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Influence of *Pseudomonas putida* AF7 inoculation on soil enzymes

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Abstract There has been some concern about the environmental impact of microbial agents. *Pseudomonas* may be used as bioremediator and as biopesticide. In this study, we report the use of soil enzyme assays as biological indicator of possible negative effects in soil functioning after the *P. putida* AF7 inoculation. For that, *P. putida* AF7 was originally isolated from the rizosphere of rice and was inoculated on three soil types: Rhodic Hapludox (RH), Typic Hapludox (TH); and Arenic Hapludult (AH). The acid phosphatase, β-glucosidase and protease enzymes activities were measured for three period of evaluation (7, 14 and 21 days). In general, the enzymatic activities presented variation among the tested soils. The highest activities of β-glucosidase and acid phosphatase were observed in the RH and AH soils, while the protease activity was higher in the TH soil. Also, the soil characteristics were measured for each plot. The activity of enzymes from the carbon cycle was positively correlated with the N and the P and the enzyme from the nitrogen cycle was negatively correlated with N and C.org. The presented data indicate that soil biochemical properties can be an useful tool for use as an indicator of soil perturbations by microbial inoculation in a risk assessment.

Keywords *Pseudomonas putida* · Soil enzymes · Bioremediation · Biopesticide · Biological indicator · Risk assessment

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Introduction

The possible effects, produced by the introduction of a microbial agent can be summarized in direct and indirect damages on non-target organisms of the local community, including the flora and fauna representatives of economical, ecological and or social importance. For example, there is some concern that introduction of these agents into the environment may cause adverse perturbations of the native soil microbiota and the nutrient turnover processes they are involved in (Johansen et al. 2005). In whatever manner, the test done to demonstrate whether a microbial agent is able to survive or replicate in the environment generally includes an evaluation of the growth of the agent when introduced into a new environment.

A wide range of isolated natural bacteria with the potential for biocontrol of seed and soil-borne pathogens have been described and *Pseudomonas* spp. are specially well studied (Whipps 2001). *P. putida* is an ubiquitous soil bacterium that has significant potential for use in biochemical areas (Schneider and Dorn 2001). *Pseudomonas* has potential in degradation of a wide range of xenobiotics (Walia et al. 2002), and also may be used as a biopesticide for management of different plant diseases (Altindag et al. 2006).

The strategies used by this agent in many cases involve production of antibiotics with a rather broad range of action against target organisms, but there is an increasing published scientific evidence that microorganisms being used as biological controls can have significant, measurable effects, both direct and indirect, on non-target organisms (Johansen et al. 2005).

The soil environment is the focus of many concerns associated with the potential environmental effects of biocontrol agents. Most attempts to monitor the effects of

microbial introductions have been centered on microbial enumeration of specific populations. Transient perturbations have been observed in indigenous bacterial (Natch et al. 1997), fungal (Glandorf et al. 2001); and protozoa (Austin et al. 1990) populations.

The measurement of perturbations with soil biochemical variables, such as enzyme activities, may be an alternative way of monitoring overall effects of the introduced bacteria on the ecosystem. The use of soil enzymes to determine the extent of perturbations undergone by a soil has proved a successful approach to study the impact of the different soil management systems but has received little attention so far to investigate the effect caused by the inoculation of a microorganism.

Naseby and Lynch (1997a) studying the impact of *Pseudomonas fluorescens* genetically modified for lactose utilization in rhizosphere of pea, observed that both acid and alkaline phosphatase, phosphodiaesterase and the carbon cycle enzyme activities increased substantially with the addition of lactose to the soil. Naseby and Lynch (1997b) found perturbations in several soil enzyme activities following the introduction of *Pseudomonas fluorescens* into the rizosphere of wheat long-term, large-scale microcosm studies. Large perturbations were also found in short-term microcosm studies using the same strain (F113) (Naseby and Lynch 1998a).

In this paper, we report the use of soil enzyme assays to evaluate the effect of introduced *Pseudomonas putida* AF7 on functioning of the soil. To that end, the current analysis aims to assess the utility of the biochemical analysis as biological indicator for the study of impact of microbial agents introduced into the soil.

Materials and methods

Pseudomonas putida isolation and characterization

Pseudomonas putida AF7 was originally isolated from the rizosphere of rice, grown cultivated on soils exposed historically to the herbicide propanil at Massaranduba City, Santa Catarina State, Brazil. The strain was previously characterized by the fatty acid methyl esters procedure (Svec et al. 2004). In all experiments *P. putida* was grown in King' B medium (Difco Laboratories), for 24 h at 30°C.

Soils and treatments

The experimental conditions were designed to create controlled experimental environmental conditions close to a field situation. The soils used, Rhodic Hapludox (RH), Typic Hapludox (TH); and Arenic Hapludult (AH), presented the chemical and physical characteristics described in Table 1. Ten subsamples of each soil were taken at random and collected at 0–10 cm depth. In the laboratory, the subsamples were mixed and homogenized to constitute a composite sample, which was air-dried, sieved (2 mm mesh) and maintained under cold storage at 4°C until use. The soil pH, moisture, and water holding capacity (WHC) were determined according to the methodology proposed by Embrapa (1997). The total organic carbon (C.org) content was measured by sulfuric-acid potassium dichromate oxidation method (Nelson and Sommers 1982) and N level was determined by Kjeldahl method (Bremner and Mulvaney 1982). The phosphate was extracted with bicarbonate (Olsen and Sommers 1982).

Table 1 Physicochemical properties of RH, TH and AH soils

Parameters	Soils[a]		
	RH	TH	AH
Sand (2.00–0.053 mm) (%)	45.6	46.5	68.1
Silt (0.053–0.002 mm) (%)	10.0	16.2	10.4
Clay (<0.002 mm) (%)	44.4	37.3	21.5
pH	4.0	4.8	4.1
Cation exchange capacity (CEC, mmol/dm^3)	93.2	51.1	71.0
Water holding capacity (WHC, %)	28.9	19.7	22.8
Organic matter (%)	4.93	2.07	3.03
C.org (%)	2.87	1.20	1.76
N-total (mg/kg)	1,952	879	1,135
P (mg/kg)	7.20	13.40	9.65
Soil base saturation (V, %)	5.0	46.0	18.0

[a] Rhodic Hapludox (RH), Typic Hapludox (TH) and Arenic Hapludult (AH)

Two hundred grams were taken from the composite sample and were incubated in Erlenmeyer flasks (500 ml). Before the addition of the bacterium to the experimental units, the soil remained in the flasks for seven days, already with humidity corrected to 70% WHC, and were maintained at 27°C. The lighting regime was set at a photoperiod of 12 h. At the end of this period, *P. putida* AF7 (3.5×10^4 cfu g^{-1} dry weight soil (concentration 1) and 3.5×10^5 cfu g^{-1} dry weight soil (concentration 2)), was then applied to the soils. The soils were sampled periodically for moisture evaluation, and the moisture was maintained at a standard level with the application of deionized water. Soil samples without inoculation of *P. putida* were used as controls. At 7, 14 and 21 days after the incubation, samples were taken to evaluate the activities of β-glucosidase, acid phosphatase and protease. Three flasks were replicated for each treatment. The influence of the inoculation of AF7 on the growth of indigenous microorganisms was considered negligible since its volume was very low to introduce enough organic matter, P and N or other nutrient in soil.

Enzyme assays

Phosphatase activity

Acid phosphatase activity was determined by a standard analytical method (Alef et al. 1995c). One gram field-moist soil was mixed with 1 ml of *p*-nitrophenyl phosphate (substrate solution) and 4 ml of buffer maleate pH 6.5, while replicates for blank were made without substrate. The samples and the control (blank) were incubated for 1 h at 37°C. Released *p*-nitrophenol was extracted after addition of 1 ml of 0.5 M $CaCl_2$ solution and 4 ml 0.5 M NaOH. The samples were filtered through Whatman no 2. Intensity of yellow colour was determined at 410 nm against the blank on Shimadzu spectrophotometer. The concentration of produced *p*-nitrophenol was calculated in μg g^{-1} dry weight soil with the calibration curve.

β-Glucosidase activity

The activity of this enzyme was measured as described by Alef and Nannipieri (1995a). 4 ml of modified universal buffer (MUB) 0.1 M (pH 6.0) and 1 ml of 25 mM *p*-nitrophenyl-*β*-D-glucoside (PNG) were added to 1 g of soil and then incubated at 37°C per 1 h. After that, it was jointed 0.5 M $CaCl_2$ and Tris buffer, pH 12. The *p*-nitrophenol (PNP) was determined by a spectrophotometer in 400 nm. All measurements were carried out in triplicate with one blank. The results were expressed as μg de *p*-nitrophenol released after the incubation of soil for 1 h.

Protease activity

This activity was measured as described by Alef and Nannipieri (1995b) and involves the determination of amino acids released after incubation of soil with sodium caseinate (2%) and 50 mM Tris (hydromexymethyl) aminomethane (THAM) buffer at 50°C for 2 h.

Statistical analysis

ANOVA analysis was performed for enzyme activity measurements where it was quantified the effects of concentration, soil, evaluation data and its interactions. The effect of *P. putida* AF7 concentrations at each soil × date combination was evaluated by F tests for contrasts (Zar 1999), utilizing the SLICE option of LSMEANS statement (SAS Institute, Inc 2000). Same procedure was used to quantify the enzymatic activity temporal variability at each soil × concentration combination.

Results

The influence of the inoculation of *P. putida* AF7 on the following enzymes activities: acid phosphatase, *β*-glucosidase, and protease, for three period of evaluation and three soil types are presented in Tables 2 and 3 and Fig. 1. In general, the enzymatic activities presented variation among the soils tested. The highest activities of *β*-glucosidase and acid phosphatase were observed in the RH and AH soils, while the protease activity was higher in the TH soil.

The effect of *P. putida* AF7 concentrations on *β*-glucosidase activity was observed only at the following cases: RH soil (14 and 21 days) and AH soil (7 and 14 days). In the RH soil, a significant reduction ($P < 0.05$) of *β*-glucosidase activity was observed for concentrations 1 (c1) (approximately 32% at 21st day) and concentration 2 (c2) (∼14 and 21% on days 14 and 21, respectively) when compared to control. In the AH soil, the activity of that enzyme was increased at approximately 117%, at c2 on day 7, and 27% at c1 on day 14 after the inoculation (Table 2).

The treatment with the bacterial strain also reduced the acid phosphatase activity in the AH soil, that was significant at c1. The activity was decreased up to 10% on 14 and 21 days after the inoculation. The highest activity was detected in the same soil, varying from 310 to 359 μg *p*-nitrophenol g^{-1} solo h^{-1}. There was no evidence ($P > 0.08$) of acid phosphatase activity temporal variability in the RH and TH soils, for none of *P. putida* concentrations

Table 2 Means of *β*-glucosidase activity corresponding to the three *P. putida* AF7 concentration evaluated for RH, TH and AH soils

P. putida (cfu ml^{-1})	RH[a]			TH			AH		
	7	14	21	7	14	21	7	14	21
0	53.75	63.24	104.06	39.72	39.03	50.71	23.96	58.71	48.47
3.5×10^4	39.48	77.78	78.29[b]	37.85	42.52	56.14	36.77	74.53[b]	43.50
3.5×10^5	50.21	28.52[b]	91.17	39.76	39.28	53.72	51.98[b]	43.02	41.87

[a] Rhodic Hapludox (RH), Typic Hapludox (TH) and Arenic Hapludult (AH)

[b] Evidence of concentration effect in relation to control, although not significant

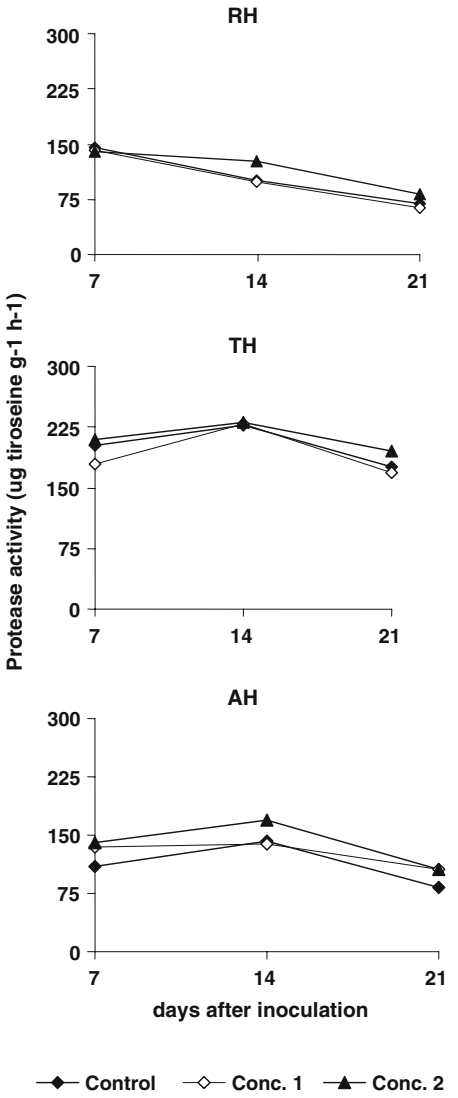

Fig. 1 Temporal variation of protease activity for different concentrations of *P. putida* (control), 3.5×10^4 (concentration 1) and 3.5×10^5 cfu ml^{-1} (concentration 2), in three types of soil: Rhodic Hapludox (RH), Typic Hapludox (TH) and Arenic Hapludult (AH)

studied. For the remaining soil, the enzymatic activity varied across time ($P < 0.05$) at lower concentration of *P. putida* (Table 3). The protease activity was not affected.

The available nutrients were similar in all treatments related to concentrations of the bacterium and in the control (Table 4). Significant positive correlations were found between levels C.org and the activities of β-glucosidase ($r = 0.66$; $P < 0.05$) and protease ($r = 0.65$; $P < 0.05$), till the end experimental period; while the same was not observed to β-glucosidase activity and protease activity that was negatively correlated with P total (-0.74; $P < 0.05$) and N total (-0.64; $P < 0.05$), respectively. The protease activity variation about 54% could be explained by the variation in P total, while 44% in β-glucosidase

Table 3 Means of acid phosphatase activity corresponding to the three *P. putida* AF7 concentration evaluated for RH, TH and AH soils

Soil[a]	Days	*P. putida* (cfu ml^{-1})		
		0	3.5×10^4	3.5×10^5
RH	7	251.68 ± 11.51	268.39 ± 22.60	261.05 ± 19.70
	14	244.36 ± 2.65	238.47 ± 3.44	255.23 ± 8.16
	21	244.99 ± 28.89	264.16 ± 14.03	246.88 ± 6.61
TH	7	140.21 ± 27.88	147.38 ± 11.00	152.43 ± 1.92
	14	108.88 ± 6.75	129.97 ± 2.50	121.18 ± 14.76
	21	218.56 ± 28.96	224.65 ± 13.77	235.31 ± 53.51
AH	7	324.65 ± 21.35	335.02 ± 14.59	337.59 ± 21.81
	14	359.25 ± 27.74	326.71 ± 27.79[b]	353.78 ± 8.74
	21	343.65 ± 20.55	310.90 ± 10.01[b]	354.53 ± 12.75

[a] Rhodic Hapludox (RH), Typic Hapludox (TH) and Arenic Hapludult (AH)

[b] Evidence of concentration effect in relation to control, although not significant

could be explained by the variation in N total. However, no evidence of correlation ($P > 0.84$) was found between the activity of acid phosphatase and these properties of soils.

Discussion

For decades bacteria that occur naturally in soil have been isolated and characterized in the laboratory with the aim of studying and improving their performance after release into the soil as biopesticides or bioremediation agents. However, not all species survive well in soil, either because they are not indigenous in soil or because they respond differently to diverse soil types. Potential deleterious effects of microbial agents on the indigenous soil microbiota may arise as a consequence of antagonism and competition for resources, and as a result of foreign bacteria introduction, both the structure and function of the indigenous microbial community might change. Based on these observations, the soil enzymes activities were measured to determine impacts of the microbial agent *P. putida* AF7 introduction.

The responses of inoculant bacterial *P. putida* AF7 was an effective population of approximately 1.8×10^{-5} cfu g^{-1} soil in rice rhizosphere, after 75 days of inoculation (Procópio 2007). The organism was capable of using herbicide propanil as the sole source of C, presenting up 60% of degradation (Procópio et al. 2007). These strain also presented capability of auxin production.

It was observed that, when inoculated in soil, the bacteria addition at the highest concentration resulted on an effect on the activity of β-glucosidase in the RH and AH soils. Since this activity is directly involved in the C cycle, in a short-term, microbial activity could be positively

Table 4 RH, TH and AH soil content of organic carbon (%), N total and P total at the end of experimental period for different concentrations of *P. putida*

Soil type	Concentration (cfu ml^{-1})	Parameters		
		C.org (%)	N total (mg/kg)	P (mg/kg)
RH[a]	0	2.64	1,713	5.2
	3.5×10^4	2.43	1,743	5.5
	3.5×10^5	2.55	1,848	5.7
TH	0	1.04	852	13.4
	3.5×10^4	1.39	778	14.8
	3.5×10^5	1.27	888	15.5
AH	0	1.78	1,174	12.0
	3.5×10^4	1.72	1,088	11.0
	3.5×10^5	1.77	1,232	12.4

[a] Rhodic Hapludox (RH), Typic Hapludox (TH) and Arenic Hapludult (AH)

affected by the organic matter. The strong positive correlations found between β-glucosidase and organic carbon support these results. The finding of significant positive correlation between β-glucosidase and Corg also confirmed the observations of the other study (Landgraf and Klose 2002). In due time, however, the available carbon could be decomposed (during the first two weeks) and the β-glucosidase activity stabilized at the end of incubation, in the AH soil. These results suggest that the AF7 treatment had a significant impact on the carbon cycle at short-time.

Also, a significant effect was found at the AF7 c1 inoculation in acid phosphatase activity in the AH soil. This reduction can be attributed to a direct or indirect effect of the bacteria introduction, resulting in a displacement of the communities that produce this enzyme in this soil. This result may also be due to a soluble phosphate form predomination since the increase of available inorganic soluble phosphate is known to decrease soil phosphatase activity (Tadano et al. 1993).

In the literature, the results related to the toxic metabolite of the bioremediator strain *P. putida* presented reduction in the soil respiration (Short et al. 1991) and the antibiotic produced by *P. fluorescens* reduced soil microbial activity (Brimecombe et al. 1998). Also, Naseby and Lynch (1997b) found perturbations in several soil enzyme activities following the introduction of *P. fluorescens* into the rhizosphere of wheat on long-term microcosm studies.

The effects of *P. fluorescens* F113 on soil enzyme activities in pea microcosms was studied by Naseby and Lynch (1998b), (1999) and Naseby et al. (2001). Generally, soil enzyme activity related to the carbon cycle decrease following introduction of F113, while enzyme activities involved in the cycle of phosphorus, sulfur and nitrogen increased. In the presence of *Pythium*, the effects of *P. fluorescens* strains F113, CHAO, SBW25 and Q-87 were reversed: the N-, P-, and S-cycle enzymes were decreased compared to an untreated control (Naseby et al. 2001).

Other studies that assessed modified biological control agents in laboratory, microcosm and field trials in order to observe the soil enzyme activities did not present results significantly affected by modified strains compared to wild type inoculants. The authors demonstrated that simply growing a plant using established farming practices had a far great impact on the soil microflora than inoculation with microbial inoculants (Research impact 2007). Meanwhile, the differences in soil enzymes caused by the soil heterogeneity may be supposed greater than the possible effect caused by the microbial agent inoculation.

Because of the importance of soil properties, the soil characteristics were measured for each plot to characterize soils chemical heterogeneity. These measures allowed the differentiation between the treatments and soils chemistry influence on enzyme activities. The activity of enzymes from the carbon cycle (β-glucosidase) was positively correlated with the N and the P while the enzyme involved in the nitrogen cycle (protease activity) was negatively correlated with N and C.org. This indicates that, since hydrolases are inducible enzymes, their activity are regulated by the presence of available substrates (Burns 1982).

There has been some concern about the impact of microbial agents on the environmental scenarios. Appropriate data of putative impacts is an important step to improving the scientific basis for its risk assessments. Our studies suggest some ways to evaluate the potential interactions that could occur before microbial agent introduction in the environment. Thus, the result-interpretation approach drawn from the data obtained are useful to establish baseline information to risk assessment.

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