Response of model plant *Arabidopsis thaliana* to Plant growth promoting rhizobacteria & phosphate concentration

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Abstract. Several reports have shown that various rhizobia can interact with non-hosted plant species, enhancing mineral nutrition and promoting plant growth. To further investigate the effects of such non-host interactions on plant growth and phosphate nutrition, we inoculated *Arabidopsis thaliana* with the model rhizobacterium *Pseudomonas fluorescens* at three phosphate concentrations in the nutrient medium. *In vitro*, we showed that root colonization by *Pseudomonas fluorescens* contributes to an increase in the amount of available phosphate that is important in plant growth, especially in the shoots, in all concentrations used in the study. In addition to improving plant growth as well as increasing plant biomass production.

Keywords. *Arabidopsis thaliana*, rhizobacteria, phosphate concentration.

1. Introduction

*Arabidopsis thaliana* belong to Brassicaceae is a world famous model in plant biology and genetics. It was the first plant to have its genome sequenced as it has a relatively small genome, which makes it a very useful model, as it is considered a popular tool for understanding the molecular biology of many plant traits [1]. Therefore, scientists consider it a vegetarian test mouse. Its popularity stemmed from the smallness of its genomic component. It has many sites for this plant on the World Wide Web and it is the most published plant for scientific research. The Arabidopsis thaliana plant is distinguished by its short life cycle, as the plant grows rapidly to reach maturity in six weeks, and produces thousands of seeds, and the plant is self-pollinating, so the genetic makeup of the plant is not affected by the succession of its generations, and hybrid seeds are not expensive and available, as well as can be stored. The seeds are for a long period of up to five years, as they are relatively small genome of approximately 135 mega base pairs (Mbp), and they can be cultivated easy (in vitro or in the field) and inexpensive, making the genetic testing of tens of thousands of plants easy [2]. Plant growth is affected by many external and internal factors that may improve or inhibit growth due to the interference of these factors with the plant's physiological processes. Among the factors that are in relationships with other organisms such as microorganisms that have a relationship with plants roots. The great richness of a rhizosphere in organic matter promotes the development of an abundant population of microorganisms. There is an abundant population of bacteria. It is estimated that the
density of bacteria present in a rhizosphere is on average 107-109 CFU / gr soil [3]. They develop either near the roots, in contact with the roots (which colonized the rhizoplane) or inside the roots (between cells and more rarely in a cell: endophytes). This enrichment in microorganisms attracts other animals (earthworms) which will feed on bacteria or fungi [4]. The rhizosphere is a small-scale model of the primary role that a plant plays in the structuring and functioning of an ecosystem. Types of symbiosis are classified according to the degree to which each species benefits from the interaction between plant and microorganisms [5]. There are beneficial bacteria and on the other hand, pathogenic bacteria, for example certain plants establish a symbiotic relationship with rhizobacteria, enabling them to produce nodules that facilitate the conversion of atmospheric nitrogen to ammonia [6]. Plants are also affected by other factors such as nutrition, which directly contribute to the improvement and growth of plants, especially the availability of Macro-elements, many Plant Growth Promoting Rhizobacteria (PGPRs) can influence the acquisition of macro and microelements, for example, some PGPRs are free fixers of atmospheric nitrogen (Azospirillum brasilense Sp245) [7]. Present near the roots, their activated will help to provide the plant with inorganic nitrogen. In some PGPRs belonging to the Pseudomonas fluorescens species, there is production of a non-ribosomal oligopeptide, pyoverdin (at the origin of the fluorescence of the strains), which acts as a siderophore and which promotes the iron nutrition of plants [8]. Finally, others are able to solubilize phosphate by excreting organic acids in the medium [5]. Their presence in the rhizosphere improves the solubilization of phosphate for the benefit of bacteria as well as plant obviously, the activity of these different microorganisms contributes to modifying the organization of the soil in the vicinity of the roots and consequently, the development of the roots [9]. PGPRs on nutrition can be more extensive and include plant harvesting activity, for example, an increase in the influx of nitrate is observed in rapeseed plants inoculated with PGPRs belonging to the rhizobial family [10]. The effects of PGPRs on plants may also include carbonaceous nutrition. However, interpreting this effect is difficult because if the inoculation with Bacillus subtilis GB03 improves the photosynthesis of Arabidopsis, cultivated in vitro [11], the inoculation of Arabidopsis thaliana plantlets by the PGPR Phyllobacterium brassicacearum STM196 on the contrary decreases photosynthesis [12]. The indirect effects that PGPR can have on plants also fall into two categories. Either the effect is obtained when PGPR restricts the progression of a pathogenic microorganism, or the effect is obtained by activating the defense reactions of plants [13]. In the genome of many PGPR strains belonging to the Pseudomonas family, there are genes encoding the synthesis of 2, 4-diacetylphloroglucinol (DAPG; for review [14]). It is a compound with an antimicrobial power that can limit the growth of one or more phytopathogenic bacteria. Finally, many PGPRs also it can stimulate the activity of antioxidant defense system of plants against attacks from phytopathogenic bacteria. This is called ISR (Induced Systemic Resistance) [13]. This mechanism relies on the activation of a signaling pathway involving ethylene and jasmonic acid which often results in the activation of expression of genes encoding Defensins, which have an antimicrobial function [15]. Numerous bacteria which are beneficial to plant growth and present in their rhizosphere have phosphate solubilization activity. It is mainly dependent on the activity of microorganisms to secrete protons and organic acids [16]. Galonic acid by fixing cations such as Ca2 is arguably the one that is most directly involved in the solubilization of phosphate. This is what makes gram-negative bacteria, such as for example Pseudomonas fluorescens which directly oxidize glucose to galonic acid in the presence of glucose dehydrogenase (GCD), excellent solubilizers of inorganic Phosphate (Pi). Also, their use is gradually popularizing in agriculture. An important component of the plant environment is formed by microorganisms. They can be pathogenic / parasitic, commensal or so beneficial (symbiosis) [17]. Studying the effect of symbioses on plants is a way to study how plants interact with their environment, but also a way to see how these symbioses could help plants better protect and adapt to climate change. This is why the study of all types of symbiosis is important today [18]. The main objective of our work was to unravel potential cross talks in the control of growth of the model plant Arabidopsis thaliana in response to different concentrations Pi and in response to the presence of Pseudomonas fluorescens.
2. Materials and Methods

2.1. Biological material

The experiments were carried out using a biological system composed by the model plant Arabidopsis thaliana as the wild type (WT) and gpt2 mutant (glucose6-Phosphate/phosphate transporter2), and at the same time by strains of rhizobacteria PGPR *Pseudomonas fluorescens*.

2.2. Culture medium and plant culture

All of the experiments were conducted in-vitro. The plants are cultivated on a minimum solid mineral medium (0.5 mM CaSO\(_4\)(H\(_2\)O)\(_2\), 2 mM KNO\(_3\), 0.5 mM MgCl\(_2\)(H\(_2\)O)\(_6\), 1 mM KH\(_2\)PO\(_4\), 0.05 mM Na\(_2\)FeEDTA, 2.5 mM MES, 0.03 μM (NH\(_4\))\(_6\)Mo\(_7\)O\(_24\)(H\(_2\)O)\(_5\), 1 μM CuSO\(_4\)(H\(_2\)O)\(_2\), 1 μM ZnSO\(_4\)(H\(_2\)O)\(_7\), 15 μM MnCl\(_2\)(H\(_2\)O)\(_4\), 50 μM H\(_3\)BO\(_3\)) supplemented by 1.2% (p/v) agar. The pH of the medium is adjusted to 5.7 with a KOH solution then aliquoted in the bottle and finally autoclaved at 120 °C for 15 min. Before sowing, the seeds are mixed in a disinfection solution (40 mL of osmosed water, 3 drops of tween, and 2 mL of sodium hypochlorite) for 15 min. They are then rinsed 5 times with sterile water. The seeds are sown one by one and spaced 1 cm apart under laminar air flow host in square Petri dishes containing solid culture medium, using a pipette. The cover closed with thick of adhesive plaster microporeTM 1.25 cm wide. The sown boxes are placed in the dark at 4°C for at least 2 days to promote uniform germination. At the end of this treatment, the boxes are placed vertically in the culture chamber set for long days (16h day / 8h night) at 21 °C and delivering a light output of 20,000 lux.

2.3. Preparation of the inoculum and inoculation of the plants

The strains of rhizobacteria PGPR *Pseudomonas fluorescens* were cultivated in the culture media which was most suitable KingB (20g bacto-tryptone, 10 glycerol, 1.5g K\(_2\)HPO\(_4\) (anhydrous) 1.5g MgSO\(_4\),) at 28 ° C for at least 3 days. The plants are inoculated by contact during their growth in the culture medium. For this, a known amount of inoculum is included in plant culture medium which has been kept liquid by incubation at 52 °C after autoclaving. The inoculum is obtained by adding 8 ml of sterile plant culture medium to the Petri dishes containing the bacteria culture. The medium is incubated under this condition for 15 min. The bacteria growing on the culture medium are then resuspended. The inoculum thus formed is taken using a sterile pipette and collected in a falcon tube. The number of bacteria contained in the inoculum is determined by measuring the turbidity of the 600nm solution. The plant culture medium is inoculated with the necessary amount of inoculum to obtain a final optical density of 0.07, which corresponds on average to 108CFU/ml of culture medium. Once the plant culture medium has cooled and been solid, eight-day-old seedlings are transferred to it. Each Petri dish then containing 10 seedlings is sealed with MicroporeTM type tape, then installed in a semi-vertical position in the culture chamber.

2.4. Plant growth

The shoot fresh weight and the root fresh weight were measured separately on a precision balance (ACL SBS-LW-300A, USA). To measure the fresh weight, all the root systems present in one plate (10 in total) were carefully collected (to avoid any sampling of agar). The result was normalized the number of root system sampled. Shoot fresh weights were measured one by one. For each genotype, the root and shoot fresh weight, and the content of soluble Pi has been repeated independently at least 5 times (50 seedlings).
2.5. Soluble phosphate content quantification

The amount of soluble/inorganic Pi was determined in the shoot, root and in aliquots of the different media 10 days after transfer. All individual samples were weighed, collected in 2 ml Eppendorf tubes, and suspended in 1ml of ultrapure water. All the samples were then heated during 1h at 95°C. We measured the amount of soluble Pi in 50 μl aliquots of each extract using the standard molybdenum blue method in conjugation with UV-visible spectrophotometer [19].

2.6. Statistical analysis

All data were collected on spreadsheets and standard statistics methods were used to describe the samples. The significance of the effect of a treatment on a variable has been tested by one - way ANOVA. The existence of differences between the observed means was tested by an LSD test at the threshold of P = 0.05. On the histograms presented in the result, the bars bearing the same letter are not considered to be significantly different. Arabidopsis thaliana seedlings were inoculated with a rhizobacteria Pseudomonas fluorescens. Ten days after inoculation, we measured the effect of the inoculation on the growth of the plant shoot and root at different concentrations of phosphate.

3. Results and Discussion

3.1. The presence of P. fluorescens in the Arabidopsis rhizosphere does affect the amount of soluble Pi present in plants

It is also noted in figure. 1 that the presence of Rhizobacterium Pseudomonas fluorescens an effect on the amount of phosphate absorbed by the plant after 10 days of inoculation into the agricultural medium for plant growth, as the presence of Rhizobacterium Pseudomonas fluorescens increased the amount of phosphate present in the shoot system in all phosphate levels used in the study and even at the level of deficient phosphate compared to the absence of Rhizobacteria in wild type plants (Figure 1. A), but at the level of roots, there are no significant differences at the level of normal phosphate and phosphate deficiency in both the presence and absence of Rhizobacteria in the phosphate content for the roots of wild plants, while there is a significant difference in the level of concentration 3 times phosphate by the presence of rhizobacteria (Figure 1. B).
Figure 1. Phosphate content in shoot and root of Arabidopsis depends on Pi availability. Plants were transferred on different type of media: standard medium (2mM Pi), Pi deficiency medium (- Pi), and 3 time phosphate concentration medium, either with non-inoculated (- *P. fluorescens*) or inoculated (+ *P. fluorescens*). Ten days after the transfer, we measured the amount of soluble Pi. (A) Average (± SD) soluble Pi concentration in the shoot of wild type (Col 0), (B), average (± SD) soluble Pi concentration in the root of wild type (Col 0), (C) Average (± SD) soluble Pi concentration in the shoot of mutant (gpt2), (B), average (± SD) soluble Pi concentration in the root of mutant (gpt2).
Also, the effect of Rhizobacteria *Pseudomonas fluorescens* was observed with the amount of phosphate present in the mutant plants (gpt2), the presence of *Pseudomonas fluorescens* led to increase in the phosphate content present in the triple concentration of phosphate significantly and greatly, as well as the normal concentration and the level of phosphate deficiency compared to the amount of phosphate present in the shoot. In the absence of rhizobacteria (Figure 1. C), at the root level of this mutant (gpt2), it can also be seen that the rhizobacteria led to a significant increase in all phosphate levels used in the study (Figure 1. D). From all these data, it can be confirmed that the presence of Rhizobacteria *Pseudomonas fluorescens* contributes to an increase in the amount of dissolved phosphates in the plant, but the mechanics are uncertain, it may have been by producing substances that contribute to increase the phosphates availability for absorption and converting complex phosphates into simple forms that can be absorbed through channels on the roots, thus increase its quantity within the shoot system, because phosphate has a great role in the biological processes inside the plant [20].

3.2. *Inoculation with Pseudomonas fluorescens stimulates plant growth*

After 10 days of culture under the different conditions, it was found that the Pi and inoculation with *Pseudomonas fluorescens* led to beneficial effects on the growth of the plant. For Col - 0, Pi deficiency decreases the growth of the shoot whereas, on the contrary, inoculation stimulates the growth of the shoot (Figure 2. A). Whether the plants grew in the medium depleted in Pi or on the contrary enriched in Pi, the magnitude of this stimulation is similar. However, the reverse is not true: in the presence of *Pseudomonas fluorescens*, we observe that the Pi deficiency has a slightly inhibitory effect on the shoot while the excess Pi has a slightly stimulating effect. The root is directly affected by the concentration of phosphate in the nutrient medium, and we did not notice any effect of rhizobacteria *Pseudomonas fluorescens* in increasing or improving its growth (Figure 2. B).
Figure 2. *P. fluorescens* promotes plant growth in vitro, regardless of Pi availability, eight days old WT Arabidopsis plantlets were. Plants were grown as described in Figure 1, ten days after the transfer, we measured the amount of soluble Pi. (A) average (± SD) shoot fresh weights of wild type (Col 0), (B), average (± SD) root fresh weights of wild type (Col 0), (C) Average (± SD) shoot fresh weights of mutant (gpt2), (B), a average (± SD) root fresh weights of mutant (gpt2).

Purely additive mechanism with regard to the gpt2 mutant, it is observed that, as for Col-0, the medium with low Pi reduces the growth of the shoot (Figure 2. C). On the other hand, the presence of *Pseudomonas fluorescens* stimulates the growth of the shoot. While we noticed that the roots of the mutant gpt2 had an increased concentration of dissolved phosphate when the bacteria were present in the col-0 roots, and this increase was also affected by the concentration of phosphate directly with the nutrient medium (Figure 2. D). this is due to the capability of *Pseudomonas fluorescens* to solubilize insoluble P via two possible mechanisms: proton extrusion by ammonium assimilation and production of organic acids [21].

3.3. Correlation analysis reveals a dichotomy between plant growth and accumulation of Pi

To determine whether there are relationships between free Pi content in plants and fresh weight gains, we set out to systematically look for both linear (Pearson) correlations in all of our data. The goal was to represent the data as a network in which each node is a measured variable and each link is the correlation. For this type of analysis to provide information, it is necessary to have both dependent and independent variables. These variables must be in large number (to constitute a network with enough nodes so that the figure is interpretable) and be easily measurable. These variables must also be
measured under a very large number of conditions, if possible contrasting. In our case, these conditions are distributed between discrete characters (one mutant and a wild type plant in the same genetic background, treatment or not by a microorganism), but also continuous characters (3 different diets of phosphate nutrition). All these conditions do not necessarily have very strong contrasting effects. The analysis that we carried out allows us to identify strong relationships between the content of Pi in the leaves and the fresh weight of the shoot (Figure 3. A) Whereas at the roots level and the fresh weight there is no significant correlation (Figure 3. B). each of these correlations is expected because they are variables that are partly dependent on each other. For example, it is logical to observe that the P deficiency will be even more stress on the plant. In addition, some of the relationships confirm observations reported in the literature. For example, it is widely described that, in a situation of Pi deficiency, the main root stops growing [22]. These expected relationships, or so known in the literature, can be considered as positive witnesses.

| Figure 3. Correlations between plant growth (fresh weight) and phosphate concentration in the plant. For the shoot part (A) and the root part (B). These variables were correlated with the phosphate concentration in the plant calculating the r of a Pearson correlation (linear correlation). To test the significance of each treatment, their p-value was also calculated. The calculations were performed using R using the Base package. |
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4. Conclusion

In conclusion, a medium with low Pi inhibits root and shoot growth while the presence of *Pseudomonas fluorescens* stimulates the shoot growth. The gpt2 gene plays no role in the shoot and root response.
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