

INFLUENCE OF SOIL SALINITY ON MICROBIOME OF FLUORESCENT PSEUDOMONADS AND SIDEROPHORE PRODUCTION IN SUNFLOWER RHIZOSPHERE

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Abstract:

*Soil salinity is one of the most serious agricultural problems. Also it disturbs plant–microbe interaction. The abilities of fluorescent pseudomonads to colonise and survive in the rhizosphere of sunflower grown under the effect of three salinity levels (2.5, 7.5 and 12.5 dS/m) in four different soil types were studied. The results of greenhouse pot experiment indicated that the soil types is the dominating factor responsible for the diversity of the fluorescent pseudomonads populations associated with sunflower roots. In comparison with untreated soil, and soil inoculated by four salt tolerant strains (FPC03, FPW03, W99 and B77) producing siderophore, it was found that under salinity stress, soil inoculation with fluorescent pseudomonads *P. fluorescens* W99 or B77 significantly increased the plant dry weight and P content of sunflower in chernozem meadow soil (Szeged) better than in kovárványos brown forest soil (Nyíregyháza), clay loamy brown forest soil (Gödöllő), and Ramann-type brown forest soil (Keszthely). In vitro, the siderophore production was affected by higher soil salinity. Among the strains, *P. fluorescent* FPW03 exerted the greatest effect on plant growth, and dry weight of sunflower in saline conditions. Therefore, in saline condition, strains of fluorescent pseudomonads can be used for promoting growth and yield of sunflower.*

Keywords:

Fluorescent pseudomonads, siderophore production, soil biotechnology, soil quality, salinity, soil type

1. INTRODUCTION

All soils contain soluble salts with major dissolved inorganic ions of Na^+ , K^+ , Mg^{2+} , Ca^{2+} , Cl^- , SO_4^{2-} , HCO_3^- , and CO_3^{2-} . Soils are considered saline when they contain high levels of soluble salts, which can have negative impacts on soil fertility and crop growth through the reduction in water availability to the plant or toxic effects of individual ions such as H_2BO_3^- and Ba^{2+} under hypersaline conditions. Soil salinity is a measure of the saltiness of the soil. Saline soils constitute a serious problem for crops production by suppressing plant growth (Figure 1). Soil salinity is a stress factor relating to microbial selection process and can reduce bacterial diversity and control microbial abundance, composition and functions. Use of soil halotolerant or halophilic bacterial strains which can either fix atmospheric nitrogen will be environmentally begin approach for nutrient management and ecosystem function for saline soils [1]. For purposes of definition, saline soils are those which have an electrical conductivity of the saturation soil extract of more than 4 dS/m at 25°C [2].

Nearly 40% of world's surface has salinity problems [3]. Active microbiological processes in soil enhance the rate of synthesis and mineralization of organic matter which then leads to better plant nutrition. Some of the microorganisms, particularly valuable bacteria and fungi can develop plant performance under stress condition and, therefore, improve yield [4]. At high salinity level, it was found that treatments supplied by biofertilization with yeast decreased the adverse effect of salinity. Halophilic microorganisms are already in use for some biotechnological processes, such as commercial production of β -carotene, polymers, enzymes, compatible solutes [3].

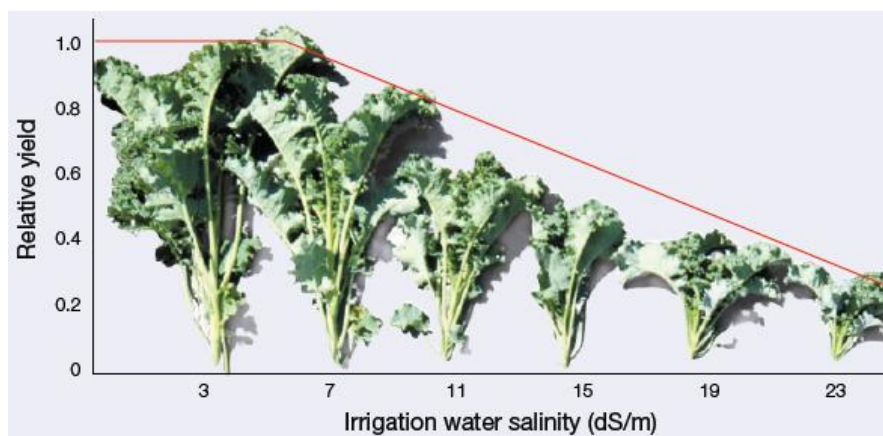


Figure 1 Growth of kale plants in response to irrigation water salinity. Plants were grown outdoors in individual sand tanks at electrical conductivity levels of 3 to 23 deciSiemens per meter (dS/m).

Salinity is the major problem in agriculture due to its negative effect on the sustainability of beneficial microorganisms associated with the rhizosphere. The beneficial attributes of these microbiomes such as antagonism against phytopathogens, nutrient recycling in the rhizosphere and root colonization or rhizosphere competence is often considered a limiting factor for biocontrol in the rhizosphere. Fluorescent pseudomonads are among the most influencing plant growth-promoting rhizobacteria (PGPR) in plants rhizosphere. Beneficial rhizosphere organisms are generally classified into two broad groups based on their primary effects: (i) microorganisms with direct effects on plant growth promotion and (ii) biological control agents that indirectly assist with plant productivity through the control of plant pathogens. In addition to their primary effects on plant productivity and health, respectively, recent work has shown that these beneficial microorganisms possess secondary, i.e., more recently discovered effects that may bestow them increased interest for plant growers (Figure 2)

Fluorescent pseudomonads are an important part of the soil microbiota, frequently found in association with plant roots. The soil gains importance, especially in saline agricultural soils, where high salinity results from irrigation practices and application of chemical fertilizer. This effect is always more pronounced in the rhizosphere as a result of increased water uptake by the plants due to transpiration. Hence, the rhizobacteria form a group of the best adapted microorganisms [4]. Fluorescent pseudomonads are known for their inhibitory activity due to their ability to produce siderophores, antibiotics and cyanides. Current topics in soil biotechnology research have focused on the optimum control of plant-microorganisms interactions beneficial to plant roots for protecting the plants from various soil-borne diseases, particularly those associated with intensive cultivation with high input. Intensive cultivation systems, like those in greenhouses, have generally caused soil salinity due to the high rates of chemical fertilizer applied [5]. Thereby, the high concentration of inorganic ions accumulated in the plant rhizosphere is likely to induce stress on both the root itself and the microorganisms associated with the root system. Thus, ecological studies on the effect of soil salinity on beneficial microorganisms are essential for optimum utilization.

There are numerous reports on the beneficial effects of rhizosphere microorganisms on plant growth [6]. These effects were closely correlated with root colonization by the microorganisms [7]. Strains of fluorescent pseudomonads, particularly *Pseudomonas putida* and *Pseudomonas fluorescens*, belong to

a major group of PGPR. However, there are few studies on the effect of soil salinity associated with intensive cultivation on the PGPR.

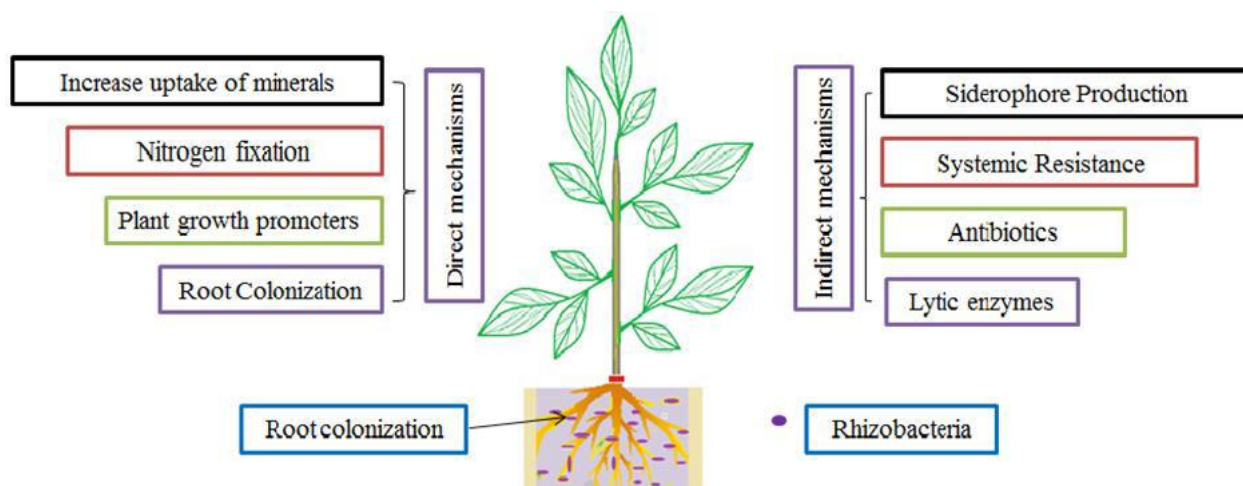


Figure 2 Potential modes of action of plant growth promoting microorganisms with primary and secondary beneficial effects on plants. Source: [8].

Bioremediation in natural ecosystems is dependent upon the availability of micronutrients and cofactors, of which Fe is one of the essential elements. Under aerobic and alkaline conditions, Fe oxidizes to Fe^{3+} creating Fe deficiency. Iron is an essential element to virtually all forms of life and plays an important role in different physiological processes such as respiration, photosynthesis, DNA synthesis and defence against reactive O_2 species [9]. Fe in an aerated environment exists in the Fe^{3+} form and so is highly insoluble in neutral or alkaline soil. To solve this problem, microorganisms are generally observed to utilize a high-affinity Fe transport system. The synthesis and secretion of a low-molecular weight Fe^{3+} -specific chelation agent to solubilize Fe is termed siderophore. Microbial siderophore may stimulate plant growth directly by increasing the availability of Fe in the soil surrounding the roots or indirectly by competitively inhibiting the growth of plant pathogens with less efficient Fe-uptake system. However, its availability is extremely limited by very low solubility of $\text{Fe}(\text{OH})_3$ complexes at neutral pH [10]. To survive in such an environment, plant-associated PGPR have different strategies of obtaining Fe from the soil, which include the synthesis of siderophores which are selective Fe^{3+} ion chelators. These low molecular weight compounds are secreted in response to Fe deficiency [9, 10]. Siderophores synthesized by fluorescent *Pseudomonads* have received much attention over the past years, because of their role in the biological control of soil-borne plant pathogens and in disease suppressive soil. Specific strains of the *P. putida* group have been used as seed inoculants on crop plants to promote growth and increase yields of various crops.

This study aimed at clarifying the effect of soil salinity on the microbial communities in the root-free soil and rhizosphere soil of sunflower plants, with emphasis placed on fluorescent *pseudomonads*.

2. MATERIALS AND METHODS

In greenhouse, sunflower plants were grown in pot of 2 kg capacity containing four various soil types: chernozem meadow soil (Szeged), Clay loamy brown forest soil (Gödöllő), Kovárványos brown forest soil (Nyíregyháza) and Ramann-type brown forest soil (Keszthely) with different levels of soil salinity

(0, 2.5, 7.5 and 12.5 dS/m) for 6 weeks for isolating fluorescent pseudomonads. On the same way, another set of pots were inoculated with different strains of fluorescent pseudomonads isolated from different plant rhizosphere, highly tolerant to NaCl salt and siderophore-producers. Soil salinity is determined by measuring the electrical conductivity of a soil-water mixture (1:5). The higher the salinity of the soil, the higher the conductivity of this mixture will be. *Electrical Conductivity (EC)*: Measures the ability of the soil solution to conduct electricity and is expressed in decisiemens per meter (dS/m, which is equivalent to mmhos/cm). Because pure water is a poor conductor of electricity, increases in soluble salts result in proportional increases in the solution EC. The standard procedure for salinity testing is to measure EC of a solution extracted from a soil wetted to a "saturation paste." Increasing soil salinity was done by the addition of NaCl to the soil solution to reach 2.5, 7.5 and 12.5 dS/m.

Isolation of fluorescent pseudomonads were isolated from sunflower plant rhizosphere using serial dilution technique. Intact root systems of sunflower plants were collected and shaken gently to remove the excess of soil. These root portions with just a layer of closely-adhering rhizosphere soil were then transferred to 100 ml sterile saline (0.85%) water and shaken at 150 rpm for 30 min. Suspensions were diluted up to 10^{-4} with three replications, and plated on King's B agar medium to enrich fluorescent pseudomonads. Fluorescent pseudomonads are easily isolated using semi-selective growth media such as King's B [11] agar medium and incubated at 28°C with rifampicin (50 µg/ml) [12]. After two days, the colonies were observed under the UV transilluminator and colonies that produced fluorescence were selected as fluorescent pseudomonads. The selected colonies were further sub-cultured on King's B agar medium. All the fluorescent pseudomonads was maintained in glycerol stocks (65%) at 4°C [13].

Solution of King's B agar medium was prepared with different NaCl concentrations (equivalent to 2.5, 7.5 and 12.5 dS/m) and fluorescent pseudomonads were inoculated. Fluorescent pseudomonads inoculated King's B agar plates were observed for growth after two days of incubation.

- The separated single yellowish green water-soluble pigment producing rhizobacterial colonies were isolated and grown on King's B agar medium
- The ability of isolates to produce fluorescent siderophore was tested by plating the isolated bacterial colonies on King's B agar and incubating for 2 days at 28°C.
- Plates were then inspected under 336-nm UV light, and fluorescence was compared visually to those of *P. fluorescens* (B77 and W99) as positive control.
- Production of siderophores was evaluated by Chrome Azurol-S Assay on CAS agar plates by observation of yellow-orange zone around the colonies according to SCHWYN & NEILANDS [14].
- The cultures were compared to the control strains.

Screening for Siderophore Producers: Siderophore production was determined using CAS assay [14]. The medium deferred by adding 8-hydroxyquinoline dissolved in chloroform to ensure complete removal of Fe [15]. The isolates were spot inoculated, and plates were incubated till 72 hours, and the isolates forming yellow-orange zone were selected for further studies.

Determination of available phosphorus was determined according to methods for P extraction and analysis in soils as detailed by OLSEN and SOMMERS [16].

3. RESULTS AND DISCUSSION

SAKAI et al. [17] Use of PGPR for stimulating plant growth and for the biological control of soil-borne diseases is necessary mainly in fields with intensive cropping. The intensive cultivation systems in horticulture have generally led to soil salinity due to the high rates of fertilizer application. Therefore,

the high concentration of inorganic salts accumulated in the plant rhizosphere is likely to be a factor of salinity stress affecting both roots and root-associated rhizobacteria. In Recent work, e.g., MATSUGUCHI and SAKAI [18] observed that high salinity of soil had induced a significant decrease in the populations of fluorescent pseudomonads in spinach roots. Furthermore, the adverse effect of high salinity of soil was more pronounced on *P. fluorescens* strains than on *P. putida* strains, resulting in an alteration of the composition of fluorescent pseudomonads in the roots where *P. putida* predominated. Our results indicated that the distribution of fluorescent pseudomonads, *P. fluorescens*, *P. putida*, *P. aeruginosa* and non-fluorescent pseudomonads were varied according to the physicochemical properties of each soil type. Figure 3 shows that the highest enumeration of fluorescent pseudomonads, *P. fluorescens* and *P. putida* were found in chernozem meadow soil, while the highest counting of non-fluorescent pseudomonads was observed in clay loam brown forest soil.

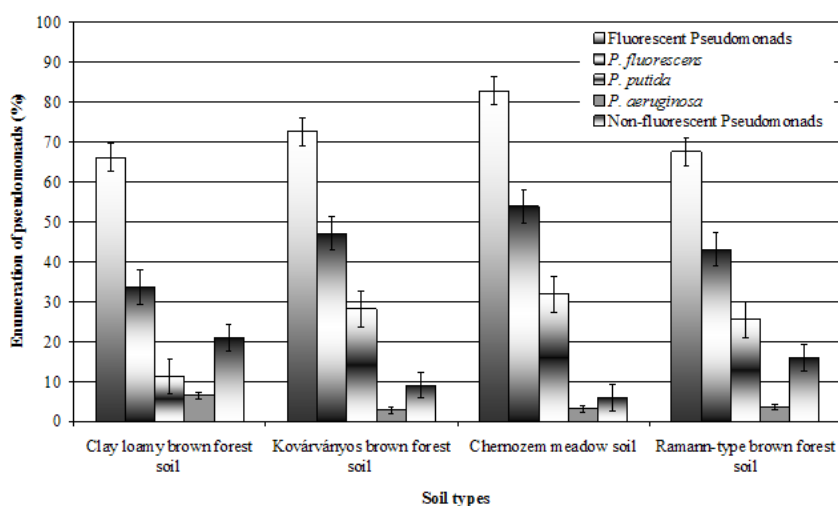


Figure 3 Enumeration of fluorescent and non-fluorescent pseudomonads in different soil types

By introducing well characterized PGPR strains of *P. fluorescens* (FPC03, W99 and B77) and *P. chlororaphis* (FPW03) into the four used soil types, Figure 4 illustrates that these strains were comparatively grown and their relative populations were varied among the soil types. It was found that maximal multiplication and survival of *P. fluorescens* FPC03 and *P. chlororaphis* FPW03 were found in all soil types except Ramann-type brown forest soil. While *P. fluorescens* strains W99 and B77 well established in chernozem meadow and Ramann-type brown forest soil types, respectively. Generally, it was found that chernozem meadow soil type was favourable for all strains.

After adjusting the salinity degrees of the soil solutions to 2.5, 7.5 and 12.5 dS/m in addition to the control soil types, Our results (Figures 5-8) showed that the frequency of the distribution of fluorescent pseudomonads, *P. fluorescens*, *P. putida*, *P. aeruginosa* and non-fluorescent pseudomonads were varied according to the physicochemical properties of each soil type and soil salinity degree. Under the effect of salinity variations in the clay loam brown forest soil (Gödöllő), higher variations in the distribution frequency of fluorescent pseudomonads, *P. fluorescens*, *P. putida*, *P. aeruginosa* and non-fluorescent pseudomonads were observed.

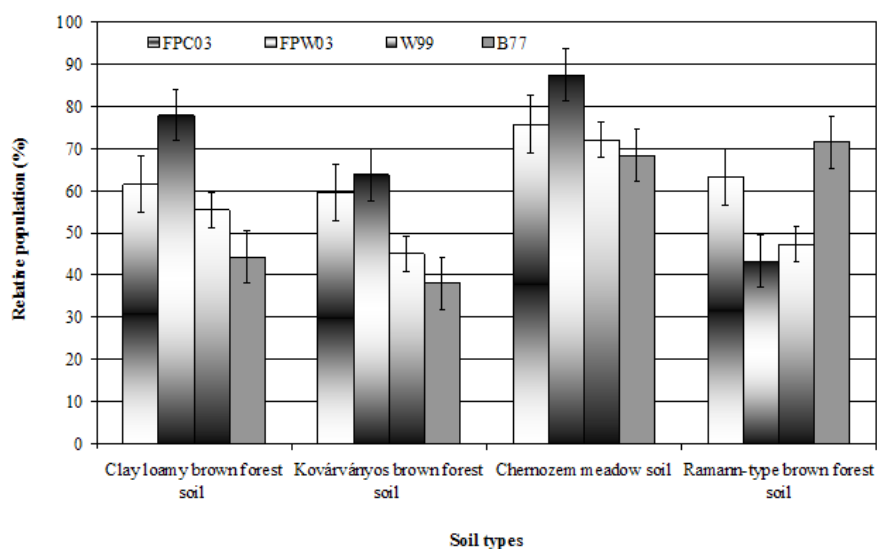


Figure 4 Relative population of four fluorescent pseudomonads strains in different soil types

Figure 5 also shows that total fluorescent pseudomonads was at maximal distribution at 2.5 and lower at control, 7.5 and lowest at 12.5 dS/m. The viable counts of *P. fluorescens* and *P. putida* were increased by increasing the soil salinity degrees and higher than in control soil even at 12.5 dS/m. While *P. aeruginosa* was at maximum counting at 2.5 and 7.5 dS/m.

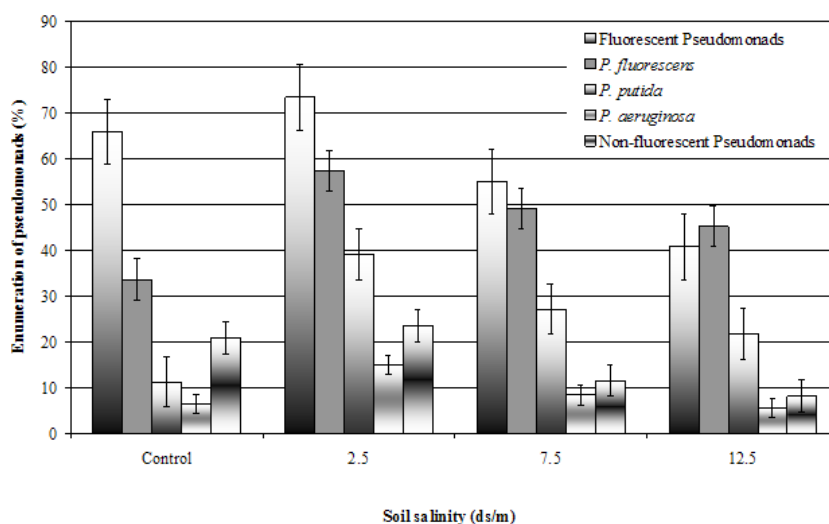


Figure 5 Effect of soil salinity (dS/m) on enumeration of fluorescent and non-fluorescent pseudomonads in clay loam brown forest soil (Gödöllő)

Non-fluorescent pseudomonads population was not significantly increased over the control at 2.5 dS/m and the decreased by increasing the level of soil salinity.

In Kovárvány brown forest soil (Nyíregyháza), it was found that total fluorescent pseudomonads had the highest multiplication at 7.5 dS/m the at 12.5 dS/m is lower than in control soil (Figure 6). The viable counts of *P. fluorescens*, *P. putida* and *P. aeruginosa* were higher in soil of 12.5 dS/m than in control soil. But the non-fluorescent pseudomonads was well established at 2.5 dS/m and better than in control soil and at 7.5 and 12.5 dS/m does not show significant increased in comparison with control.

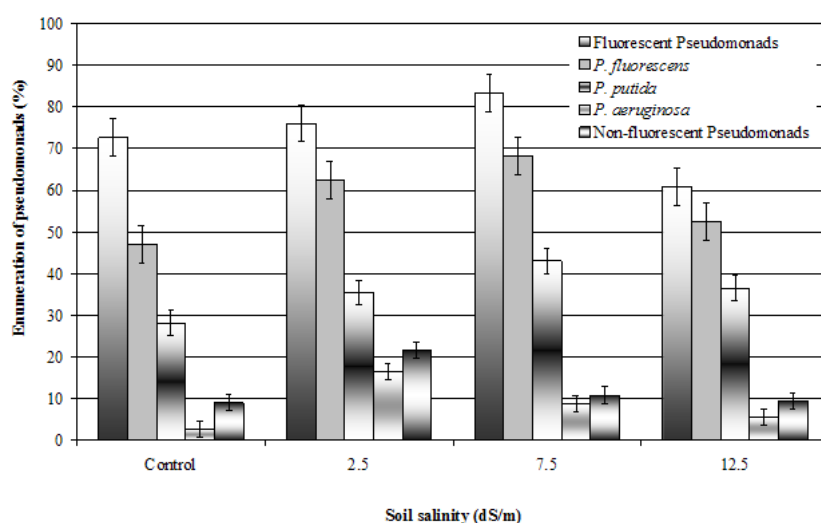


Figure 6 Effect of soil salinity (dS/m) on enumeration of fluorescent and non-fluorescent pseudomonads in Kovárvány brown forest soil (Nyíregyháza)

Figure 7 demonstrates the distribution frequency of fluorescent pseudomonads, *P. fluorescens*, *P. putida*, *P. aeruginosa* and non-fluorescent pseudomonads in chernozem meadow soil (Szeged) which were higher at 2.5 and 7.5 dS/m than in control soil. But the populations of *P. aeruginosa* and non-fluorescent pseudomonads at 12.5 dS/m were higher than in control soil.

In case of Ramann-type brown forest soil (Keszthely), it was found that the population rates (Figure 8) of fluorescent pseudomonads, *P. fluorescens*, *P. putida*, *P. aeruginosa* and non-fluorescent pseudomonads were varied according to the degree of soil salinity. Fluorescent pseudomonads, *P. fluorescens*, *P. putida* and *P. aeruginosa* were higher at 2.5 dS/m and decreased by increasing the soil salinity. But the enumeration of non-fluorescent pseudomonads was lower than control.

Figure 9 illustrates the rate of siderophore production in the four different soil types under the stress of three level of salinity. The highest rates of siderophore production were measured in chernozem meadow followed by Kovárvány brown forest, clay loam brown forest and Ramann-type brown forest soils. In all four soil types, maximum rates of siderophore production were observed at 2.5 dS/m, and also, observed at 7.5 in chernozem meadow and Kovárvány brown forest soils. Generally, soil salinity of 12.5 dS/m decreased the production of siderophore.

Figure 10 shows the effect of saline soil extraction of soil types on the siderophore production by four fluorescent pseudomonads strains. It was found that the lowest siderophore production was recognized by the strain FPW03 followed by FPC03 which produced more siderophore at 12.5 dS/m than in control soil. While, the strain B77 was produced more siderophore at 2.5 and 7.5 dS/m than the strain W99 which produced more siderophore at 12.5 dS/m than B77. Over the all, 7.5 dS/m salinity level stimulated the four strains to produced more siderophore than any level else.

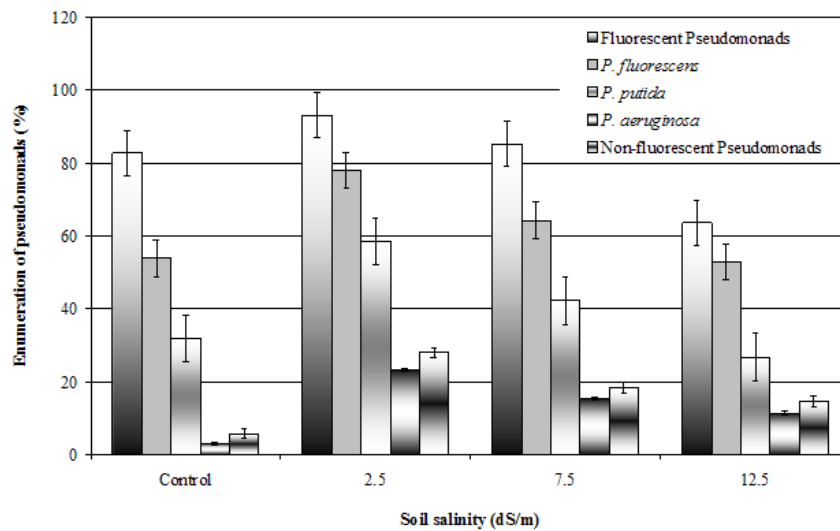


Figure 7 Effect of soil salinity (dS/m) on enumeration of fluorescent and non-fluorescent pseudomonads in chernozem meadow soil (Szeged)

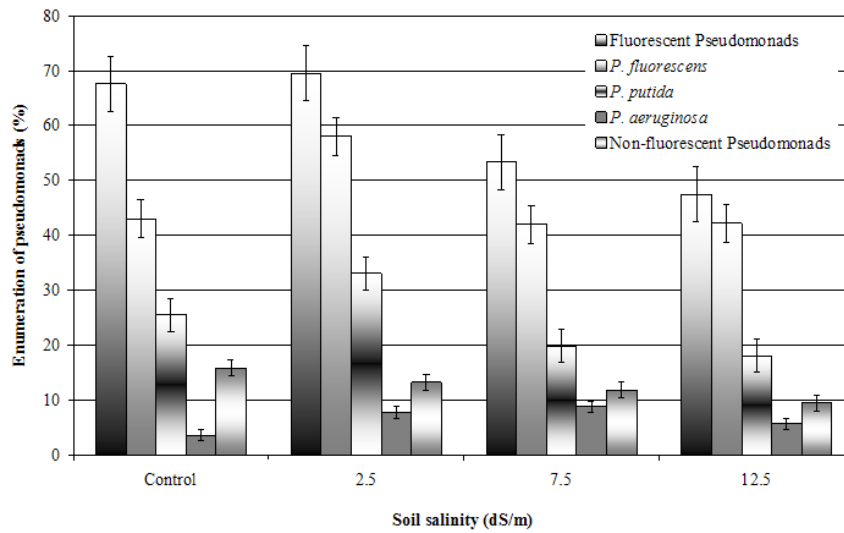


Figure 8 Effect of soil salinity (dS/m) on enumeration of fluorescent and non-fluorescent pseudomonads in Ramann-type brown forest soil (Keszthely)

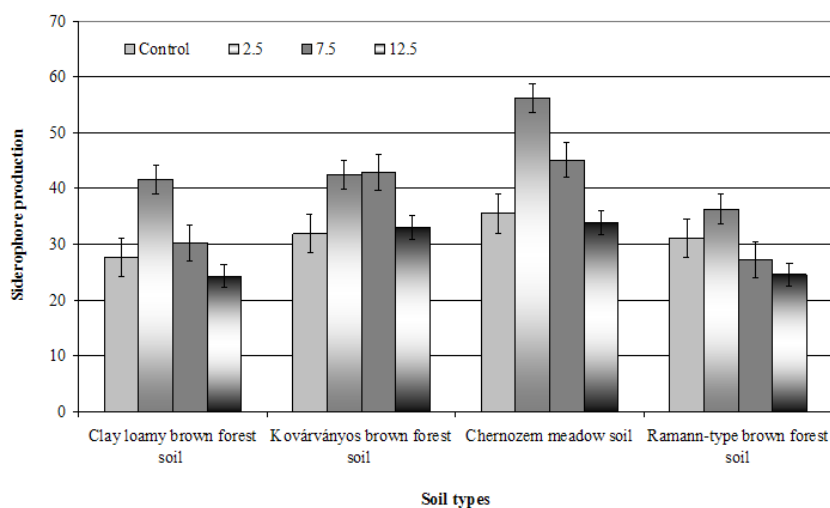


Figure 9 Effect of soil salinity (dS/m) on siderophore production

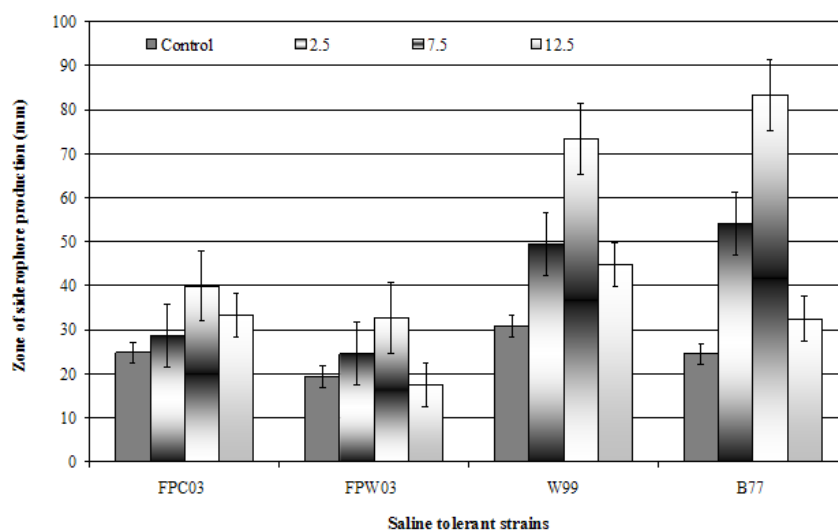


Figure 10 Effect of saline soil extraction of different soil types on the siderophore production by four fluorescent pseudomonads strains

Figure 11 illustrates the effect of soil inoculation by four strains of fluorescent pseudomonads as biofertilizers on the sunflower root dry weight grown in the four different soil types under the stress of salinity levels. It was found that soil inoculated by the strain W99 stimulate the sunflower plant roots in the four soil types followed by B77, FPC03 and the lowest plant root dry weight was determined in the soil inoculated by FPW03. Relatively, the maximum root dry weight was measured from the sunflower plant grown in chernozem meadow soil followed by Kovárvány brown forest soil and Ramann-type brown forest soil. The lowest plant root dry weight was observed from the plant grown in clay loam brown forest soil.

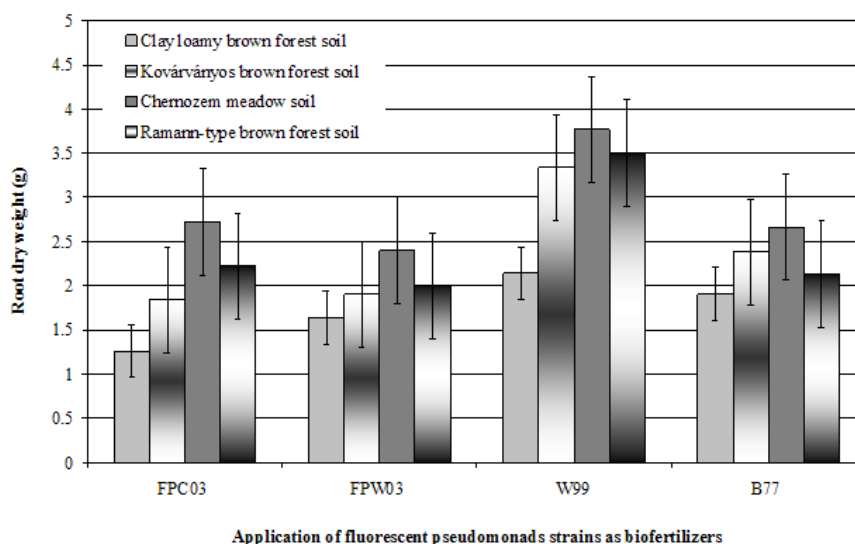


Figure 11 Effect of soil inoculation by fluorescent pseudomonads as biofertilizers on the sunflower root dry weight grown in different soil types

Figure 12 demonstrates the effect of soil inoculation by fluorescent pseudomonads strains as biofertilizers on the sunflower dry weight grown in four different soil types. Results indicated that the strain B77 had more stimulative activity to produce higher dry weights of sunflower plants grown in chernozem meadow soil and clay loam brown forest soil, while the strain FPW03 was able to stimulate the dry weights of the plant grown in Kovárvány brown forest soil and the strain W99 was improved the dry weights of the plant grown in Ramann-type brown forest soil.

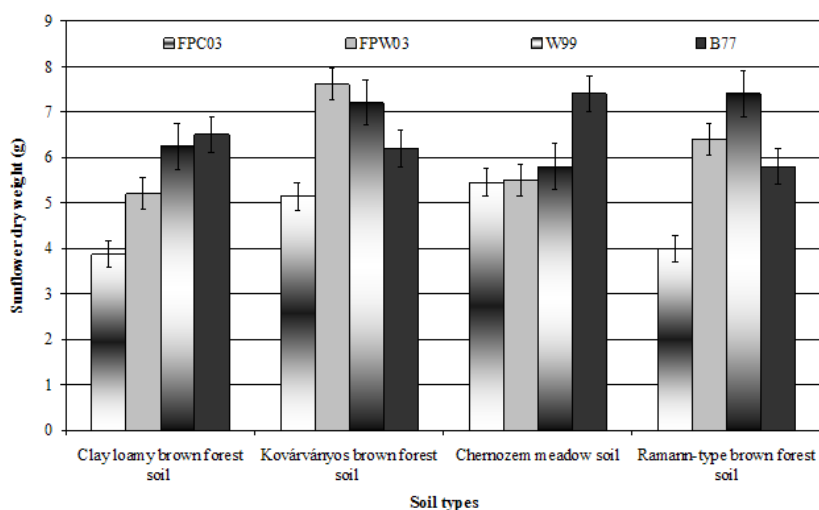


Figure 12 Effect of soil inoculation by fluorescent pseudomonads as biofertilizers on the sunflower dry weight grown in different soil types

The fluorescent pseudomonad strain FPC03 had no ability to improved the dry weights of the plants in all investigated soil types.

Figure 13 shows the effect of soil inoculation by fluorescent pseudomonads on the phosphorus content in the rhizosphere of the sunflower plants grown in the four different soil types. It was found that strains FPW03 improved the P content in the rhizosphere of the plants grown in clay loam brown forest, chernozem meadow, Ramann-type brown forest and Kovárvány brown forest soil types. The rhizosphere of sunflowers grown in Kovárvány brown forest and Ramann-type brown forest soil types had the lowest P content in comparison with the soils inoculated by FPW03. Strain B77 almost gave the similar reaction regarding to the P content in all soil types.

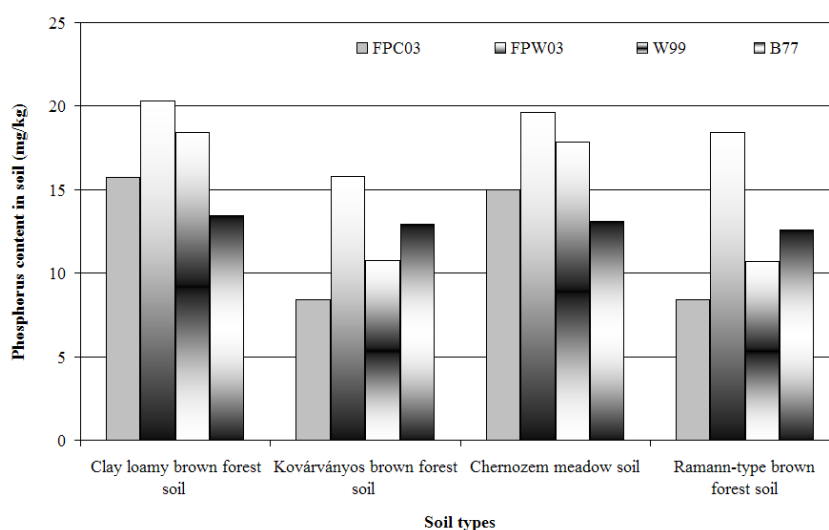


Figure 13 Effect of soil inoculation by fluorescent pseudomonads as biofertilizers on the phosphorus content in different soil types

A few papers have reported the effect of soil salinity on the microbial populations. LOPER et al. [19] reported that the sensitivity to osmotic stress was a limiting factor for the root-colonization potential of certain fluorescent pseudomonad strains. To date, fluorescent pseudomonads, a major group of PGPR, have been found to consist of two physiological groups, *Pseudomonas putida* and *Pseudomonas fluorescens*. Nevertheless, the effect of environmental stress on each group has not been separately studied.

The salinity of the soil plays a prominent role in the microbial selection process as environmental stress has been shown to reduce bacterial diversity [20]. This requires the bacterial population to be salt-tolerant in order to be able to grow and survive, which is reflected in the salt tolerance studies that were carried out for all these strains.

Many species of bacteria are able to solubilize phosphates in vitro and most of them live in the plant rhizosphere. At present, bacilli, rhizobia and pseudomonads are the most studied phosphate-solubilizers [21]. PGPR may enhance mineral uptake including N, P, K, and microelements more efficiently from the soil, not only as a consequence of the increase in root surface area, but also by stimulating the ion uptake systems [22].

Conclusion

The electrical conductivities of the 1:5 (w/w) soil: water suspensions at were 2.5, 7.5, and 12.5 dS/m were adjusted. The unfavourable effects of salinity on soil fertility are e.g., uptake of nutrients, absorption of moisture and soil structure are well known. Use of soil microorganisms which can either phosphate solubilizers, phytohormone-producer or by enhancing the decomposition of plant residues to release vital nutrients and increase humic content of soils, will be environmentally begin approach for nutrient management and ecosystem function. We can point out the following points:

- The effect of soil type that has different nutrient status on the stimulatory efficiency of PGP fluorescent pseudomonads rhizobacterial inoculants may be important for successful root inoculation and plant growth stimulation.
- The results of greenhouse pot experiment carried out with 4 different soil types indicate that the soil types is the dominating factor responsible for the diversity of the fluorescent pseudomonads bacterial populations associated with plant roots of sunflower.
- The inoculation with the fluorescent pseudomonads strains frequently increased the mentioned indices at all salinity doses.
- Among the strains, *P. fluorescent* FPW03 exerted the greatest effect on plant growth, plant height, and dry weight of sunflower shoots in saline conditions.
- Therefore, in saline condition, strains of fluorescent pseudomonas can be used for promoting growth and yield of sunflower.

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