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Relation between soil microbial activity and the effect of seed inoculation with the rhizopseudomonad strain 7NSK2 on plant growth

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Summary. The relationship between the microbial activity in the soil and the effect of seed inoculation with the rhizopseudomonad strain 7NSK2 was evaluated in a series of pot experiments under greenhouse conditions. The microbial activity in plain soil, as measured by the respiratory activity, was significantly increased by the growth of the plants. Both the respiration rate of the microorganisms and the density of the bacteria and fungi in the bulk soil increased with increasing duration of the plant growth. Upon repeated short-term growth of plants on the same soil, a similar stimulation was noticed.

The effect of seed inoculation on the growth of the maize cultivar Beaupré and the barley cultivar Iban was most pronounced in the microbiologically more active soils. The results suggest that the increase of the plant growth by seed inoculation is probably due to the inhibition of deleterious root microorganisms.

Key words: Rhizopseudomonads – Seed inoculation – Microbial activity – *Zea mays* – *Hordeum vulgare*

The growth and the morphology of plants, especially of the plant root system, are considerably influenced by microorganisms present in the rhizosphere of plants (Rovira 1972; Lynch and Clark 1984). The stimulatory effect of rhizosphere bacteria from the genus *Pseudomonas* on growth of plants has been demonstrated for many crops, such as sugar beet (Suslow and Schroth 1982a), wheat (Elliott and Lynch 1985), rice (Kundu and Gaur 1984) and rough lemon (Gardner et al. 1984). The poor knowledge of the overall ecology of the interaction between rhizopseudomonads and the plant host is at the moment the main drawback for the practical application of seed bacterization and the widespread agricultural and commercial development.

This study was set up to evaluate the influence of the soil microbial density and activity on the beneficial effect of seed inoculation with a rhizopseudomonad strain. The microbial activity in the soil was stimulated through growing plants (barley). The soil, pretreated in this way, was subsequently used in seed bacterization experiments.

Materials and methods

Pretreatments of the soil. Tiegem sandy loam soil (pH 6.5, organic C 1.7%) was used in this experiment. Top soil (0–20 cm depth) was collected from the experimental plots of Clovis Matton N.V., Tiegem, Belgium, at the beginning of February 1984. No plants were growing on the site of sampling. The soil was subsequently air-dried to a water content of about 18% on a dry weight basis. Twelve kilograms of soil were placed into a plastic container after being sieved through a 2-mm sieve. The stored containers were covered with polyethylene plastic sheets. Different levels of microbial activity in the soils were obtained by growing barley plants according to the scheme presented in Table 1.

The soil samples were amended with Long Ashton nutrient solution (Hewitt 1952). Five seeds of the barley cultivar Iban were sown in the soil. The plants were watered with demineralized water. Pretreatments were programmed in such a way that all the pretreated soils became available at the same time.

Microbial density on the roots of the plants used for the pretreatment of the soil. To determine the number of total bacteria and fungi on the roots of plants coming out of the soil pretreatment program, the roots were collected, freed from the adhering soil

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Table 1. Soil pretreatment scheme: stimulating microbial activity in the soil in various ways through the root exudates of the barley cultivar Iban

Pretreatment of the soil	Description
I. Long-term growth	
A	Control, soil stored without growing plants in it
B	Barley plants harvested after 1 month of growth
C	Barley plants harvested after 2 months of growth
D	Barley plants harvested after 3 months of growth
E	Barley plants harvested after 4 months of growth
II. Short-term growth	
F	Control, soil stored without growing plants in it
G	Plants grown for 1 month
H	Plants grown for 1 month 2 consecutive times
I	Plants grown for 1 month 3 consecutive times
J	Plants grown for 1 month 4 consecutive times

particles and washed carefully with running tap water until the recognizable soil particles were removed. Subsequently, the roots were washed with sterile demineralized water. The cleaned roots were chopped into small pieces and placed in a 250-ml Erlenmeyer flask containing 50 ml sterile physiological solution (8.5 g NaCl/l). Five grams wet weight of root pieces were ground with a Polytron ultramixer. The water content of the root pieces was determined. The suspension thus obtained was used to make a ten-fold dilution series. Two dilution series from each sample were prepared and the appropriate dilutions of each dilution series were plated out in duplicate on nutrient agar and Martin agar. The Petri dishes were incubated at 28° C for 3 days.

Microbial activity in the bulk pretreated soil. Soil respiration was determined according to the jar method. Two hundred grams of moist soil was placed in a 1-l jar. Two small beakers, one containing 5.0 ml of 0.2 *N* KOH (freshly prepared) and the other containing 20 ml water to counteract desiccation, were put into the jar. The jars were closed airtight and incubated at 28°C for 7 days. At the end of the incubation period, the amount of CO_2 produced was determined titrimetrically. Each treatment had three replicates. For controls, jars without soil were used.

Source of the rhizospeudomonad strain 7NSK2. The strain 7NSK2 was isolated from a hydroponic culture (Long Ashton nutrient solution) of the barley cultivar Iban in which Melle soil (0.1%) (sandy loam, pH 5.1) served as source of microorganisms. The nutrient solution contained 40 μ*M* bathophenantrolinedisulphonic acid disodium salt hydrate. The strain 7NSK2 was isolated on modified King B medium containing (grams per liter): proteose peptone 5; K_2HPO_4, 1.2; $MgSO_4 \cdot 7H_2O$, 1.5; and glycerol, 4 ml (pH adjusted to 7.2).

Seed inoculation experiments on the pretreated soils. Twenty milliliters of a 5 times concentrated Long Ashton nutrient solution was added to pots containing 600 g moist pretreated soil.

One hundred milliliters of a 3-day-old liquid culture of the rhizopseudomonad strain 7NSK2 was centrifuged at 10 000 g. The bacterial cells were washed with sterile 0.1 *M* $MgSO_4 \cdot 7H_2O$, then resuspended in 20 ml of the same solution. Twenty milliliters of sterile 2% methylcellulose was added to the resuspended bacterial cells and subsequently mixed until a homogeneous bacterial suspension was obtained. The bacterial suspension thus obtained was divided into two parts. The first part was autoclaved at 120°C for 20 min and used to treat the seeds of the control treatments. The second part was used to treat the seeds with viable cells. Four milliliters of the bacterial suspensions was used to treat 40 maize seeds or 50 barley seeds.

The seeds of the maize (*Zea mays*) cultivar Beaupré and the barley (*Hordeum vulgare*) cultivar Iban were used in this experiment. The seeds were first surface sterilized with 0.1% $HgCl_2$ and then washed with sterile demineralized water and inoculated as described previously. The numbers of bacterial cells per seed of maize and barley were 5×10^6 and 6×10^5 CFUs respectively. Three seeds of maize or five seeds of barley were drilled per pot. Each treatment had five replicates. The experiments were not replicated over time. The number of plants per pot was reduced to two for maize and to four to barley plants. The intensity of the light used was 120 $\mu E/m^2/s$ with a 16-h irradiance time per day. The temperature was 25°C during the day and 18°–20°C during the night. The plants were grown for 3 weeks. Harvesting was done by cutting the plants, followed by drying at 105°C for 24 h. The dry weight of the plants was determined.

Results

The microbial density on the roots of the barley coming out of the pretreated soils and the soil respiration are presented in Table 2. The data from the long-term growth pretreatments summarize the changes in the numbers of microorganisms in the different growth stages of the plants. The number of total bacteria and fungi increased up to 3 months, and then decreased. The density of total bacteria and of fungi for 1-month-old plants (B) was significantly lower than those for the older plants (C, D and E). In the case where the barley plants were repeatedly grown for periods of 1 month (pretreatments H, I and J), the number of total bacteria and fungi was significantly higher than after a single period of 1 month (G). The total bacterial count on the roots of barley plants from pretreatment H, I or J was at least 3 times higher than in pretreatment G. Microbial activity, as could be expected, was lowest in the soils without plants (controls A and F). The effect of plants on the microbial activity increased when the plants had been grown longer, up to a period of 3 months (D). From pretreatment D, it appears that after 3 months of growth barley is very active in releasing root exudates to the soil. When barley had been growing for 4 months under greenhouse conditions, it matured. The leaves became yellow and dry. It is likely that the exudation was less and that the microbial activity decreased. The microbial activity in the soil with matured barley (pretreatment E) was lower

Table 2. Soil respiration and microbial density on the roots of the barley plants according to pretreatment of the soil

Pretreatment of the soil	Total bacteria[a]	Fungi[b]	Respiration[c]
I. Long-term growth			
A	–	–	1.669
B	38	9	1.973
C	86	65	2.954
D	890	610	3.490
E	79	231	2.504
$LSD_{0.05}$	39	51	0.245
II. Short-term growth			
F	–	–	1.669
G	43	9	1.973
H	179	217	2.932
I	130	390	2.732
J	230	156	2.653
$LSD_{0.05}$	35	49	0.230

Values are the averages of four replicates; –, no sample

[a] 10^7 CFUs/g root dry weight
[b] 10^4 CFUs/g root dry weight
[c] mg CO_2–C/kg soil/day

Table 3. Effect of seed inoculation with the rhizopseudomonad strain 7NSK2 on growth of maize cultivar Beaupré, grown in soils with different levels of microbial density and activity

Pretreatment of the soil	Plant growth (g/pot)		Increase
	Control	Inoculated	(%)
I. Long-term growth			
A	1.351	1.380	2.1
B	1.342	1.491	11.1*
C	1.195	1.341	12.2**
D	1.292	1.480	14.6**
E	1.093	1.198	9.4
$LSD_{0.05}$	0.130	0.115	
II. Short-term growth			
F	1.351	1.380	2.1
G	1.342	1.491	11.1*
H	1.369	1.532	11.9*
I	1.262	1.414	12.0**
J	1.117	1.296	16.3**
$LSD_{0.05}$	0.090	0.125	

Values are the average of five replicates
* significantly different at the 10% level; **significantly different at the 5% level

than in pretreatment D (3 months), but it was still 50% above that of the control (A). The microbial activities as measured by soil respiration in the soils planted two (H), three (I) and four (J) consecutive times with barley were not different from each other, but were all about 60% above the level of the control (F).

Table 4. Effect of seed inoculation with rhizopseudomonad strain 7NSK2 on growth of barley cultivar Iban, grown in soils with different levels of microbial density and activity

Pretreatment of the soil	Plant growth (g/pot)		Increase
	Control	Inoculated	(%)
I. Long-term growth			
A	0.437	0.459	5.0
B	0.394	0.414	5.1
C	0.360	0.410	16.9*
D	0.350	0.410	18.0**
E	0.335	0.389	16.0*
$LSD_{0.05}$	0.039	0.041	
II. Short-term growth			
F	0.437	0.459	5.0
G	0.394	0.414	5.1
H	0.370	0.418	13.0
I	0.340	0.409	20.3*
J	0.343	0.393	14.9**
$LSD_{0.05}$	0.042	0.045	

Values are the averages of five replicates.
*significantly different at the 10% level; **significantly different at the 5% level

The effect of seed inoculation with the rhizopseudomonad strain 7NSK2 on the growth of maize grown in soils with different levels of microbial activity is presented in Table 3. The results of the same experiment with barley are presented in Table 4. In pretreatment A (control), inoculation with the rhizopseudomonad strain 7NSK2 resulted in an increase of plant dry weight (2.1% for maize and 5.0% for barley), but these increases were not statistically significant (Tables 3, 4). The beneficial effect of the inoculant on plant dry weight was more pronounced in the more active soils and was most striking in treatment D. In this pretreated soil, with the highest microbial density and activity, increases in plant dry weight of maize and barley were 14.6% and 18.0%, respectively. A significant increase in plant growth was also observed in treatments C, H, K and J.

The effect of seed inoculation on the growth of the maize cultivar Beaupré was statistically more pronounced than the effect on the growth of barley cultivar Iban. This was at least in part due to the fact that the experimental error in the experiment with barley was higher than in the experiment with maize.

Discussion

Using growing plants to increase the microbial activity in the soil offers the important advantage that the microbial activity is stimulated by a group of com-

pounds which are actually excreted under field conditions. However, it should be noted that many biotic and nonbiotic factors affect the quantity and quality of root exudates released, including the age of the plant (Hale and Moore 1979; Kraffczyk et al. 1984; Beck and Gilmour 1983). Furthermore, microorganisms have been reported to produce substances that increase the permeability of cell membranes (Norman 1955; Martin 1958), so they may stimulate exudation. The pretreatments used are limited in time and soil volume and can therefore only be considered as approximations of actual field situations.

In general, the number of bacteria and fungi on the roots of plants increased with increasing plant age. The highest numbers of bacteria and fungi were found on the roots of the 3-month-old barley plants (pretreatment D, Table 1). This is probably due to the fact that plants grew as far as possible in their vegetative stage (3 months in the greenhouse conditions) and the amount of organic compounds released increased concomitantly. It is also likely that at the age of 3 months barley produces not only the highest amount of root exudates, but also a wide range of organic compounds. When the plants grew older than 3 months, their photosynthetic activity decreased. Related to that, microbial activity decreased, probably because of a decline in the amount of organic compounds released.

The change in the numbers of bacteria and fungi on the roots of the plants was more pronounced for the different stages of growth than for repeated periods of growth in the same soil. In the pretreatments where the plants had been grown consecutively 2 (H), 3 (I), and 4 (J) times for a period of 1 month, the organic compounds were released discontinuously and the total amount was probably less. In this case there was a period without supply of organic compounds from the roots. Obviously, this must have affected the microbial activity.

It is well established that the roots of cereals, particularly barley and wheat, are colonized by deleterious bacteria like *Pseudomonas* and *Enterobacter* (Kleeberger et al. 1983). Reduction of the dry weight of sugar beet by a group of deleterious rhizobacteria was reported by Suslow and Schroth (1982b). They reported further that the deleterious rhizobacteria on sugar beet were members of the genera *Enterobacter, Klebsiella, Citrobacter, Flavobacterium, "Achromobacter", Arthrobacter*, and fluorescent *Pseudomonas*. The fluorescent *Pseudomonas* bacteria were identified as being similar to *P. cichorii* and *P. viridiflava*.

The development of deleterious microorganisms most probably lies at the basis of the yield depressions in high-frequency potato cropping as reported by Geels and Schippers (1983a, b). It is known that the development of deleterious bacteria in the rhizosphere can be counteracted by seed inoculation with certain fluorescent *Pseudomonas* spp. In the currently described experiment the plant yield in the pretreated soils (control treatments in Table 4) was declining in function of the amount of precropping cycles with barley. Seed inoculation with 7NSK2 counteracted this effect, resulting in a rise of the beneficial effect of the inoculant. These results fit the hypothesis that a narrow crop rotation can stimulate the development of deleterious microorganisms and that this effect can be neutralized by the inoculation with a proper beneficial rhizopseudomonad. However, the same experiment also shows that other factors are involved. In the soil in which barley was precropped up to the age of 3 months (pretreatment D), seed inoculation with rhizopseudomonad strain 7NSK2 also resulted in an increase of plant growth of 18% (Table 4). In this case, the development of deleterious microorganisms through a narrow crop rotation cannot be the underlying mechanism. In this experiment with "long-term growth of barley" as pretreatment, there was a linear correlation between the overall soil respiration and the increase in plant growth due to inoculation with the strain 7NSK2 (Table 5). As summarized in Table 5, this was also the case in the other experiments. So the development of microorganisms in the soils which are not directly linked to the practice of narrow crop rotation may as well be responsible for a deleterious effect on plant growth. This hypothesis finds additional evidence in the inoculation experiments with maize. In these experiments, plant growth stimulation shows results similar to the experiments with barley. However, in both experiments, the soil was pretreated with barley in the same way. The mere stimulation of the overall microbial activity seems to be responsible for a decrease in the plant growth. Inoculation with 7NSK2 could counteract the effect. The present findings do not, however, exclude the possibility that the development of deleterious microorganisms grown on the exudates of barley are deleterious to maize as well, which should mean that they are aspecific.

It must be acknowledged that in the pot experiments, due to the confinement of the roots, the

Table 5. Correlation coefficient (r) between soil respiration and increase in plant growth as an effect of seed inoculation with the strain 7NSK2

Soil microbial activity	% Increase in plant growth			
	Long-term growth		Short-term growth	
	Maize	Barley	Maize	Barley
Soil respiration	0.810*	0.914*	0.806*	0.899*

*Significantly correlated at the 0.05 level; $n = 10$

amount of root biomass per unit of soil is much higher than under field conditions. Consequently, under field conditions the chances of development of plant deleterious microorganisms are probably lower, thus reducing the possibility of revealing the beneficial effect of the strain. In addition, the migration of the cells through the soil is less problematic in the pot experiments than in the field. Therefore, the results obtained do not permit extrapolation to practice as yet.

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