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INFLUENCE OF PSEUDOMONAS PUTIDA AF7 INOCULATION ON SOIL ENZYMES

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ABSTRACT
Pseudomonas may use as bioremediator and as biopesticide. The use of soil enzymatic assays as biological indicator of possible negative effects in soil functioning was evaluated after *P.putida* AF7 inoculation. For that, AF7 was originally isolated from the rizosphere of rice and was inoculated on three soils: Rhodic Hapludox (RH), Typic Hapludox (TH); and Arenic Hapludult (AH). Soil characteristics were measured in each plot. Acid phosphatase, β-glucosidase and protease activities were measured at 7, 14 and 21 days. The enzyme activity waved during the experimental period but there is a significant reduction of β-glucosidase activity in RH soil on day 14. Corg was positively correlated to the activities of β-glucosidase and protease. The presented data indicate that soil biochemical properties may be useful as indicator of soil perturbations.

1. INTRODUCTION
P. putida is a ubiquitous soil bacterium that has significant potential for use in biochemical areas, such as the production of natural compounds, the bioremediation of numerous compounds of polluted habitats, and the use of strains to fight plant diseases [8]. *Pseudomonas* may has potential in degradation of a wide range of xenobiotics [10], and also may be also used as a biopesticide for management of different plant diseases [4].
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 [5]. 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. 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, in a more sensitive and comprehensive way. In this paper, the use of soil enzyme assays was studied 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 microbial agents' impact at its soil introduction.

2. MATERIAL AND METHODS
2.1. Pseudomonas putida isolation and characterization
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. In all experiments AF7 was grown in King' B medium for 24h at 30°C.

2.2. Soils and treatments
The experimental conditions were designed to create controlled experimental environmental conditions close to a field situation. The soils used were Rhodic Hapludox (RH), Typic Hapludox (TH); and Arenic Hapludult (AH). Ten sub samples of each soil were taken at random and collected at 0-10 cm depth. In laboratory, the sub samples were mixed and homogenized to constitute a composite sample, which were air-dried, sieved and maintained under 4°C until use. The soil characterizes as pH, moisture, water holding capacity (WHC), total organic carbon (Corg) content, phosphate and N level was determined. Before the addition of the bacterium to the experimental units, the soil remained in incubator Erlenmeyer flasks for seven days, already with humidity corrected to 70 % WHC at 27°C. The lighting regime was set at a photoperiod of 12 hours. Then, two concentrations of 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), were then applied to the soils. The soils moisture was maintained at a standard level with the application of deionized water. Soil samples without inoculation 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 at three replications for each treatment.

2.3. Enzyme assays
Acid phosphase [1] β-glucosidase [2] and protease activities [3] were determined by standard analytical methods. ANOVA analysis followed by F test for contrasts was performed for enzyme activity measurements where it was quantified the effects of concentration, soil, evaluation data and its interactions.

3. RESULTS

The soil characteristics and the influence of the inoculation of AF7 on enzymes activities are presented in Table 1 and 2 respectively. In general, the enzymatic activities presented variation among the soils tested. In RH soil, a significant reduction ($p<0.05$) of β-glucosidase activity was observed for concentration 2 on day 14. In the AH soil, its activity was increased at concentration 2 on day 7 about 103 % and 27% at concentration 1 on day 14, although not significantly.

TABLE 1. Physicochemical properties of RH, TH and AH soils.

Parameters	Soils [a]		
	RH	TH	AH
Sand (2,00-0,053mm)	456	465	681
Silte (0,053-0,002mm)	100	162	114
Clay (<0,002mm)	444	373	221
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
Corg (%)	2,87	1,20	1,76
N-total (mg/kg)	1952	879	1135
P (mg/kg)	7,2	13,4	9,65
Soil Base Saturation (V, %)	5	46	18

The treatment with the bacterial strain reduced slightly the acid phosphatase activity in the AH soil at concentration 1. The activity was decreased 10.5 % after 21 days from the inoculation. The protease activity waved during the experimental period but not significantly. For example, at concentration 2, the activity was enhanced 20 till 33 % in RH soil during the 21 days of the test. Also, it was reduced on day 7 in TH soil at concentration 2. However, on days 14 and 21 in AH soil, the enzyme activity was reduced 16.8 and 12% respectively. There was no evidence ($p>0.08$) of acid phosphatase activity temporal variability in the RH and TH soils. For the remaining soil the enzymatic activity varied across time ($p<0.05$) at lower concentration of AF7. The available nutrients were similar in all treatments related to concentrations of the bacterium and in the control (Table 3).

TABLE 2. Enzymes activities (means and standard deviation) corresponding to the three *P. putida* AF7 concentrations (control – 0, concentration c1 - 3,5 x10^4 and c2 - 3,5 x 10^5).

AF7 (cfu. mL^{-1})	RH			TH			AH		
	7	14	21	7	14	21	7	14	21
	β-glucosidase activity (µg p-nitrophenol g-1 h-1)								
0	46.19 ±17.65	53.55 ±18.46	92.61 ±23.50	39.72 ±2.99	39.03 ±2.11	50.71 ±2.72	31.06 ±12.57	58.71 ±12.23	44.05 ±8.77
C1	48.18 ±16.96	77.78 ±8.21	78.29 ±2.65	37.84 ±2.85	42.53 ±2.96	56.14 ±9.30	46.76 ±17.59	74.54 ±6.76	43.51 ±3.77
C2	55.33 ±9.63	23.69* ±9.37	91.17 ±9.50	39.76 ±1.79	39.28 ±2.53	53.72 ±5.04	62.98 ±19.06	54.72 ±20.28	41.87 ±2.98
	acid phosphatase activity (µg p-nitrophenol g^{-1} h^{-1})								
0	251.68 ±11.51	244.36 ±19.91	244.99 ±15.55	140.21 ±27.88	108.88 ±6.75	218.56 ±21.65	324.65 ±21.35	333.97 ±48.33	343.65 ±34.45
C1	268.39 ±22.61	238.47 ±3.44	264.16 ±13.07	147.38 ±11.00	129.97 ±2.50	224.65 ±21.88	335.02 ±14.59	326.71 ±14.87	310.90 ±9.09
C2	261.34 ±16.66	255.23 ±8.16	246.88 ±18.75	152.43 ±1.92	121.18 ±14.76	235.31 ±22.41	337.59 ±21.81	353.68 ±5.20	354.53 ±12.53
	protease activity (µg tiroseine g-1 h-1)								
0	145.52 ±21.11	100.43 ±8.05	68.24 ±0.75	201.64 ±6.64	227.45 ±27.29	163.86 ±21.23	140.36 ±56.25	167.13 ±45.36	128,31 ±79.55
C1	196.39 ±94.01	99.14 ±9.43	69.79 ±13.85	179.60 ±7.71	228.14 ±15.72	181.50 ±26.63	135.03 ±11.64	139.16 ±28.60	112,99 ±14.65
C2	196.90 ±100.1	110.50 ±35.24	82.87 ±6.50	267.56 ±102.5	230.38 ±25.13	177.62 ±29.50	122.72 ±30.79	169.19 ±4.15	123,06 ±29.41

TABLE 3. RH, TH and AH soil content of Corg (%), N total and P total at the end of experimental period.

Soil type	Concentration (cfu mL^{-1})	Parameters		
		Corg(%)	N total (mg/kg)	P (mg/kg)
RH	0	2.64	1713	5.2
	3.5×10^4	2.43	1743	5.5
	3.5×10^5	2.55	1848	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	1174	12.0
	3.5×10^4	1.72	1088	11.0
	3.5×10^5	1.77	1232	12.4

Significant positive correlations were found between levels Corg 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 could be explained by the variation in P total, while the β-glucosidase results 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.

4. DISCUSSION

After release into the soil as biopesticides or bioremediation agents, 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 [7]. In this way, appropriate data of putative impacts is an important step to improving the scientific basis for risk assessments in relation to the impact of microbial agents on the environmental scenarios.

Because of the importance of chemical and physical soil properties, the soil characteristics were measured for each plot to characterize their heterogeneity. These measures allowed the differentiation between the treatments and soil influence on enzyme activities. In the same direction, 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.

Since the activity of β-glucosidase is directly involved in the C cycle, in a short-term, microbial activity could be positively affected by the organic matter. The finding of significant positive correlation between β-glucosidase and Corg also confirmed the observations of the other study [6]. During the first two weeks, however, the available carbon could be decomposed and the β-glucosidase activity stabilized at the end of incubation. These results suggest that the AF7 treatment had a significant impact on the carbon cycle at short-time. The phosphatase results in AH soil may be due to a soluble phosphate form predomination since the increase of available inorganic soluble phosphate is known to decrease soil phosphatase activity [9]. The fluctuation values in enzyme activity found after AF7 inoculation may be attributed to a direct or indirect effect of the bacteria introduction, resulting in a displacement of the communities that produce them.

Our studies suggest some ways to evaluate the potential interactions that could occur before microbial agent introduction in the environment. The presented data indicate that soil biochemical properties may be useful as indicator of soil perturbations, although the variance found in experimental data, expressed by the large standard deviations obtained. Thus, the result-interpretation approach from the data obtained is useful to establish baseline information to risk assessment.

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