Recovery of *Fusarium oxysporum* Fo47 Mutants Affected in Their Biocontrol Activity After Transposition of the *Fot1* Element

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ABSTRACT

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To investigate the biocontrol mechanisms by which the antagonistic *Fusarium oxysporum* strain Fo47 is active against Fusarium wilt, a *Fot1* transposon-mediated insertional mutagenesis approach was adopted to generate mutants affected in their antagonistic activity. Ninety strains in which an active *Fot1* copy had transposed were identified with a phenotypic assay for excision and tested for their biocontrol activity against *F. oxysporum* f. sp. *lini* on flax in greenhouse experiments. Sixteen strains were affected in their capacity to protect flax plants, either positively (more antagonistic than Fo47) or negatively (less antagonistic). The molecular characterization of these mutants confirms the excision of *Fot1* and its reinsertion in most of the cases. Moreover, we demonstrate that other transposable elements such as *Fot2*, *impala*, and *Hop* have no transposition activity in the mutant genomes. The phenotypic characterization of these mutants shows that they are affected neither in their in vitro growth habit nor in their competitiveness in soil compared with wild-type strain Fo47. These results show that mutants are not impaired in their saprophytic phase and suggest that the altered biocontrol phenotype should likely be expressed during the interaction with the host plant.

Fusarium oxysporum Schlechtend.:Fr. is a common soilborne fungus, well represented in every type of soil throughout the world (9). It includes a large diversity of strains, all of which are successful saprophytes, i.e., able to grow and survive for long periods on organic matter in soil and in the rhizosphere of many plant species (20). Many strains are parasitic but nonpathogenic, i.e., able to invade to some extent plant tissues without inducing symptoms, and some of them are pathogenic and induce either root rot or tracheomycoses. The wilt-inducing strains of *F. oxysporum* cause severe damage on a wide range of economically important crops (46). They exist in many specialized forms and are grouped into formae speciales and physiological races depending on their pathogenicity toward particular plant species or cultivars (5).

Nonpathogenic strains of *F. oxysporum* play an important role in soil microbial ecology and especially in the natural phenomenon of soil suppressiveness to diseases induced by pathogenic strains (1,2). The first evidence of a possible role of nonpathogenic *Fusarium* spp. in suppressive soils (45,50) resulted from the observation that soils suppressive to Fusarium wilt supported a large population of nonpathogenic *Fusarium* spp. It was confirmed experimentally by demonstrating that suppressiveness disappeared after elimination of *Fusarium* spp. by heat treatment and was partly restored after their reintroduction into the heat-treated soil (42). Not all the *Fusarium* spp. isolated from suppressive soils are able to induce suppressiveness in disinfected soil, but most of the isolates of *F. oxysporum* studied showed some ability to limit the incidence of Fusarium wilt (48,49).

Until now, the mechanisms by which nonpathogenic strains control the disease have been studied by comparing soilborne pathogenic strains with the nonpathogenic ones. In this respect, the strain Fo47 is probably the most studied nonpathogenic isolate. Three major mechanisms were reported: (i) competition for nutrients (11,28); (ii) competition for infection sites (30) and root colonization (6,21,38); and (iii) induced systemic resistance (19, 39). However, it is likely that these approaches, which are all based on the analysis of microbial interactions in the soil, the rhizosphere and the root tissues, do not account for all the potential mechanisms implicated in the biocontrol activity of nonpathogenic *F. oxysporum*. One possibility to enlarge the screening of other putative mechanisms is to generate *F. oxysporum* isogenic mutants affected in their biocontrol activity and to characterize their molecular differences.

At present, several methods are available to generate fungal mutants. Bouhot (7) reported that mutants obtained by chemical or UV mutagenesis presented a low stability and their analysis could be complicated by the presence of multiple sites of mutation events. Therefore, approaches generating a single mutation are envisaged. Recent developments in mutagenesis strategies in *F. oxysporum* offer the opportunity to tag genes without any a priori knowledge about their function. There is a huge emerging literature concerning the use of the so-called "black box" approach to isolate genes involved in fungal pathogenicity as reviewed by Hensel and Holden (23), Brown and Holden (8), Yoder and Turgeon (54), Hamer et al. (22), or Idnurm and Howlett (24). Restriction enzyme-mediated integration mutagenesis is efficient in tagging an arginine biosynthesis gene involved in *F. oxysporum*

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f. sp. *melonis* pathogenicity (37). An *Agrobacterium*-mediated transformation strategy permitted to generate *F. oxysporum* mutants in which fungal genes tagged by T-DNA could be cloned using a thermal asymmetric interlaced-polymerase chain reaction (PCR) approach (36). Finally, the efficiency of *impala* transposition in generating *F. oxysporum* mutants affected in their pathogenicity has been reported (34).

Whereas insertional mutagenesis has been extensively used to tag genes involved in the biocontrol activity of bacteria (4,35), no data are available concerning the use of such a method to identify functions implicated in the *F. oxysporum* biocontrol activity. Integrative transformation of plasmids into the *F. oxysporum* genome is inefficient in this respect due to plasmid loss either in vitro or in situ (32). Recently, the transposition of the autonomous *Fot1* element in *F. oxysporum* strain Fo47 has been described and proposed as a gene tagging system (33). We describe here the use of this approach to generate Fo47 mutants affected in their biocontrol activity against *F. oxysporum* f. sp. *lini*, incitant of Fusarium wilt disease on *Linum usitatissimum* L. Because the biocontrol activity of Fo47 is mainly based on mechanisms of competition involving a great saprophytic ability, the mutants have been carefully characterized for their capacity to grow and compete in soil.

MATERIALS AND METHODS

Fungal strains. Unless otherwise specified, all strains were first grown on potato dextrose agar (PDA, Sigma Chemical, St. Louis) at 25°C. After 7 days, a portion of the agar, covered by mycelium, was cut and immediately transferred under mineral oil. All the strains were stored under these conditions at 4°C.

The nonpathogenic strain of *F. oxysporum* Fo47 was isolated in 1976 from the soil of Châteaurenard (France), naturally suppressive to Fusarium wilt (2). Its efficacy in reducing the incidence of Fusarium wilt has been demonstrated regularly for the last 25 years (1,2,12,27,28). Moreover, the strain Fo47 presents the advantage of being free of endogenous *Fot1* transposable elements (17,33).

A highly pathogenic isolate of *F. oxysporum* f. sp. *lini*, coded Foln3, was used in the bioassays. Strain Foln35 is a benomyl resistant mutant obtained from isolate Foln3 after UV exposure and was used only to test the ability of Fo47 mutants to compete with a pathogenic strain in soil.

Obtaining cotransformants. A spontaneous nitrate reductase deficient mutant (*nia*$^-$) of Fo47, coded *nia1* (mutant 155), was selected on chlorate medium (13) by Migheli et al. (33). It showed thin growth on a minimal medium with nitrate as the sole nitrogen source (MM-nitrate) (10). This mutant was then cotransformed as described (33) and presented in Figure 1 with two plasmids: one containing the *nia*D (nitrate reductase) gene from *Aspergillus nidulans* (29), disrupted by a copy of the transposon *Fot1* inserted within the third intron (*nia*D::*Fot1*), the other carrying a selectable marker (hygromycin B resistance). The cotransformants (strains that have integrated the two plasmids in their genome) showed thin growth similar to that of the mutant 155, due to their genotype *nia*$^-$ and *nia*D$^-$ (inability to utilize nitrate as a nitrogen source). Three *F. oxysporum* cotransformants, coded 63, 69, and 77, initially coded 280, 281, and 272, respectively (33), which contain a single copy of the *nia*D::*Fot1* construct, were previously obtained from the nonpathogenic isolate Fo47 (33) and used to generate mutants. Fungal strains and cotransformants used in this study are listed in Table 1.

Obtaining and selecting mutants. To generate mutants, a phenotypic assay was used to identify the excision events of the *Fot1* transposable element. Briefly, the assay consisted in cultivating the cotransformant strain 77 on a selective liquid medium (LMM-nitrate [15], a minimal medium containing nitrate as the unique nitrogen source). A *Fot1* excision event from the *nia*D gene in the cotransformant restores the nitrate reductase activity (Fig. 1), allowing an aerial growth of the mutants on MM-nitrate (33). These strains correspond to revertants because they regain prototrophy but, with the aim to simplify and clarify the understanding, we decided to call them just mutants.

Single spore isolates from cotransformant 77 were grown for 5 days in malt extract liquid medium (10 g liter^{-1}) (Biokar Diagnostics, Beauvais, France) at 25°C on a rotary shaker (150 rpm). A conidial suspension was obtained by filtering through a sintered glass funnel (maximum pore size 40 to 100 μm) to remove mycelial mats. Microconidia left in the filtrate were pelleted by centrifugation at 5,000 × *g* for 10 min (Model GR20.22; Jouan, France) and rinsed three times with sterile distilled water. The conidial density of the suspension was determined by direct observation on a hemacytometer and adjusted by dilution with sterile distilled water. The conidia were inoculated (10^6 spores ml^{-1}) in liquid MM-nitrate (15). Excision events were identified after 14 to 21 days of incubation at 25°C by the appearance, in the liquid culture, of the wild-type phenotype (mycelium mats). Single spore colonies from mutants were then cultured onto MM-nitrate medium plus 1.5% agar (MMA-nitrate) for further analysis. Several monoconidial strains for each mutant were retained, but only one was used in subsequent analyses. Moreover, 19 mutants isolated previously (33) from cotransformants 63, 69, and 77 were already available to identify those affected in their antagonistic activity.

Screening of mutants affected in their biocontrol activity. The antagonistic efficacy was evaluated in bioassays using flax (*Linum usitatissimum* L.) cv. Opaline and its pathogen *F. oxysporum* f. sp. *lini* under gnotobiotic conditions. This pathosystem was chosen because flax presents the following advantages: it shows typical Fusarium wilt symptoms (yellowing of the leaves which then turn completely yellow, wilt and dry up); it has a small size, interesting to run many plants needed for statistical analysis; and it is well characterized, especially during the phases of root colonization by strain Foln3 (52).

Firstly, a large-scale screening including 90 mutants was conducted to select those affected in their antagonistic potential on flax. Conidial suspensions of *F. oxysporum* f. sp. *lini* strain Foln3, the nonpathogenic strain Fo47, cotransformants and mutants were prepared as described by Steinberg et al. (47). Briefly, fungal strains and mutants were grown in malt broth (10 g liter^{-1} malt extract) (Biokar Diagnostics) for 5 days on a rotary shaker (150 rpm) at 25°C. The culture was filtered through a sintered glass funnel (maximum pore size 40 to 100 μm) to remove mycelial mats. Microconidia in the filtrate were pelleted by centrifugation at 5,000 × *g* for 10 min (Model GR20.22) and washed twice with sterile distilled water to remove traces of nutrients. The concentration of microconidia in each suspension was measured by microscopic observation under a hemacytometer and adjusted to the given concentration required for the bioassays.

Heat-treated (100°C for 1 h) soil from Dijon (35.1% clay, 47% loam, 15.1% sand, 1.22% organic C, and 0% $CaCO_3$ [pH 6.9]) was distributed in 50-ml plastic pots and inoculated with a mixture of conidial suspensions of the nonpathogenic strain (wild-type strain Fo47, cotransformant or mutant) and of the pathogen Foln3 in the 100:1 (nonpathogen/pathogen) ratio. The strain Fo47 and its mutants were introduced at 1×10^5 conidia ml^{-1} of soil, whereas the pathogenic isolate Foln3 was introduced at 1×10^3 conidia ml^{-1}. Three flax seeds were deposited at the soil surface between two layers of disinfected clay granules (Terra-green, oil dri US special type 3R, Brenntag Bourgogne, Montchanin, France). Flax were grown in the greenhouse at 15 to 17°C for 10 days, and then at 22 to 25°C. Four days after inoculation, flax were thinned to one plant per pot. Three replicates of 12 independent pots with one plant per pot were inoculated with each mutant in random block design. Starting 2 weeks after inoculation, the plants were checked once a week for 7 weeks, and the plants exhibiting typical yellowing of the leaves indicative of Fusarium wilt were cut. The test was interrupted 63 days after inoculation.

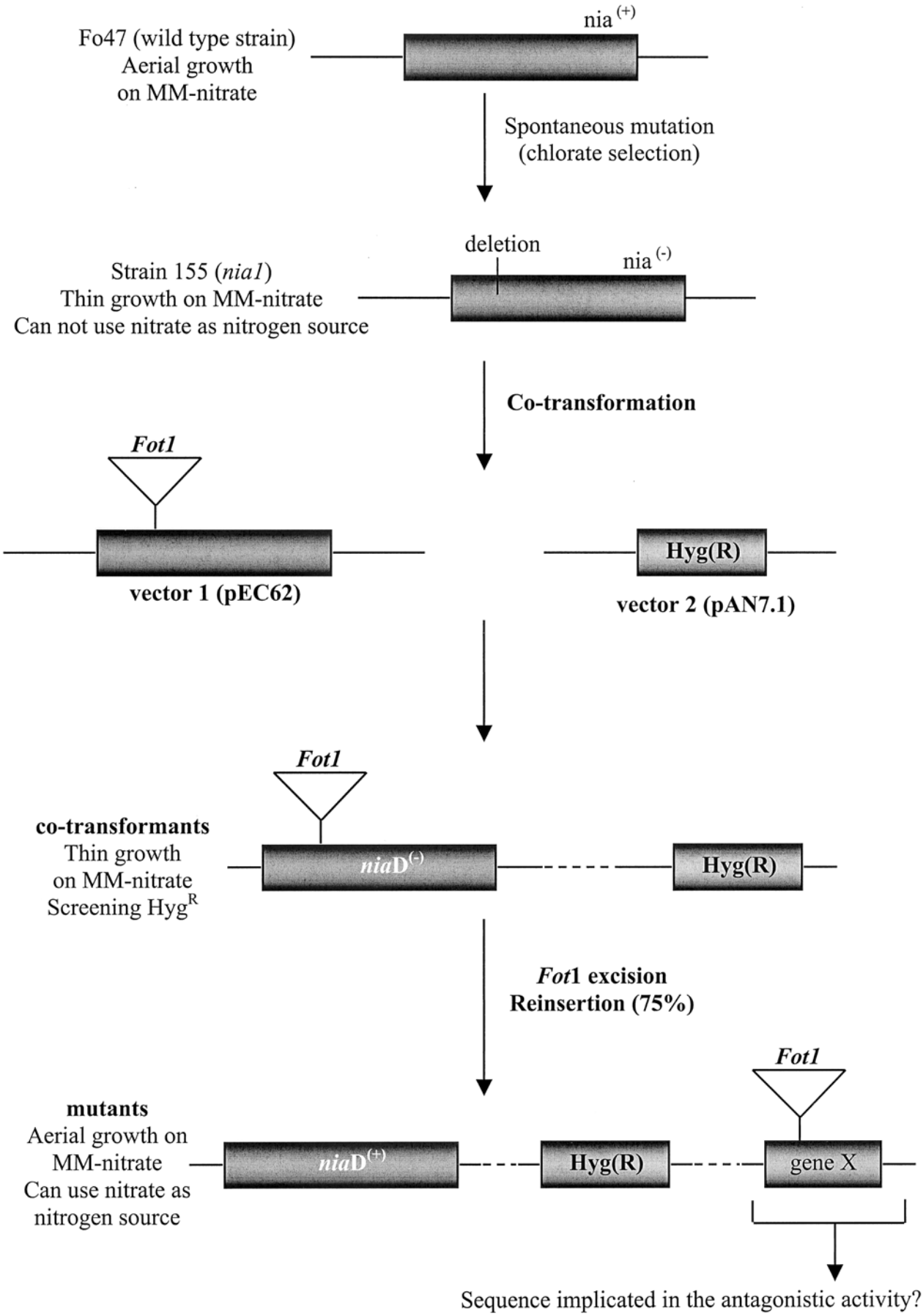


Fig. 1. Scheme of the molecular strategy used to obtain Fo47 mutants as described by Migheli et al. (33).

Only mutants that were significantly affected in their antagonistic activity compared with the wild-type strain Fo47 were retained for subsequent antagonistic tests to confirm impairment of antagonism.

To select the most affected mutants, new bioassays were performed following the same protocol as described previously, with three population ratios (nonpathogen/pathogen): 10:1, 100:1, and 1,000:1, the concentration of the pathogen being kept at 10^3 conidia ml^{-1} of soil.

In both experiments, negative and positive controls consisted of seedlings inoculated with water (healthy control) or with the pathogenic strain Foln3 alone (diseased control). All screening experiments were repeated.

To identify mutants significantly affected in their biocontrol activity, a survival data analysis method was performed. This analysis estimates the survival function $S(t)$, which is the probability that the failure time (typical symptom appearance) is at least t for each plant (25). A mean survival time (MST) was then evaluated for the flax plants inoculated with a given mutant, provided that at least 1 plant among the 12 exhibited symptoms (51). MST in the mutant-protected treatment was compared with that in Fo47 and the diseased control using analysis of variance (ANOVA) ($P < 0.05$).

DNA preparation and Southern blot analysis. DNA for Southern blot analysis was obtained by the extraction method described by Edel et al. (18). Approximately 6 µg of DNA was digested in the presence of 30 units of the restriction enzymes *Eco*RI (Roche, Mannheim, Germany) or *Ava*II (Biolabs, Oty, UK), which have no restriction site within the *Fot1* sequence, and then subject to electrophoresis in 0.8% agarose gels. Southern transfer was performed by standard methods (44) onto 0.45-µm membranes (Biodyne Plus; Pall Gelman Laboratory, Pall Life Sciences, Ann Arbor, MI) and fixed by heating 30 min at 120°C. DNA hybridization and probe detection were done with a nonradioactive DNA labeling and detection kit (Roche) according to the supplier's instructions. Five different probes obtained by PCR were used. The first one corresponded to the gene *nia*D. Primers used for the amplification of the *nia*D specific probe (probe A) were *nia*D144 (5′-GTTCATGCCGTGGTCGCTGCG-3′) and *nia*D145 (5′-CCCGGCCAAAGCCTCGAATTCG-3′). The PCR amplification was performed according to Saiki et al. (43) in a total volume of 50 µl by mixing the template DNA (50 ng of genomic DNA from the strain *F. oxysporum* f. sp. *melonis* containing the *nia*D gene [34] with 0.1 µM each primer); 100 µM each of dATP, dCTP, dGTP, and dTTP; 1 unit of *Taq* DNA polymerase (Appligene-Oncor, Illkirch, France); and PCR reaction buffer (10 mM Tris-HCl [pH 9.0], 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, and 0.2 mg of bovine serum albumin per ml). Amplifications were conducted in a thermal cycler (GeneAmp PCR System 9600; Perkin-Elmer Applied Biosystems, Foster City, CA) with 30 cycles of 90 s at 94°C, 60 s at 60°C, and 90 s at 72°C followed by an additional extension step of 15 min at 72°C. The specificity and efficacy of amplification were checked by electrophoresis with 5-µl aliquots of PCR products in a 0.8% agarose gel.

Four transposons were used as probes: *Fot1*, *Fot2*, *impala*, and *Hop*. *Fot2* and *Hop* probes were prepared from PCR products provided by M.-J. Daboussi (Institut de Génétique et Microbiologie, Université Paris-Sud, Orsay, France). The probes *Fot1* (probe B) and *impala* were obtained by PCR amplification of genomic DNA from the strain Foln3. A unique primer, deduced from the inverted terminal repeat sequence, was used (14,26). These amplifications were performed as described previously. In all cases, PCR products were digoxigenin-labeled with a random primer extension kit (Roche) following manufacturer's instructions. The presence and location of the *nia*D gene and of the *Fot1* element was assessed using labeled PCR products as probes A and B, respectively. Hybridization was performed using standard conditions (68°C and 0.2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]).

PCR detection of the *Fot1* element in mutants. For all mutants, PCR amplifications were performed in the same conditions as described previously with 100 to 300 ng of total genomic DNA. The presence of the *Fot1* element was checked by electrophoresis on 0.8% agarose gels.

Phenotypic characterization of mutants significantly affected in their biocontrol activity. To evaluate radial growth, strains were first grown on PDA medium (Sigma Chemical) at 25°C. After 7 days, they were first subcultured on MM-nitrate (10) to eliminate any residue of PDA medium. After 7 days, they were subcultured again on MMA-nitrate in 10-cm-diameter petri dishes using straw, sized plugs (3-mm diameter). Three replicates per mutant were prepared. The colony diameters of each of the repli-

TABLE 1. Description of the fungal strains used to obtain Fo47 mutants or applied in antagonistic and phenotypic assays

Fungal strain number[a]	Origin	Phenotype
Fo47	Nonpathogenic: isolated from the suppressive soil from Châteaurenard (France)	nia$^+$: no endogenous *Fot1* copy
155	Spontaneous nitrate reductase mutant of Fo47	nia$^-$: no *Fot1* copy
COTR 63	Cotransformant of the strain 155: pAN7.1 (Hyg^R), pEC136 (*nia*D disrupted with *Fot1*)	*nia*$^-$: *nia*D$^-$: Hyg^R: 1 copy of *Fot1* (within *nia*D gene)
COTR 69	Cotransformant of the strain 155: pAN7.1 (Hyg^R), pEC136 (*nia*D disrupted with *Fot1*)	*nia*$^-$: *nia*D$^-$: Hyg^R: 1 copy of *Fot1* (within *nia*D gene)
COTR 77	Cotransformant of the strain 155: pAN7.1 (Hyg^R), pEC62 (*nia*D disrupted with *Fot1*)	*nia*$^-$: *nia*D$^-$: Hyg^R: 1 copy of *Fot1* (within *nia*D gene)
Foln3	Pathogenic on flax, isolated from a diseased flax plant	nia$^+$: multiple copies *Fot1*
Foln35	Pathogenic on flax, benomyl resistant mutant obtained from Foln3 after UV mutagenesis	nia$^+$: multiple copies *Fot1* benomyl^R

[a] Strains 155, COTR 63, 69, and 77 correspond to strains *nia*1, 280, 281, and 272, respectively, in Migheli et al. (33).

TABLE 2. Estimation of mean survival time (MST) of flax seedlings inoculated either with the pathogenic strain Foln3, the nonpathogenic strain Fo47, or any of the 16 mutants altered in their antagonistic activity in greenhouse experiments

Fungal strain	MST (days)	Class[a]
Foln3	37.07	
Foln3 + Fo47	50.13	1
69 (COTR 281)	46.50	1
77 (COTR 272)	46.11	1
76	54.30	2
81	54.70	2
83	57.96	2
119	56.22	2
193	56.60	2
289	56.61	2
354	56.03	2
406	54.90	2
835	55.05	2
977	58.36	2
94	43.40	3
503	42.61	3
505	42.10	3
614	43.19	3
782	43.78	3
885	43.75	3

[a] Each class grouped strains for which the MST is not significantly different at $P < 0.05$.

cates were measured twice a day for 10 days. The data were analyzed using ANOVA.

The ability of fungal strains to compete with the pathogen in soil was assessed by using the technique described by Couteaudier and Alabouvette (11) by using polypropylene tubes (9 ml) containing 5 g of soil from Dijon, sterilized by γ irradiation, and infested by a mixture of pathogenic and nonpathogenic strains of *F. oxysporum*. Briefly, a concentration of 1×10^3 conidia g^{-1} of nonpathogenic strains was mixed with different concentrations of the benomyl resistant pathogenic strain Foln35 in order to obtain five initial ratios (nonpathogenic/pathogenic strains) of inoculum densities: 0.1, 0.2, 1, 2, and 10. Three replicates of sterilized soil were infested in each case. In previous experiments, Zegerman et al. (55) showed that a strain growing in sterilized soil at 25°C reached its plateau between 10 and 15 days. Based on these data, the number of propagules of each strain was determined after 20 days of incubation at 25°C by using the soil dilution plate technique and by culturing on both 1% malt agar to reveal the pathogenic and nonpathogenic strains and 1% malt agar amended with benomyl (5 mg $liter^{-1}$) to specifically reveal the pathogenic strain. The difference between the two counts was assumed to be the population density of the nonpathogenic strain. This time-consuming experiment was conducted with mutants 83 and 94, cotransformant 77, and strain Fo47 to compare their competitiveness toward the pathogenic isolate Foln35.

RESULTS

Obtaining mutants. From 1,000 initial cultures of cotransformant 77, a total of 71 mutants were obtained showing the wild-type phenotype (aerial growth on MMA-nitrate). In addition, 19 mutants obtained by Migheli et al. (33) were included, accounting for a total of 90 mutants.

Selection of mutants significantly affected in their antagonistic activity. On susceptible flax cv. Opaline, the first typical symptoms of wilt induced by isolate Foln3 appeared 28 to 35 days after sowing. The MST observed for plants inoculated with Foln3 alone was 37 days, whereas it reached 50 days in coinoculation with Fo47 (Table 2). Comparison of the ability of each mutant to protect the plants more or less than the wild-type strain Fo47 was performed based on MST.

In the first screening, using the ratio 100:1, 16 mutants (obtained from cotransformants 69 and 77) significantly affected in their capacity to protect flax from the pathogen were obtained, according to the kinetics of wilt symptom development (Fig. 2). The statistical analysis performed by ANOVA ($P < 0.05$) on the MST enabled us to define 3 classes of strains with significantly different MST. Mutants in class 1 were not significantly different from Fo47, mutants in class 2 were more antagonistic than Fo47, and mutants in class 3 were less antagonistic than Fo47. The first class grouped Fo47 and the two cotransformants 69 and 77. The second class grouped mutants showing an MST between $54 < MST < 58.5$ significantly different from MST of Fo47. Finally, the third class grouped mutants showing an MST between $40 < MST < 44$ significantly different from MST of Fo47 (Table 2).

The second bioassay allowed us to select two mutants, coded 83 and 94, which showed marked differences with respect to their antagonistic activity whatever the density ratio (10, 100, or 1,000) as shown in Figure 3. Mutant 83 inoculated in ratio 10:1 was as effective as strain Fo47 inoculated in ratio 1,000:1, whereas mutant 94 inoculated in ratio 1,000:1 was no more effective than strain Fo47 inoculated in ratio 10:1. Only these two most affected strains (83 and 94) were retained for phenotypic experiments such as strain competitiveness measurement in soil.

Molecular characterization of mutants. Based on previous results (33) by Southern blot analysis, we expected to observe a

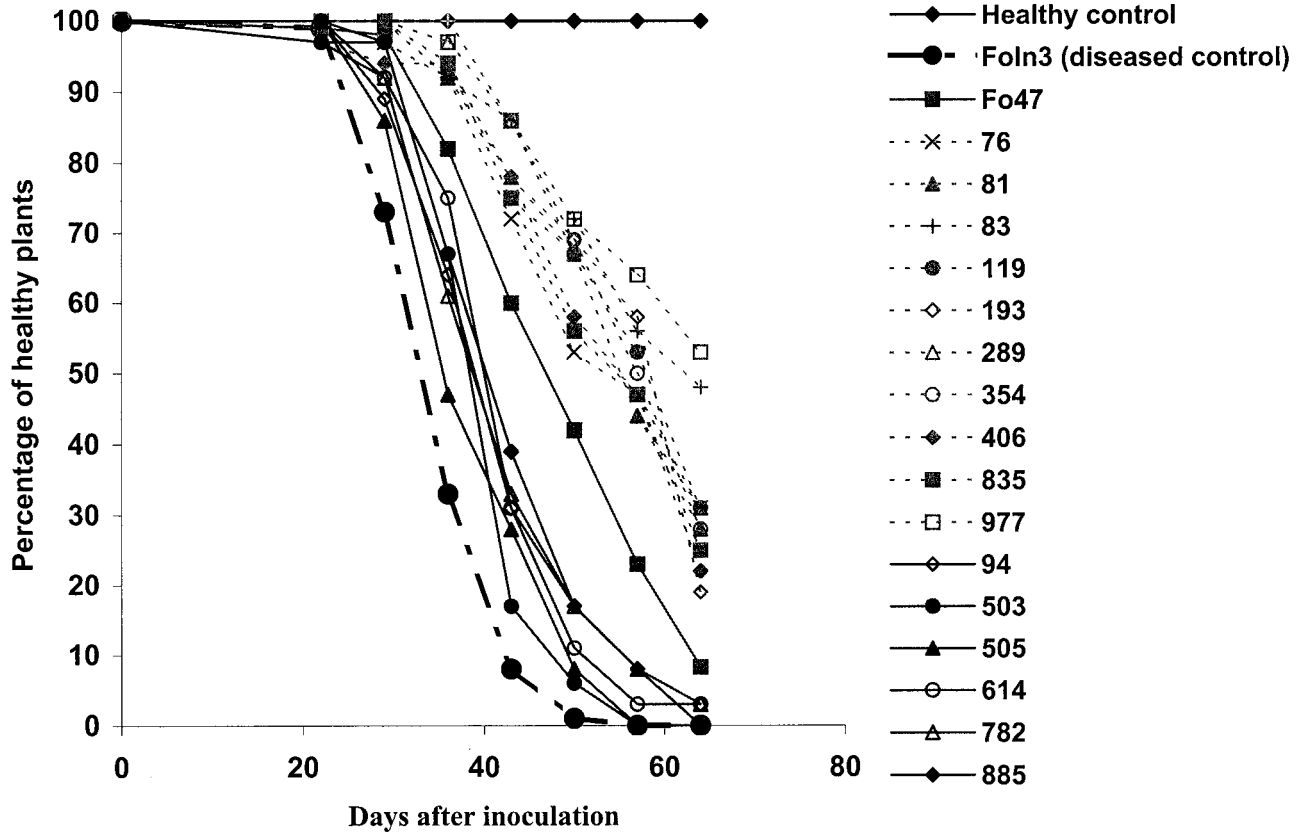
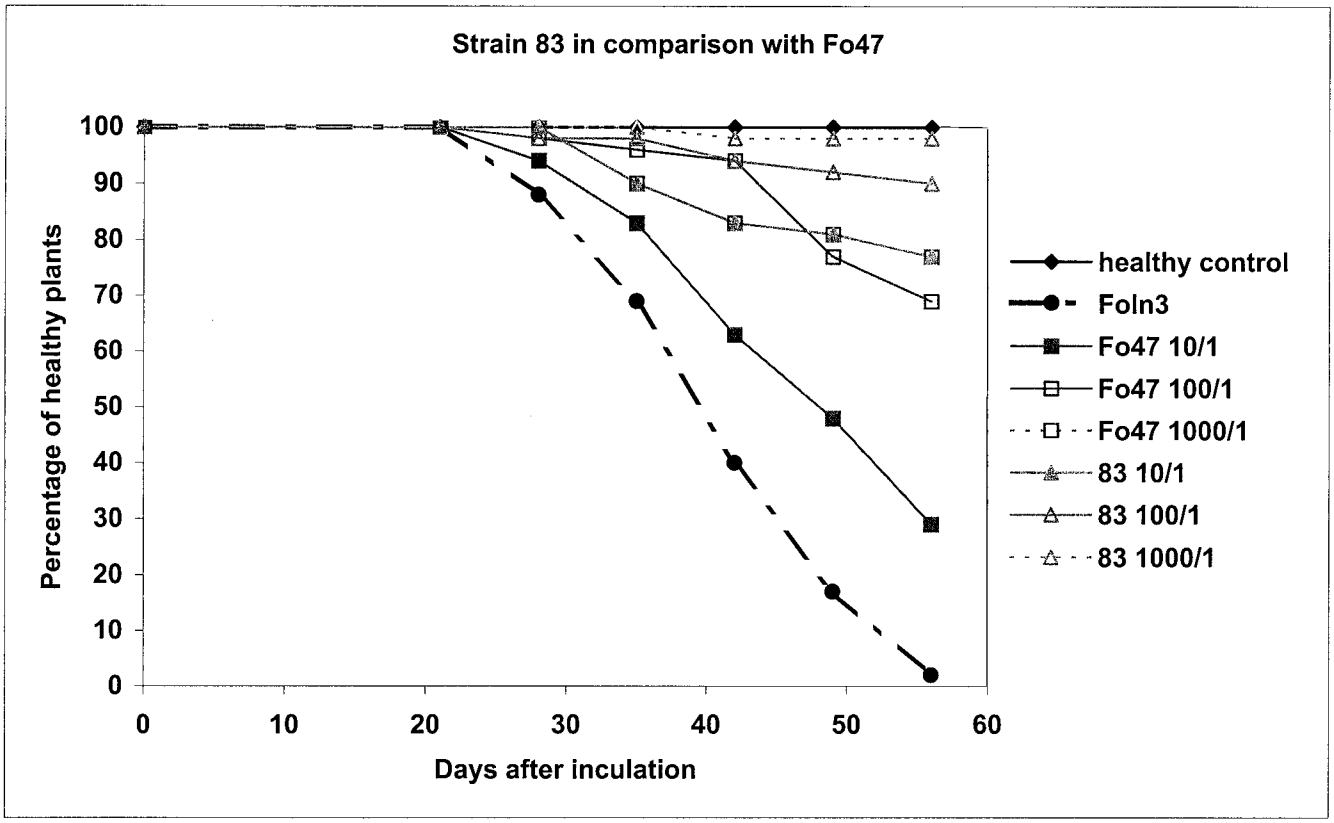


Fig. 2. Ability of 16 different mutants of the nonpathogenic *Fusarium oxysporum* Fo47 to control Fusarium wilt of flax in greenhouse experiment. The data represent the mean percentage of the three replicates of 12 plants. Only the mutants that were significantly different from the wild-type strain are represented.



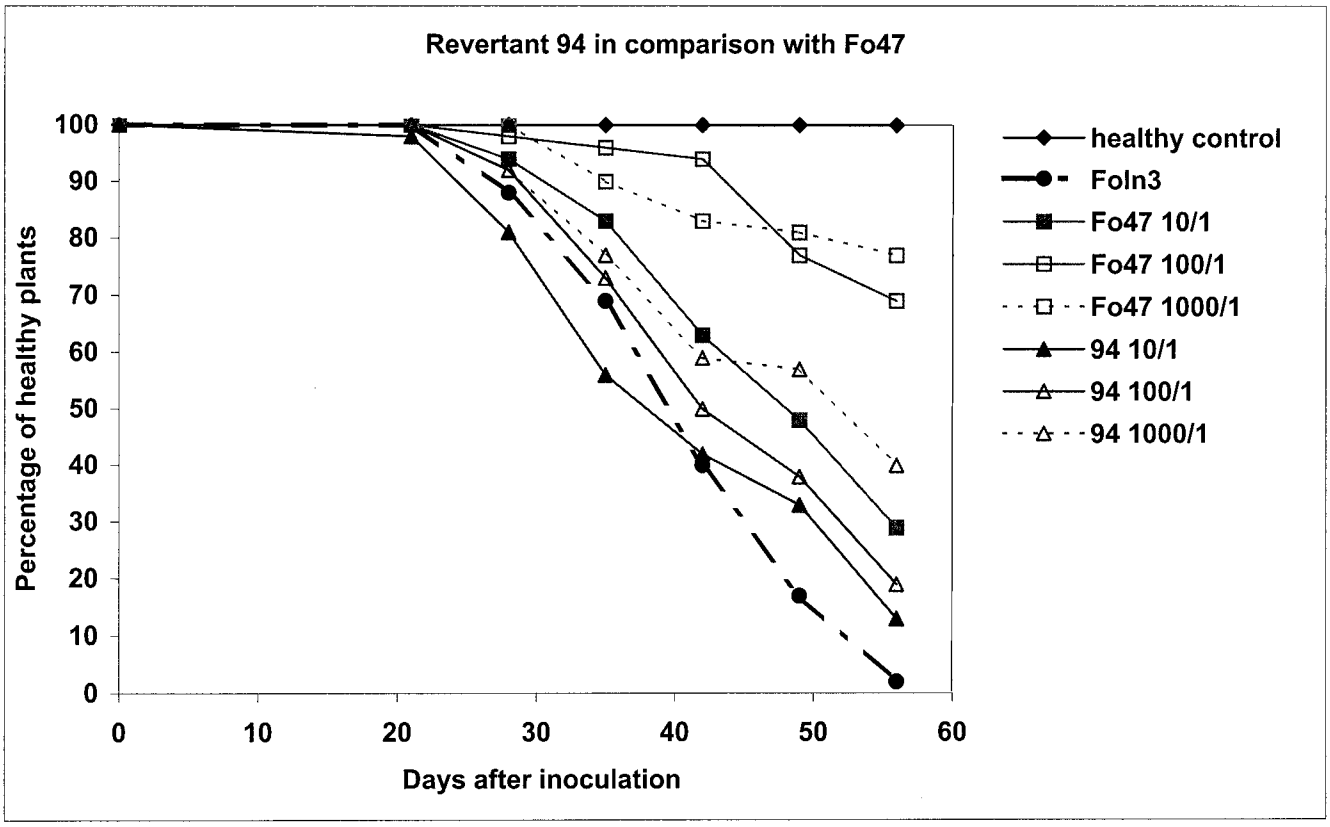


Fig. 3. Ability of the two most affected mutants of nonpathogenic *Fusarium oxysporum* Fo47 to control Fusarium wilt of flax in greenhouse experiments at different inoculum concentration ratios. The data represent the mean percentage of the three replicates of 12 plants.

band at 4.7 kb when *Fot1* was inserted in the *nia*D gene and a band at 2.6 kb when it was excised from this gene. Figure 4A shows that only cotransformant 77 kept the *Fot1* copy in the *nia*D gene, whereas this transposable element was excised in cotransformant 69. For all 16 mutants, *Fot1* was excised from the *nia*D gene (Fig. 4A), and in 12 cases (75%), it was reintegrated in a new genomic position (Fig. 4B). This frequency was confirmed by the restriction enzyme *Ava*II (data not shown) and by the amplification of the *Fot1* element (Fig. 5). However, the pattern of reinsertion of the transposed *Fot1* copy was different for most of the mutants analyzed, indicating that reintegration occurred at different sites in the genome and that each mutant corresponds to an independent transposition event. Moreover, each mutant displayed hybridization patterns similar to those of the insertion mutant (cotransformant) after probing with other transposable elements such as *Fot2* or *impala* (Fig. 6), suggesting that the phenotype observed was not linked to the transposition of these elements. Finally, it is interesting to note that Fo47 has no endogenous copies of the *Hop* element (Fig. 6).

Phenotypic characterization of affected mutants. As shown in Figure 7, cotransformants (69 and 77) and mutants had the same ability to grow at 25°C as wild-type strain Fo47. ANOVA showed that there was no significant difference ($P < 0.05$) in their capacity to elongate mycelium on mineral medium.

The soil population densities at the plateau in the three replications of each treatment were not significantly different. Results showed that all the strains actively colonized the disinfested soil. Population density at the plateau on malt agar reached 5.4×10^5 CFU g^{-1} of soil regardless of the initial concentration. A regression analysis was performed between the inoculum density ratio at the plateau (x/y) and the inoculum density ratio at the time of soil infestation (x_0/y_0), where x represents the nonpathogenic and y the pathogenic inoculum density. The high R^2 values, ranging from 0.96 to 1.00, indicated a significant linear relationship between inoculum density at the plateau and inoculum density ratios at the time of inoculation of the fungi into soil. As described by Couteaudier and Alabouvette (11), the slope of the regression lines (data not shown) represents the competitiveness of the nonpathogenic strain compared with the pathogenic one. In the case of the four strains considered in the present study, the slopes were higher than 1.00, indicating that mutants 83 and 94 were not affected in their ability to compete with Foln35 in soil.

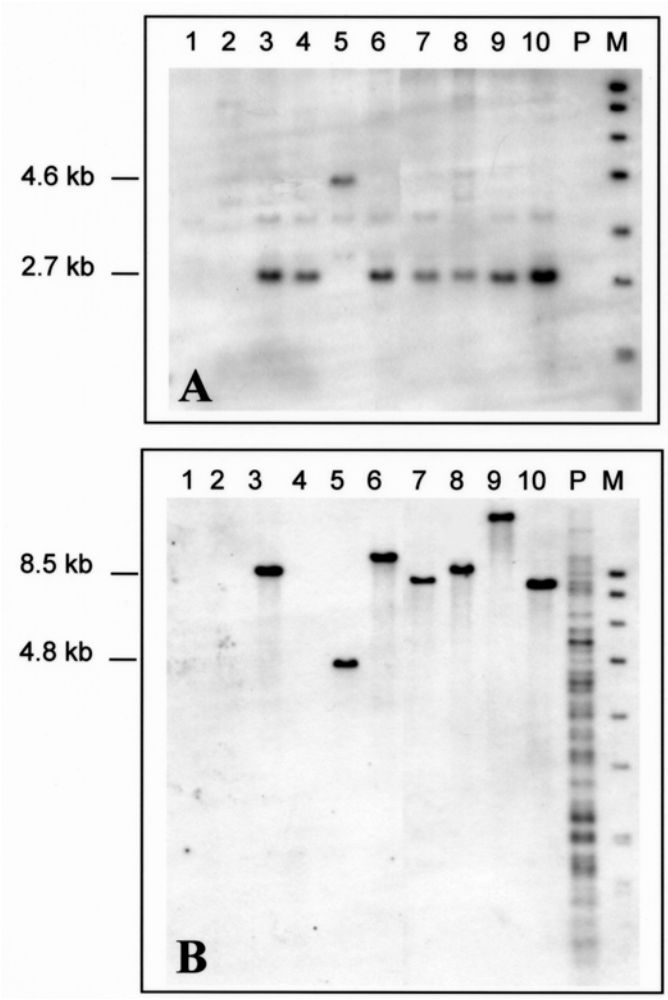


Fig. 4. Hybridization patterns using *nia*D or *Fot1* probes. Each lane contains genomic DNA digested with *Eco*RI and probed with the polymerase chain reaction product of **A,** *nia*D or **B,** *Fot1*. From left to right; lane 1, Fo47 showing no copy of either *nia*D and *Fot1*; lane 2, 155 (Fo47 *nia*$^-$) showing the same patterns as Fo47; lanes 3 and 5, cotransformants 69 and 77, respectively; lane 4, mutant from cotransformant 69 presenting the lack of reinsertion of the *Fot1* copy; lanes 6 to 10, independent mutants from transformant 77 resulting from the excision of the *Fot1* copy in the *nia*D gene and its reinsertion in different genomic positions; lane P, pathogenic strain Foln3; and lane M, molecular weight marker bands.

DISCUSSION

The first authors who discovered the narrow host specificity of the wilt-inducing strains of *F. oxysporum* also noticed that preinoculation of a plant with an incompatible strain results in the mitigation of symptoms when the plant is subsequently inoculated with a compatible strain (31). Following these first observations, several nonpathogenic strains of *F. oxysporum* have been selected for their biocontrol capacity and are being developed as biocontrol agents. Despite efforts made to identify the mode of action by which these nonpathogenic strains protect the plant against the compatible forma specialis, the basic mechanisms are far from being well understood. The biocontrol activity of a nonpathogenic strain results from a tripartite relationship involving the plant, the pathogen, and the biocontrol agent. Indeed, several studies have

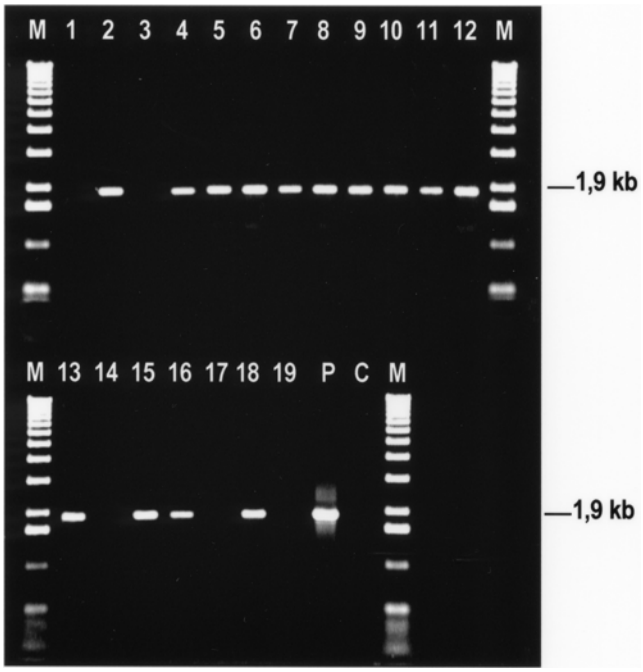


Fig. 5. Polymerase chain reaction amplification of the *Fot1* copy, using the FT1 primer, from template DNAs of the 16 *Fusarium oxysporum* Fo47 mutants significantly affected in their antagonistic potential. From left to right: lane 1, Fo47; lanes 2 and 4, cotransformants 69 and 77, respectively; lane 3, mutant from cotransformant 69; lanes 5 to 19, independent mutants from transformant 77; lane P, pathogenic strain Foln3; lane C, control; and lane M, molecular weight marker bands (1-kb ladder).

shown that nonpathogenic strains of *F. oxysporum*, including strain Fo47, express three major mechanisms by which they suppress Fusarium wilt: (i) competition for nutrients (11,28); (ii) competition for the infection site (30) and root colonization (6, 21,38); and (iii) induced systemic resistance (19,39). The efficacy of a biocontrol strain of nonpathogenic *F. oxysporum* results from the combination of these mechanisms expressed separately, successively, or together (3). In order to further progress in the understanding of these modes of action, to determine their molecular basis and possibly to identify new mechanisms, it was necessary to use an experimental approach based on the production of mutants affected in their biocontrol activity. This approach has proved useful to study the determinants of the pathogenicity of several plant pathogenic fungi, but to our knowledge, it has never been used with nonpathogenic *F. oxysporum*.

By means of a transposon-mediated insertional mutagenesis approach, several mutants of Fo47 affected in their biocontrol activity have been obtained. It is important to stress that these mutants were either less or more antagonistic than the wild-type strain. Moreover, the cotransformants were not significantly different from wild-type strain Fo47, suggesting that the cotransformation event was not responsible for the affected phenotype. These results demonstrate for the first time the usefulness of this strategy to generate mutants affected in their antagonistic potential, thus offering a unique opportunity to understand the basis of the antagonistic activity of the nonpathogenic strains of *F. oxysporum*.

All mutants presented a *Fot1* excision event and the majority showed a *Fot1* reinsertion in a different location of the genome.

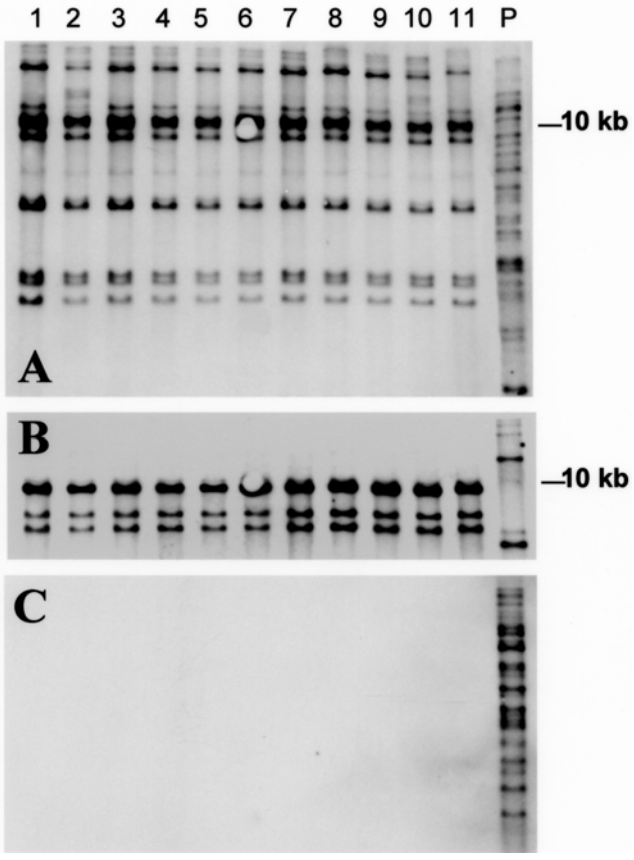


Fig. 6. Hybridization profiles of *Fot2*, *impala*, and *Hop* in *Fusarium oxysporum* Fo47-derived mutants. Each lane contains genomic DNA digested with *Eco*RI and probed with the digoxigenin-labeled polymerase chain reaction product of **A,** *Fot2*, **B,** *impala*, or **C,** *Hop*. From left to right: lane 1, Fo47; lane 2, 155 (Fo47 *nia*$^{-}$); lanes 3 and 5, cotransformants 69 and 77, respectively; lane 4, mutant from cotransformant 69; lanes 6 to 11, independent mutants from transformant 77; and lane P, pathogenic strain Foln3.

We also demonstrated that other elements such as *Fot2*, *impala*, or *Hop* had no transposable activity in the mutant strains. These results indicate the possibility to tag the genetic corresponding sequences involved in the affected phenotype, as it was described by using the same strategy in *Magnaporthe grisea* (53). However, given that 25% of significantly affected mutants did not present a *Fot1* reinsertion and that Villalba et al. (53) recently reported the low proportion of genes tagged using transposon mutagenesis, it is not possible to directly link the tagged sequences and the altered phenotype. Moreover, the high frequency of Fo47 mutants altered in their biocontrol property suggests that alternative mechanisms, such as large deletions or chromosomal translocations caused by ectopic recombination between homologous transposon copies, may be responsible for dramatic phenotypic changes, as recently demonstrated in *F. oxysporum* (16). A complementary strategy to characterize the affected mutants involves a proteomic approach, as described by Recorbet et al. (41).

Through Southern blot analysis, we showed that the antagonistic strain Fo47 has no copies of transposable elements such as *Fot1* or *Hop*. Moreover, it is noteworthy that Fo47 contains a limited number of *impala* copies compared with a pathogenic *F. oxysporum* such as Fom24 (34). This low number of transposable elements might help to explain the very stable phenotype of Fo47, which was isolated 25 years ago and still is a very efficient biocontrol agent.

Because most hypotheses related to the mode of action of the biocontrol strains of *F. oxysporum* emphasize competition-based mechanisms, the mutants were characterized for their saprophytic traits. Mutants 83 and 94, the most significantly affected in their biocontrol capacity, have the same ability to grow and elongate hypha on MMA-nitrate as wild-type strain Fo47. The high population densities observed at the plateau after soil infestation with a conidial suspension showed that both mutants were able to germinate and actively colonize a disinfested soil. Moreover, under these conditions, they were as competitive as Fo47 when confronted to pathogenic isolate Foln35. Even mutant 94, which has almost totally lost the capacity to protect the plant against Foln3, is still competitive. It is also not possible to establish any correlation between the saprophytic competitiveness in soil and the biocontrol capacity of these mutants. These results raise the hypothesis that the most important phenomena leading to biological control should take place during the interaction within the plant.

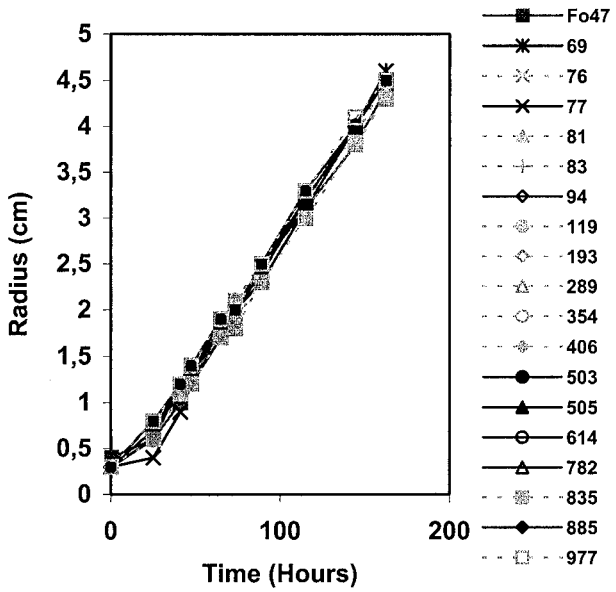


Fig. 7. Radial growth on minimal medium for the 16 mutants significantly affected in their capacity to protect flax.

Indeed, both the nonpathogenic and pathogenic strains are able to colonize the root surface and penetrate into the root cortex. Based on preliminary experiments showing that both mutants could be isolated from surface-disinfected flax root (data not shown), one can assume that these mutants are still able to colonize the root system to some extent. If this is the case, their different ability to control Fusarium wilt should be related to differences in the plant–fungus interaction at the cellular and molecular level. To address this question, it will be necessary to accurately study the localization of the fungi within the flax root, to characterize the plant defense reaction in the tissues, and dissect early reaction events (pH variation, active oxygen species production, and calcium flux) induced by the mutants on plant cell cultures as described by Olivain et al. (40).

These results confirm that transposon mutagenesis is a powerful tool to generate *F. oxysporum* mutants affected in their biocontrol ability and suggest that different mechanisms are involved in the biocontrol activity, since affected mutants may be generated by independent *Fot1* insertion events throughout the genome.

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