Kroon and Elgersma, 1991). Selection of mutants after a low mutagenic dose is expected to reduce the likelihood of accompanying mutational changes (Andrews, 1986), but fitness and stability of the resistance marker of the mutants have to be evaluated before the mutants can be used in ecological studies. It has not been established which screening methods are most convenient and yield relevant information about the *in vivo* ecological properties. Illustrative is a benomyl-resistant mutant of *Chaetomium globosum*, which was as antagonistic as its wild type in growth chamber experiments, but showed a decreased survival under field conditions (Cullen and Andrews, 1985). Often the estimation of fitness in competition with other isolates is more sensitive than evaluating isolates seperately. For example, TBZ-resistant isolates of *Penicillium digitatum* showed the same pathogenicity as sensitive isolates, but if inoculated in competition with the sensitive isolates, they were less fit (Seidel et al., 1990).

This paper describes the screening of benomyl-resistant mutants of three antagonistic and one pathogenic *Fusarium*-isolate. Stability of the resistance marker and maintenance of ecological important properties, e.g. growth rate, competition, saprophytic ability, antagonism and pathogenicity were assessed. The aim of the research was to critically evaluate the use of benomyl-resistant mutants of *Fusarium* in studies on the population dynamics of pathogenic and nonpathogenic strains.

Materials and methods

Fungal strains. Two *Fusarium* strains antagonistic to fusarium wilt in carnation (618-12 and Fmon), as well as a virulent isolate of *F. o.* f.sp. *dianthi* race 2 (WCS816), were used to produce benomyl-resistant mutants. These isolates have been described previously (Postma and Rattink, 1992; Rattink, 1987). Also included in this study were the nonpathogenic *F. oxysporum* Fo47 (Alabouvette et al., 1987), and its benomyl-resistant mutant Fo47B10, both obtained from C. Alabouvette (INRA, Station de Recherche sur la Flore Pathogène dans le Sol, Dijon, France). Fo47B10 was selected after UV radiation (254 nm) and was first described by Eparvier et al. (1991).

Production of benomyl-resistant mutants. Survival rates of microconidia of isolate 618-12 and Fmon were determined at different doses of UV irradiation. A suspension with microconidia, harvested from plates with potato dextrose agar (PDA; 39.0 g PDA (Oxoid) in 1 l deionized water), was spread on PDA plates, so that each plate contained between 50 and 100 conidia. After removing the lid, the plates were exposed for different lengths of time to a UV source (Philips 15 W, 253.7 nm) at 70 cm distance and incubated in the dark at 25 °C. The number of colonies per plate was determined after 3, 4 and 5 days.

For the production of mutants, dense suspensions of microconidia of 618-12, Fmon and WCS816 were spread on PDA containing 10 mg/l benomyl (Benlate, 50% a.i.). About 0.5–1 x 10^7 conidia per plate were treated by UV as described above. A radiation time of

1 or 2 minutes was used at which 50% of the conidia survived. About 50 benomyl-resistant colonies were chosen and monospore cultures were prepared. The mutants were first selected for their growth on PDA with 10 mg/l benomyl. Then, the mutants were grown on PDA and only mutants with a similar growth rate and colony morphology as the wild type were chosen. From these mutants, a conidial suspension harvested from PDA was spread on PDA and on PDA with 10 mg/l benomyl. Only mutants which showed the same number of colonies on both media were selected. The selected benomyl-resistant mutants were kept on Czapec Dox agar with 5 mg/l benomyl at 4 °C.

Growth, in vitro competitive ability and germination of mutants and wild types on agar media. Growth rate of the selected benomyl-resistant mutants and their wild types were determined on PDA and PDA with 10 mg/l benomyl. A mycelial disk (5 mm) was placed on the medium and radial growth at 25 °C was measured between day 2 and 6 (5 replicates).

Competitive ability of the mutants with respect to their wild types was determined by placing a mycelial disk (5 mm) of the mutant next to a disk of the wild type in the middle of a Petri dish containing PDA (juxtaposition). After 7 to 9 days at 25 °C, the angle of the mutant colony was measured (5 replicates). An angle less than 180 was interpreted as less competitive.

The maximum benomyl concentration tolerated by mutants was determined by assessing germination after spreading a conidial suspension with 50 to 200 microconidia per plate on PDA with different benomyl concentrations (0, 5, 10, 20, 30, 50, 100 mg/l). The influence of the medium on the benomyl resistance was studied with mutant 618-12B17 on PDA, selective fusarium agar (SFA) and water agar (WA; 20.0 g agar (no. 3, Oxoid) in 1 l deionized water). SFA contained: 20.0 g D-(+)-glucose, 0.5 g KH_2PO_4, 2.0 g $NaNO_3$, 0.5 g $MgSO_4.7H_2O$, 1.0 g yeast-extract (Oxoid), 0.01 g $FeSO_4.7H_2O$, 20.0 g agar, 25 mg dichloran (Allisan, 50% a.i.), 100 mg streptomycin, 10 mg tetracycline, in 1 l deionized water.

Saprophytic ability and stability of benomyl resistance in sterilized soil. Saprophytic growth of the selected mutants and their wild types was compared in sterilized soil, using separate and combined inoculations. Glass cylinders, 4 cm in diameter, 8 cm high and closed with aluminium caps, were filled with 15.5 g moist potting soil (5 g dry weight) and sterilized by γ-radiation (4 Mrad). Two ml of a conidial suspension of the wild type, the mutant or a combination, each containing about 5×10^4 conidia/ml, was added aseptically to the soil (5 replicates per treatment). After inoculation, the substrate contained 71% moisture. After 14 days incubation in the dark at 25 °C, the soil portions were suspended by shaking the soil for 30 min at 450 rpm in 90 ml of a 0.1% sodium pyrophosphate solution containing 10 g gravel (2–4 mm diameter). Appropriate dilutions were plated in triplicate on SFA and on SFA with 10 mg/l benomyl with a spiral plater (Spiral Systems, Inc., Cincinnati, Ohio, USA). Contamination of the soil portions was checked on diluted tryptone soya agar (Postma and Rattink, 1992).

The population dynamics of 618-12 and 618-12B17 were determined in duplicate after incubation periods of 1, 2, 8, 15, and 34 days. Inoculation and sampling procedure was as described above.

Stability of benomyl resistance in different substrates. The stability of the benomyl resistance of mutant 618-12B17 was determined by comparing cfu on SFA and SFA with benomyl. Propagules of the mutant harvested from media containing benomyl, were added at low inoculum densities to different sterile substrates without benomyl. The

substrates used were (1) 150 ml liquid medium (33.4 g Czapek Dox (Oxoid) and 1.0 g yeast extract (Oxoid) per l) in a 500 ml Erlenmeyer, (2) vermiculite (2 g dry weight), (3) vermiculite with 4 mg glucose and 19 mg yeast extract, (4) vermiculite with 190 mg carnation roots and (5) potting soil (3.2 g dry weight). The liquid medium was autoclaved and the solid substrates were sterilized by γ-radiation (4 Mrad). Moisture contents of vermiculite and potting soil were resp. 80 and 68% before inoculation and 82 and 71% after inoculation (g water per g moist substrate). The liquid medium was aerated by sterile air. All media were incubated in the dark at 25 °C. Long-term stability was determined in non-sterile vermiculite with growing carnation plants (cultivar Lena and Pallas). After different incubation periods, *Fusarium* and benomyl-resistant *Fusarium* propagules were enumerated on respectively SFA and SFA with 10 mg/l benomyl. The liquid medium was plated after dilution, whereas the solid media were first suspended by shaking with gravel as described above. Each substrate was sampled in triplicate.

Antagonism and pathogenicity in glasshouse experiments with fusarium wilt in carnation. Antagonism and pathogenicity of the selected mutants were tested twice in 3 separate glasshouse experiments. The antagonistic ability of the mutants 618-12B17, FmonB1 and Fo47B10 and their wild types was compared from January to June 1991 (experiment I). Rooted carnation (*Dianthus caryophyllus* L.) cuttings of Lena and Pallas (Van Staaveren B.V., Aalsmeer), a susceptible and a moderately resistant cultivar, respectively, were planted in 9 cm pots with steamed potting soil (Trio no. 17 from Trio BV, Vroomshoop, the Netherlands). Plants were grown in the glasshouse at 23 °C, watered every 2 or 3 days and extra light was given (16 h, approximately 100 μmol $sec^{-1}m^{-2}$). The experiment contained 11 treatments: a disease-free control, the wild type of the pathogen only, the nonpathogenic benomyl-resistant mutants only, pre-inoculation with the nonpathogenic benomyl-resistant mutants or wild types followed by inoculation with the pathogen. All treatments consisted of 3 replicates, with 6 plants each of the 2 cultivars. The nonpathogenic isolates were added to the soil three times, 7, 12 and 19 days after planting, by pipetting 3 ml of 6×10^6 conidia/ml around the stem base. The pathogen, 3 ml of 10^5 conidia/ml, was added to the soil 26 days after planting. Conidia were harvested from PDA, or from PDA with 5 mg/l benomyl in case of benomyl-resistant mutants. Wilt symptoms were examined weekly, using wilt indices from 0 to 5 (Baayen and Niemann, 1989). The percentage diseased plants was calculated as the proportion of plants with wilt indices $\geqslant 2$.

The antagonistic ability of the nonpathogenic isolates and the mutants was evaluated at a slightly higher inoculum dose of the pathogen in a second experiment. In addition, the pathogenicity of the mutants WCS816B9 and WCS816B13 and the wild type WCS816 were compared. This experiment (II) was performed with the susceptible cultivar Lena from January to June 1992. The conditions were similar to those described above. The experiment contained 10 treatments: a disease-free control, wild type or benomyl-resistant mutants of the pathogen, pre-inoculation with the nonpathogenic benomyl-resistant mutants or wild types followed by inoculation with the wild type of the pathogen. The treatments were randomized within 5 blocks, with 6 plants per unit. The nonpathogenic isolates were added 4 days after planting by soil inoculation with 3 ml of 3×10^7 conidia/ml and the pathogenic isolates were added 18 days after planting by soil inoculation with 3 ml of 3×10^5 conidia/ml. Wilt symptoms were examined weekly.

Pathogenicity of wild type and benomyl-resistant mutants WCS816B1 and WCS816B9 were compared in a third glasshouse experiment (III, June-November 1992). Wild type and mutants were inoculated 21 days after planting by pipetting 3 ml of 6×10^5 conidia/ml around the stem base. Treatments were randomized within 3 blocks, with 6 plants (cv. Lena) per unit.

Colonization and stability of benomyl resistance of antagonistic and pathogenic isolates in plants and soil. Colonization of stem and soil by the nonpathogenic benomyl-resistant mutants in experiment I was assessed 59, 102, and 144 days after planting. Colonization of wilting stems by wild type and benomyl-resistant mutants of the pathogen was assessed at the end of experiment II and III. Healthy noninoculated plants were used as control. Stem pieces 1 cm long from different heights were surface sterilized and placed on SFA or SFA with 10 mg/l benomyl (Postma and Rattink, 1992). Suspensions of the stems were prepared by blending them in 100 ml sterile water in a Braun blender (2 min at maximum speed). Soil suspensions were prepared as described above. Suspensions were plated in triplicate on SFA and SFA with 10 mg/l benomyl with a spiral plater.

Statistical analyses. Analyses of variance were carried out with the statistical programme Genstat 5. Least significant differences (LSD) were calculated at a significance level of $P = 0.05$. Numbers of propagules in soil or carnation stems were compared by analysis of variance after logarithmic ($\log_{10}$) transformation. Disease incidence and severity in glasshouse experiments were assessed by analyses of variance at different days after planting. Treatments without wilt symptoms were excluded from the analyses.

Results

Production of benomyl-resistant mutants. The highest number of benomyl-resistant mutants were obtained after 1 or 2 minutes of UV radiation, lethal for 30 to 70%. After a rough screening of growth rate, colony morphology and germination percentage, only a few mutants were selected.

Growth, in vitro competitive ability and germination of mutants and wild types on agar media. Wild types showed almost no growth on PDA with 10 mg/l benomyl, whereas the growth rate of most mutants, with the exception of WCS816B1 and WCS816B9, on

Table 1. Growth rate on PDA with and without 10 mg/l benomyl (B) and the angle of colonies of the benomyl-resistant mutants in competition with their wild-type strains on PDA at 25 °C (mean ± SD, $n = 5$).

Fusarium-isolate	UV (min)	Growth (mm/d)		Angle PDA
		PDA	PDA+B	
618-12	0	4.5 ± 0.2	0.0 ± 0.0	
618-12B17	2	4.7 ± 0.4	4.4 ± 0.1	184 ± 4
Fmon	0	4.5 ± 0.2	0.9 ± 0.2	
FmonB1	2	3.7 ± 0.2	4.1 ± 0.1	148 ± 6
Fo47		5.7 ± 0.5	0.0 ± 0.0	
Fo47B10		4.6 ± 0.5	4.3 ± 0.1	107 ± 4
WCS816	0	3.4 ± 0.6	0.0 ± 0.0	
WCS816B1	1	3.6 ± 0.7	0.8 ± 0.1	180 ± 14
WCS816B9	1	3.0 ± 0.4	2.1 ± 0.2	178 ± 6
WCS816B13	1	2.7 ± 0.1	2.9 ± 0.3	156 ± 6

Table 2. The level of the benomyl resistance of 618-12B17 in relation to the composition of the medium; percentage germination as compared to germination on PDA without benomyl.

Medium	Benomyl(mg/l)					
	0	10	30	50	70	100
PDA	100	114	47	5	0	0
SFA	113	106	113	78	0	0
WA	68	51	17	0	0	0

LSD (0.05) = 19.

Table 3. Logarithmic number of colony-forming units per g dry soil (log cfu/g) and percentage of benomyl-resistant colonies (%Bres) after benomyl-resistant mutants and their wild types (wt) were inoculated separately or together into sterilized soil (mean ± SD, $n = 5$).

Fusarium-isolate	inoculum[1]		14d after inoculation	
	log cfu/g	%Bres	log cfu/g	%Bres
618-12 wt	4.05	0	6.67 ± 0.11	0
618-12B17	3.96	102	6.66 ± 0.04	113 ± 18
wt + B17	4.01	46	6.62 ± 0.03	65 ± 7
Fmon wt	4.09	0	7.47 ± 0.09	0
FmonB1	3.99	88	7.72 ± 0.11	113 ± 26
wt + B1	4.04	39	7.58 ± 0.12	19 ± 4
Fo47 wt	4.33	0	6.97 ± 0.17	0
Fo47B10	4.32	⩾100	6.98 ± 0.07	100 ± 35
wt + B10	4.32	50	7.00 ± 0.17	32 ± 12
WCS816 wt	4.28	0	6.46 ± 0.11	0
WCS816B1	4.29	105	6.77 ± 0.33	103 ± 8
WCS816B9	4.31	103	6.41 ± 0.14	*
WCS816B13	4.29	105	6.37 ± 0.10	87 ± 40
wt + B1	4.28	52	6.68 ± 0.04	55 ± 16
wt + B9	4.29	53	6.50 ± 0.08	49 ± 7
wt + B13	4.28	52	6.39 ± 0.05	44 ± 3

[1] The inoculum concentration enumerated just before inoculation was calculated to the equivalent value in log cfu/g dry soil.
* Problems with plate counts.

PDA with benomyl was similar to that on medium without benomyl (Table 1). Growth rates of the mutants FmonB1, Fo47B10, and WCS816B13 on PDA were slightly lower than that of their wild types, whereas 618-12B17, WCS816B1 and WCS816B9 had the same growth rates as their wild types. Growth of the mutants on PDA in competition with their wild type showed corresponding results: FmonB1, Fo47B10 and WCS816B13 formed colonies with an angle significantly smaller than 180°, whereas the angle of the other mutants was not significantly different from 180° (Table 1). Control plates with wild types in intra-specific competition showed an angle of 180°.

All mutants showed 100% germination of conidia on media up to 10 mg benomyl/l PDA or more. The wild type isolates did not germinate on PDA with 10 mg/l benomyl. On a nutrient-poor medium (WA), germination was lower compared to germination of 618-12B17 on PDA, but the sensitivity for benomyl was not significantly different (Table 2). The sensitivity of 618-12B17 for benomyl was not increased by the presence of several fungicides and antibiotics in SFA; germination was still 100% at 30 mg/l benomyl.

Saprophytic ability and stability of benomyl resistance in sterilized soil. After inoculation of sterilized potting soil with low inoculum levels, populations of all mutants and wild types increased up to 0.5-5 x 10^7 cfu/g dry soil in 14 days and none of the mutants showed a lower population density than its wild type (Table 3). Cfu of wild types and mutants were 0% and 100% benomyl-resistant, respectively, just before inoculation as well as after 14 days incubation in soil. The percentage of benomyl resistance in the combined inoculations with 618-12B17, WCS816B1, and WCS816B9 was still 50% after 14 days of incubation, whereas the combinations with FmonB1, Fo47B10, and WCS816B13 showed percentages of benomyl resistance that were lower than 50%. Population dynamics of 618-12 and 618-12B17 were similar: an increase during the first 8 days followed by stabilization at about 10^7 cfu per g dry soil until day 34 (Fig. 1). If inoculated together, 618-12B17 was present until the end of the experiment.

Stability of benomyl resistance in different substrates. In all sterile substrates tested, the number of cfu of 618-12B17 increased substantially, but the benomyl resistance was not lost in any of the substrates (Table 4). In the non-sterile system, the resistance was not significantly reduced during 84 days incubation.

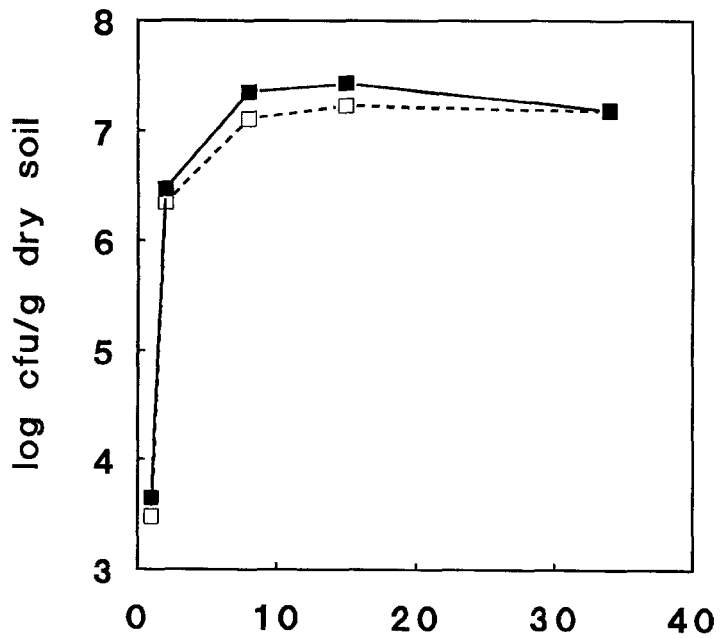

Fig. 1. Population dynamics of 618-12 (□) and 618-12B17 (■) after inoculation into sterilized potting soil. LSD (0.05) = 0.02.

Table 4. Recovery of the benomyl resistant 618-12B17 after incubation in different sterile (st) and nonsterile (ns) substrates, given as logarithmic number of colony-forming units (log cfu) and the percentage of benomyl resistant cfu (mean ± SD) after different incubation periods.

	log cfu/g dry substrate				% benomyl-resistant cfu		
incubation (days)	0*	4	7		4	7	
st liquid medium[1]	0.6	7.0 ± 0.4	7.9 ± 0.5		105 ± 23	106 ± 1	
incubation (days)	0*	7	14	20	7	14	20
st vermiculite	2.0	6.1 ± 0.1	6.4 ± 0.1	6.5 ± 0.1	100 ± 7	95 ± 4	104 ± 16
st vermiculite + nutrients	2.0	7.0 ± 0.1	7.7 ± 0.1	5.8 ± 0.2	129 ± 90	89 ± 14	117 ± 52
st vermiculite + roots	2.0	6.6 ± 0.1	6.5 ± 0.2	5.2 ± 0.5	122 ± 15	101 ± 16	162 ± 53
st potting soil	2.0	6.3 ± 0.0	6.9 ± 0.1	6.8 ± 0.1	90 ± 17	112 ± 15	124 ± 30
incubation (days)	0*	15	42	84	15	42	84
ns vermiculite, cv. Lena	5.2	5.1 ± 0.2	4.5 ± 0.1	4.8 ± 0.2	119 ± 27	87 ± 4	101 ± 1
ns vermiculite, cv. Pallas	5.2	5.1 ± 0.1	4.9 ± 0.2	4.9 ± 0.3	110 ± 9	75 ± 8	85 ± 34

* = Inoculum dose.
[1] = log cfu/ml instead of log cfu/g dry substrate.

Antagonism of the benomyl-resistant mutants. In experiment I, symptoms in cv. Lena became visible 89 days after planting. At the end of the experiment (day 145), 33% of the plants in the treatment with only pathogen showed wilt symptoms. All other treatments showed lower percentages of diseased plants. Incidence (% plants with disease severity index ≥ 2) and severity (disease severity index) showed the same tendency, but differences between treatments were most pronounced if the disease severity index (Fig. 2) was used. Pre-inoculation with 618-12, 618-12B17, Fmon, FmonB1 and Fo47 significantly reduced the disease severity at day 125, 132 and 145 (Fig. 2). Pre-inoculation with Fo47B10 reduced disease severity, but this effect was only significant at day 125. The biological control effects of mutants and wild types of the antagonists were not significantly different. Inoculation with the benomyl-resistant antagonists alone, resulted in only 5 to 10% disease, which was not significantly different from the disease-free control. In the partly resistant cultivar Pallas, only 11% of plants showed wilt symptoms and pre-inoculations did not significantly reduce the disease incidence or severity.

In experiment II, with a higher disease pressure compared to experiment I, symptoms became visible at day 54. At the end of the experiment, 87% of the plants inoculated with WCS816 showed wilt symptoms. The disease severity index (Fig. 3) showed the same tendency as the percentage of diseased plants. Again 618-12, 618-12B17, Fmon, FmonB1 and Fo47 significantly reduced the disease severity. Pre-inoculation with Fo47B10 did not result in any antagonistic effect and was significantly different from Fo47.

Pathogenicity of the benomyl-resistant mutants. Compared to the wild type, pathogenicity of the benomyl-resistant mutants of WCS816 in carnation was lower (Table 5).

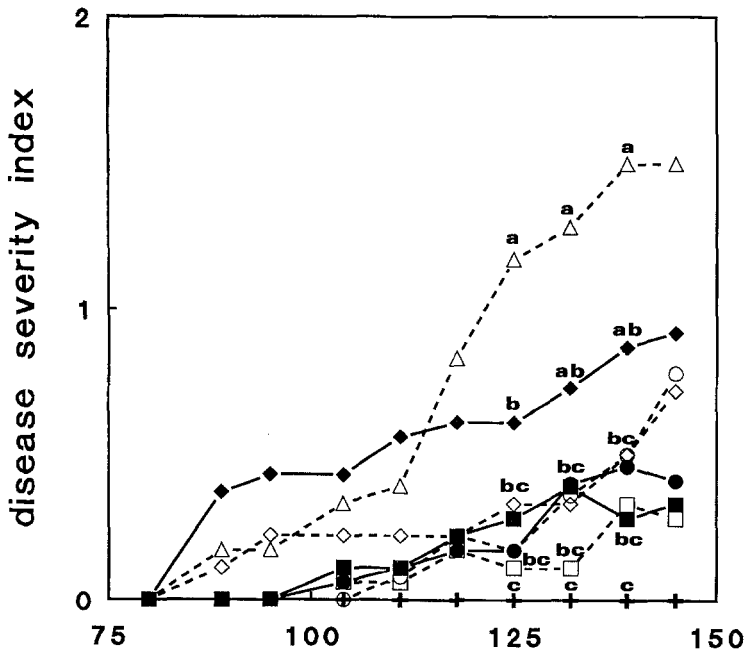

Fig. 2. Disease severity of fusarium wilt in carnation (cv. Lena, experiment I) after inoculation with water (+) or WCS816 (△). Prior to inoculation with WCS816, wild types (open symbols) and benomyl-resistant mutants (closed symbols) of 618-12 (□, ■), Fmon (○, ●) and Fo47 (◇, ◆) were added to the soil. Disease severity indices at selected assessment times with the same letter are not significantly different at $P > 0.05$.

Colonization and stability of benomyl resistance of antagonistic and pathogenic isolates in plants and soil. Colonization of the soil by the benomyl-resistant 618-12B17, FmonB1 and Fo47B10 (inoculated alone) was similar. The percentage of benomyl-resistant cfu was not significantly lower than 100% on the different sampling days, except for FmonB1 on day 102 (Table 6). In the plant, the percentage of benomyl-resistant cfu of

Table 5. Disease severity index of fusarium wilt in carnation (cv. Lena) in experiment II and III after inoculation with WCS816 or benomyl-resistant mutants.

Fusarium-isolate	Experiment II		Experiment III	
	days after planting		days after planting	
	126	152	126	151
H_2O	0.03	0.07	0.00	0.00
WCS816	3.07	3.67	1.50	1.95
WCS816B1	nd	nd	0.67	1.00
WCS816B9	2.47	3.03	0.17	0.28
WCS816B13	1.07	1.50	nd	nd
LSD (0.05)	1.52	1.53	0.74	1.22

nd = not detected.

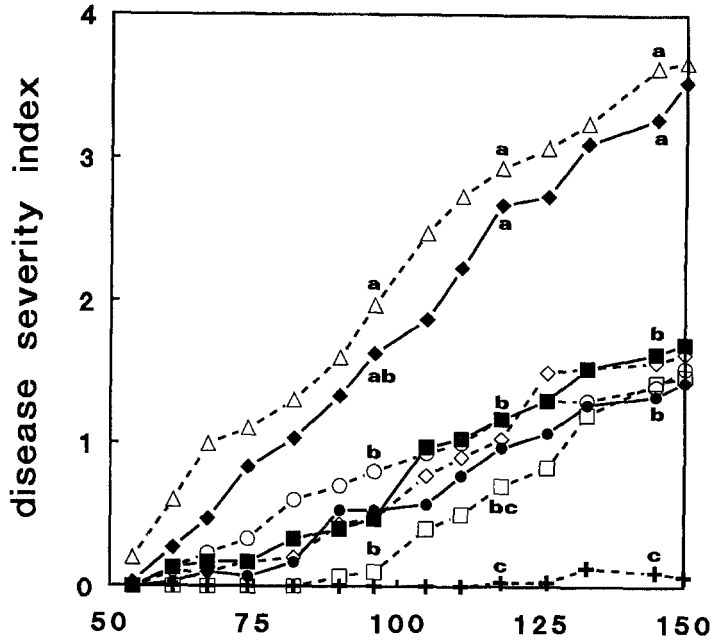

Fig. 3. Disease severity of fusarium wilt in carnation (cv. Lena, experiment II) after inoculation with water (+) or WCS816 (△). Prior to inoculation with WCS816, wild types (open symbols) and benomyl-resistant mutants (closed symbols) of 618-12 (□, ■), Fmon (○, ●) and Fo47 (◇, ◆) were added to the soil. Disease severity indices at selected assessment times with the same letter are not significantly different at $P > 0.05$.

618-12B17, FmonB1 and Fo47B10 (inoculated alone) was calculated from carnation stems with detectable numbers of cfu/stem ($\geqslant 5 \times 10^3$). In experiment I these numbers were all close to the detection limit and variations were large. There were no significant differences between isolates, and the mean percentage of benomyl-resistant cfu was 65 ± 42 ($n = 18$).

In contrast to the poor colonization of the stem in experiment I, wild types as well as mutants of the pathogen reached 1.5×10^6 cfu/stem in wilted plants in experiment II and

Table 6. Presence of the benomyl-resistant 618-12B17, FmonB1 and Fo47B10 in potting soil during experiment I, inoculated into soil in the absence of the pathogen. Summarized data of cvs. Lena and Pallas are given.

Fusarium-isolate	log cfu/g soil (SFA)			%Bres (SFA+B/SFA)		
	days after planting			days after planting		
	59	102	144	59	102	144
618-12B17	4.89	4.46	4.60	173	116	72
FmonB1	5.08	5.20	4.64	99	32	59
Fo47B10	4.77	4.27	4.41	126	95	79
LSD (0.05)		0.33			51	

Table 7. Presence and benomyl-resistance of the pathogen WCS816 and its mutants in carnation stems with disease indices ⩾ 3 at the end of experiment II and III (day 152 and 151, resp.) (mean ± SD, $n = 5$).

Fusarium-isolate	Experiment II		Experiment III	
	log cfu/plant	%Bres	log cfu/plant	%Bres
WCS816	6.21 ± 0.39	0 ± 0	6.17 ± 0.35	0 ± 0
WCS816B1	nd	nd	6.17 ± 0.51	1 ± 2
WCS816B9	6.26 ± 0.23	33 ± 20	6.60 ± 0.40	37 ± 50
WCS816B13	6.16 ± 0.16	31 ± 12	nd	nd

nd = not detected.

Table 8. Number of carnation stem pieces from different heights of 15 plants containing non-resistant (-res) or benomyl-resistant (Bres) Fusarium, and the mean disease severity index (DSI) of the sampled plants (experiment II, day 152).

Height (cm)	Water + WCS816		618-12B17 + WCS816		FmonB1 + WCS816		Fo47B10 + WCS816	
	-res	Bres	-res	Bres	-res	Bres	-res	Bres
60	3	0	7	0	2	0	4	0
50	5	1	6	0	2	2	8	0
40	6	0	6	0	3	1	9	0
30	7	0	7	1	0	3	10	0
20	8	0	8	1	2	2	9	0
10	10	0	8	0	2	3	10	1
5	13	0	8	1	3	6	12	0
0	13	1	1	12	3	12	11	4
DSI	4.20		2.07		1.53		3.73	

III (Table 7). At the end of experiment II and III, the percentage of benomyl-resistant cfu, however, had significantly decreased from 100% to approx. 30%, and had even disappeared in the treatment with WCS816B1. The noninoculated control plants did not contain substantial numbers of *Fusarium*.

After combined inoculations in experiment II, the nonpathogenic benomyl-resistant mutants could be detected in the presence of the pathogen. Isolation of the benomyl-resistant mutants at different heights in 15 plants per treatment showed that the nonpathogenic mutants were most frequently present in the stem at soil level (Table 8). FmonB1 was more often present at higher levels in the stem compared to 618-12B17, whereas Fo47B10 was rarely isolated.

Discussion

Benomyl-resistant mutants of antagonistic and pathogenic *Fusarium* isolates were easily obtained after UV radiation of microconidia on agar plates. The mutation frequency at a UV dosis at which 50% of the conidia survived, was $1–5 \times 10^{-7}$. The selected mutants were all able to grow on a medium that contained 10 mg/l benomyl or more, a concentration on which the wild types did not grow. *In vitro* tests showed that the benomyl-resistant mutants were stable. Nonpathogenic benomyl-resistant mutants were still resistant after

reisolation from carnation stems, but the pathogenic mutants tested lost a substantial part of their resistance after proliferation in wilting carnations. Thus, stability of benomyl resistance in the *in vitro* tests reflecting saprophytic conditions, provided insufficient information about what takes place under pathogenic conditions. Colonization and reisolation of benomyl-resistant nonpathogenic and pathogenic *Fusarium* mutants from soil and rhizosphere have been described frequently (Cugudda and Garibaldi, 1987; Gordon et al., 1989; Couteaudier and Alabouvette, 1990a, 1990b; Garibaldi et al., 1991), but information about stability of the marker after growth in soil or plants is scarce.

In addition to the stability of the benomyl resistance, the maintenance of important ecological properties is a prerequisite for the application of mutants in biological control studies. In *in vitro* tests, the mutants 618-12B17, WCS816B1 and WCS816B9 were similar to their wild types regarding growth rate, competition on PDA and saprophytic ability in sterilized soil. The growth rate and competitive ability on PDA of FmonB1, Fo47B10 and WCS816B13, however, was decreased compared to their wild types (Table 1). Although the population size of these mutants was similar to that of their wild type after single inoculation in sterilized soil, the competitive saprophytic ability in sterilized soil of these mutants was less (Table 3). Measurements of the relative fitness is a more sensitive parameter than comparing absolute fitness properties, corresponding results of Seidel et al. (1990). Competitive saprophytic interaction of isolates is a measure for nutrient use efficiency, whereas the final population level in a substrate is dependent on the amount of available nutrients and of the yield coefficient of the isolate (Couteaudier and Alabouvette, 1990b).

For all mutants tested, the competitive ability on PDA correlated with the competitive saprophytic ability examined in sterilized soil. Measurement of the competitive ability on PDA was faster, less laborious, and resulted in smaller standard deviations than measurement of the saprophytic ability in soil, and is therefore recommended.

Antagonism of the mutants examined in two glasshouse experiments with carnation, showed that the benomyl-resistant mutants 618-12B17 and FmonB1 controled wilt symptoms of *F. o.* f.sp. *dianthi* to the same extent as their wild types. The ability of the mutant 618-12B17 to control wilt in carnation confirms the earlier results of Postma and Rattink (1992). The antagonistic ability of Fo47B10 was not significantly reduced compared to its wild type if applied three times in combination with a relatively low disease pressure (experiment I). However, if the disease pressure was high and Fo47B10 was applied only once (experiment II), the antagonistic ability of Fo47B10 was significantly less compared to its wild type. Lemanceau et al. (1992) described the antagonistic effect of Fo47B10 against fusarium wilt in carnation grown in rock-wool (a reduction from 90% to 60% wilted plants), but the efficacy of wild type and mutant were not compared.

Maintenance and loss of antagonism of 618-12B17 and Fo47B10, respectively, correlated with the results of the *in vitro* tests. The decreased growth rate and lesser competitive ability of FmonB1, did not result in a diminished antagonistic effect in the glasshouse experiments. The *in vitro* tests used were based on competitive saprophytic ability, whereas antagonism by nonpathogenic *Fusarium* isolates can also be the result of other mechanisms, e.g. induced resistance. Therefore, potential antagonistic mutants which have a different mode of action can be lost with such *in vitro* tests.

Pathogenicity of the mutants of WCS816 was obviously affected when compared with the wild type and did not correlate with the results of the *in vitro* tests. Screening under saprophytic conditions probably yields insufficient information about maintenance of pathogenicity, and pre-screening of large numbers of mutants under pathogenic conditions might be necessary.

Colonization by the nonpathogenic isolates was successfully assessed in the presence

of the pathogen by using the selected benomyl-resistant mutants. The occurrence of the nonpathogenic benomyl-resistant mutants in the carnation stems reflected their biological control effect (Table 8). Isolates 618-12B17 and FmonB1 were present in most of the plants, whereas Fo47B10 was only present in 4 out of 15 plants. However, it is not known if the wild type Fo47, which had the same control effect as the other antagonists, colonized the stems more abunduntly than Fo47B10.

UV-mutagenesis and other forms of non-recombinant DNA mutations are rather uncontrolled approaches and may implicitly lead to an array of unknown mutations resulting in alterations of pathogenicity or antagonism. Therefore, recombinant DNA mutations are to be conceptually prefered when specific marker-mutants are required. Yet, this study has shown that UV-induced benomyl-resistance is a valuable marker to distinguish between different *Fusarium* isolates. It allows quantitative studies, but intensive screening of the mutants used, including comparative approaches with wild type and mutant, is a prerequisite. The *in vitro* methods described, especially competition of mutant and wild type juxtaposed on PDA, are useful pre-screening methods. However, *in vitro* screening methods alone are insufficient since (1) antagonists with mechanisms not related with competitive saprophytic ability might be lost and (2) mutants of the pathogen, which were selected under saprophytic conditions, showed decreased pathogenicity and lost their resistance under pathogenic conditions.

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