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Effects of antagonistic *Fusarium oxysporum* on functional groups of indigenous bacteria in soil

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Abstract

Before planning the commercial use of microorganisms, genetically manipulated or not, in agricultural environments, their behavior and potential impact on soil ecosystems should be carefully studied as part of risk assessment. The influence of added inoculum of antagonistic *Fusarium oxysporum* strains, genetically manipulated (T26/6) or not (233/1) on nitrogen and carbon functional groups were evaluated in three soils from different sites at Albenga in Northwestern Italy, one natural and two other previously solarized, and in a fourth soil obtained from a site at Carignano (Northwestern Italy) with a 10-year-old poplar stand. There were no detectable effects of the antagonistic *Fusarium* strains on ammonifiers, ammonium oxidizers, nitrite oxidizers, nonsymbiotic anaerobic nitrogen fixers, nonsymbiotic aerobic nitrogen fixers and denitrifiers evaluated with the most probable number (MPN) method. Also, no effects were observed on amylolytic, proteolytic, cellulolytic, pectolytic and total bacteria evaluated with the plate count (PC) method. Survival and population dynamics of the antagonistic *Fusarium* strains were monitored during the experiments, and a decline over time of the introduced microorganisms was assessed. © 1997 Elsevier Science B.V.

Keywords: *Fusarium oxysorum*; Risk assessment; Soil microflora

1. Introduction

Saprophytic *Fusarium* spp., isolated from *Fusarium*-suppressive soils, have been exploited for their activity against various *Formae speciales* (f. sp.) of *Fusarium oxysporum*, incitants of vascular wilts of numerous economically important crops (Garibaldi et al., 1992; Alabouvette et al., 1993). However, before planning commercial use of such organisms, genetically manipulated or not, in agricultural environments, their behavior and potential impact on soil ecosystems should be carefully studied as part of risk assessment (Office of Technology Assessment, 1988; Tiedje et al., 1989; Cairns and Orvos, 1992; Stotzky et al., 1993). Concern has been expressed regarding the potential for introduction of transgenic organisms to displace resident species in the receiving community, particularly species performing key functional roles such as nitrogen processing and fixation, organic carbon decomposition, or pesticide degradation (Levin and Harwell, 1986; Domsch et al., 1983). Qualitative and quantitative alterations of the micro-

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bial 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 release (Cairns and Orvos, 1992; Stotzky et al., 1993; Kluepfel, 1992). Displacement of given microbial groups can be dramatic if the introduced microorganisms possess a high saprophytic fitness; such an effect is envisaged and expected for biological control agents. Unwanted effects may occur when displaced microorganisms play an important role in the geochemical cycling of nutrients, thus having broad consequences on the ecosystem (Lindow, 1992; De Leij et al., 1994).

Bacteria belonging to definite functional groups can indicate the occurrence of certain ecological processes in the ecosystem, such as transformations of various compounds of carbon, nitrogen and phosphorus. Cellulolytic, amylolytic, proteolytic and pectolytic bacteria are among the groups involved in detritus mineralization, and their presence is characteristic of a certain type of degradation in the system. Bacteria from each functional group can be isolated from their natural habitat and detected by growth on selective media with either plate count (PC) or most probable number (MPN) methods (Herbert, 1990). These methods have proven to be inexpensive, yet reliable (Olsen and Bakken, 1987; Orvos et al., 1990; Scanferlato et al., 1990).

The objective of this study was to determine the potential effects of antagonistic *F. oxysporum* strains, genetically manipulated or not, released in different soils on bacterial functional groups involved in carbon and nitrogen mineralization cycles.

2. Materials and methods

2.1. Microorganisms

Two antagonistic benomyl resistant strains of *F. oxysporum* 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 hygromicin B, was obtained by transforming strain 233/1 C5 (a dark red-pigmented mutant of 233/1), with plasmid PAN-1, which contains the *Escherichia coli* hygromycin B phosphotransferase gene (*hph*), encoding for hygromycin B resistance (Migheli et al., 1996).

Table 1
Characteristics of the soils used in the experiments

Soil characteristics	A-S94	A-S95	A-N	C
pH	7.8	7.8	8.02	7.68
Electrical conductivity (μS cm^{-1})	0.85	0.80	0.24	0.15
Organic matter (%)	3.9	ne[a]	2.6	1.7
Total N (%)	0.19	ne[a]	0.15	0.14
P soluble (μg P g^{-1} soil)	133.1	48	210.3	24.6
Clay (%)	12	11	7	6
Silt (%)	19	25	34	58
Fine sand (%)	31	31	28	28
Coarse sand (%)	36	32	39	6

[a]Not evaluated.
A-S94: Albenga solarized 1994; A-S95: Albenga solarized 1995; A-NS: Albenga nonsolarized; C: Carignano.

The strains were grown on liquid casein hydrolysate medium, with shaking on a rotary shaker at 100 rpm for 5 days at 28°C with a 12-h photoperiod. The conidial suspensions were obtained by filtering cultures, centrifuging at 3000 rpm for 10 min, and resuspending the pellets in sterile distilled water. The survival of the antagonists in different soils 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 De Nemours, USA). Petri dishes were incubated at 20°C for 5–6 days. Colony counts of *Fusarium* were averaged from 10 plates and converted to log colony forming units (CFU) per gram on a dry weight soil basis.

2.2. Soils

Four soils, whose characteristics are shown in Table 1, were used in all experiments. Carignano soil was collected from a field with a 10-year-old poplar stand in Piedmont (Northwestern Italy). The three Albenga soils, solarized and nonsolarized, were collected from different sites at a horticulture experimental station in Liguria (Northwestern Italy); two had been previously solarized (maximum temperature 45°C at 10 cm depth). 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 the beginning of the experiments. These

four soils are hereafter referred to as Carignano (C), Albenga-nonsolarized (A-NS), Albenga-solarized 1994 (A-S94) and Albenga-solarized 1995 (A-S95).

Soil microcosms, plastic tubes, 50 ml, were prepared and infested with 1×10^5 CFU g^{-1} soil of the antagonists individually. This concentration of the antagonists was proved to be the most effective against *F. oxysporum* f. sp. *dianthi* (Aloi et al., 1990). Then they were sealed 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-h photoperiod. Three tubes (replicates) for each treatment were made. Soil moisture was adjusted to 70% of maximum water holding capacity (MWHC).

2.3. Evaluation of functional groups

Ammonifiers, ammonium oxidizers, nitrite oxidizers, denitrifiers and nitrate reducers, aerobic and anaerobic nitrogen fixers were evaluated with the MPN technique (Herbert, 1990) on appropriate selective media (Pochon and Tardieux, 1962; Alexander, 1965). The degree of dilutions of the sample required and the number of positive tubes at different dilutions were used to determine the MPN of organisms by referring to statistical tables of McCrady, modified by Allievi et al. (1983). The number of microorganisms calculated were converted to $\log_{10}$ MPN per gram on a dry weight soil.

Cellulolytic, pectolitic, amylolytic, proteolitic and total bacteria were evaluated with the spread plate count (SPC) method (Herbert, 1990) on appropriate media (Rodina, 1972; Cuppels and Kelman, 1974). Soil samplings were carried out at day 7 and 21 from infestation. Colony counts of each group were averaged from 10 plates and converted to log colony forming units (CFU) per gram on a dry weight soil basis.

2.4. Statistical analysis

Analysis of variance was carried out and statistical significance between groups was evaluated with the Duncan test ($P = 0.05$). Statistical analysis was carried out with SPSS software (SPSS for Windows 5.01, 1992).

3. Results

Survival of the antagonistic strains of T26/6 and 233/1 of *F. oxysporum* was confirmed over the duration of the experiment in the soils tested (Fig. 1). The population dynamics of the antagonistic strains were similar in C, A-NS and A-S95 soils; they declined significantly over the duration of the experiments (21 days). In A-NS and A-S95, the number of CFU g^{-1} of soil at day 7 from soil infestation was still at 1×10^5 in both treatments. At day 21 from soil infestation with strain 233/1 and with strain T26/6, the number of CFU declined respectively to 3.89×10^2 and to 3.4×10^2 for A-NS, to 1.9×10^4 and 8.5×10^3 for A-S95. In the A-S94 soil, the number of CFU recovered at 7 and 21 days from soil infestation was not statistically different, showing no decline over the period of time tested. However, this did not happen in A-S95, where the population at day 7 from soil infestation was higher than in A-S94 at the same sampling date, but similar to A-S94 at day 21 from soil infestation. In soil C, the number of CFU recovered was always lower than in the other three soils; at day 7 the number of CFU g^{-1} of soil had already declined to 2.5×10^2 and to 1×10^2 when soil infestation was carried out with strain 233/1 and T26/6, respectively.

The effects on ammonifiers due to the release of antagonistic strains of *F. oxysporum* 233/1 and T26/6 were not observed (Fig. 2). In the soils tested, the population estimated was always higher than 1×10^{14} MPN g^{-1} of soil. The decreases of the population to 5.1×10^{11} MPN g^{-1} of soil observed in A-S94 after 21 days from soil infestation with strain T26/6 was similar but significantly different from the decrease of population in the control microcosms (3.16×10^{10} MPN g^{-1} of soil).

A significant increase of ammonium oxidizers, from 8.1×10^3 to 1.3×10^4 MPN g^{-1} of soil, was observed in soil C in the microcosms infested with strain T26/6 21 days from soil infestation. In A-NS and A-S94 soils, no change in the population dynamics was observed. In A-S95 soil the population decreased equally in natural and infested soils (Fig. 3).

A statistically significant change in the population of nitrite oxidizers was not detected in soils C and A-NS (Fig. 4). In A-S94, the population estimated was higher in the microcosms infested with 233/1

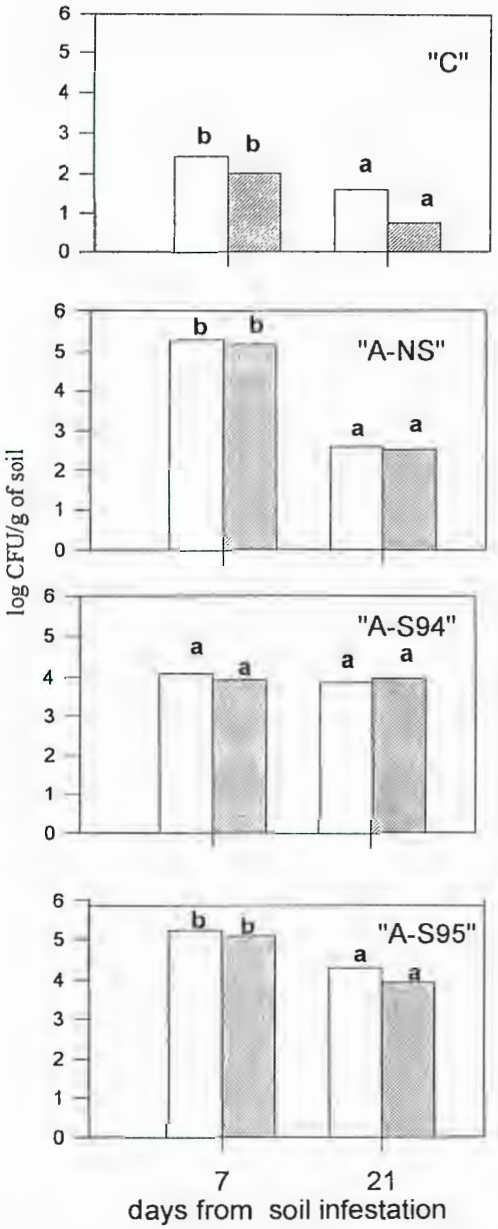

Fig. 1. Survival of antagonistic *F. oxysporum*, strain 233/1 (□) and strain T26/6 (///) released in closed microcosms (50 ml capacity) at 1×10^5 CFU g^{-1} soil, containing Carignano soil (C) (Piemonte, Northwestern Italy); Albenga (Liguria, Northwestern Italy) nonsolarized soil (A-NS); Albenga solarized soil in 1994 and 1995 (A-S94 and A-S95). Isolations were carried out on Komada *Fusarium*-selective medium (1975). Bars with the same letter are not significantly different (Duncan test, $P = 0.05$).

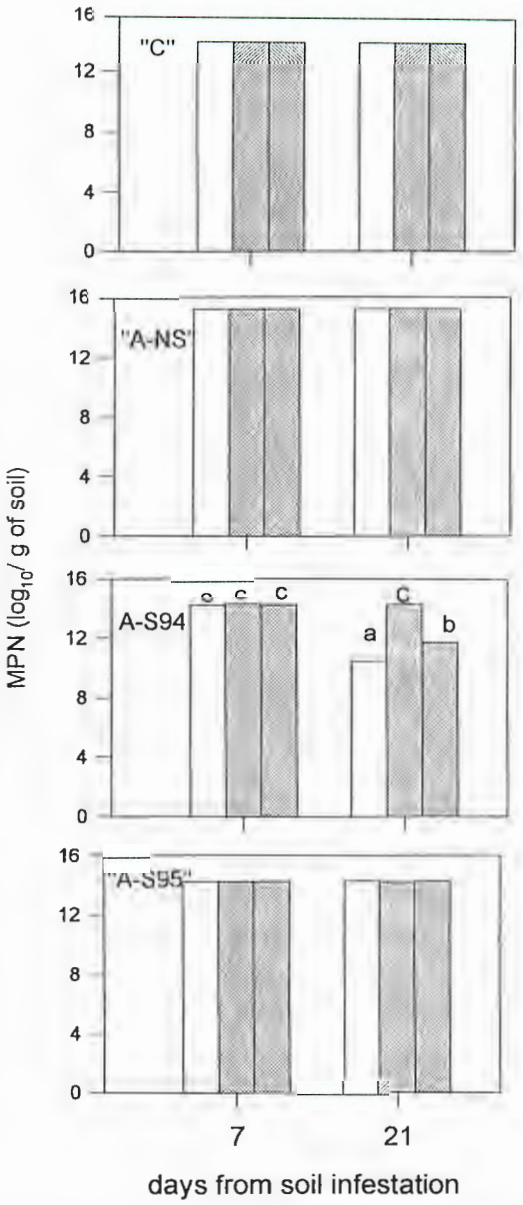

Fig. 2. MPN (log_{10}) of ammonifiers detected in soil microcosms (50 ml capacity) containing Carignano soil (C) (Piemonte, Northwestern Italy); Albenga (Liguria, Northwestern Italy) nonsolarized soil (A-NS); Albenga solarized soil in 1994 and 1995 (A-S94 and A-S95) natural (□) and infested with antagonistic *F. oxysporum*, strain 233/1 (///) and strain T26/6 (\\\), at 1×10^5 CFU g^{-1} soil. Bars with the same letter are not significantly different (Duncan test, $P = 0.05$).

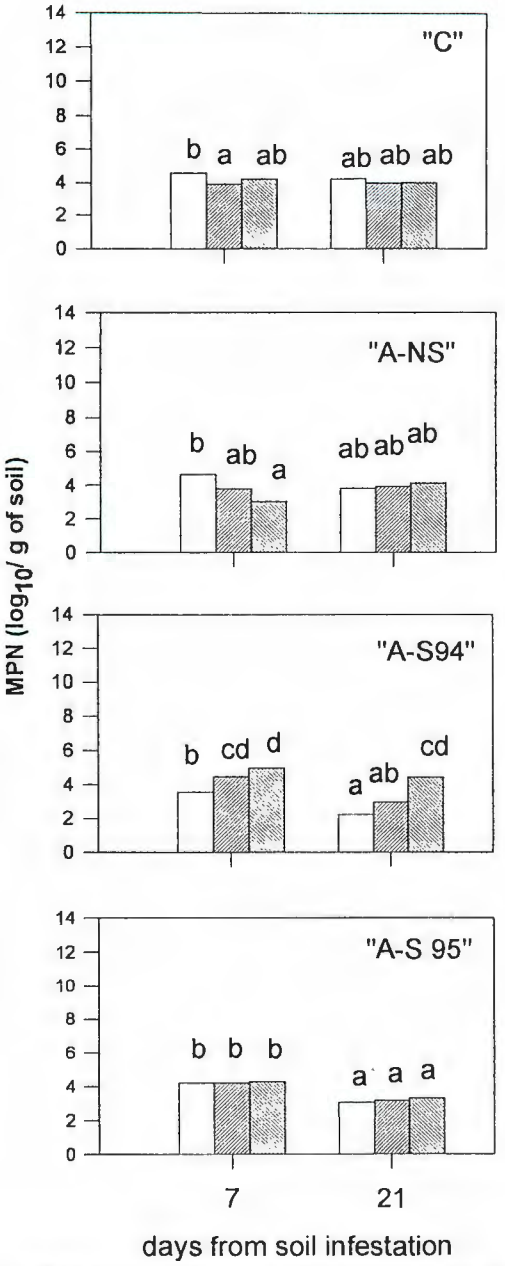

Fig. 4. MPN (log_{10}) of nitrite oxidizers detected in soil microcosms (50 ml capacity) containing Carignano soil (C) (Piemonte, Northwestern Italy); Albenga (Liguria, Northwestern Italy) nonsolarized soil (A-NS); Albenga solarized soil in 1994 and 1995 (A-S94 and A-S95) natural (□) and infested with antagonistic *F. oxysporum*, strain 233/1 (///) and strain T26/6 (\\\), at 1×10^5 CFU g^{-1} soil. Bars with the same letter are not significantly different (Duncan test, $P = 0.05$).

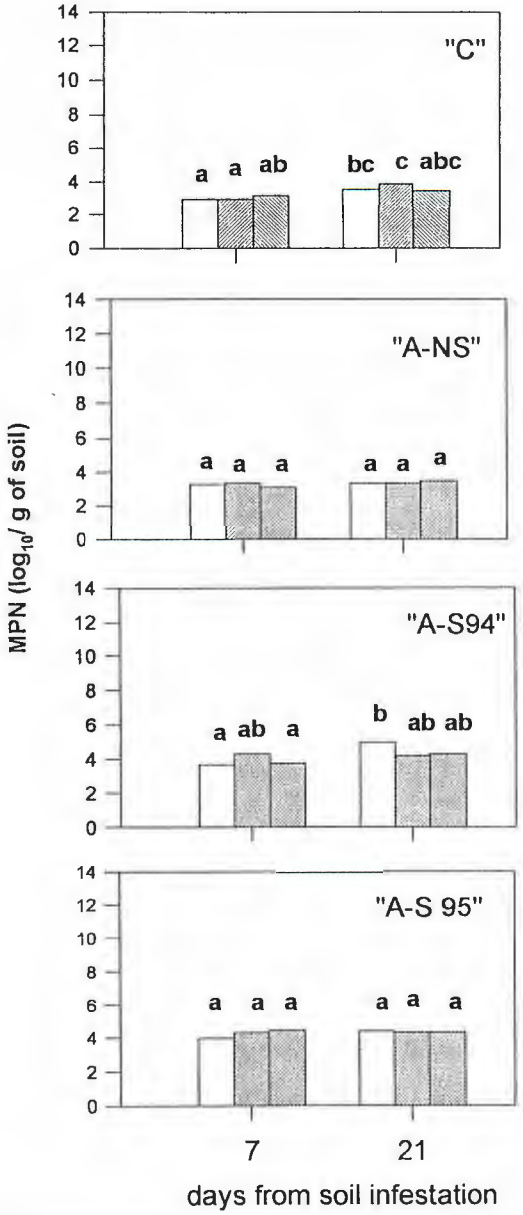

Fig. 5. MPN (log_{10}) of nonsymbiotic anaerobic nitrogen fixers detected in soil microcosms (50 ml capacity) containing Carignano soil (C) (Piemonte, Northwestern Italy); Albenga (Liguria, Northwestern Italy) nonsolarized soil (A-NS); Albenga solarized soil in 1994 and 1995 (A-S94 and A-S95) natural (□) and infested with antagonistic *F. oxysporum*, strain 233/1 (///) and strain T26/6 (\\\), at 1×10^5 CFU g^{-1} soil. Bars with the same letter are not significantly different (Duncan test, $P = 0.05$).

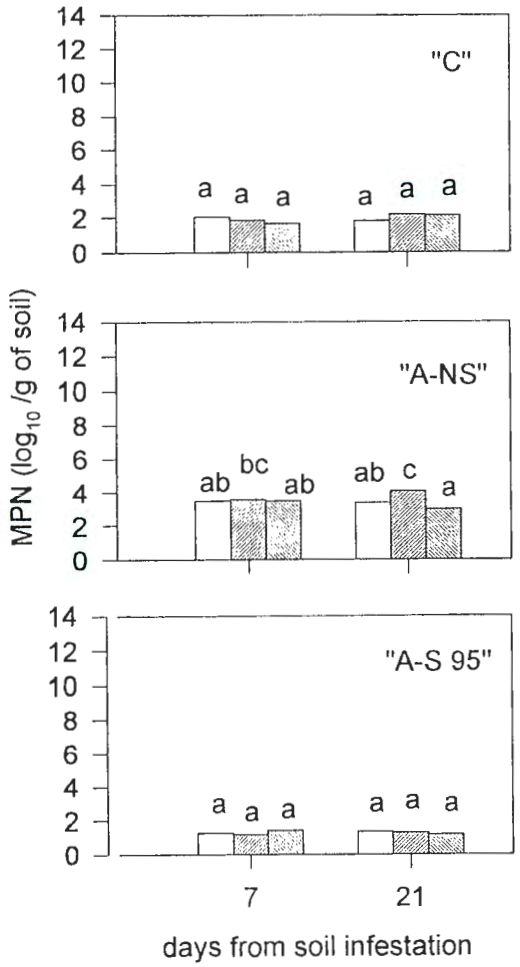

Fig. 6. MPN ($\log_{10}$) of nonsymbiotic aerobic nitrogen fixers detected in soil microcosms (50 ml capacity) containing Carignano soil (C) (Piemonte, Northwestern Italy); Albenga (Liguria, North western Italy) nonsolarized soil (A-NS); Albenga solarized soil in 1994 and 1995 (A-S94 and A-S95) natural (□) and infested with antagonistic *F. oxysporum*, strain 233/1 (///) and strain T26/6 (\\\), at 1×10^5 CFU g^{-1} soil. Bars with the same letter are not significantly different (Duncan test, $P = 0.05$).

sessed before by Cairns and Orvos (1992). The evaluation of functional effects is the most ambiguous category, since lack of effect upon a particular end-point may indicate a poor end-point choice rather than a lack of functional effect. The results obtained previously by Gullino et al. (1995), on the interactions with indigenous microorganisms, and by Ghini et al. (1996) on the effects on total biomass, can help avoid the risk of coming to wrong conclusions. In

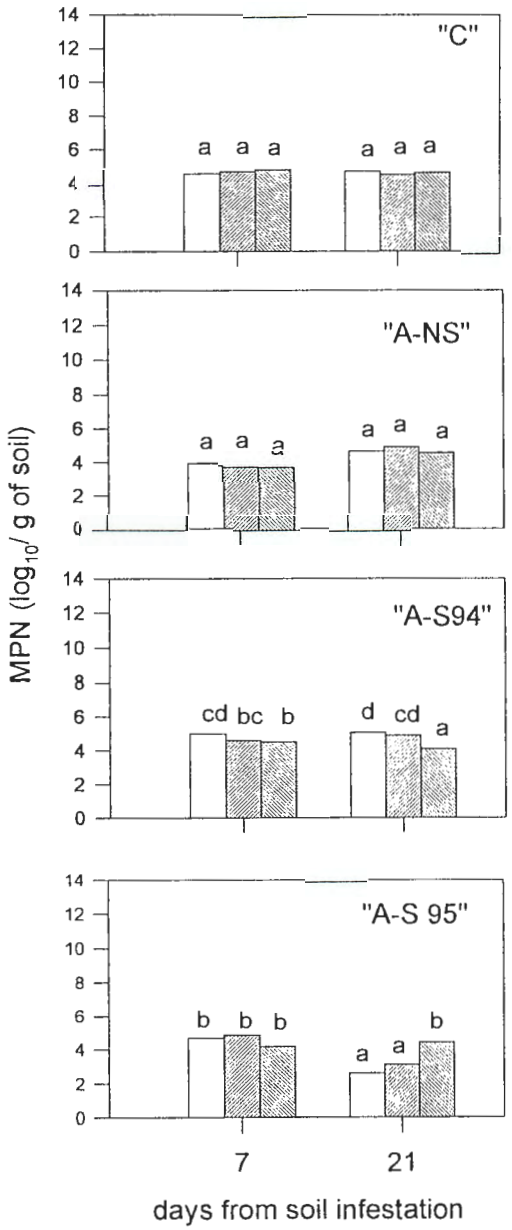

Fig. 7. MPN ($\log_{10}$) of denitrifiers detected in soil microcosms (50 ml capacity) containing Carignano soil (C) (Piemonte, Northwestern Italy); Albenga (Liguria, Northwestern Italy) nonsolarized soil (A-NS); Albenga solarized soil in 1994 and 1995 (A-S94 and A-S95) natural (□) and infested with antagonistic *F. oxysporum*, strain 233/1 (///) and strain T26/6 (\\\), at 1×10^5 CFU g^{-1} soil. Bars with the same letter are not significantly different (Duncan test, $P = 0.05$).

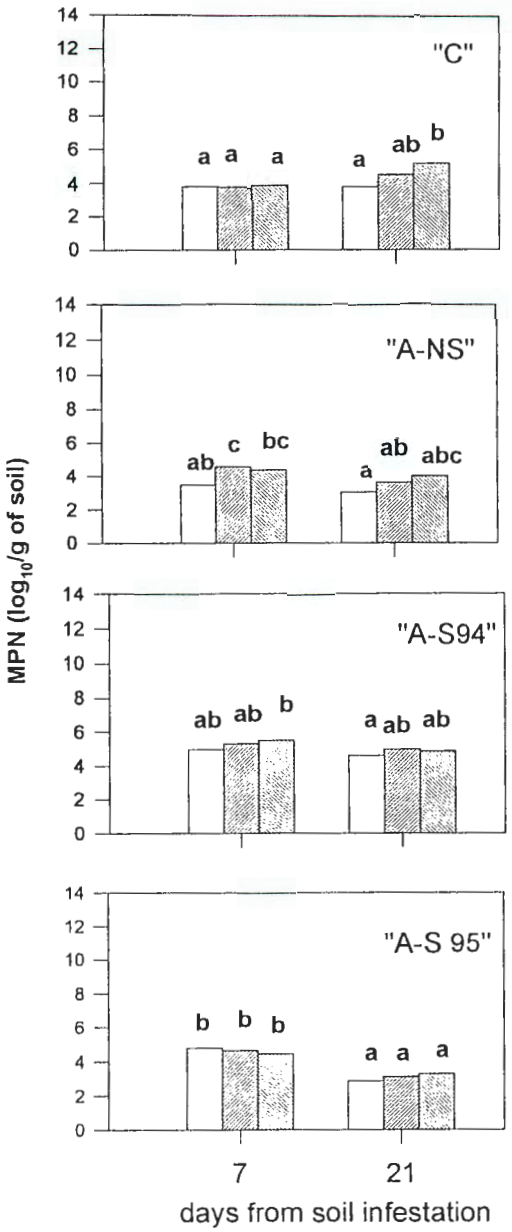

Fig. 3. MPN (log_{10}) of ammonium oxidizers detected in soil microcosms (50 ml capacity) containing Carignano soil (C) (Piemonte, Northwestern Italy); Albenga (Liguria, Northwestern Italy) nonsolarized soil (A-NS); Albenga solarized soil in 1994 and 1995 (A-S94 and A-S95) natural (□) and infested with antagonistic *F. oxysporum*, strain 233/1 (///) and strain T26/6 (\\\), at 1×10^5 CFU g^{-1} soil. Bars with the same letter are not significantly different (Duncan test, $P = 0.05$).

(2.8×10^4 MPN g^{-1} of soil) and T26/6 (8.9×10^4 MPN g^{-1} of soil) at day 7 from soil infestation. At day 21, it was significantly different only in the soil microcosms infested with T26/6 (2.6×10^4 MPN g^{-1} of soil). In soil A-S95, the population tested decreased after 21 days from soil infestation. The same was observed in the control and infested microcosms (Fig. 4).

In the soil microcosms infested with strain 233/1 and T26/6 (Fig. 5), no consequence was observed on the populations of nonsymbiotic anaerobic nitrogen fixers; furthermore, no effects were observed on nonsymbiotic aerobic nitrogen fixers in the four soils tested infested with strains 233/1 and T26/6 (Fig. 6).

The population of denitrifying bacteria decreased significantly ($P = 0.05$) only in A-S94 in the microcosms infested with strain T26/6 from 3.3×10^4 to 2.6×10^4 MPN g^{-1} of soil. In C and A-NS no effect was observed. In A-S95, the population decreased significantly in the control microcosm and in the microcosm infested with strain 233/1. The population dynamics did not change in the presence of strain T26/6 in this soil (Fig. 7).

During the 21-day period, no effects on amylolytic, proteolytic, cellulolytic, pectolytic and total bacteria (Table 2) were observed due to the release of antagonistic *F. oxysporum*.

4. Discussion

The results obtained on survival of antagonistic *F. oxysporum* isolates genetically manipulated or not confirmed the results obtained previously by Mezzalama et al. (1994). The different population dynamics observed in soil C can be explained by the higher indigenous microbial activity published for this soil in previous work (Ghini et al., 1996).

The results obtained showed that there are no deleterious effects due to the release of the antagonistic strains of *F. oxysporum*, T26/6 and 233/1, genetically manipulated or not, on the indigenous microbial groups involved in nitrogen and carbon nutrient cycling. This confirms what has been as-

on a case-to-case basis, since they depend not only on the characteristics of the microorganism, but also on soil type. The results obtained during this study on solarized and natural soils may be extrapolated to describe the possible ecological effects that can occur during practical applications of these microorganisms in integrated control strategies.

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