DETECTION AND ENUMERATION OF ENZYMATIC ACTIVITIES AND FLUORESCENT PSEUDOMONADS POPULATION IN THE RHIZOSPHERE OF WHITE CLOVER DUE TO SALINITY

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Abstract

In the greenhouse, *Trifolium repens* was grown in an agroecosystem using acidic brown forest soil treated with different NaCl doses. *In vitro,* different techniques were used for counting fluorescent pseudomonads, as well as the measurement of enzymatic activities. The result indicates that the population density of fluorescent pseudomonads in the rhizosphere was directly affected by the degree of salinity. The population was maximum at 0.2% comparing with other saline treatments. Strains of *Pseudomonas putida* and *P. fluorescens* were the most dominant fluorescent pseudomonads at this concentration. It was found that the NaCl tolerated strains of the fluorescent pseudomonads have high survival and root colonizing activity in the rhizosphere, especially in NaCl high treated soil. The addition of NaCl successfully increased the activities of dehydrogenase, catalase and urease between 0.2 - 0.4%, while for protease, it was laid between 0.1 - 0.2% NaCl. The activities of phosphatase and β-glucosidase were lower than the control by increasing NaCl concentrations.

Introduction

The physico-chemical properties of soils, their abilities to support plant growth and the diversity of microbial populations, their numbers and activities in soil are particularly important in any agroecosystem. Soil biological activity is the aggregate results of various microbial processes and the impact of salinity on them has a direct bearing on the productivity of soils. The metabolic activities of microorganisms vary considerably with changes in their growth environments. One of the parameters affecting microbial physiology is the medium osmolarity. Gram positive bacteria have been reported to accumulate the neutral amino acids proline (BROWN 1978) in response to increasing salt stress, whereas Gram negative bacteria accumulate glutamate (HUA *et al*. 1982). SAKAI *et al*. (1995) investigated the effect of inorganic ion in culture on the growth of *Pseudomonas putida* and *P. fluorescens* isolates from spinach roots grown in soils with high-salinity and low-salinity levels. Among the inorganic ions tested (K^+, Na^+, Ca^{2+}, Mg^{2+}, Cl^-, NO^{3-}, and SO_4^{2-}), Ca^{2+} was found to be the most inhibitor to the growth of isolates. The higher tolerance to Ca^{2+} of the isolates from the high-soil may have been acquired through selections under increasing Ca^{2+} stress in the field. It is concluded that the Ca^{2+} tolerance of the fluorescent pseudomonad strains may account for their survival and root colonizing activity in soils with Ca^{2+} accumulation.

Enzymes produced by soil microorganisms catalyzed biochemical processes involved in nutrient cycling in soil and may provide an index of total microbial activity. Dehydrogenase activity is an indicator of biological redox-systems, and can be taken as a measure for the intensity of microbial metabolism in soil (TABATABAI 1982). Soil catalase was used to characterize soil microbial activities. The hydrolysis of proteins is an important step in the organic N cycle of soil, and is essential to maintain soil fertility. Urease in soil acts on the hydrolysis of amido compounds that are supplied to the soil from plants, animals and microorganisms. The importance of phosphatase for plant nutrition has repeatedly been pointed out. In most soils, the organically bound P-fraction is higher than the inorganic. Phosphatases are excreted by plant roots and by microorganisms. β-Glucosidases play an important role in the total degradation of cellulose to glucose. β-glucosidase activity is considered as on indictor for biomass turnover. BATRA & MANNA (1997) determined dehydrogenase activity in typical saline, alkaline, and saline-alkaline sandy soils of India. A pot experiment was conducted to evaluate the influence of salinity on the population density of fluorescent pseudomonads and the enzymatic activities in the rhizosphere of white clover.

Materials and Methods

Detection of fluorescent pseudomonads:

The soil samples used in these experiments were characterized as acidic (pH_{KCl} 4.67) sandy, humus (1.2%) of brown forest in Gödöllő region, Hungary. NaCl (molecular weight 58.44 with 99.5 % Purity) salt was used to salinize the soil at different levels: 0, 0.1, 0.2, 0.4 and 0.8% (w/w). In the greenhouse, 25 seeds of white clover (*Trifolium repens* L) were planted / pot and covered with a layer of approximately 20 mm of soil. After two weeks emergence, the seedlings were thinned to 15 seedlings. Seedlings were watered with sterile distilled water when required. Plants grown under natural illumination (14h photoperiod) at around 23 ± 2°C. After 50 days, plants were carefully uprooted and thoroughly washed with light tap water (to remove all loosely adhering soil particles) followed by sterile 0.85% (wt / vol) saline Milli Q water (MQW). The roots were cut sharply. The roots were macerated in 0.85% saline MQW with a mortar and paste. Serial dilution technique was carried out for all macerated-root samples in sterile distilled water. From each of the homogenate sample, 1 ml was plated on King-B agar and Pseudosel agar for isolating fluorescent pseudomonad strains. Cultural media were supplemented with cycloheximide (100 µg/ml) and benomyl (30 µg/ml active ingredient) to inhibit fungal growth. The plates were incubated at 30°C for bacterial cultures and were counted after 2 days. The pure colonies were stored in respective medium and maintained at 4°C for further studies. The selected bacterial colonies were purified using dilution technique and streak plate method. The identification of isolated fluorescent pseudomonads was done according to Bergey's Manual of Systematic Bacteriology (1984), BBL Crystal Programme for identification systems and microflora associated with the rhizosphere were identified according to NAUTIYAL & DION (1990).

Measurement of the enzymatic activities:

The activities of the following enzymes were detected. ***Dehydrogenase activity*** was assessed in 1 g of soil, exposed to 0.2 ml of 4% 2-P-iodophenyl-3-P-nitrophenyl-5-phenyl-tetrazolium chloride (INT) at 22°C in darkness. The iodonitrotetrazolium formazan (INTF) formed was extracted with a mixture of 1:1.5 ethylene chloride/acetone by shaking vigorously and filtrated. INTF was measured spectrophotometrically at 490 nm (GARCÍA *et al*. 1993). The activity is expressed as μg of INTF/g dry soil. ***Catalase activity*** was determined by measuring the amount of O_2 consumed by $KMnO_4$ after addition of H_2O_2 to the soil samples (TABATABAI & BERMNER 1970). The activity is expressed as μmol O_2/min/g dry soil. ***Urease activity:*** 2 ml of phosphate buffer (pH 7) and 0.5 ml of 6.4% urea were added to 0.5 g soil sample which was incubated at 30°C for 90 min and then the volume was made up to 10 ml with sterile distilled water. The NH^+_4 released after addition of 0.1 ml of 10 M NaOH was measured using an NH^+_4 selective electrode. A control without urea was carried out for each sample (NANNIPIERI *et al*. 1980). The activity is expressed as μmol of NH^+_4-N released/g soil/h. ***Protease activity:*** 2 ml of phosphate buffer (pH 7) and 0.5 ml of 0.05M N-α-benzoyl-L-arginamide substrate were added to 0.5 g soil sample. The mixture was incubated at 37°C for 90 min and then diluted to 10 ml with sterile distilled water. The release of NH^+_4 was measured in the same way as for urease (NANNIPIERI *et al*. 1980). The activity is expressed as μmol of NH^+_4-N released/g dry soil/h. ***Phosphatase activity:*** 2 ml of 0.1M maleate buffer (pH 6.5) and 0.5 ml of 0.115M P-nitrophenyl phosphate were added to 0.5 g of soil sample and incubated at 37°C for 90 min. Cooling to 2°C for 15 min stopped the reaction. Then 0.5 ml of 0.5M $CaCl_2$ and 2 ml of 0.5M NaOH were added and the mixture was centrifuged to 4000 rpm for 5 min. P-nitrophenol (PNP) was determined in a spectrophotometer at 398 nm (TABATABAI & BERMNER 1969). Controls were made in the same way, although the substrate was added before the $CaCl_2$ and NaOH. The activity is expressed as μmol of PNP/g dry soil/h. ***β-glucosidase activity:*** 2 ml of 0.1M maleate buffer (pH 6.5) and 0.5 ml of 50mM P-nitrophenyl-β-D-glucopyranoside (PNG) were added to 0.5 g of soil sample. The rest of the method was the same as for phosphatase activity (MASCIANDARO *et al*. 1994). The activity is expressed as μmol of PNP/g dry soil/h.

Statistical analysis:

The experiments were layout in a complete randomized block design in triplicates. ANOVA was used to determine the statistical differences among treatments and LSD at $P \leq 0.05$ was calculated.

Results

Among soil microorganisms, Bacteria are particularly suitable for quantification by counting. It should be considered, however, that bacteria are never uniformly distributed in the soil and that their spatial arrangement varies even in neighbouring microsites. Furthermore, the bacterial biomass may fluctuate drastically within short periods. As culture techniques record only viable bacteria that are able to proliferate on nutrient media, the term “viable counts” is

often used to distinguish results from "total counts" derived from microscope examination. With the plate count technique it cannot be excluded that a colony originates from more than one individual cell. For this reason, results are expressed nowadays correctly as the number of "colony forming units" (cfu). Table (1) shows the percentage of the population densities of *P. fluorescens* and *P. putida* among the bacterial population in the rhizosphere of white clover. Meanwhile the populations of the two *Pseudomonas* species estimated as 40.1 and 23.8, respectively of the total fluorescent pseudomonads that occupied about 46.3% of total Gram negative rod shaped bacteria.

Table 1. Percentage of rhizobacterial population in white clover rhizosphere

Rhizobacterial Population	Percentage
Gram negative	79.9
Rod shape	87.2
Fluorescent pseudomonads	46.3
Pseudomonas fluorescens	40.1
Pseudomonas putida	23.8
Gram positive	20.1

Moreover, the effect of NaCl added to the rhizosphere can also changes the population of fluorescent pseudomonads (Table 2). It was found that by increasing the level of NaCl in the rhizosphere the number of these fluorescent bacteria increased significantly up to 0.4% NaCl. The most abundant population was found at 0.2% which was 3.9 x 10^4 cfu/g soil. Also, the concentration 0.8% did not inhibit the abundance of these bacteria, but it was not significant comparing with the control soil.

Table 2. Changes of the count number of fluorescent pseudomonads due to salinity in the rhizosphere of white clover

NaCl dose (%)	CFU X 10^4 of fluorescent pseudomonads
0.0	1.9
0.1	2.8*****
0.2	3.9*****
0.4	3.6*****
0.8	2.1
LSD	**0.25**

The number labelled with (*****) within the row is significant difference with control at $P \leq 0.05$.

The detection of the enzymatic activities in the rhizosphere is shown in the Table (3). Dehydrogenase indicates the metabolic activity of rhizosphere microorganisms and catalase refers to the number of aerobic microorganisms in the rhizosphere of salinized soil. Table (3) gives a comparison between the activities of enzymes in the rhizosphere. The activities of dehydrogenase and catalase were positively affected even when the concentration of NaCl reached 0.4%.

Table 3. Effect of salinity on enzymatic activities in rhizosphere of white clover (*Trifolium repens* L)

ENZYMES		Enzymatic activities at different NaCl doses (%)[a]					
		0.0	0.1	0.2	0.4	0.8	**LSD**
Dehydrogenase	(µg INTF/g soil)	147	159	183*****	167*****	121*****	**14.6**
Catalase	(µmol O_2/min/g soil)	1.9	2.3*****	2.7*****	2.6*****	1.8	**0.25**
Protease	(µmol NH_3/g soil/h)	2.3	2.7*****	3.1*****	2.4	2.1	**0.25**
Urease	(µmol NH_3/g soil/h)	2.1	2.5*****	3.3*****	2.9*****	1.8	**0.38**
Phosphatase	(µmol PNP/g soil/h)	144	113*****	97*****	84*****	61*****	**19.5**
β-glucosidase	(µmol PNP/g soil/h)	219	192*****	181*****	173*****	151*****	**15.7**

The number labelled with (*****) within the row is significant difference with control at $P \leq 0.05$.
(a) Values are means of three randomly chosen replicates.

Thus, an increased in the enzymatic activities were observed at higher NaCl concentrations (0.2 - 0.4%) with a maximum reaction at 0.2% NaCl. In these cases, the increased salinity of the plant rhizosphere of salinized soil did not have a negative effect on the metabolic activity of the microorganisms. At 0.8% NaCl, the activity of both enzymes decreased compared with control. According to the results, oxidoreductases are affected differently by various levels of salinity in the soil, indicating that there must be different physiological responses of the microorganisms to the stress created by NaCl addition. Data in Table (3) illustrates the activities of protease and urease in the rhizosphere. Hydrolyses related to the nitrogen cycle (protease and urease) were determined as well as with the phosphorus cycle (P-ase) and with the carbon cycle (β-glucosidase). Maximum activities of protease and urease in the rhizosphere were found at 0.2% NaCl. Also the activities of these enzymes were higher than controls at 0.4% and slightly inhibited at 0.8% NaCl. Also, result shows a comparison between the activities of phosphatase and β-glucosidase in the rhizosphere. It was found that the activity of the phosphatase was decreased by the increasing of NaCl doses applied to the soil more than the activity of β-glucosidase at the same level of NaCl. The decreases in the activities were significant at $P \leq 0.05$ at all concentrations.

Discussion

MATSUGUCHI & SAKAI (1995) investigated the effect of soil salinity with intensive cultivation on microbial communities in the soil-root system in a growth chamber experiment. Microbial populations, including fluorescent pseudomonads, in 3 sites of soil-root system, namely root-free soil, rhizosphere soil and rhizoplane, of 3-week-old spinach plants were compared in relation to the levels of soil salinity. In the root-free soil, populations Gram-negative bacteria (GN) did not change significantly with salinity, while the populations of total fluorescent pseudomonads (FP) apparently increased. These results are in an agreement with our results. In the rhizosphere soil, however, soil salinity induced changes in the populations depending on the microbial groups; GN population increased while the FP population was not effected. We are agreed with the result of Gram-negative but not with the population of fluorescent pseudomonads. It is concluded that soil salinity markedly modified the populations of FP in the soil-root system, such that the populations of the *P. putida* group became dominant in the

rhizosphere and soil-root system that was not subjected to salinity. Those findings support our results shown in the Tables (1). In the case of the fluorescent pseudomonads the percentages was 46.3% in the rhizosphere.

The determination of soil biological activity by tested enzymes showed that it is low at 0.8% NaCl. The stimulating effect of NaCl on the activities of some enzymes was evident at all concentrations up to 0.4%. The activities of phosphatase and β-glucosidase were more negatively affected by salinity than dehydrogenase, catalase, urease and protease. GARCÍA & HERNÁNDEZ (1996) found that irrigation of agricultural land with saline waters could lead to soil degradation. FRANKENBERGER & BINGHAM (1982) have pointed out the inhibition of different enzymatic activities caused by soil salinity. DASH & PANDA (2001) established that NaCl salt stress induced changes in the growth and enzyme activities in blackgram (*Phaseolus mungo*) seeds. Our results up to some extend are in agreement with the above mentioned results. It is concluded that salinity had negative affects on biological features of the soil rhizosphere studied at higher concentrations. This negative effect is greater on hydrolases (phosphatase and β-glucosidase) than on oxidoreductases. This means that the microbial activity of the rhizosphere is harmed by the continuous and high degree of soil salinization.

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