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Sewage sludge effect on management of *Phytophthora nicotianae* in citrus

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Abstract

Greenhouse and field experiments evaluated the effect of sewage sludge incorporation to the soil against *Phytophthora nicotianae* in cravo lemon plants. Six sludge doses, ranging from 0 to 30% (v/v), were tested per assay on plants at different developmental stages and with different pathogen inoculum levels. The increase in sewage sludge dose resulted in pH reduction, electric conductivity and soil microbial activity increases (evaluated by FDA hydrolysis and microbial respiration), and reduction in *P. nicotianae* recovery, both from the soil and from the plant roots. The pathogen recovery was significant and negatively correlated with soil microbial activity and electric conductivity. Better plant development was observed with sludge incorporation up to 20%. These results indicate that the incorporation of sewage sludge can suppress *P. nicotianae*, by nonchemical management of the pathogen and is a potential means of disposal of this residue.

Keywords: Organic matter; *Citrus limonia*; Soil pathogen; Biosolid

1. Introduction

Damping-off, gummosis, and root rot caused by *Phytophthora* spp. are among the most economically important fungal diseases in citrus, occurring in nearly all producing regions. The main *Phytophthora* species predominant in Brazil are *P. nicotianae* (sin. = *P. parasitica*) and *P. citrophthora* (Feichtenberger, 2001).

The management of diseases caused by *Phytophthora* is based on the integration of several preventive and curative control measures, which may vary depending on plant age and disease manifestation (Erwin and Ribeiro, 1996; Wilcox et al., 1999). The many problems resulting from the use of chemical control, especially those associated with impacts on the agroecosystem, have led to a search for alternative control methods. In addition, despite the fact that soil plant pathogens hardly develop resistance to fungicides, there have been reports of *P. parasitica* resistance to metalaxyl (Ferrin and Kabashima, 1991; Timmer et al., 1998).

One alternative to the management of soil-borne pathogens is the use of organic matter sources, both incorporated to the soil and as mulches, and also as a vehicle for biological control agents. Organic matter contributes toward a more effective control of pathogens due to an increase in microbial activity and to improved physical and chemical soil properties (Baker and Cook, 1974; Casale et al., 1995; Chung et al., 1988; Hoitink and Boehm, 1999).

Several studies have been conducted for *Phytophthora* spp. management through the application of organic matter sources (Casale et al., 1995; Erwin and Ribeiro, 1996; Hoitink and Boehm, 1999; Lumsden et al., 1983; Widmer et al., 1998). In addition to improving the

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physical and chemical properties of soil, organic matter use is based on the low saprophytic and competitive capacity of the pathogen in relation to other microorganisms. Based on studies dealing with soils that are suppressive to *Phytophthora*, several microorganisms have been reported as partly responsible for this property. Among them are fungi in the genera *Trichoderma*, *Clonostachys*, *Myrothecium*, and *Penicillium*; bacteria in the genera *Bacillus*, *Enterobacter*, and *Pseudomonas*; and actinomycetes in the genus *Streptomyces* (Erwin and Ribeiro, 1996).

At present, the sewage sludge generated in sewage treatment stations constitutes one of the organic matter sources available in ever-increasing amounts, and is rich in nutrients for plants. Despite this fact, no information is available concerning its effect on suppressing soil pathogens in citrus. With regard to *Phytophthora*, Millner et al. (1981) achieved control of *P. capsici* in a greenhouse test, while Utkhede (1984) observed that sludge application resulted in an increase of the incidence of *P. cactorum* in apple trees. However, they concluded that the disease is positively correlated with the amount of nitrogen applied and not with its organic or inorganic origin. Kim et al. (1997) conducted tests in the field to evaluate the effects of organic compounds in the control of *P. capsici* in pepper, and verified that sewage sludge composted with gardening residues did not reduce the pathogen population nor the disease symptoms.

The objective of the present work was to evaluate the effect of sewage sludge incorporation to the soil on cravo lemon plants growth at different development stages, soil microbial activity, soil chemistry, and the recovery of *P. nicotianae*, under greenhouse and field conditions. The two development stages tested were the plantlet (up to 3 months of age) and the seedling stage (from 3 months of age).

2. Material and methods

The IAC 01/95 *Phytophthora nicotianae* Breda de Haan (1896) (sin. *P. parasitica* Dastur (1913)) isolate employed in the experiments was provided by Centro de Citricultura "Sylvio Moreira"—Instituto Agronômico de Campinas (CCSM—IAC). The isolate was maintained in sterilized distilled water at room temperature and in the absence of light until used. The inoculum for the experiments was produced on wheat grains autoclaved inside polypropylene bags. Polypropylene bags (30 × 40 cm) containing 350 g of wheat grains and 200 ml of distilled water were autoclaved at 121 °C for 40 min. After 24 h, 150 ml of distilled water were added by bag, followed by a autoclavation at 121 °C for 20 min. Mycelial disks were placed into the bags (40 discs of 5 mm diameter per bag) and incubated at 25 °C for a month (Leoni and Ghini, 2003).

Table 1
Characteristics of the sewage sludge used in the experiments

Humidity (%) (65 °C)	83.3
pH (water)	6.4
C ($g\,kg^{-1}$)	374.4
N Kjeldal ($g\,kg^{-1}$)	50.8
N-ammoniacal ($mg\,kg^{-1}$)	119.5
N-nitrate-nitrite ($mg\,kg^{-1}$)	54.8
P ($g\,kg^{-1}$)	21.3
K ($g\,kg^{-1}$)	0.99
Ca ($g\,kg^{-1}$)	16.8
Mg ($g\,kg^{-1}$)	2.5
S ($g\,kg^{-1}$)	13.3
Mo ($mg\,kg^{-1}$)	<1
B ($mg\,kg^{-1}$)	7.1
Na ($g\,kg^{-1}$)	0.6
Cr ($mg\,kg^{-1}$)	1325
Mn ($mg\,kg^{-1}$)	267.4
Fe ($mg\,kg^{-1}$)	31706
Ni ($mg\,kg^{-1}$)	74.7
Cu ($mg\,kg^{-1}$)	359.2
Zn ($mg\,kg^{-1}$)	1590
Al ($mg\,kg^{-1}$)	33550
Cd ($mg\,kg^{-1}$)	2
Pb ($mg\,kg^{-1}$)	118.8
Ar ($mg\,kg^{-1}$)	<1
Se ($mg\,kg^{-1}$)	0
Hg ($mg\,kg^{-1}$)	<1

Determined according EPA (1986).

The sewage sludge utilized was obtained from the Sewage Treatment Station in Franca, SP. This sludge is from a residential area, with a low heavy metal content (Table 1). The Red–Yellow Oxysol, clayey phase, used in the experiments was obtained at the experimental field of Embrapa Meio Ambiente (Jaguariúna, SP), with 25 $g\,dm^{-3}$ of organic matter and a pH in $CaCl_2$ of 5.1.

2.1. Test involving cravo lemon plantlets grown in seedling tubes in the greenhouse

The commercial substrate (Plantmax®) treated with sewage sludge at the proportions of 0%, 5%, 10%, 15%, 20%, and 30% (v/v) was placed in 45 ml capacity seedling tubes until approximately one-half of that volume, infested with 0, 1.5, or 3 g of *P. nicotianae* inoculum per seedling tube; the volume was then completed. After 1 day, germinated cravo lemon seeds (*Citrus limonia* (L.) Osbeck) were sown and maintained under greenhouse conditions. The germinated seeds were obtained placing the seeds into trays containing wet vermiculite, at 27 °C, until total germination. In the third month, fresh matter weight of the above-ground part and roots of the plantlets, as well as the pathogen presence, were determined for all seedling tubes. The pH and electric conductivity of substrates in different

treatments were determined in substrate mixes of seedling tubes from the same plot.

The pathogen presence in the substrate and roots was evaluated through the citrus (*Citrus* spp.) leaf bait test, modified from Grimm and Alexander (1973). Plastic Petri dishes 9 cm in diameter received 5 g of the substrate (two dishes per seedling tube) or the complete root system (one dish per plantlet), 30 ml distilled water, and 20 citrus leaf fragments measuring 3×3 mm, previously disinfested for 1 min in 70° alcohol. The plates were maintained at 27 ± 2 °C under continuous fluorescent light for 48 h. In order to evaluate percentage of pathogen recovery, the baits were transferred to blades containing water, covered with glass slides, and observed under the optical microscope. During the experiment, dead plantlets were also evaluated with regard to *P. nicotianae* recovery or not from the roots and substrate.

Two experiments were conducted; in the first, in addition to the no sludge treatment, a no sludge treatment with weekly foliar applications of nutrients (Ajifol®, 2.5 ml l^{-1}) was also used as a control. The experimental design was randomized blocks, with four replicates of each plot. Each plot consisted of four seedling tubes in the first experiment and six in the second.

2.2. Test involving cravo lemon seedlings grown in pots in the greenhouse

In the first experiment, the soil was treated with sewage sludge at the proportions of 0%, 5%, 10%, 15%, 20%, and 30% (v/v). After 1 day, the soils were placed in 4.5 l capacity pots, until approximately one-half of this volume, infested with 0, 8, or 15 g of *P. nicotianae* inoculum, and the pots were again filled with soil until the volume was completed. Next, 3-month-old commercial cravo lemon seedlings were transplanted to the pots. Pots-containing soil without sewage sludge and fertilized weekly with foliar applications of nutrients were used as controls, as described for the seedling tube test, and then infested with 0, 8, or 15 g of *P. nicotianae* inoculum per pot. Seedlings were maintained under greenhouse conditions and irrigated regularly. The experimental design was organized as random blocks, with four replicates, and each plot consisted of six pots with one seedling per pot.

In the second experiment, 4-month-old commercial cravo lemon seedlings were used; sewage sludge was applied at the proportions of 0%, 5%, 7.5%, 10%, 15%, 20%, and 30% (v/v), and the soil was infested with 30 g of inoculum per 4.5 l capacity pot. As a control, seedlings were transplanted to pots containing soil without inoculum and without sewage sludge. The experimental design consisted of completely randomized plots, with four replicates, and each plot consisted of six pots containing one seedling per pot.

In both experiments, evaluations included: fresh matter weight of the above-ground part and roots, pathogen presence in the substrate and roots by means of the citrus leaf bait test, pH, and electric conductivity. The evaluations were performed 150 days after treatment for the first experiment, and after 120 days, for the second experiment. The microbial activity of the substrates containing 15 g of inoculum was evaluated by fluorescein diacetate hydrolysis (FDA) using the methodology described by Boehm and Hoitink (1992), and by microbial respiration measured through CO_2 release, according to a method described by Grisi (1978).

In the first experiment, leaf samples were collected (100 leaves), 150 days after treatments, for nutritional status determination by means of leaf tissue analysis. The macronutrients results (N, P, and K) were analyzed through the DRIS (Diagnosis and Recomendation Integrated System) indexes described by Sumner (1986), and indexes were calculated using the standard values for $K/N = 0.358$; $P/N = 0.056$, and $K/P = 6.3929$ (Embleton, 1973, cited by Marchal, 1984) and a coefficient of variation of 20%. The index is higher and has a positive value when a relative excess of the element exists, and vice versa when it is negative. A value of zero indicates that the nutrients ratio is close to the standard, at a distance that is smaller than the standard deviation. The algebraic sum of the indexes must be zero. The sum of the absolute values of the indexes is a measurement of the global balance of the leaf composition under study (Table 2).

2.3. Test involving cravo lemon seedlings grown in the field

Three-month-old cravo lemon seedlings, obtained from a commercial nursery, were transplanted to 9 m^2 plots (3×3 m) treated the previous day with sewage sludge at the proportions of 0%, 5%, 7.5%, 10%, or 15% (v/v). Soil infestation was performed simultaneously with transplanting, with 0 or 1,250 g of *P. nicotianae* inoculum per plot (20 g of inoculum per seedling; 138.9 g m^{-2}). Infested or non-infested plots were installed as controls, and were fertilized with urea (85 g N per plot per month, 510 g during the entire experiment) and leaf fertilizer, as described for the seedling tube test. The amounts of fresh sewage sludge incorporated to the soil at a 20 cm depth, in the entire area of plots, were 0%, 5%, 7.5%, 10%, and 15% (v/v), and were equivalent to 0, 372, 558, 744, and 1,116 g of N, respectively. Seedlings were irrigated by sprinkling during the experiment.

The experimental design was organized as completely randomized blocks, with four replicates. Soil microbial activity was determined by FDA hydrolysis and by CO_2

Table 2
Effect of sewage sludge on leaf tissue composition of cravo lemon seedlings (*Citrus limonia*) at 150 days after transplanting, in the first greenhouse experiment, and at 182 days after transplanting in the field experiment

Greenhouse experiment								Field experiment						
Sewage sludge (%)	N	K	P	DRIS index[a]				N	K	P	DRIS index			
	g kg^{-1}	g kg^{-1}	g kg^{-1}	N	P	K	Sum of absolute value	g kg^{-1}	g kg^{-1}	g kg^{-1}	N	P	K	Sum of absolute value
Treatments without Phytophthora nicotianae *inoculum*								*Treatments without* P. nicotianae *inoculum*						
0[b]	21.8[c]	21.0	1.2	−42.27	−43.43	85.70	171.41	25.5[c]	12.4	1.7	−8.96	0	8.96	17.92
0 + A[d]	29.5	12.2	1.9	0	0	0	0	27.6	9.4	1.6	0	0	0	0
5	23.7	8.8	1.8	−8.91	16.60	−7.69	33.19	27.0	9.9	1.7	0	0	0	0
7.5	—	—	—	—	—	—	—	26.5	9.4	1.8	−5.32	5.32	0	10.65
10	27.2	9.3	1.9	−6.18	13.84	−7.65	27.67	27.2	9.9	1.7	0	0	0	0
15	28.2	7.8	1.6	7.36	7.78	−15.14	30.28	27.5	9.4	2.0	−7.47	16.47	−9.00	32.94
20	32.0	8.3	1.5	9.51	0	−9.51	19.01	—	—	—	—	—	—	—
30	33.7	8.8	1.3	20.57	−11.29	−9.27	41.13	—	—	—	—	—	—	—
Treatments with 8 g of P. nicotianae *inoculum per pot*								*Treatments with 1250 g of* P. nicotianae *inoculum per plot*						
0	21.8	18.5	1.4	−34.26	−26.68	60.94	121.87	25.2	9.9	1.7	−5.12	5.12	0	10.23
0 + A	29.8	11.7	1.9	0	0	0	0	27.7	10.4	1.5	0	0	0	0
5	23.5	9.8	1.8	−9.19	9.19	0	18.39	25.5	9.9	1.7	0	0	0	0
7.5	—	—	—	—	—	—	—	26.5	9.9	1.8	−5.32	5.32	0	10.65
10	27.8	8.3	1.8	0	9.66	−9.66	19.32	27.2	9.4	1.9	−6.18	13.49	−7.30	26.98
15	31.1	8.8	1.8	6.63	7.69	−14.32	28.64	27.4	10.4	2.0	−7.59	7.59	0	15.17
20	31.5	8.8	1.5	7.04	0	−7.04	14.07	—	—	—	—	—	—	—
30	34.5	8.3	1.5	19.40	−7.20	−12.20	38.80	—	—	—	—	—	—	—
Treatments with 15 g of P. nicotianae *inoculum per pot*														
0	22.7	17.6	1.4	−29.14	−24.16	53.30	106.61	—	—	—	—	—	—	—
0 + A	28.3	9.8	1.3	0	0	0	0	—	—	—	—	—	—	—
5	24.1	8.8	1.9	−10.20	19.70	−9.51	39.41	—	—	—	—	—	—	—
7.5	—	—	—	—	—	—	—	—	—	—	—	—	—	—
10	27.3	9.3	1.7	0	0	0	0	—	—	—	—	—	—	—
15	28.3	8.3	1.9	0	11.59	−11.59	23.17	—	—	—	—	—	—	—
20	32.0	8.8	1.4	14.55	7.00	−7.55	29.09	—	—	—	—	—	—	—
30	35.9	8.3	1.4	24.61	−10.90	−13.71	49.22	—	—	—	—	—	—	—
Reference values[e]														
Low	<23.9	<6.9	<1.1	—	—	—	—	<23.9	<6.9	<1.1	—	—	—	—
Optimum	24–26	7–10.9	1.2–1.6	—	—	—	—	24–26	7–10.9	1.2–1.6	—	—	—	—
High	>27	>11	>1.7	—	—	—	—	>27	>11	>1.7	—	—	—	—

[a]DRIS index = "Diagnosis and Recomendation Integrated System" index.
[b]Sewage sludge doses incorporated to the soil (% v/v).
[c]Data were obtained from one compound sample (100 completely developed and healthy leaves) per treatment.
[d]Treatment without sewage sludge incorporation to the soil and with mineral fertilization (A).
[e]Reference values were obtained from Embleton, 1973 (cited by Marchal, 1984), for leaves in non-fruiting branches.

release at 5, 15, 29, 43, 82, 118, 147, and 182 days after sewage sludge incorporation to the soil. At 29, 82, 118, 147, and 182 days after sewage sludge incorporation, determinations were made for fresh matter weight in the above-ground part and roots of the seedlings (except roots at 182 days), pathogen's presence in the soil and roots, pH, and electric conductivity of the soil solution. At the end of the experiment (182 days after treatments), a compound sample of seedling leaves was collected to determine their nutritional status by means of tissue analysis, and results were analyzed using the DRIS indexes for N, P, and K.

2.4. Statistical analyses

The statistical analyses were performed using the SAS for Windows statistical package, Version 6.12, by S.A.S. Institute, Cory NC, USA.

3. Results

3.1. Test involving cravo lemon plantlets grown in seedling tubes in the greenhouse

Significant differences among treatments were observed for fresh matter weight in the above-ground part of plantlets, with positive increases when sewage sludge doses increased (Table 3, Figs. 1A and C). With respect to fresh matter weight of roots, significant differences were only observed in the second experiment (Table 3, Figs. 1B and D). However, a tendency of reduction in growth was observed with the application of sludge doses higher than 20%. Electric conductivity was directly proportional to sludge doses (Table 3, Figs. 2B and D), while pH was inversely proportional (Table 3, Figs. 2A and C).

The percentages of *P. nicotianae* recovery from plantlets and substrates were inversely proportional to the sewage sludge concentrations (Table 3, Figs. 3A–D). The pathogen recovery values in the first experiment for the no-sludge and mineral fertilization treatments were the highest observed in the experiment, i.e., 46% and 55% for root recovery, and 38% and 50% for substrate recovery, in treatments containing 1.5 and 3.0 g of inoculum per seedling tube, respectively.

In the first experiment, the pathogen recovery data from roots was negatively correlated with electric conductivity values ($r = -0.49$; $P = 0.024$), and positively correlated with pH values ($r = 0.513$; $P = 0.017$), and the same tendency was maintained in the second experiment, but without significance. The pathogen recovery data from the substrate showed the same tendency as the recovery data from roots, but were not significant in any of the experiments.

P. nicotianae recovery from dead plantlets and their corresponding substrates was 60% and 100% in the first and second experiments, respectively.

3.2. Test involving cravo lemon seedlings grown in pots in the greenhouse

In the first experiment, fresh matter weight for the above-ground part and roots of citrus seedlings was directly proportional to sludge dose increase up to the 20% application (Table 3, Figs. 1E and F), in a similar way as observed in the second experiment with plantlets in seedling tubes (Table 3, Figs. 1C and D). However, in the second experiment, this tendency was not observed (Table 3, Figs. 1G and H). In the first experiment, treatments involving mineral fertilization were only superior to treatments without sludge for variables that reflect seedling development.

In the first experiment, pH in water did not show significant differences between treatments (Table 3, Fig. 2E). In the second experiment, however, despite the absence of significance for the regression curve ($R^2 = 0.16$), significant differences were observed among treatments (Duncan Test, at the 5% probability level), with a tendency to decrease when sludge doses increased, with values of 5.94 for treatments without sludge and without inoculum, and 5.25 for treatments with 30% sludge and 30 g of inoculum per seedling (Table 3, Fig. 2G).

The substrate electric conductivity values were directly proportional to sludge doses and showed significant differences among treatments in both experiments (Table 3, Figs. 2F and H).

Soil microbial activity, evaluated through FDA hydrolysis and microbial respiration, showed significant differences between treatments, with positive increases when sludge concentrations increased (Table 3, Fig. 4).

P. nicotianae recovery from seedling roots and from the substrate was low in both experiments (Table 3, Figs. 3E–G). No pathogen recovery from the substrate was obtained in the first experiment, except in the treatment with 20% sludge and 8 and 15 g of inoculum, with values of 7.5% and 5%, respectively. Although low, in the first experiment, the pathogen recovery values from roots and from the soil were negative and significantly correlated with FDA hydrolysis ($r = -0.94$ and $r = -0.99$; $P < 0.01$) and microbial respiration values ($r = -0.82$; $r = -0.90$; $P < 0.01$), and were positive and significantly correlated with pH values ($r = 0.74$; $r = 0.60$; $P < 0.01$, respectively), but were not correlated with electric conductivity values.

From the leaf tissue analysis and DRIS index results for N, P, and K, it can be seen that treatments without fertilization showed the greatest imbalances, with relative deficiencies of N and P and a relative excess of K (Table 2). However, treatments involving mineral

Table 3
Regression equations of sewage sludge effect on fresh matter weigh of the above-ground and roots of plantlets and seedlings, pH and electric conductivity of substrate and soil, *Phytophthora nicotianae* recovery from roots and soil, microbial soil activity evaluated by fluorescein diacetate hydrolysis (FDA) and microbial respiration (CO_2), in the experiments performed with plantlets and seedlings

Experiment[a]	Inoculum level (g)	Regression equation		*p* value	
		Linear	Quadratic	Linear	Quadratic
Fresh matter weigh of the above-ground plant part					
Plantlets 1	0	$y = 0.58 + 0.015x$	$y = 0.47 + 0.041x - 0.00088x^2$	0.0109	0.0135
	1.5	$y = 0.59 + 0.015x$	$y = 0.57 + 0.019x - 0.00013x^2$	0.0214	0.0739
	3.0	$y = 0.72 + 0.006x$	$y = 0.75 - 0.0013x + 0.00025x^2$	0.3118	0.5630
Plantlets 2	0	$y = 0.54 + 0.022x$	$y = 0.32 + 0.075x - 0.00178x^2$	<0.0001	<0.0001
	1.5	$y = 0.33 + 0.011x$	$y = 0.28 + 0.025x - 0.00045x^2$	0.0260	0.0597
	3.0	$y = 0.22 + 0.007x$	$y = 0.22 + 0.008x - 0.00004x^2$	0.0768	0.2160
Seedlings 1	0	$y = 54.69 + 1.27x$	$y = 34.77 + 6.05x - 0.16x^2$	0.0110	<0.0001
	8.0	$y = 63.48 + 1.27x$	$y = 49.31 + 4.69\ x - 0.11x^2$	0.0019	<0.0001
	15.0	$y = 51.51 + 1.95x$	$y = 35.26 + 5.85x - 0.13x^2$	<0.0001	<0.0001
Seedlings 2	30.0	$y = 46.69 + 0.61x$	$y = 54.34 - 1.16x + 0.06x^2$	0.2281	0.2559
Fresh matter weigh of roots					
Plantlets 1	0	$y = 0.50 + 0.004x$	$y = 0.49 + 0.007x - 0.00008x^2$	0.3592	0.6544
	1.5	$y = 0.48 + 0.004x$	$y = 0.54 - 0.011x + 0.00045x^2$	0.4549	0.4968
	3.0	$y = 0.59 - 0.002x$	$y = 0.67 - 0.012x + 0.00059x^2$	0.7306	0.5950
Plantlets 2	0	$y = 0.40 + 0.004x$	$y = 0.28 + 0.032x - 0.00093x^2$	0.3075	0.0567
	1.5	$y = 0.19 + 0.006x$	$y = 0.14 + 0.018x - 0.00040x^2$	0.0802	0.1149
	3.0	$y = 0.12 + 0.003x$	$y = 0.12 + 0.003x - 0.00003x^2$	0.2482	0.5172
Seedlings 1	0	$y = 39.24 + 0.63x$	$y = 31.46 + 2.496x - 0.06227x^2$	0.0184	0.0028
	8.0	$y = 39.79 + 0.59x$	$y = 33.55 + 2.088x - 0.04993x^2$	0.0103	0.0026
	15.0	$y = 37.21 + 0.70x$	$y = 28.70 + 2.743x - 0.06811x^2$	0.0037	<0.0001
Seedlings 2	30.0	$y = 62.72 + 0.60x$	$y = 67.40 - 1.692x + 0.3687x^2$	0.2672	0.4428
pH					
Plantlets 1	0	$y = 5.37 - 0.025x$	$y = 5.47 - 0.048x + 0.00076x^2$	0.0134	0.0394
	1.5	$y = 5.44 - 0.020x$	$y = 5.42 - 0.014x - 0.00020x^2$	0.0092	0.0550
	3.0	$y = 5.34 - 0.024x$	$y = 5.36 - 0.029x + 0.00016x^2$	0.1810	0.950
Plantlets 2	0	$y = 5.25 - 0.019x$	$y = 5.38 - 0.053x + 0.00110x^2$	0.0436	0.0431
	1.5	$y = 5.13 - 0.007x$	$y = 5.24 - 0.033x + 0.00088x^2$	0.3419	0.2727
	3.0	$y = 4.87 + 0.006x$	$y = 4.87 + 0.007x - 0.000004x^2$	0.4993	0.8241
Seedlings 1	0	$y = 5.39 + 0.010x$	$y = 5.36 + 0.019x - 0.000277x^2$	0.2343	0.4795
	8.0	$y = 5.76 - 0.010x$	$y = 5.81 - 0.021x + 0.000381x^2$	0.2857	0.5289
	15.0	$y = 5.83 - 0.010x$	$y = 5.85 - 0.015x - 0.000173x^2$	0.2809	0.5583
Seedlings 2	30.0	$y = 5.36 - 0.046x$	$y = 5.49 - 0.036x + 0.00106x^2$	0.3600	0.0771
Electric conductivity					
Plantlets 1	0	$y = 0.116 + 0.0043x$	$y = 0.099 + 0.008x - 0.0001x^2$	0.031	0.097
	1.5	$y = 0.127 + 0.0043x$	$y = 0.1057 + 0.009x - 0.0001x^2$	0.040	0.1006
	3.0	$y = 0.084 + 0.0065x$	$y = 0.0928 + 0.045x + 0.00007x^2$	0.0019	0.0156
Plantlets 2	0	$y = 0.108 + 0.0036x$	$y = 0.078 + 0.0102x - 0.00024x^2$	0.0675	0.0147
	1.5	$y = 0.129 + 0.0020x$	$y = 0.114 + 0.0548x - 0.00012x^2$	0.0529	0.0553
	3.0	$y = 0.174 + 0.00001x$	$y = 0.174 + 0.00001x + 0.0000001x^2$	0.3891	0.792
Seedlings 1	0	$y = -0.032 + 0.0020x$	$y = 0.059 - 0.00013x + 0.00073x^2$	<0.0001	<0.0001
	8.0	$y = -0.002 + 0.0021x$	$y = 0.093 - 0.0023x + 0.00076x^2$	<0.0001	<0.0001
	15.0	$y = -0.035 + 0.0020x$	$y = 0.007 - 0.0039x + 0.00083x^2$	<0.0001	<0.0001
Seedlings 2	30.0	$y = 0.041 + 0.0107x$	$y = 0.038 + 0.0113x - 0.000022x^2$	<0.0001	<0.0001
P. nicotianae *recovery from roots*					
Plantlets 1	1.5	$y = 36.18 - 0.82x$	$y = 38.59 - 1.403x + 0.0193x^2$	0.2403	0.4930
	3.0	$y = 35.18 - 1.04x$	$y = 42.85 - 2.887x + 0.0614x^2$	0.0512	0.0815
Plantlets 2	1.5	$y = 8.25 - 0.17x$	$y = 8.31 - 0.185x + 0.0005x^2$	0.3425	0.6439

Table 3 (*continued*)

Experiment[a]	Inoculum level (g)	Regression equation		*p* value	
		Linear	Quadratic	Linear	Quadratic
	3.0	$y = 12.76-0.32x$	$y = 15.93-1.008x+0.0224x^2$	0.0921	0.1271
Seedlings 1	8.0	$y = 7.98-0.18x$	$y = 6.10+0.272\ x-0.0151x^2$	0.4765	0.6723
	15.0	$y = 3.01-0.09x$	$y = 2.36+0.064\ x-0.0052x^2$	0.3817	0.6176
Seedlings 2	30.0	$y = 5.46-0.23x$	$y = 9.40-1.142\ x+0.0310x^2$	0.1048	0.0228
P. nicotianae *recovery from soil*					
Plantlets 1	1.5	$y = 34.93-0.81x$	$y = 36.42-1.164x+0.0119x^2$	0.2541	0.5232
	3.0	$y = 42.85-0.78x$	$y = 49.47-2.373x+0.0530x^2$	0.3738	0.5775
Plantlets 2	1.5	$y = 8.64-0.13x$	$y = 4.81+0.783x-0.0305x^2$	0.5389	0.3254
	3.0	$y = 18.62-0.47x$	$y = 24.82-1.813x+0.0437x^2$	0.1095	0.0942
Seedlings 1	8.0	No recovery			
	15.0	No recovery			
Seedlings 2	30.0	$y = 3.70+0.25x$	$y = 5.41-0.149\ x+0.0134x^2$	0.1503	0.2719
FDA hidrolisys					
Seedlings 1	15.0	$y = 2.40+0.03x$	$y = 2.41+0.029x+0.00006x^2$	<0.0001	<0.0001
Seedlings 2	30.0	$y = 2.12+0.021x$	$y = 1.90+0.071x-0.00171x^2$	0.0013	<0.0001
CO_2					
Seedlings 1	15.0	$y = 0.25+0.009x$	$y = 0.26+0.006x+0.0001x^2$	<0.0001	<0.0001
Seedlings 2	30.0	$y = 0.52+0.027x$	$y = 0.35+0.066x-0.0013x^2$	<0.0001	<0.0001

[a]Plantlets 1 = First experiment with plantlets in seedling tubes; Plantlets 2 = Second experiment with plantlets in seedling tubes; Seedlings 1 = First experiment with seedlings in pots; Seedlings 2 = Second experiment with seedlings in pots.

fertilization showed an excellent nutritional balance (values near the reference values), with values of zero in each DRIS index for nutrients. For treatments involving sewage sludge incorporation, a relative excess of N was observed in treatments with more than 15% sludge, a relative P deficit in treatments containing 30% sludge, and a relative K deficiency, because sewage sludge is, in general, deficient with reference to this nutrient.

3.3. Test involving cravo lemon seedlings grown in the field

Since there was no significant effect of the factor inoculum concentration, treatment means with and without inoculum were obtained; these are shown in Fig. 5. Fresh matter weight of the above-ground part and roots of seedlings, in general, showed a positive response to sewage sludge concentration increases (Figs. 5A and B), even though not always statistically significant differences were observed on the different evaluation dates.

The values for electric conductivity and pH in soil solution water showed statistically significant differences among treatments, which are explained by the sludge factor for electric conductivity, and by the factors sludge and blocks for pH, on different evaluation dates (Figs. 5C and D).

For pH, the no sludge and no mineral fertilizer treatment showed the highest values, with a steady trend along the experiment (Fig. 5C). The soil without sludge and with fertilizer showed steady pH values up to 43 days after treatments, decreasing from that point until 182 days, reaching, together with treatments containing 15% sludge, the smallest values in the experiment. In general, there was a tendency for pH reduction as sludge concentration in the soil increased.

Electric conductivity values increased up to 43 days after all treatments, and then decreased right afterward, with a tendency to become stable from 118 days, but always with values higher than the initial values (Fig. 5D). It can be observed that, in general, the electric conductivity values increased as sewage sludge concentrations increased, and only treatments involving mineral fertilization surpassed those containing sludge from 118 days after treatments.

In general, the leaf tissue analysis results for seedlings showed that differences between treatments with and without sludge are small (Table 2). Nutritional optima were obtained in treatments with mineral fertilization and 5% sewage sludge, either inoculated or not, and in the treatment without inoculum containing 10% sewage

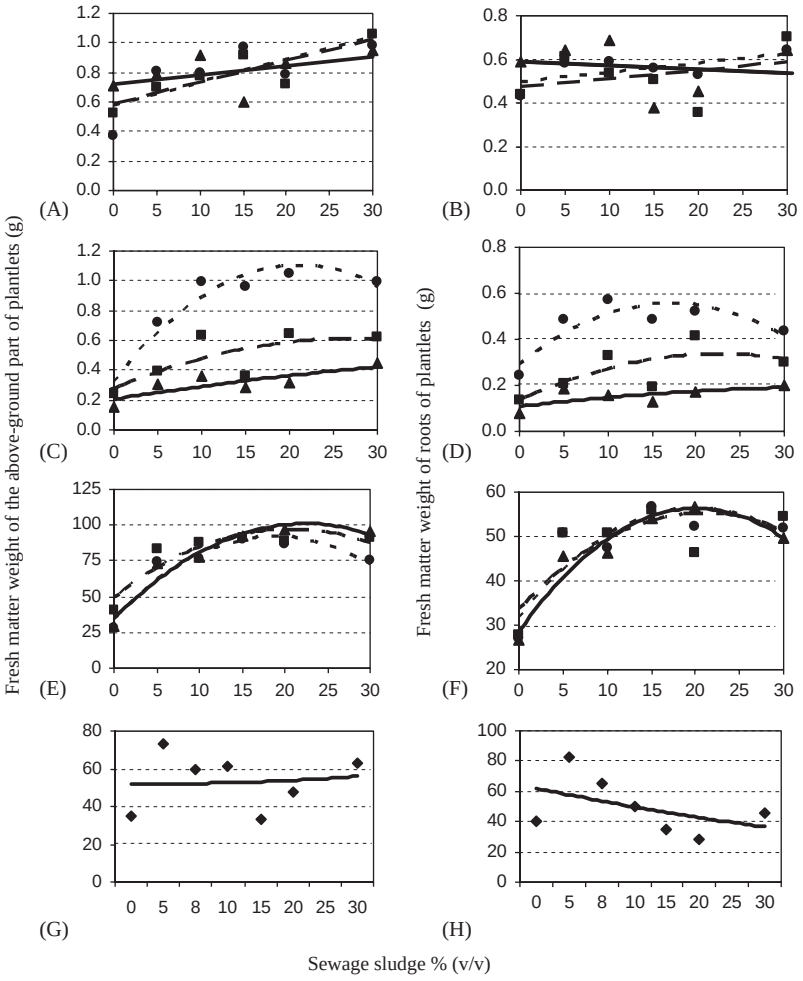

Fig. 1. Sewage sludge effect on fresh matter weight of the above-ground part and roots of plantlets (first experiment: A and B; second experiment: C and D) and seedlings (first experiment: E and F; second experiment: G and H) of cravo lemon (*Citrus limonia*) in greenhouse experiments, for different inoculum levels (0 -●-, 1.5 -■-, and 3.0 -▲- g per seedling tube, or 0 -●-, 8.0 -■-, 15.0 -▲-, and 30.0 -◆- g per pot). Dots are means of each treatment.

sludge. With respect to P, a relative excess of this nutrient can be observed when sludge doses were equal to or higher than 7.5%, usually in association with N deficiencies. With regard to K, the DRIS index in treatments with sludge incorporation indicated a balance of this nutrient with N and P, and relative deficiencies only occurred in treatments containing 10% and 15% sludge, with and without inoculum, respectively.

Soil microbial activity, evaluated through FDA hydrolysis and microbial respiration, showed statistically significant differences among treatments on different evaluation dates (Fig. 5). These differences were due to the factors sludge and block for the FDA hydrolysis variable, and to the factor sludge for respiration, with positive responses to sewage sludge concentration increases. Like with the other variables, the factor inoculum was not significant on any of the evaluation dates. Microbial activity evolution with time showed maximum activity at 5 and 15 days for CO_2 release and FDA hydrolysis, respectively (Fig. 5).

P. nicotianae recovery from the soil was only possible at 82 days after seedling transplanting, with low values, which, however, showed a tendency to decrease when sewage sludge values increased (Fig. 3H). On the other dates, no pathogen recovery was obtained, either from roots or from the soil. The values for pathogen recovery from the soil were correlated with FDA hydrolysis values ($r = -0.2819$; $P = 0.052$), but not with microbial respiration, nor with soil electric conductivity and pH.

4. Discussion

In the greenhouse experiments, it was observed that *P. nicotianae* recovery from the soil and roots was, in

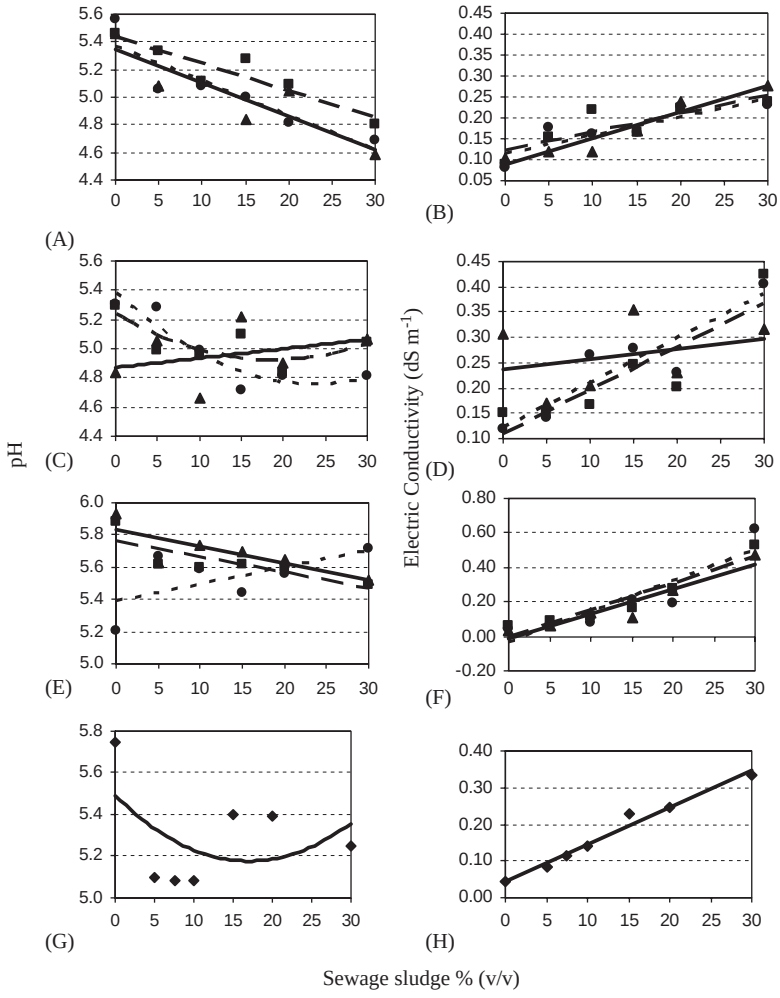

Fig. 2. Sewage sludge effect on water pH and on soil electric conductivity in an experiment conducted with cravo lemon (*Citrus limonia*) plantlets in seedling tubes (first experiment: A and B; second experiment: C and D) or seedlings in pots (first experiment: E and F; second experiment: G and H), in the greenhouse, for different inoculum levels (0 -●-, 1.5 -■-, and 3.0 -▲- g per seedling tube, or 0 -●-, 8.0 -■-, 15.0 -▲-, and 30.0 -◆- g per pot). Dots are means of each treatment.

general, smaller when sewage sludge concentration increased (Table 3, Fig. 3). These results coincide with those obtained by other authors, in the management of diseases caused by both *Phytophthora* and by other pathogens, in several crops (Bettiol and Krügner, 1984; Casale et al., 1995; Chung et al.; 1988; Costa et al., 1996; Erwin and Ribeiro, 1996; Hoitink and Boehm, 1999; Kim et al., 1997; Lewis et al., 1992; Lumsden et al., 1983; Millner et al., 1981; Widmer et al., 1998). The processes involved are complex and include biotic and abiotic factors, some of which were evidenced in this work, such as alterations in the soil chemical properties (electric conductivity and pH), improvement in seedling development conditions, and microbial activity increase (Table 3, Figs. 1, 2, 4, and 5).

Electric conductivity increased as a response to increases in the amount of sludge incorporated to the substrate (Table 3, Fig. 2); however, the values attained are within those recommended for agricultural use (Widmer et al., 1998). Similarly to this work, Workneh et al. (1993) established negative correlations between electric conductivity and the presence of *P. parasitica* or the incidence of the disease in tomato plants.

In the greenhouse and field experiments, the pH values showed a decreasing trend when sewage sludge levels increased (Table 3, Figs. 2 and 5). According to Carmo (2001), the reduction in pH values in the soil solution is due to the release of $N–NH_4^+$ during the sludge mineralization process in the soil, and the high $N–NH_4^+$ contents could indicate a greater release of H^+ to the medium, promoting acidification. Tsao (1959) observed that low pH contents reduced the incidence of root rot caused by *P. nicotiana* in citrus. Downer et al. (2001) suggested that suppressiveness to *P. cinnamomi* is favored by low pH values, which favor the action of enzymes produced by antagonists to the pathogen.

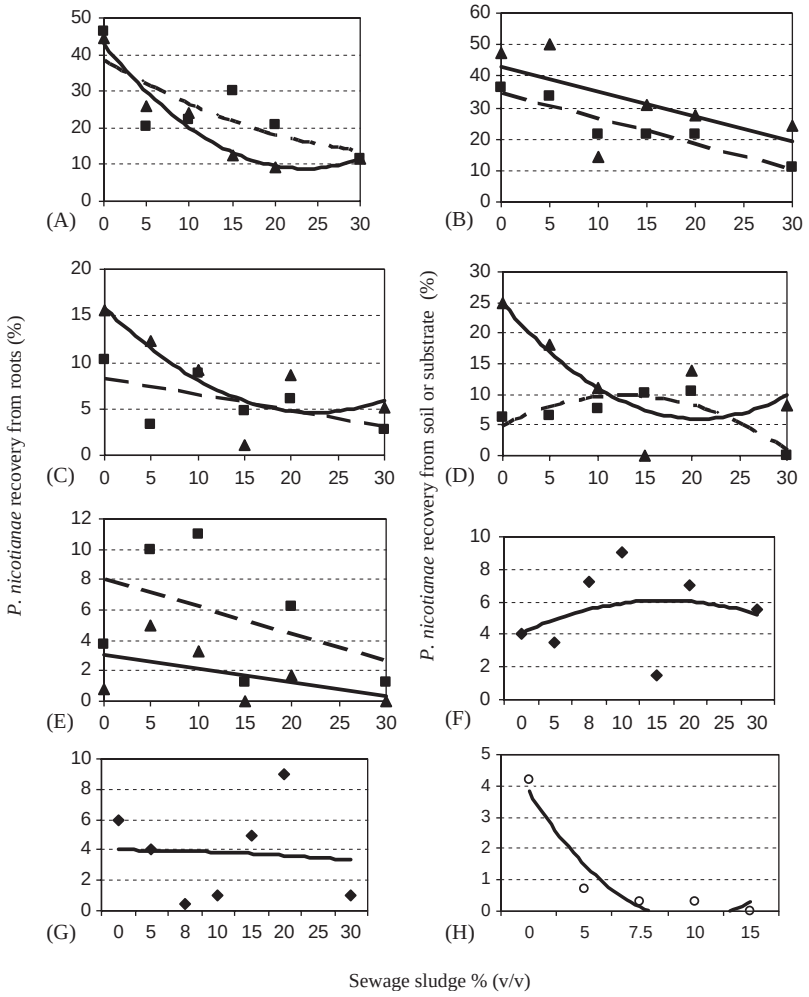

Fig. 3. Sewage sludge effect on *Phytophthora nicotianae* recovery from roots and from soil or substrate, by means of the citrus leaf test, in experiments with cravo lemon (*Citrus limonia*) plantlets in seedling tubes (first experiment: A and B; second experiment: C and D), or seedlings in pots (first experiment: E; second experiment: F and G), in the greenhouse, and in the field (H), for different inoculum levels (1.5 -■-, and 3.0 -▲- g per seedling tube, or 8.0 -■-, 15.0 -▲-, or 30.0 -◆- g per pot, or 20.0 -○- g per plant). Dots are means of each treatment.

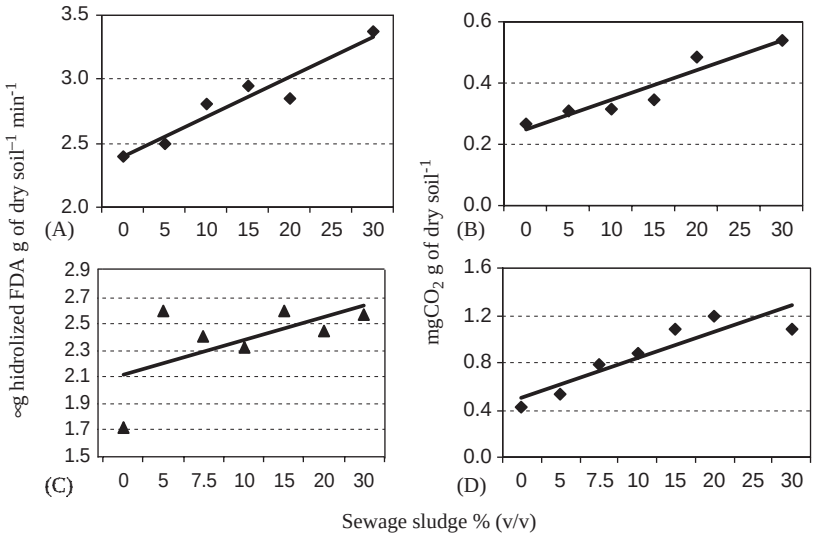

Fig. 4. Sewage sludge effect on soil microbial activity, evaluated by fluorescein diacetate hydrolysis (FDA) and microbial respiration (CO_2) in the first (A and B) and second experiments (C and D) with cravo lemon seedlings (*Citrus limonia*) in the greenhouse.

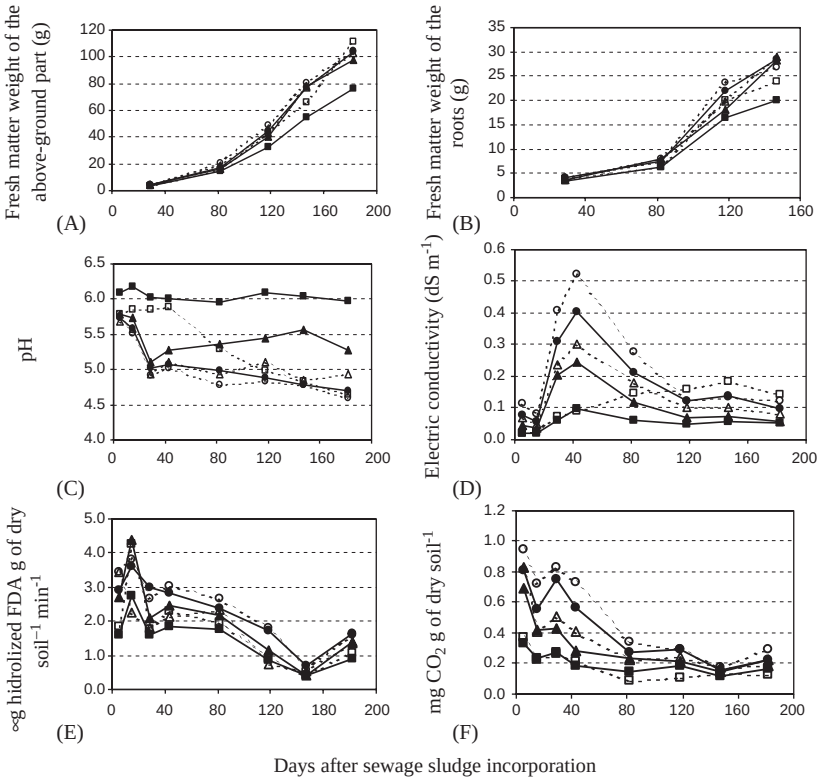

Fig. 5. Effect of sewage sludge applied at the proportions of 0 (-■- without mineral fertilization; -□- with mineral fertilization), 5 (-▲-), 7.5 (-△-), 10 (-●-) e 15 (-○-) % (v/v), in the field experiment (means with and without inoculum), on fresh matter weight of the above-ground part (A) and roots (B) of cravo lemon seedlings (*Citrus limonia*), water pH (C), soil electric conductivity (D), soil microbial activity, evaluated by fluorescein diacetate hydrolysis (FDA) (E), and microbial respiration (CO_2)(F).

Sludge showed a significant and positive effect on seedling development (Table 3, Figs. 1 and 5). These results agree with several papers that suggest that plants attain better development when growing in soils with incorporation of organic matter from various sources (Bettiol and Krügner, 1984; Kim et al., 1997; Pascual et al., 2000). Improvements in soil infiltration and drainage are among the factors involved, favoring root development and limiting the possibility of soil saturation by excess water, and providing a more balanced plant nutrition, thus compensating imbalances.

A tendency of reduction in fresh matter weight of the above-ground part and roots was observed with sewage sludge incorporation at concentration of 30% (v/v) (Table 3, Fig. 1), suggesting a possible phytotoxicity effect as reported by other authors when large volumes of organic matter are incorporated to the soil and/or when they are not completely composted (Aryantha et al., 2000; Casale et al., 1995; De Vleeschauwer et al., 1981; Widmer et al., 1998). According to Widmer et al. (1998), this negative effect may disappear with time, and stimulate crop development in the long run. De Vleeschauwer et al. (1981) studied the phytotoxic components from fresh city refuse composts and affirmed that the main phytotoxic substance was acetic acid, followed by organic acids (propionic, isobutyric, butyric, and isovaleric), which reached non-toxic levels to plants after composting for five months.

Increases in soil microbial activity are mentioned by several authors as one of the main factors that could explain suppression of *P. nicotianae*, where microbial communities would establish biological control by means of the classic mechanisms described by Baker and Cook (1974): competition, antibiosis, parasitism, and resistance induction. Success in the control of *Phytophthora* by the microbial community is based, among other factors, on its low saprophytic and competitive capacity (Erwin and Ribeiro, 1996). Malajczuk (1983) suggested that the most important mechanisms involved in the control of *Phytophthora* spp. are competition by nutrients and antibiosis. However, Downer et al. (2001) suggested that cellulase and laminarinase production is the chief mechanism involved in suppressing *P. cinnamomi* in a system developed in Australia for root rot control in avocado trees, based on the application of large amounts of organic material. The authors stated that destruction of the pathogen zoospores and other propagules is a consequence of the activity of enzymes produced by the community of fungi, including *Penicillium* sp. and *Aspergillus* sp.

An increase in soil microbial activity was verified in the present work, with positive responses of fluorescein diacetate hydrolysis (FDA) and microbial respiration to sewage sludge incorporation to the soil (Table 3, Figs. 4 and 5). These data coincide with results by several authors who reported significant correlations between incidence of the disease or presence of the pathogens and increases in FDA values (Aryantha et al., 2000; Boehm and Hoitink, 1992; Costa et al., 1996; Ghini et al., 1998; Kim et al., 1997; Workneh et al., 1993). The relationship between microbial activity and suppressiveness to pathogens has been demonstrated by several authors: CO_2 release (Costa et al., 1996; Ghini et al., 1998); activity of dehydrogenase (Lewis et al., 1992) and other enzymes such as phosphatase, urease, β-glucosidase, galactosidase, *N*-acetyl-glucose-aminidase (Pascual et al., 2000); and microbial biomass (Hoitink and Boehm, 1999). Hoitink and Boehm (1999) suggested that the level of FDA hydrolysis is a good indicator of the suppressiveness of soils, but considered that the success of biological control against *Pythium* sp. and *Phytophthora* sp. also depends on the amount and quality of the organic matter that will provide energy to the microorganisms involved in biological control.

The importance of studies on disposing of sewage sludge in agriculture has increased considerably in Brazil and in other countries, because a great number of cities are treating their sewage and generating sludge. In addition, many cities are beginning the construction of treatment stations, because it is crucial to collect and treat sewage in order to reduce public health and water pollution problems. These results demonstrate the potential use of sewage sludge in citrus, as well as the necessity for additional studies in other pathosystems. Moreover, they show the need for interdisciplinary research studies involving the utilization of urban–industrial residues in agriculture to be carried out. Considering that sewage sludge amendments carry in their composition, different forms of pollutants such as: heavy metals, organic chemical compounds and pathogens that may represent threat to human life, rigorous control in their agricultural use is recommended.

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