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The effect of storage and activation respectively on plant growth promoting rhizobacteria pseudomonas sp.
Effet de la conservation et de l'activation sur la rhizobactérie pseudomonas amélioratrice de la croissance des plantes

AGYEKUM Sammy Foh

Soil Research Institute, Academy Post Office, Kwadaso-Kumasi, Ghana.

INTRODUCTION

Hofte *et al*. (1990) have reported that *Pseudomonas aeruginosa* strain 7NSK2 and *P. fluorescens* strain ANP15 are natural root isolates with plant growth-promoting capacities. Earlier, Iswandi *et al*. (1987) have also noticed that the effects of these strains on plant growth are especially evident in soils in which high microbial activities decrease plant growth. Further work has shown that strain 7NSK2 promote the growth of various cereals (maize, wheat and barley) and vegetables (spinach, cornsalad and cucumber) (Hofte *et al*., 1991). The beneficial effect of the ANP15 strain has been demonstrated on barley (Iswandi *et al*., 1978d), maize and pea (Hofte *et al*., 1990).

Since the inception of research on strains 7NSK2 and ANP15 at the Laboratory of Microbial Ecology, University of Gent, some of them have been maintained frozen at - 70 ^{o}C whilst others are being used for routine work (Dr. Boelens, personal communication). It was considered worthwhile, therefore, to assess the effect of storage on these plant-growth promoting pseudomonas and, with that in view, the present investigation was initiated to evaluate whether it is good to store strains before they are used or whether storage has adverse effects on the strains.

In a later development, it was noted that certain detergents may prove to be effective selected substrate since they satisfy many of the criteria for selection as put forward by Lajoie *et al*. (1991). So a second experiment was also undertaken to determine the effectiveness of the selective substrate (detergents) as compared to no substrate amendment, for creating a niche for the strains 7NSK2 and ANP15 in a soil environment.

MATERIALS AND METHODS

Effect of storage on plant growth promoting Pseudomonas

Bacterial strains: *Pseudomonas aeruginosa* strain 7NSK2, a plant growth promoting bacterium (Hofte *et al*, 1991), was isolated from barley roots (Iswandi *et al*, 1987a). *P. fluorescens* strain ANP15, a plant growth promoting bacterium (Iswandi *et al*, 1987d) was also isolated from barley roots. Both isolates that are used routinely (R) and those that have been maintained frozen (F) were used in this experiment.

Soil Incubation: Ardoyen soil (sandy; pH 6.5; org-C: 1.7%) was used for the first experiment. The soil was sieved through a 2-mm sieve. Recognizable plant debris and gravel were removed from the soil. The water content of the composite soil was determined with an infrared Sartorius balance (Sartorius GMBH Gottingen, Type 7393A). The soil was then divided into two parts and the microbial activity in one part of the soil was stimulated by adding sugars (glucose and galactose) equivalent to 660 mg C/kg soil. The C/N ratio of this amendment was adjusted to 10 by adding NH_4NO_3. In the other part of the soil there was no addition of sugars or NH_4NO_3, the control. These soils were put separately in two plastic bags. The incubation took place at 28 C for four weeks. The soils were mixed weekly.

Preparation of dry inoculum

The bacterial strains, as previously described, were used in the preparation of the dry inoculum. Each dry inoculum was prepared as follows: four hours prior to centrifugation at 10000 x g (10 minutes), 1 g/L glutamic acid was added to 600 ml of densely grown FP culture (Shaken at 165 rpm, 2 days) grown in the MKB medium. [1.5g/L, $MgS0_4.7H_20$; 1.2g/L, K_2HP0_4; 2m/L, glycerol; 5.g/l, proteose peptone No..3 (Difco); pH adjusted to 7.2]. After centrifugation, cells were washed in 75 ml 0.1 M $MgSO_4.7H_20$ + 0.01% peptone and 7.5 ml of a 2% CMC (carboxyl methyl cellulose) solution. The suspension was mixed with 50 g sterile talc, dried overnight in a laminar flow and ground until a fine powder was obtained that could be stored at 2-4 ^oC. For the control, the preparation of dry inoculum was exactly the same as described above except that no bacterial cells were added.

Enumeration of bacteria and fungi: The total bacterial count, number of pseudomonas (on MKB), and number of fungi (on Martin agar) [5.0g/L, peptone; 10.0g/L glucose; 1.0g/L KH_2 PO_4; 100.0ml/L, Bengalic rose; 20.0g/l Agar (Bacto); pH adjusted to 5.5] in soil were determined. To obtain a 10^{-1} dilution, 10 g soil was placed in a 250 ml Erlenmeyer flask containing 90 ml sterile physiological solution (8.5 g NaC1/1).

Plant experiment: After an incubation period of 4 weeks, the soil pretreated with sugars and NH_4NO_3 and the control soil, each ca. 500 g, was put into a plastic pot (diameter: 11 cm; height 7.5. cm). Two grams of the appropriate dry inoculum was mixed thoroughly and carefully with the soil to avoid contamination from other treatments. Twenty ml of a 5-time concentrated Long Ashton nutrient solution (Hewitt, 1966) was added into each pot. Then the soil was brought to a water content of 3/4 field capacity (18% on dry weight basis). The plant used in this experiment was maize (*Zea mays*) cultivar Issa (N.V. Clovis Matton, Belgium).

The seeds were sorted to obtain the same size of seeds. They were surface sterilized with 150 ml of Na0C1 (bleaching liquor) for two minutes, washed several times with sterile demineralized water and dried with a sterile blotting paper in the laminar flow. The surface sterilized seeds were planted into each pot. Five seeds were sown in each pot. Each treatment had five replicates.

After emergence, the number of plants per pot was reduced to four. The plants were watered daily with demineralized water. The plants were grown in a growth chamber with an irradiation energy of about 120 $\mu E\ m^{-2} S^{-1}$. The irradiance time was 16 hours a day. The temperatures during the day and the night were 24 ^oC and 14 ^oC respectively. The relative humidity was 85% during the day and 80% during the night. The plants were harvested after 21 days of growth. The dry weight of the plants was determined (oven: 105 ^oC, 24 hours). Data were analyzed using ANOVA and means were separated using Duncan's new multiple-range test.

Root colonization (after harvesting of plants): After harvesting, three replicates of the roots from the soil were collected. The roots were washed three times with 250 ml of tap water until no recognizable soil particles were left on the roots. For the final wash, sterile demineralized water was used. The clean roots were cut into small pieces with a sterile pair of scissors.

The root pieces were divided into two parts in order to determine the number of FP colonizing the roots or maize. The first part was used to determine the water content of the root sample. The second part of ca. 1 g was weighed and put in an acid washed porcelain mortar. It was ground by using a Polytron ultramixer. The ground roots was put into a 9 ml physiological solution (8.5 g NaC1/L) and a 10-fold dilution series was subsequently prepared. Total bacteria was plated on MKB medium and incubated at about 4 ^oC. The whole work was done aseptically in the laminar flow chamber.

Effect of the PGPR on the growth of maize in soil treated with detergents as carbon sources.

Bacterial strains: The stored two plant growth-promoting strains, ANP15 and 7NSK2, were used throughout this second experiment.

Detergents: Two anionic and two nonionic detergents were utilized as carbon sources. The abbreviations used in this experiment for these detergents are shown in Table 1 below:

Training of the strains: The bacteria, ANP15 and 7NSK2, were trained in order to grow in some of the detergents. The mineral medium used was MM9.

In a complete MM9 medium (7.52 g/L, $Na_2HPO_4.2H_20$; 3.0 g/L, $KH_2\ PO_4$; 0.5 g/L, NaCl; 2.0ml/L, 1M Mg $SO_4.7H_20$; 1.0ml/L, 1M $CaCl_2$; 4.0 ml/L, 25% NH_4Cl; 10.0ml/L, 20% Glucose 20%), glucose serves as carbon source. In this experiment, instead of glucose, the detergents were used as carbon sources. For instance, 7NSK2 was trained to grow in MM9 medium in which the carbon source was Co-720. The strain was allowed to grow in an initial detergent concentration of 2.0g/L for one week. After one week 1 ml of the old culture was transferred into a fresh MM9 medium where the detergent concentration was 1.0 g/L. Subsequent transfers of 1ml old cultures were made to fresh MM9 medium but the detergent concentration was reduced to 0.5 g/L during the third week. Below is a summary of subsequent transfers of old cultures to fresh MM9 medium:

Week	Detergent concentration	Volume of old culture transferred
1	2.0 g/L	-
2	1.0 g/L	1ml
3-4	0.5 g/L	1ml
5-6	0.5 g/L	1ml

Treatments: There were eight 100-ml flasks each containing "MM9" medium. 0.5ml of detergents were put into the flasks containing the MM9 medium. Each flask was inoculated with 1ml of either ANP15 or 7NSK2. In total, there were eight treatments from the above combination.

Next, 0.5ml detergent was placed in four flasks containing MM9 medium. These flasks were not inoculated with the strains and they therefore served as controls for each of the four detergents, i.e Tw 80, Co 720, LSC and DSS.

Lastly, a control consisting of only MM9 medium (no bacteria and no detergent) was set up for all the treatments. This was labelled as control. These thirteen treatments were put into the shaking room for shaking and incubation for one week prior to the pot experiments.

Pot experiment: The number of replications was four and therefore there were fifty two pots. Each pot was filled with 500g of soil. Prior to sowing, 20 ml of each treatment was mixed thoroughly with the soil in the pots. The rest of this pot experiment was exactly like the first pot experiment as previously described.

RESULTS

The number of total bacteria and fungi in the soil after pretreatment of the soil is presented in Table 2. As can be seen from the Table, in the sugar treated soil the number of total bacteria increased from 0 week to the 4th week and it was at least one log unit higher than the number of total bacteria in the control soil. Again, Table 2 shows that the number of fungi also increased from 0 week to the 4th week and with the exception of the 0 week, this increase was at least one log unit higher than that of the control soil.

The beneficial effect of the rhizopseudomonad strains, 7NSK2 and ANP15, on the growth of the maize cultivar Issa as influenced by storage under frozen condition at -70 ^{o}C (F) or routinely transfers on solid media (MKB) every two weeks kept at 2 - 4 ^{o}C (R) is presented in Table 3. The increases in plant dry weight (%) as an effect of storage have also been presented in Table 3. The addition of both isolates to the soil resulted in significant maize shoot dry matter yields. In the control soil (Table 3) the strains, 7NSK2 (R) and 7NSK2 (F) had no significant effect on the shoot dry weight of the maize, except ANP15 (F). The significant increase on plant growth with the ANP15 (F) strain was 17.2%. However, the means of all the strains were not significantly different at P=0.05. Numerically, the percent increase over the control treatment has been graded as follows:

ANP15 (F) > ANP15 (R)
7NSK2 (F) > 7NSK2 (R)

In the sugar treated soil, all the strains had significant effect on the growth of maize (Table 4), except 7NSK2 (R). The strain with the highest percent increase (20.7%) was ANP15 (F) and the percent increase over the control has also been graded as follows:

ANP15 (F) > ANP15 (R)
7NSK2 (F) > 7NSK2 (R)

The root colonization of the rhizopseudomonad strains ANP15 and 7NSK2 under storage condition is presented in Table 3 and Table 4. The root colonization of all the strains, with the exception of ANP15 (R), was not significantly different at the 5% level of probability.

In the second experiment, prior to the pot experiment, the two plant growth promoting Pseudomonas spp., ANP15 and 7NSK2, were trained to grow at the expense of two of the detergents, Igepal Co-720 and Dioctyl sulfosuccinate. The result of this experiment is shown in Table 5. The two pseudomonas strains both grew well in both detergents as evidenced by an increase in turbidity (by visual observation) in the detergent enrichment cultures and, more importantly, by the enumeration of total bacteria (Table 5). The Table shows that the growth of the strain 7NSK2 in Igepal Co-720 was the highest.

The beneficial effect of the strains, ANP15 and 7NSK2, on the growth of the maize cultivar Issa as influenced by the detergents is presented in Table 6. The increases in plant dry weight (%) as an effect of the strains and the detergents are also shown in Table 6. There was significant difference among the treatment means at the 1% level of probability. The experiment thus provides evidence of real differences among treatment means.

Table 7 shows the comparative performance of the strains with the detergents in increasing the shoot dry weight of the maize plants. The strain and detergent combination which gave the highest percent increase over control was DSS + 7NSK2 (20.9%). ANP15 also performed well in DSS (16.8% increase). However, Tween 80 was poor as carbon source for the strain ANP15. ANP15 in Tween 80 could not increase the shoot dry weight of the maize plants. Table 7 can also be considered as an overview of the influence of the strains in the various detergents on the maize plants. The beneficial effect of the two strains in the detergents on the maize ranges from 6.9% to 20.9%. The root colonization of the strains ANP15 and 7NSK2 in the various detergents is shown in Table 6. Although differences exist among treatment means for total bacteria, pseudomonas and fungi, they were not found to be significant at the 5% level of probability.

DISCUSSION

This work demonstrates that the plant growth-promoting pseudomonas strains 7NSK2 and ANP15 are able to increase the dry weight of maize. This is in correspondence with earlier work with these strains (Iswandi *et al*, 1987d; Hofte *et al*, 1991). In the present study, the effects of the strains on the growth of the maize plants were especially evident in the pretreated soil. This was not unexpected since the microbial activity in this soil was highly activated by adding readily available organic matter like sugars and by adjusting the C/N ratio to 10 with NH_4NO_3.

A relationship is evident between the storage under deep freezing condition and the utility of the strains ANP15 and 7NSK2 as plant growth promoters (Table 3 and Table 4). The strains that have been kept under storage at -70 ^{o}C performed better than the ones that are used routinely. Earlier, Chanway and Nelson (1991), working on tissue culture bioassay for plant growth promoting rhizobacteria, maintained their isolates frozen at -80 ^{o}C. They reported that inoculation with one of their two isolates, Pseudomonas strain G 11-32, significantly increased the callus biomass. Strain G 2-8, they observed, decreased the callus biomass without vitamins but the effect was not significant in any one single experiment. It is interesting to note that they did not consider storage under frozen condition as having any adverse effects on the performance of the strains they used.

Iswandi *et al*, (1987) noted that the beneficial effect of rhizopseudomonads decreased considerably during storage. they reported that only 3 out of the 9 rhizopseudomonad strains tested maintained their stimulatory effect after 6 months storage. Here, the picture is different from this present experiment in that they obtained their data just after isolation of the strains and, more importantly, the temperature of the incubation was constant at 28 ^oC and the MKB they used in their work was considered as a relatively rich medium. They admitted that these two factors probably enhanced the deterioration of rhizopseudomonads tested. But even then their results corroborate the present study since, interestingly, 2 out of the 3 strains whose stimulatory effect on plant growth were not affected by storage under laboratory conditions were strains 7NSK2 and ANP15.

That the bacterial strains which were maintained frozen did not lose their beneficial effects on plant growth is not unexpected: presumably no genes are lost under frozen condition.

Table 6, shows that both strains, 7NSK2 and ANP15, performed well in some of the detergent-amended soil. Thus the experimental results demonstrate the importance of supplying exogenous substrate for plant growth promoting rhizobacteria. This finding holds the attention because it means that some of these detergents, used as substrate, have the ability to select for the strains ANP15 and 7NSK2 in a competitive environment, indicating that the strains are able to use the detergents as a selective substrate as carbon and energy sources at acceptable rates. In a screening of organism-substrate combinations experiment, Lajoie *et al*, (1991) identified five of the six organisms integral to these organism-substrate combinations as members of the genus *Pseudomonas***.** Strains ANP15 and 7NSK2 are also members of the genus *Pseudomonas*, as has been pointed out throughout this study.

Table 7 shows the percent increase in shoot dry weight of the maize plants due to strains ANP15 and 7NSK2. From the results of the present experiment, it is possible to assess the relative efficiencies of these detergents as soil carbon and energy sources and they can be graded as: DSS+7NSK2, TW-80+7NSK2 and DSS+ANP15 the better combinations, TW-80+ANP15, LSC+7NSK2, LSC+ANP15, Co-720+ANP15 and Co720-7NSK2 being the less efficient of the eight combinations. The poorer performance of most of the combinations might have been due to a number of factors, as previously reviewed (Lajoie *et al*., 1991).

Many researchers, as reported by Iswandi *et al*., (1987), have shown the importance of the colonizing capacity of a strain in relation to plant growth stimulation on sugar (Suslow and Schroth, 1982), on potatoes (Kloepper *et al*., 1980), and on wheat (Weller and Cook, 1983). In the case of fluorescent pseudomonas, Weller (1983) reported a critical number corresponding o about 10^4 CFU/g dry root. In the present study, in all cases of significant stimulation of plant growth, the number of bacterial cells on the roots surpassed 10^6 CFU/g dry root (Tables 3, 4 and 6).

In conclusion, it can be stated that many authors have reported strain degeneration under laboratory conditions and that this phenomenon is encountered in the industrial production of starter cultures, antibiotics and enzymes. The finding that strains do not lose their beneficial effects on plant growth when maintained frozen could be of immense benefit to research and industry. Again, the production of highly competent strains of microorganisms for the introduction of beneficial genetic capabilities into the environment is indispensable to environmental and agricultural biotechnology. Therefore any technology that offers competitive advantage to strains in a highly competitive environment must be given priority. Selective substrate-strain combinations is one of such technology that deserves much attention.

Table 1. Detergent abbreviations, trade names and structure

Detergent.	Abbreviation	Trade name	Structure
Non-ionic	Tw 80	Tween 80	Polyoxyethylene (20) sorbitan monooleate
	Co-720	Igepal Co-720	4-(C_9H_{19}) C_6H_4O ($CH_2CH_20)_{12}H$
Anionic	LSC	N-Lauroylsarcosine	N-Dodecanoyl-N- methylglycine
	DSS	Dioctylsulfo- succinate	Sulfosuccinic acid bis-[2-ethyl-hexyl] ester

Source: Adapted from Lajoie *et al*. (1991)

Table 2. Number of bacteria and fungi after pretreatment of soil with sugar

Treatment (Week)	Control	Sugar treated
	Total Bacteria (Log CFU/g)	
0	6.01 (0.02)	6.54 (0.21)
1	6.51 (0.18)	7.86 (0.29)
2	6.64 (0.01)	7.85 (0.34)
3	6.20 (0.03)	8.29 (0.02)
4	6.30 (0.02)	9.14 (0.05)
	Fungi	
0	4.38 (0.10)	4.51 (0.05)
1	4.39 (0.08)	5.62 (0.13)
2	4.42 (0.05)	5.81 (0.03)
3	4.48 (0.01)	5.87 (0.05)
4	4.58 (0.02)	6.00 (0.02)

Values inside the bracket indicate the standard error

Table 3. Influence of Pseudomonas strains that are kept in storage (F) and those that are used routinely (R) on growth of maize grown in the control soil

Treatment	Shoot dry weight (g/pot)	% Increase	Root colonization (Log CFU/g dry root)	
			TB	FP
Control	1.3175 a	0.0	5.90 a	0.0
ANP15 (R)	1.5145 b	15.0*	7.34 b	6.53
ANP15 (F)	1.5437 b	17.2*	6.20 a	6.08
7NSK2 (R)	1.3891 ab	5.4	5.99 a	5.18
7NSK2 (F)	1.4925 ab	13.3	6.08 a	5.26

Table 4. Influence of Pseudomonas strains that are kept in storage (F) and those that are used routinely (R) on growth of maize grown in the soil pretreated with sugars and NH_4NO_3

Treatment	Shoot dry weight (g/pot)	% Increase	Root colonization (Log CFU/g dry root)	
			TB	FP
Control	1.3307 a	0.0	5.15	5.00
ANP15 (R)	1.5727 b	18.2*	7.11	6.53
ANP15 (F)	1.6068 b	20.7*	6.45	6.28
7NSK2 (R)	1.5158 ab	13.9	7.04	6.32
7NSK2 (F)	1.5943 b	19.8*	6.52	6.45

Note for Table 3 and 4. (R): strains used routinely, (F): strains kept in storage at -70 °C and freshly used, TB: total bacteria, FP: fluorescent pseudomonas. Values are means of five replicates. Within column, numbers followed by the same letter are not significantly different at P = 0.05. (*): significant at P = 0.05

Table 5. The growth of ANP15 and 7NSK2 strains in detergents as carbon sources

Detergent + strain	Total counts (CFU x 10 /ml)
Igepal Co-720 + ANP15	1.53 (0.03)
Igepal Co-720 + 7NSK2	18.20 (0.04)
DSS + ANP15	1.05 (0.04)
DSS + 7NSK2	4.80 (0.02)

For symbols see Materials and Methods.

Table 6. Effect of treatment of soil with strain ANP15 and 7NSK2 grown in detergents on maize

Treatment	Shoot dry weight (g/pot)	% Increase	Root colonization (Log CFU/g dry root)		
			TP	FP	TF
Control	2.2613 ab	0.0	7.01	0.0	4.00
Detergent:					
DSS	2.4119 cd	6.7*	7.11	7.03	4.61
Tw-80	2.1491 a	-5.0	7.10	7.03	4.60
LSC	2.5100 d	11.0*	7.06	7.00	4.57
Co-720	2.3156 bc	2.4	7.12	7.00	4.62
Detergent+PGPR:					
DSS+7NSK2	2.8266 f	25.0*	7.10	7.00	4.60
DSS+ANP15	2.6796 ef	18.5*	7.08	6.98	4.58
Tw-80+7NSK2	2.7136 f	20.0*	7.17	7.07	4.67
Tw-80+ANP15	2.1700 a	-4.0	7.13	7.05	4.63
LSC+7NSK2	2.4750 cd	9.5*	7.11	7.02	4.61
LSC+ANP15	2.5313 de	11.9*	7.08	6.99	4.59
Co-720+7NSK2	2.3795 bc	5.2	7.04	6.96	4.54
Co-720+ANP15	2.4148 cd	6.9*	7.01	6.95	4.52

Values are means of four replicates. Within column, numbers followed by the same letter are not significantly different at P = 0.05. TB: total bacteria, FP: fluorescent pseudomonas, FT: total fungi. (*): significant at P = 0.05.

Table 7. Comparative performance of the strains in the detergents in increasing the shoot dry weight of the maize plants

Treatment	% Increase over control	% Increase due to strains
DSS + 7NSK2	25.0* f	18.3 (7NSK2)
DSS + ANP15	18.5* ef	11.8 (ANP15)
DSS (control)	6.7* cd	-
Tw 80 + 7NSK2	20.0* f	25.0 (7NSK2)
Tw 80 + ANP15	-4.0 a	1.0 (ANP15)
Tw 80 + (control)	-5.0 a	-
LSC + ANP15	11.9* de	0.9 (ANP15)
LSC + 7NSK2	9.5* cd	-1.5 (7NSK2)
LSC + (control)	11.0* d	-
Co-720 + ANP15	6.9* cd	4.5 (ANP15)
Co-720 + 7NSK2	5.2 bc	2.8 (7NSK2)
Co-720 (control)	2.4 bc	-

Within column, numbers followed by the same letter are not significantly different at = 0.05. (*): significant at P = 0.05.

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