FUSARIUM OXYSPORUM STRAINS AS BIOCONTROL AGENTS AGAINST *FUSARIUM* WILT: EFFECTS ON SOIL MICROBIAL BIOMASS AND ACTIVITY[1]

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ABSTRACT - Before planning the large-scale use of nonpathogenic strains of *Fusarium oxysporum* as biocontrol agents of *Fusarium* wilt, their behaviour and potential impact on soil ecosystems should be carefully studied as part of risk assessment. The aim of this work was to evaluate the effects of antagonistic *F. oxysporum* strains, genetically manipulated (T26/6) or not (233/1), on soil microbial biomass and activity. The effects were evaluated, in North-western Italy, in two soils from different sites at Albenga, one natural and the other previously solarized, and in a third soil obtained from a 10-year-old poplar stand (*Popolus* sp.), near Carignano. There were no detectable effects on ATP, fluorescein diacetate hydrolysis, and biomass P that could be attributed to the introduction of the antagonists. A transient increase of carbon dioxide evolution and biomass C was observed in response to the added inoculum. Although the results showed only some transient alterations, further studies are required to evaluate effects on specific microorganism populations.

Index terms: biological control, soilborne pathogen, risk assessment.

LINHAGENS DE *FUSARIUM OXYSPORUM* COMO AGENTES DE BIOCONTROLE DA MURCHA-DE-*FUSARIUM*: EFEITOS NA BIOMASSA E ATIVIDADE MICROBIANA DO SOLO

RESUMO - Antes do uso em larga escala de linhagens não-patogênicas de *Fusarium oxysporum* como agentes de biocontrole da murcha-de-*Fusarium*, o seu comportamento e seus impactos potenciais no solo devem ser estudados como parte da avaliação de riscos. O objetivo do presente trabalho foi avaliar os efeitos de linhagens antagonistas de *F. oxysporum*, geneticamente manipuladas (T26/6) ou não (233/1), na biomassa e atividade microbiana do solo. Os efeitos foram avaliados no noroeste da Itália em dois solos de Albenga, sendo um natural e outro previamente solarizado, e em um terceiro solo obtido numa plantação de 10 anos de *Popolus* sp., em Carignano. Não foram observados efeitos significativos na quantidade de ATP, hidrólise de diacetato de fluoresceína e biomassa P, após a introdução dos antagonistas. Um aumento transitório foi observado na evolução de dióxido de carbono e no carbono da biomassa em resposta à adição dos antagonistas. Embora os resultados tenham demonstrado apenas efeitos transitórios, outros estudos são necessários para a avaliação dos efeitos em populações específicas de microrganismos.

Termos para indexação: controle biológico, patógeno de solo, análise de risco.

INTRODUCTION

Fusarium wilts, caused by *formae speciales* of the soil-borne fungus *Fusarium oxysporum*, is a major problem on many crops. The management of this disease depends on the integration of different control methods including chemical, genetic, management, physical or microbial biocontrol. On crops such as tomato, melon, carnation, cyclamen and basil (Garibaldi et al., 1992; Alabouvette et al., 1993;

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Minuto et al., 1994, 1995), good results were obtained with saprophytic *Fusarium* spp. antagonistic to the pathogen and isolated from soils suppressive to the disease or from the rhizosphere of plants grown in such soils (Schneider, 1984; Paulitz et al., 1987; Toyota et al., 1995). The mechanisms of suppression of *Fusarium* wilt by these nonpathogenic *Fusarium* strains involve competition in the rhizosphere and infection sites, as well as induction of enhanced resistance in the host (Mandeel & Baker, 1991).

The biocontrol potential of antagonistic *Fusarium* spp. can be enhanced by manipulating existing wild-type strains using conventional mutagenesis (Papavizas, 1987; Garibaldi et al., 1989), protoplast fusion (Migheli et al., 1992) or genetic transformation (Chet et al., 1993). However, before planning the commercial use of such organisms, genetically manipulated or not, in agricultural environments, their behaviour and potential impact on soil ecosystems should be carefully studied as part of risk assessment (Estados Unidos, 1988; Tiedje et al., 1989; Cairns & Orvos, 1992). A proposed strategy for environmental risk assessment includes five steps: evaluation of the trait, i.e. risk associated with the origin; location and function of recombinant DNA; assessment of survival of the microorganism under various conditions; evaluation of gene transfer; evaluation of possible structural (e.g. total biomass, ATP content) and functional effects (e.g. on selected microbial functional groups, microbial enzymes; nutrient dynamics); and the conduct of small field trials (Cairns & Orvos, 1992; Stotzky et al., 1993). Qualitative and quantitative alterations of the microbial community structure are probably the most difficult aspects of risk assessment, since a variety of studies are required to estimate probable effects resulting from planned or unplanned releases (Cairns & Orvos, 1992; Kluepfel, 1992; Stotzky et al., 1993). Displacement of given microbial groups can be dramatic if introduced microorganisms possess a high saprophytic fitness; yet such an effect is envisaged and expected for biological control agents (Lindow, 1992; Leij et al., 1994).

In previous work, the evaluation of the trait of the manipulated antagonistic *F. oxysporum* (Migheli et al., 1996), the assessment of its survival and evaluation of possible gene transfer under various conditions were carried out (Mezzalama et al., 1994; Gullino et al., 1995).

The aim of this work was to evaluate the effects of antagonistic *F. oxysporum* strains, genetically manipulated or not, on soil microbial biomass and activity.

MATERIAL AND METHODS

Microorganisms

Two *F. oxysporum* strains, previously selected for antagonism against pathogenic *Fusarium* spp. and for benomyl resistance (10 μg mL^{-1}), were used. Strain 233/1 was obtained after UV treatment of the parental strain 233 (Garibaldi et al., 1988). Strain T26/6, also resistant to hygromycin B, was obtained by transforming strain 233/1 C5 (a dark red-pigmented mutant of 233/1), with plasmid pAN7-1, which contains the *Escherichia coli* hygromycin B phosphotransferase gene (*hph*), encoding for hygromycin B resistance (Migheli et al., 1996).

The strains were grown on liquid casein hydrolysate medium (Dhingra & Sinclair, 1986), with shaking on a rotary shaker at 100 rpm for 5 days at 28°C with a 12 hour photoperiod. The conidial suspensions were obtained by filtering cultures, centrifugating at 1300 *g* for 10 minutes and resuspending the pellets in sterile distilled water.

Survival of antagonists in different soils, during the experiments, was evaluated by plating a series of tenfold dilutions on a *Fusarium*-selective medium (Komada, 1975), amended with 10 μg mL^{-1} of benomyl (Benlate Du Pont, Denemours, USA). Petri dishes were incubated at 20°C for 5-6 days. Colony counts of *Fusarium* were averaged from ten plates (5 replications of 10^{-2} and 10^{-3} dilutions) and converted to log colony forming units (CFU) per gram on a dry weight soil basis.

Soils

Three soils, with characteristics shown in Table 1, were used in all experiments. Carignano soil was collected from a field with a 10-year-old poplar stand (*Popolus* sp.) in Piedmont (North-western Italy). The Albenga solarized and non-solarized soils were collected from different sites at a horticulture experimental station in Liguria (North-western Italy). Soil solarization was obtained by covering the soil with a transparent polyethylene sheet, during one month (Katan & De Vay, 1991).

Soil samples were collected at 10 cm depth, sieved (4 mm) and kept at 4°C before use; soils were transferred at 25°C one week before beginning the experiments. These

three soils hereafter are referred to as Carignano (C), Albenga-non-solarized (ANS) and Albenga-solarized (AS).

Soil microcosms of different sizes for each experiment were prepared and infested with 10^5 CFU of strains/g of soil. Soil moisture was adjusted to 70% of maximum water holding capacity (MWHC).

Carbon dioxide evolution

This measurement was made in microcosms consisting of hermetically-sealed polyethylene boxes (6 L capacity), in which a 500 g soil (dry-weight basis) with added suspensions of conidia was kept at 22°C in the dark. Vials containing 10 mL of 0.5N KOH were placed inside and the amount of CO_2 emitted was measured by titrating with 0.1N HCl after 6, 10, 13 and 17 days of incubation (Grisi, 1978). Three boxes were used as repetitions for each treatment. Two control samples were included in this experiment: a sample without soil and soil without added *Fusarium*.

Hydrolysis of fluorescein diacetate and ATP extraction

Microcosms for these measurements, plastic tubes (50 mL) containing 50 g of soil, were kept in transparent polyethylene bags (to avoid external contaminations and to maintain soil humidity), and incubated at 26°C in a growth chamber with a 12-hour photoperiod. Three replicates for each treatment were made.

Evaluations of hydrolysis of fluorescein diacetate (FDA, Sigma Chemical Co., St. Louis, MO) were carried out weekly for four weeks according to the method described by Boehm & Hoitink (1992). Five-gram soil samples were placed into 250-mL flasks, and 20 mL of 60 mM potassium phosphate buffer (pH 7.6) was added. The reaction (FDA hydrolysis) was started by adding 400 µg of FDA. The reaction mixture was incubated for 20 min on a rotatory shaker (90 rpm) at 25ºC. The reaction was stopped by adding 20 mL of acetone to each of the flasks. Soil residues were removed from the mixture by filtration through filter paper (Whatman nº 1). The filtrate was collected in a test tube, covered and placed into an ice bath to reduce volatilization of the acetone. The concentration of free fluorescein was determined spectrophotometrically by comparing absorbancies (490 nm) against a standard curve.

Standard curves were prepared in duplicate for each soil as follows: 0, 100, 200, 300 and 400 µg of FDA were added to 5 mL of phosphate buffer in screw-capped test tubes. Test tubes were heated in boiling water for 60 min to hydrolyse the FDA. Cooled fluorescein was then added to 250-mL flasks containing 5 g samples of the soils. An additional 15 mL of phosphate buffer was used to wash the fluorescein from the tubes into the samples. The samples were processed as described above.

ATP extraction and determination was carried out after the first and third week of incubation according to the method of Eiland (1983), modified by Arnebrant & Baath (1991), using purified luciferin-luciferase enzyme from Lumac (Landgraaf, NL). The measurement was based on the amount of light emitted by the luciferin-luciferase system. Soil samples (0.5 g) were shaken with 10 mL ice cold 500 mM H_2SO_4 and 250 mM Na_2HPO_4 for 30 min on a rotary flat-bed shaker (500 rpm). Then 50 µL of the soil solution was added to 1.5 mL 250 mM Tris buffer with 4 mM EDTA, pH 7.5. A sample (50 µL) of this buffered soil solution was added to 50 µL of NRM reagent

TABLE 1. Characteristics of the Albenga and Carignano soils used in the experiments.

Soil characteristic	Albenga		Carignano
	Solarized	Non solarized	
pH	7.8	8.0	7.6
Electrical conductivity (µS/cm)	0.85	0.24	0.15
Organic matter (%)	3.9	2.6	1.7
Total N (%)	0.19	0.15	0.14
P soluble (µg P g^{-1} soil)	133.1	210.3	24.6
Clay (%)	12	11	6
Fine silt (%)	13	13	30
Coarse silt (%)	6	10	27
Fine sand (%)	31	28	28
Coarse sand (%)	36	39	6

(Lumac Cat. nº 9332-1) and was shaken for 10 s before ATP measurement. Light output was measured in a luminometer (Biocounter M 1500L). ATP (as disodium salt) was used as an internal standard added to the samples before determination.

Microbial biomass C and P

The soil microcosms for measurements of microbial biomass C and P consisted of 700 mL plastic pots prepared and incubated as described for FDA and ATP. Each microcosm contained 600 g of soil. Three replicates per treatment were made, and for each of these, three replications were developed to determine biomass C and six to determine biomass P. Analyses were carried out at one and three weeks after soil infestation and incubation, using the fumigation-extraction method for biomass C (Vance et al., 1987) and biomass P estimations (Brookes et al., 1982).

Briefly, one of a pair of soil samples was fumigated with ethanol-free $CHCl_3$ (24 hours at 25ºC), extracted (0.5 M K_2SO_4, on a rotary shaker at 200 rpm for 30 min, then filtered through a Whatman nº 1 filter paper) and organic C measured by digesting with $K_2Cr_2O_7$ and titrating with $FeSO_4$ (Allison, 1965). A second matching soil sample was not fumigated before extraction. Biomass C was calculated assuming that biomass C = 2.64 Ec, where Ec is the difference between C extracted from the fumigated and non-fumigated treatments (Vance et al., 1987).

Biomass P was calculated from the difference between the amount of P extracted by 0.5 M $NaHCO_3$, pH 8.5 (Olsen et al., 1954), from fresh soil fumigated, as described above, and the amount extracted from non-fumigated soil. Some $CHCl_3$-released P is sorbed by soil during fumigation and extraction: an approximate allowance for this was made by incorporating a know quantity of P during extraction and correcting for recovery (Brookes et al., 1982).

Experimental designs and statistical analyses

Completely randomized designs were used in all bioassays. All statistical analyses were performed using SAS procedure (SAS Institute, Cary, NC) for analysis of variance. Means were separated by Tukey's multiple range test.

RESULTS AND DISCUSSION

The results showed that, in general, no effects on soil respiration were observed after the introduction of the two strains of antagonistic *F. oxysporum* (Fig. 1). A significant increase (Tukey 1%) on CO_2 evolution was observed after adding strain 233/1 in AS soil (6 and 13 days from soil infestation) and in C soil (13 days from soil infestation), but there was no difference among treatments after 17 days from soil infestation.

Statistically significant differences (Tukey 1%) in rates of respiration were found among soils, but the rate of evolution from each soil was the same whether or not *Fusarium* inoculum was added (Fig.1). Lower rates of CO_2 evolution occurred in the Albenga solarized soil presumably because of reduced microbial activity owing to solarization. The daily rates of respiration declined over time in solarized soil, but remained almost constant in non solarized Albenga and Carignano soils (Fig.1). The exhaustion of resources during incubation promotes a reduction of microbial activity over the time under microcosm conditions (Ritz et al., 1994). This effect was probably accelerated in solarized soil due to a reduction in microbial populations promoted by the treatment.

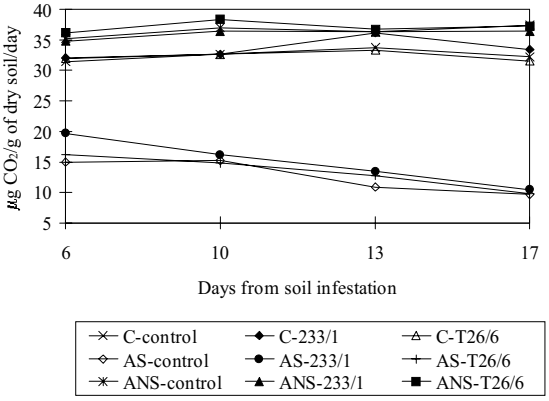

FIG. 1. Daily rates of CO_2 evolution from Albenga solarized soil (AS), Albenga non solarized soil (ANS) and Carignano soil (C), infested with the antagonistic *Fusarium oxysporum* strains, transformed (T26/6), non-transformed (233/1), and the non-infested control. DMS values (Tukey test, p ⟨ 0.01): DMS (6 days from soil infestation) = 0.8582; DMS (10 days from soil infestation) = 1.1021; DMS (13 days from soil infestation) = 1.3597; DMS (17 days from soil infestation) = 2.6457.

In general, during the four weeks evaluated, there was no significant difference (Tukey 1%) in the rates of FDA hydrolysis from each soil whether or not the antagonistic strains were added (Table 2). Similarly, no differences were found among the amounts of ATP extracted from each soil tested in the presence or absence of antagonists. In spite of the lower content of available P (Table 1), which could have lowered the ATP level, the Carignano soil presented a higher ATP amount than solarized and non-solarized Albenga soils. This result may be due to a higher clay and organic matter content in the Albenga soils, which could have interfered with the ATP extraction (Smith & Paul, 1990).

The amount of biomass C was significantly different in the three soils; the lowest quantity was found in the solarized soil, the highest in Albenga non-solarized soil (Table 3). No statistically significant interaction between soils and strains was observed. The inoculum of *F. oxysporum* (T26/6) added to the soils resulted in an increase of biomass C as compared with the control during the first week from soil infestation, but there was no difference among treatments after three weeks. No statistical difference was found among treatments in biomass P. The high quantity of phosphorous present in the Albenga soils probably interfered with the biomass P evaluation, generating a high variation among replications.

The population dynamics of strains T26/6 and 233/1 of *F. oxysporum* were not statistically different (Tukey 1%) in each soil tested (Fig. 2). The populations declined along of the experiments, confirming

TABLE 2. Amount of hydrolysed fluorescein diacetate (FDA) and ATP in Carignano (C), Albenga solarized (AS) and Albenga non-solarized (ANS) soils, infested with the antagonistic *Fusarium oxysporum* strains, transformed (T26/6) or non-transformed (233/1), and the non-infested control, after 1, 2, 3, and 4 weeks from the infestation[1].

Treatment	μg hydrolysed FDA/g dry soil				10^{-5} μg ATP/g dry soil	
	1 week	2 weeks	3 weeks	4 weeks	1 week	3 weeks
Soil						
C	98.76b	70.31a	75.78a	76.83b	13.41a	8.25a
AS	254.81b	107.98a	110.19a	78.02b	6.36b	4.28b
ANS	632.22a	144.00a	147.51a	242.34a	6.38b	4.34b
Strain						
Control	221.82a	75.48a	83.56a	79.63	9.51a	5.59a
233/1	363.65a	122.55a	112.17a	130.98	7.04a	5.43a
T26/6	400.30a	124.26a	137.76a	186.58	9.60a	5.85a
Control (C)	99.85	57.19	106.41	83.44a	14.83	8.54
233/1 (C)	103.13	80.16	83.44	73.60a	9.53	7.73
T26/6 (C)	93.28	73.60	37.50	73.46a	15.88	8.47
Control (AS)	80.91	53.36	59.98	29.02a	7.31	4.05
233/1 (AS)	197.56	118.90	158.23	66.47a	5.75	4.57
T26/6 (AS)	485.95	151.68	112.35	138.56a	6.03	4.21
Control (ANS)	484.70	115.90	84.29	126.44b	6.39	4.17
233/1 (ANS)	790.27	168.59	94.82	252.88a	5.84	3.99
T26/6 (ANS)	621.68	147.51	263.42	347.71a	6.91	4.87

[1] Values followed by the same letter in the same column do not differ among themselves (Tukey test, $p < 0.01$).

previous results obtained by Mezzalama et al. (1994). In the solarized Albenga soil, the number of CFU recovered four weeks from soil infestation was higher than in the Carignano and non-solarized Albenga soils (10^4 and 10 CFU g^{-1} of soils, respectively). This slower decline in solarized soil is probably due to a lack of competition caused by soil solarization (Katan & De Vay, 1991).

The potential ecological effects of the antagonistic microorganisms used to control plant pathogens must be evaluated on a case-by-case basis, since they depend on the characteristics of microorganism but also on soil type (Elsas & Trevors, 1991; Stotzky et al., 1993). The results obtained during this study on solarized and natural soils can partially describe the possible ecological effects that could be expected during practical applications of these microorganisms in integrated control strategies.

As suggested by several authors, more than one technique must be adopted for measuring soil biomass and activity, since each method has some limitations (Nannipieri et al., 1990; Stotzky et al., 1993; Ritz et al., 1994). The results confirmed that it is wise to use more than one independent method, wherever possible, in order to overcome natural variability and assess whether the release of an antagonist does actually modify the soil ecosystem. Among the methods adopted, the evaluation of CO_2 evolution and biomass C showed a transient increase followed by a decrease to control values, indicating that gross metabolic activity was not altered. There were no detectable effects on the other methods adopted that could be attributed to the introduction of the antagonists.

As recommended by Stotzky et al. (1993), the studies were initially conducted under laboratory conditions in confined environments (microcosms). Although the incubation conditions remained constant over time, some reduction in soil microbial biomass or activity (Fig.1), as well as the population of the antagonistic strains of *F. oxysporum* were observed (Fig.2). Field studies are necessary in order to confirm conclusions from microcosms studies.

The results obtained assessed the possible impact of the introduction of antagonistic strains of *F. oxysporum* on global microbial activities, but not on specific populations of microorganisms. Indeed, further studies are required to evaluate some transient or relatively long-term effects.

TABLE 3. Microbial biomass C and P in Carignano (C), Albenga solarized (AS) and Albenga non-solarized (ANS) soils, infested with the antagonistic *Fusarium oxysporum* strains, transformed (T26/6) or non-transformed (233/1), and the non-infested control, after 1 and 3 weeks from the infestation[1].

Treatment	Biomass C (μg C/g dry soil)		Biomass P (μg P/g dry soil)	
	1 week	3 weeks	1 week	3 weeks
Soil				
C	117.97b	122.71b	19.85b	5.45b
AS	45.70c	43.73c	22.15b	17.46b
ANS	344.73a	235.53a	82.32a	89.91a
Strain				
Control	136.76b	126.74a	52.20a	32.23a
233/1	170.62ab	125.95a	44.06a	44.87a
T26/6	201.02a	149.25a	28.06a	35.73a

[1] Values followed by the same letter in the same column do not differ among themselves (Tukey test, $p < 0.01$).

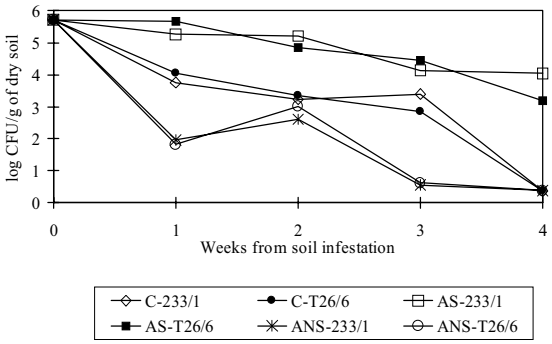

FIG. 2. Survival of antagonistic *Fusarium oxysporum* strains, transformed (T26/6) and non-transformed (233/1), in three soils: Albenga solarized (AS), Albenga non-solarized (ANS) and Carignano (C).

CONCLUSION

By the evaluation of CO_2 evolution, FDA hydrolysis, amount of ATP, biomass C and P, there are no detectable effects on the soil microbial biomass and activity that can be attributed to the introduction of antagonistic strains of *Fusarium oxysporum*, genetically manipulated or not.

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