#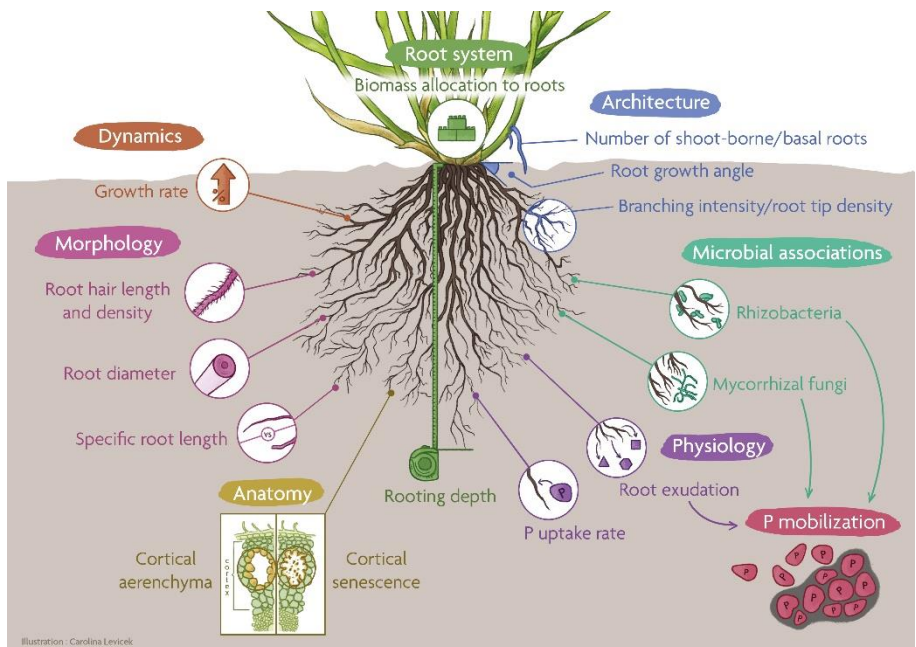 Developmental plasticity of *Brachypodium distachyon* (L.) P. Beauv. in response to P-solubilizing bacteria inoculation

Caroline BAUDSON

Illustration : Carolina Levicek

COMMUNAUTÉ FRANÇAISE DE BELGIQUE
UNIVERSITÉ DE LIÈGE – GEMBLOUX AGRO-BIO TECH

# Developmental plasticity of *Brachypodium distachyon* (L.) P. Beauv. in response to P-solubilizing bacteria inoculation

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Dissertation originale présentée en vue de l'obtention du grade de doctorat en sciences agronomiques et ingénierie biologique

Promoteurs : Pr Patrick du Jardin, Pr Pierre Delaplace
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# Abstract

**Introduction** Mineral phosphorus (P) fertilizers should be sparingly used to slow down the depletion of rock phosphate, a finite and non-renewable resource. Plants cope with stresses in their environment and heterogeneous soil conditions by adjusting their phenotype, what defines the phenotypic plasticity. The use of fertilizers in agriculture could be reduced by integrating plastic root system traits into crop breeding strategies. Bacterial bioinoculants are also considered as an interesting strategy to increase the nutrient use efficiency of plants and reach desired phenotypes. This project studied the impact of inoculation of the model plant *Brachypodium distachyon* with P solubilizing bacteria on the plant phosphorus use efficiency, focusing on enhancement of phosphorus bioavailability and modulation of the root system plasticity.

**Materials and methods** The first step of this project consisted in the characterization of the response of *B. distachyon* to inorganic P deficiency, in order to define levels of inorganic P resulting in contrasted plant phenotypes. Then the ability of selected bacterial strains to solubilize poorly available P forms (tricalcium phosphate and hydroxyapatite) was quantified. Finally, based on the results of the previous steps, the response of *B. distachyon* to contrasted P supplies and inoculation with P-solubilizing bacteria was studied by focusing on the plant developmental plasticity and P use efficiency. Allometry analyses were performed to study plasticity in the biomass allocation pattern and persistent homology analyses were conducted to detect differences in root system morphology.

**Results** A plastic response in *B. distachyon* biomass allocation pattern was observed, by prioritizing root over shoot development under poorly soluble P conditions. All the bacterial strains were able to solubilize tricalcium phosphate and hydroxyapatite in the solubilization test. However, inoculation of the plants with bacteria reduced the shoot productivity. On the other hand, the root system development was maintained. Both P condition and inoculation with bacteria impacted the root system morphology. P use efficiency in *B. distachyon* was not improved by the modulation of its developmental plasticity induced by the bacteria.

**Conclusion** The results support the hypothesis that P-solubilizing bacteria can modulate the plastic response of *B. distachyon* in response to limited P condition. The methods used to study the plant plasticity were useful and should be considered as potential tools to investigate the effects of bioinoculants on plant nutrient use efficiency. The experimental system can greatly impact plant-bacteria interaction. Experimental conditions as close as possible to agronomic ones are recommended to work with bacterial bioinoculants.

# Résumé

**Introduction** Les engrais minéraux phosphorés doivent être utilisés avec parcimonie afin de retarder l'épuisement des roches phosphatées, une resource limitée et non renouvelable. Les plantes font face à des stress dans leur environnement et à des conditions de sol hétérogènes en ajustant leur phénotype, ce qui définit la plasticité phénotypique. L'utilisation de fertilisants en agriculture pourrait être réduite en integrant des traits d'architecture racinaire plastiques dans les stratégies d'amélioration variétale. Les bioinoculants bactériens sont également considérés comme une stratégie intéressante pour améliorer l'efficience d'utilisation des nutriments des plantes et obtenir des phénotypes ciblés. Ce projet a étudié l'impact de l'inoculation de la plante modèle *Brachypodium distachyon* avec des bactéries solubilisant le phosphore sur l'efficience d'utilisation du phosphore, en s'intéressant particulièrement à l'augmentation de la disponibilité du phosphore et à la modulation de la plasticité du système racinaire.

**Matériel et méthodes** La première étape de ce projet a permis de caractériser la réponse de *Brachypodium* à la carence en phosphore inorganique, afin de définir des niveaux de phosphore inorganique engendrant des phénotypes contrastés chez la plante. Ensuite, la capacité de souches bactériennes à solubiliser des formes de phosphore peu disponibles (phosphate tricalcique et hydroxyapatite) a été quantifiée. Enfin, sur base des résultats des deux premières étapes, la réponse de *Brachypodium* à des apports contrastés en phosphore et à l'inoculation avec des bactéries solubilisant le phosphore a été étudiée, en se focalisant sur la plasticité développementale de la plante et l'efficience d'utilisation du phosphore. Des analyses allométriques ont été réalisées afin d'étudier la plasticité de l'allocation de biomasse et des analyses d'homologie persistente ont permis de détecter des différences de morphologie du système racinaire.

**Résultats** Une réponse plastique de l'allocation de biomasse chez la plante a été observée, en privilégiant le développement du compartiment racinaire au détriment de la tige en conditions de carence en phosphore soluble. Toutes les souches bactériennes testées se sont montrées capables de solubiliser le phosphate tricalcique et l'hydroxyapatite lors du test de solubilisation. Cependant, les plantes inoculées avec les bactéries ont montré une réduction de production du compartiment épigé. Le développement du système racinaire était quant à lui constant. La morphologie du système racinaire s'est montrée impactée par les conditions d'apport de phosphore ainsi que par l'inoculation de bactéries. L'efficience d'utilisation du phosphore n'a pas été augmentée par la modulation de la réponse plastique engendrée par les bactéries.

**Conclusion** Les résultats supportent l'hypothèse que les bactéries solubilisant le phosphore peuvent moduler la plasticité développementale de *Brachypodium* en réponse à la carence en phosphore. Les méthodes utilisées dans ce projet se sont

montrées adéquates pour l'étude de la plasticité développementale et devraient être envisagées comme outils potentiels dans l'étude de l'effet de bioinoculants sur l'efficience d'utilisation des nutriments. Les résultats montrent également que le dispositif expérimental peut avoir un impact important sur l'interaction plante-bactérie. Des conditions expérimentales les plus proches possibles des conditions agronomiques devraient être privilégiées dans les études avec des inoculants bactériens.

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En premier lieu, je tiens à remercier mes promoteurs Patrick du Jardin et Pierre Delaplace. Merci pour la confiance que vous m'avez accordée en me permettant d'occuper l'enrichissant poste d'assistante pédagogique. Monsieur du Jardin, vos enseignements que j'ai pu suivre dès la première année de bachelier étaient passionnants et ont sans aucun doute fait grandir mon intérêt pour les sciences des végétaux. Je sortais de vos cours en ayant le sentiment qu'une histoire m'avait été racontée, et en étant impatiente de connaître la suite. Pierre, merci pour ton aide et ton soutien exceptionnels. Tout au long de ma thèse, tu as été présent que ce soit pour des discussions scientifiques, de l'aide au laboratoire et pour l'enseignement, mais aussi pour des moments plus informels en équipe. Tu as joué un rôle très important dans la réalisation de ma thèse et dans la construction de liens forts au sein de l'équipe. Merci pour tout cela.

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# List of acronyms

ACC: 1-aminocyclopropane-1-carboxylic acid

AMF: arbuscular mycorrhizal fungi

ANOVA: analysis of variance

APase: acid phosphatase

Bd21: *Brachypodium distachyon* line Bd21

CFU: colony-forming unit

HA: hydroxyapatite

IAA: indole-3-acetic acid

L2LR: length of $2^{nd}$ order lateral roots

L2LR0-5 (5-10, 10-15, 15-20): length of $2^{nd}$ order lateral roots between 0-5 cm (5-10, 10-15, 15-20 cm) along the primary seminal root

LB: Luria-Bertani

LCNR: length of coleoptile nodal roots

N2LR: number of $2^{nd}$ order lateral roots

N2LR0-5 (5-10, 10-15, 15-20): number of $2^{nd}$ order lateral roots between 0-5 cm (5-10, 10-15, 15-20 cm) along the primary seminal root

NCNR: number of coleoptile nodal roots

NBRIP medium: National Botanical Research Institute's phosphate growth medium

P: phosphorus

PCA: principal components analysis

PERMANOVA: permutational multivariate analysis of variance

PGPR: plant growth-promoting rhizobacteria

Pi: inorganic phosphorus

PPFD: photosynthetic photon flux density

PPUE: physiological phophorus use efficiency

PSB: phosphate solubilizing bacteria

PSM: phosphate solubilizing microorganisms

PSR: phosphate starvation response

PSRL: primary seminal root length

PUE: phosphorus use efficiency

PUpE: phosphorus uptake efficiency

PUtE: phosphorus utilization efficiency

RDW: root dry weight

RMF: root mass fraction

Rpm: rotations per minute

RSA: root system architecture

SDW: shoot dry weight

SMA: standardized major axis

TCP: tricalcium phosphate

TDW: total dry weight

# Chapter 1

## Introduction

# 1.   P, an essential element for life

Phosphorus (P) is an essential element to every living cell. It is a structural element of cells, a constituent of nucleic acids carrying and translating genetic information (DNA and RNA) and also a component of phospholipids present in membranes. P has a role in energy transfer with energy-rich phosphate bonds (*i.e.* ATP) releasing and transferring the energy necessary to many biological processes (Cordell and White 2011; Hawkesford et al. 2011). Due to its major importance in biological processes, a limited P availability can have a high impact on ecosystem function and structure (Tiessen 2008). Compared to other elements, P is an abundant element in the environment with a poor availability to organisms regarding their stoichiometric requirement for it in weight concentration. Organisms adapted and developed mechanisms to acquire P from habitats with low P availability and to economize on P use in their metabolism (Raven 2008).

P is present in soil in inorganic and organic forms (Fig. 1-1). It is highly immobile due to its high reactivity and is mainly concentrated in the topsoil due to continuous deposition of plant residues on the soil surface, limited P leaching and great biotic activity in the topsoil (Lynch 2019; White et al. 2013). Unfortunately, the quantity of total P in a soil does not reflect the quantity of P available to the plant. Soil P reserves are poorly available to the plants due to the capacity of many soils to fix P (Havlin et al. 2013). Inorganic P (Pi), accounting for 35 to 70% of total phosphorus in soil, exists in different forms that constitute a complex equilibrium. Primary P minerals such as apatite are stable and release available P very slowly by weathering. Calcium, iron and aluminium phosphates are secondary P minerals that are formed by precipitation and can release P by dissolution at variable rates depending mainly on the soil pH and the size of the particles. P can be adsorbed on clays, aluminium and iron oxides under acidic soil conditions. Under neutral to alkaline soil conditions, P can adsorb on calcium carbonates and clays and can precipitate with calcium ions into dicalcium phosphate. Dicalcium phosphate can ultimately be transformed into more stable and less available forms to plants such as hydroxyapatite (Shen et al. 2011). Organic P exists in soils in forms such as inositol phosphates, phosphonates, orthophosphate mono- and diesters and organic polyphosphates. Microbes are major players in the cycling of insoluble inorganic and organic P forms (Prabhu et al. 2019). Available P can be released from organic P forms by mineralization processes mediated by roots and other soil organisms through phosphatase secretion (Shen et al. 2011).

Plant roots can take up P as $H_2PO_4^-$ or $HPO_4^{2-}$ (phosphate anions) from the soil solution (Shen et al. 2011). The concentration of phosphate anions in soil solution varies widely among soils, from $10^{-7}$ to $10^{-4}$ M  (Havlin et al. 2013) with an average of ~2 µM (Smith 2002). Phosphate ions in the soil solution are in equilibrium with ions sorbed onto minerals and colloids. Sorption reactions buffer the phosphate ions

concentration in the soil solution and maintain low concentrations of phosphate in the solution (Shen et al. 2011). Phosphate ions in the solution move to the uptake sites into roots through diffusion which is a slow process (~0.13 mm/day) (Havlin et al. 2013), creating depletion zones around plant roots in soils with low P availability (Smith 2002). A depletion shell of 0.2-1.0 mm rapidly forms around the root (Vance et al. 2003). The P concentration in soil solution required by the plant for optimal growth depends on the plant species and the expected level of production (Havlin et al. 2013). From an agronomic point of view, a sufficient available P level for plant growth is comprised between 4.6 and 7.5 mg P/100 g dry soil (for medium-textured soils at pH≥5.5, ammonium acetate-EDTA extraction method, Genot et al. 2011). The relatively low P concentration in soil solution (~2 µM phosphate anions in solution, Smith 2002) requires to continuously replenish the soil solution to supply the needs of the plants (~0.3% P in plant tissues) (Havlin et al. 2013).
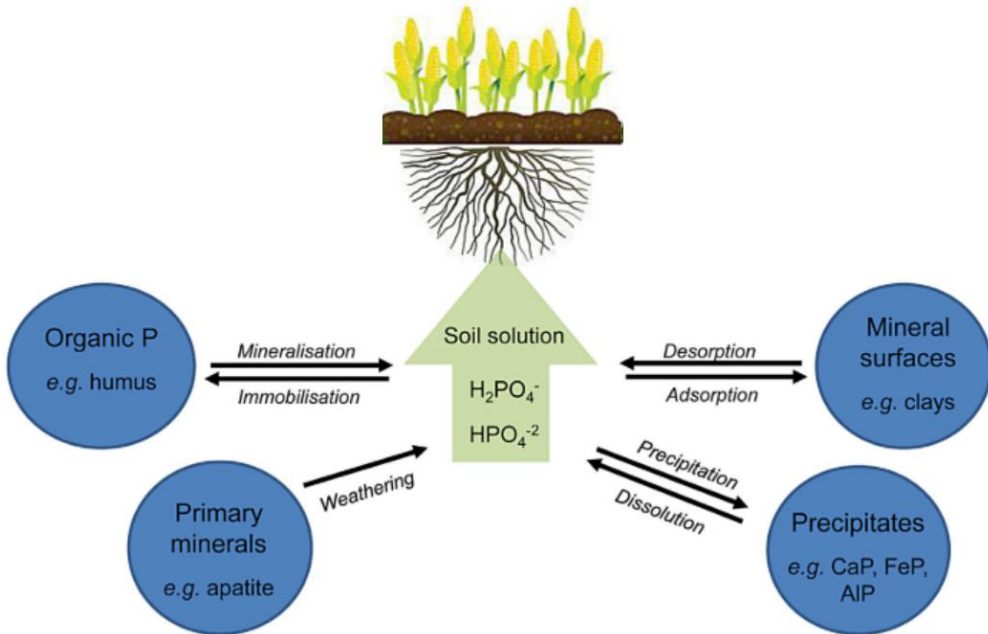


**Figure** 1-1: Phosphorus pools and dynamics in soil (modified from Owen et al. 2015).

Phosphorus cannot be substituted and is therefore a crucial input in agriculture (Steen 1998). Phosphate rock is a limited P source and has been intensively mined since the World War II (Cordell et al. 2009; Steen 1998). Similarly to other non-renewable resources (like oil), phosphate production from phosphate rock will reach a peak after which the quality and accessibility of phosphate rock resources will decline leading to an economically non-viable production and ending by total depletion (Cordell and White 2011). If the occurrence of a peak in primary production is not debated anymore in the scientific community, its timing is still uncertain.

Reserves of phosphorus are dynamic, evolving following technological development in the mining industry and in recycling processes. At some point, recycling will probably overtake the primary production (Wellmer and Scholz 2017). Recently, a massive deposit of phosphate rock was discovered in Norway. It is the world's largest listed deposit and offers to satisfy the world demand of phosphate rock for at least 50 years (Simon 2023). However, concerns about long-term phosphorus availability remain legitimate due to its limited, non-substitutable and non-renewable nature (Wellmer and Scholz 2017).

During the 20th century, the Green Revolution involved a steep increase in the input of mineral P fertilizers in European agricultural soils, while the access to fertilizers remained limited in developing countries. Disparities in application of fertilizers in the 20th century resulted in important agronomic P imbalances between areas where mineral P fertilizers and manure have been extensively applied and areas where the availability of P fertilizers is limited (Cordell et al. 2009). Macdonald et al. (2011) estimated that 29% of global cropland area had P deficits and 71% had P surpluses. While P deficit limits crop productivity, P surpluses may have environmental impact like eutrophication and degradation of water quality (Chislock et al. 2013; Dupas et al. 2015). It is hypothesized that the use of mineral P fertilizers also causes accumulation of cadmium and uranium in arable topsoils (Bigalke et al. 2017). On a global scale as well as at EU level, it was estimated that P inputs to croplands (mainly inorganic fertilizers and manure) exceeded the output (removal by harvesting crops), creating a surplus of P in soils (estimates for year 2000 by Macdonald et al. 2011; estimates for the period 2011-2019 by Panagos et al. 2022). van Dijk et al. (2016) estimated that in EU-27 in 2005, almost a half of imported P accumulated in agricultural soils via crop production and a half was lost as wastes from the different considered sectors (crop and animal production, food processing, non-food production and consumption). The annual accumulation of P in agricultural soils was estimated to be 4.9 kg P/ha in 2005 in the EU-27, with Belgium showing the highest accumulation rate of +23.2 kg P/ha (van Dijk et al. 2016). Until now, estimations of P budget of agricultural soils have not included P inputs via solubilization by microorganisms nor P contained in planted seeds, due to lack of data (Panagos et al. 2022). Agriculture is a major actor in P import and the dependency of Europe on mineral P (van Dijk et al. 2016; Ott and Rechberger 2012). However high soil P stocks resulting from long-term fertilization allow to maintain P uptake by crops and yield while reducing fertilization (Sattari et al. 2012). P stocks in agricultural soils can potentially be used in the future by reducing inputs to neutral or negative P balances, enhancing the use of residual P by the crops and avoiding losses (van Dijk et al. 2016; Ringeval et al. 2014; Sattari et al. 2012).The requirement for inorganic P fertilisers could be reduced by 50% if legacy soil P was included in nutrient management practices (Sattari et al. 2012).

When related to crop productivity, these P imbalances are translated into highly variable P use efficiency ("PUE", crop productivity per unit of P applied). An improved P-fertilizer use efficiency is essential to use P in a more sustainable way (Macdonald et al. 2011). In the sector of crop production, strategies to increase PUE encompass balanced fertilization (aligning supply and demand of P) (Withers et al. 2015), implementation of the 5R of plant nutrition (right timing, placement, type of fertilizer, application method and management) (Schröder et al. 2011; Withers et al. 2014) and crop selection for enhanced P uptake and utilization efficiency (Gaxiola et al. 2011; Rose et al. 2016).

Phosphate rock entered the list of critical raw materials for the European Union in 2014 and it is still listed in the most recent list published in 2020 (EC 2020). The main sourcing countries for Europe are Morocco, Russia and Finland. The European Union reliance on import of phosphate rock is estimated at 84% (import reliance = (import – export) / (domestic production + import – export)) (EC 2020). Nutrient balance in soils is tackled at European level with the EU Farm to Fork Strategy and Soil Strategy for 2030, setting an ambitious goal to reduce by at least 50% nutrient losses to the soils by 2030 while maintaining fertility and reducing by 20% fertilizer use. With these measures, the new European Green Deal aims at achieving a good soil health by 2050 (EC 2019), which is essential to meet the final goal to make the European Union a climate-neutral continent by 2050 (Montanarella and Panagos 2021).

## 2.   Plant plasticity

Plants respond to variations in environmental conditions by modifying their phenotype (Nicotra et al. 2010). This response capacity is called phenotypic plasticity and can take place at different levels such as physiology, anatomy and morphology. The plastic response of plants to varying environmental conditions may eventually result in enhanced plant survival and fitness (Lobet et al. 2019). However, under favourable environmental conditions, the costs for the construction and maintenance of sensory and regulatory mechanisms underlying plasticity can have a negative impact on plant performance (Dalal et al. 2017; Schneider and Lynch 2020). The cost of plasticity is defined as '*the reduction in the fitness of a genotype due to its phenotypic plasticity, as compared to fixed patterns of development that maintain homeostasis under stable conditions*' (Dalal et al. 2017). Phenotypic plasticity may also be maladaptive when environmental conditions fluctuate and there is a time lag between environmental cues and the expression of the plastic response (Schneider and Lynch 2020). Trade-offs among plastic responses exist under multiple stress conditions and may impair the plant's fitness as well. P has low mobility in soils and is present mainly in the topsoil due to the deposition of plant organic matter. In comparison with P, mobile resources like nitrate and water have a more vertical distribution in soils as they can quickly move to deeper soil layers. Therefore,

favouring traits that enable P acquisition may reduce the efficiency of plants in taking up nitrate and water (Lynch 2011). In case of multiple edaphic stresses, identifying a single phenotype that performs optimally across contrasting environments is unlikely (Rangarajan et al. 2018). However, suites of traits benefitting the acquisition of several nutrients (e.g., N, S, K, B and P) were identified and could be considered to obtain root ideotypes suitable for multiple environmental conditions (White et al. 2013).

For decades, breeding programmes have selected high-yielding varieties under constant optimal or targeted stress conditions. This strategy has resulted in reduced plasticity in crop species compared to wild ones (1.8-fold difference, among 11 species and a diversity of traits) (Des Marais et al. 2013). Cultivated genotypes, exhibiting more stable traits, may have greater susceptibility to varying or suboptimal conditions compared to more flexible wild-type genotypes (Dalal et al. 2017). Past selection also likely led to smaller root systems, enabling a reduction of the competition between crop root systems and consequently yield increases (Fradgley et al. 2020). However, in the current context, the need for crop cultivars that have sufficient productivity in low-input systems and reduced input requirements in high-input systems is emphasized (Lynch and Brown 2012). Phenotypic plasticity is an important component of plant root systems that needs to be further considered in order to achieve acceptable yields under varying conditions (Lobet et al. 2019; Reynolds et al. 2021). Root architectural plasticity was shown to be related to yield stability in response to drought and low phosphorus stress (Sandhu et al. 2016). It is also relevant for plant performance in the context of plant intra- and interspecific interactions (Yu et al. 2020; Zhang et al. 2020). Therefore, plant breeding strategies should seek 'robust' cultivars performing optimally in a broad range of suboptimal conditions and breeding programs are recovering interest in plant phenotypic plasticity.

Since plants capture only a small portion of the resources that are naturally present or applied to the soil, improving resource mobilization and acquisition should be the priority for crop selection (Lynch and Brown 2012). Selecting for root system architectural traits does not necessarily mean selecting for bigger root systems. In addition to modifying biomass distribution between shoots and roots, the ability to alter biomass distribution between root system organs is also an important characteristic that allows plants to better explore the soil environment and reach soil domains with the greatest availability of resources (Koevoets et al. 2016).

# 3.   Phosphorus use efficiency

One of the strategies to reduce the need for P fertilizers is breeding crops that acquire and/or use P more efficiently (Hammond et al. 2009). Performance indicators of the plant ability to utilize the nutrients present in their environment are used to compare species or cultivars and improve them through breeding. "Efficiency" can be defined in a general way as: '*The achievement of an intended outcome with a lowest possible input of costs*' (Reich et al. 2014). In the context of plant nutrition, the *input* is the nutrient of interest. From the agricultural point of view, the *intended outcome* is a desired yield of product. At the plant level, the nutrient use efficiency can be divided into two components: nutrient uptake efficiency and nutrient utilization efficiency (Reich et al. 2014).

The definition and terminology of P use efficiency (PUE) are not clearly established because many definitions of PUE as well as many acronyms exist and are used by authors (Rose and Wissuwa 2012). A common definition of PUE is the increase in yield or dry matter produced per unit of applied P fertilizer, also referred as agronomic P use efficiency (Hammond et al. 2009).

$$PUE = \frac{Yield_{high\,P} - Yield_{low\,P}}{\Delta P_{applied}} \quad \text{(g biomass g}^{-1}\text{ P fertilizer)}$$

The P use efficiency can be divided into P uptake efficiency (PUpE) corresponding to the tissue P content per unit of P applied and P utilization efficiency (PUtE) corresponding to the biomass produced by unit of tissue P content (Neto et al. 2016).

$$PUpE = \frac{[P] \times biomass}{P_{applied}} \text{(mg P g}^{-1}\text{ P fertilizer)}$$

$$PUtE = \frac{biomass}{[P] \times biomass} \text{(g biomass g}^{-1}\text{ P)}$$

The physiological P use efficiency (PPUE) can also be used as an indicator, defined as the yield or biomass produced divided by the tissue P concentration (Hammond et al. 2009; Neto et al. 2016).

$$PPUE = \frac{biomass}{[P]} \text{(g}^2 \text{ biomass g}^{-1}\text{ P)}$$

When characterizing plant performance, different cultivars can be classified as
- efficient/non efficient, depending on their ability to produce biomass under low P condition,
- and responsive/non responsive to applied P fertilizer, based on the response in biomass production to addition of P fertilizer (Baligar and Fageria 2015; Neto et al. 2016).

This classification allows to identify cultivars that are efficient and responsive to the applied P fertilizer, as done by Neto et al. (2016) with coffee cultivars (Fig. 1-2).
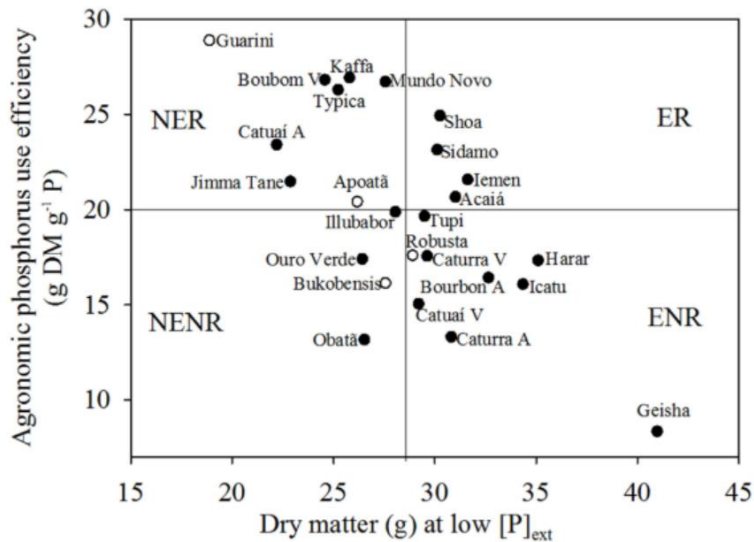
**Figure** 1-2: Classification of coffee cultivars into four groups according to the relationship between shoot dry matter at low P supply and the responsiveness to P applied measured as the agronomic P use efficiency. NER: non-efficient and responsive, ER: efficient and responsive, ENR: efficient and non-responsive, NENR: non-efficient and non-responsive (Neto et al. 2016).

# 4. Strategies of plants to cope with P deficiency

Phosphate anions are taken up by plant roots through a high-affinity system at low external P level and a low-affinity system at higher external P level. To be transferred from the apoplasm to the root symplasm, phosphate ions must pass from low concentrations in the micromolar range (~2 µM, Smith 2002) to higher concentrations (60-80 µM, as measured by Pratt et al. 2009). Active transporters are therefore necessary to take up phosphate ions against a sharp chemical potential gradient across the plasmalemma of root cells. Pi/H+ symporters, belonging to the Phosphate Transporter 1 (PHT1) gene family, operate this process (Shen et al. 2011).

Plants have developed a "phosphate starvation response" (PSR) system that regulates the external P uptake and the internal P use according to their nutritional requirements. The PSR is a complex array of morphological, physiological and biochemical/metabolic adaptations partly resulting from the coordinated induction of hundreds of phosphate starvation inducible genes (Paries and Gutjahr 2023; Plaxton and Tran 2011). Transcriptional regulators named Phosphate Starvation Response (PHR) play a central role in the PSR. Genes encoding high-affinity Pi transporters of the PHT1 family, purple acid phosphatases (a large class of plant acid phosphatases "APases", intra- and extracellular enzymes hydrolyzing phosphate from phosphate monoesters), SPX domain containing proteins (monitoring phosphate level within the plant cells) and microRNAs of the miR399 and miR827 family (targeting transcripts

of negative regulators of the PSR) are a major targets of the transcriptional regulator PHR1 (Paries and Gutjahr 2023).

One of the mechanisms of plants to face shortage of exogenous resources like P, is to allocate the production of new biomass to the organs that are involved in the acquisition of the scarcest resource. P deficiency affects carbohydrate partitioning between source and sink tissues, inducing an increase in root-to-shoot biomass ratio (Hermans et al. 2006). Level of gene expression and proteins involved in photosynthesis and sucrose synthesis is affected in plant exposed to P deficiency. The concentration of sugars and starch in leaves increases under P deficiency. Sugars play a regulatory and signalling function in carbohydrate partitioning in the plant. The leaf sugar concentration may involve transcriptional changes in P deficient plants (Hermans et al. 2006). An increase of sucrose concentration in shoot and changes in cytokinin, auxin and ethylene level in plant may be involved in the modulation of the development and biomass allocation in plants by regulating genes involved in photosynthesis and accelerating the export of sucrose from the leaves. However, a deeper understanding of these mechanisms is necessary (Hermans et al. 2006). The root system and its spatial arrangement are determinant for nutrient acquisition, plant interactions and nutrient cycling (York et al. 2013). Root traits can be linked to their functional utility, *i.e.*, resource acquisition or utilization (York et al. 2013), which are components of PUE (du Jardin 2020). Resource acquisition may be further explored by classifying the traits into two categories according to the foraging strategy: exploration of new soil domains and exploitation of the existing domains (York et al. 2013). A root strategy to enhance P acquisition comprises better exploration of soil P-rich domains and exploitation of these domains through P solubilization and uptake (Lynch 2019). Among the trait categories defined by McCormack et al. (2017), root dynamics, root system architecture ("RSA"), physiology, morphology, anatomy and microbial associations present interesting P-responsive traits (Fig. 1-3). Examples of the influence of the P context on root traits are given in Table 1-1.

Due to the poor mobility of P resulting in great spatial variation in P bioavailability, it can be argued that traits favouring soil exploration are probably of first importance in low input systems by enabling P interception by roots and locating plant exudates as well as microbial interactions in P-rich domains (Lynch 2019). Foraging in the topsoil is expected to be beneficial because bioavailability of P is generally greater in the topsoil. Exploration of the soil needs to be continuous to grow beyond the depletion zone in the rhizosphere (Lynch 2019). Trade-offs were identified among functional traits related to P-uptake strategies (Fig. 1-3). Root diameter is positively correlated to the release of P-mobilizing exudates in the rhizosphere and colonization by arbuscular mycorrhizal fungi ("AMF"), but negatively correlated to root branching intensity and specific root length in herbaceous plant species (Wen et al. 2019). Han et al. (2022) recently provided a different picture by showing that the greatest root

phosphatase activity in forest tree species was found in "do-it-yourself" species with a high specific root length and low mycorrhizal colonization rates.

In addition to adaptations of the root system, plants also have developed strategies to produce more dry matter per unit of P taken up and increase their internal PUE (Richardson et al. 2011; van de Wiel et al. 2016). P-efficient species produce biomass with low P concentrations by translocating P from metabolically inactive senescing sites to non-mature growing tissues. Internal phosphatases and high-affinity transporters are involved in the remobilisation of P from senescing tissues (Richardson et al. 2011; van de Wiel et al. 2016). Seed P content also is an interesting target to reduce crop P requirements and P content in animal waste streams but decreasing too drastically seed phytate levels can affect the seedling vigour (Richardson et al. 2011; Rose et al. 2013; van de Wiel et al. 2016). The plant metabolism is able to adapt for lower P requirement as well: phospholipids in membranes are replaced by sulfo- and/or galactolipids, the synthesis of P-free polysaccharides (such as cellulose) as cell wall constituents increases, triose-P (intermediates in the carbon-fixation cycle) are replaced by starch in plastids and sucrose in the cytosol, and the level of ribosomal RNA and in subsequent protein production is reduced at early leaf growth in the Proteaceae family (van de Wiel et al. 2016).

The PSR system is also implicated in the interaction of plants with microbes. Indeed, the PSR system regulates the plant ability to form symbioses with AMF and controls the root nodule endosymbiosis between legumes and nitrogen-fixing rhizobia bacteria. It was also demonstrated that the PSR system is regulated by association between the plant and endophytic fungi (Paries and Gutjahr 2023), bacteria and AMF (Saia et al. 2015; Sun et al. 2022). Finally, the plant response to detrimental microbes and the composition of the bacterial community in the rhizosphere are modulated by the plant immune system, which is partly governed by the PSR system (Paries and Gutjahr 2023).
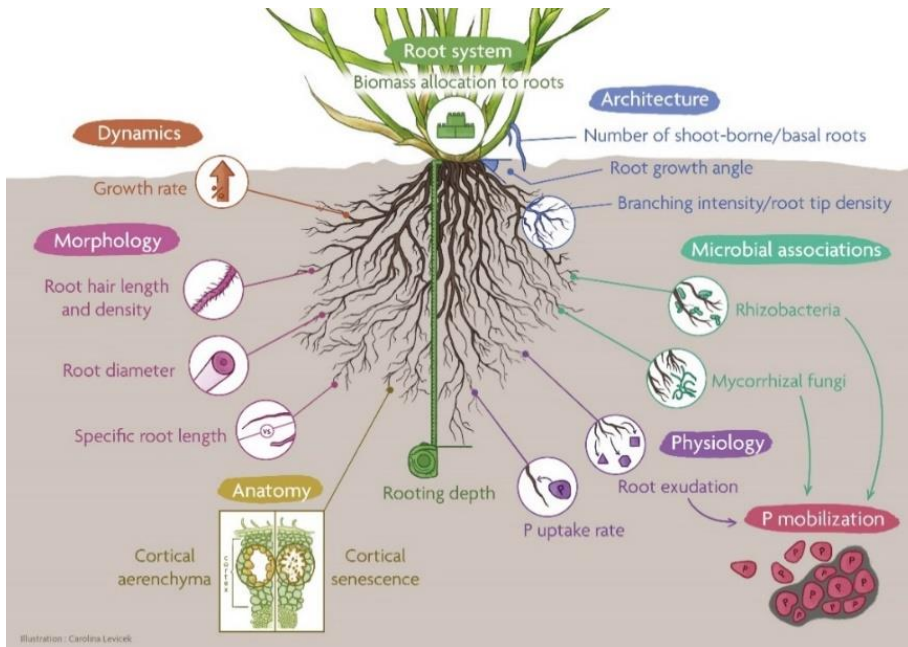
**Figure** 1-3: Root traits enhancing PUE, classified according to McCormack et al. 2017 (Baudson et al. 2023).

**Table** 1-1: Influence of P starvation and microbial context on root system traits enhancing PUE, classified according to their foraging strategy (Baudson et al. 2023)

| Trait | Foraging strategy | Influence of P starvation | Influence of microbial context |
|---|---|---|---|
| **Biomass allocation to the roots** | Exploration and exploitation | Higher root/shoot ratio in maize plants, indicating a more severe reduction of shoot growth than root growth (Mollier and Pellerin 1999) | Increase in biomass allocation to the roots in *Brachypodium* plants inoculated with bacterial strains (Baudson et al. 2021) or exposed to bacterial volatiles (Delaplace et al. 2015); greater biomass allocation to aboveground parts in inoculated switchgrass due to a lagged response of the root system to inoculation (Wang et al. 2015) |
| **Rooting depth** | Exploration | Inhibition of primary root growth in *Arabidopsis* (Q. Liu et al. 2013); reduced depth of the root system in *Brachypodium distachyon* accessions (Ingram et al. 2012); increase of seminal root length in maize (Zhu et al. 2006) | Increase of the length of the seminal roots in inoculated wheat under low and high P treatments (Talboys et al. 2014); increased root depth of wheat inoculated with PSB (Elhaissoufi et al. 2020) |
| **Phenology** | Exploration | Phenological delay in bolting and maturity in *Arabidopsis* due to a lengthened vegetative phase (Nord and Lynch 2008) | Increase of growth rate and shortened vegetative phase in inoculated *Arabidopsis* (Poupin et al. 2013); increase of growth rate in *Brachypodium distachyon* exposed to bacterial volatiles (Delaplace et al. 2015); increase of growth rate in inoculated wheat (Zaheer et al. 2019) |
| **Shoot-borne or basal roots** | Exploration | High number of basal root whorls and hypocotyl-borne roots in common bean | Precocious development of adventitious roots in *Brachypodium distachyon* |

| | | (Burridge et al. 2019; Miguel et al. 2013; Rangarajan et al. 2018; Walk et al. 2006) | exposed to bacterial volatiles (Delaplace et al. 2015); stimulation of adventitious root development in *Prunus* and hazelnut exposed to bacterial metabolites (Luziatelli et al. 2020) |
|---|---|---|---|
| **Branching intensity** | Exploration and exploitation | Increase in lateral root number and density in cotton (Zhang et al. 2021); slight reduction of root branching in maize (Mollier and Pellerin 1999) | Increase in production of lateral roots in inoculated wheat under low and high P treatments (Talboys 2014); promotion of lateral root formation in *Arabidopsis* exposed to bacterial volatile organic compounds (Bailly et al. 2014; Li et al. 2021) |
| **Root growth angle** | Exploration | More vertical lateral root orientation in *Arabidopsis* (Bai et al. 2013); greater biomass production and tissue P content in common bean genotypes with shallow basal root growth angle (Miguel et al. 2015) | No information found |
| **Root hairs** | Exploitation | Increase of biomass production in common bean with long and dense root hairs (Miguel et al. 2015); increase of length of root hairs in pasture species (Haling et al. 2016) | Increase of root hair number and length in inoculated *Arabidopsis thaliana* (Poupin et al. 2013; Spaepen et al. 2014; Sun et al. 2022); burst in root hair formation in inoculated *Dianthus caryophyllus* (Gang et al. 2018) |
| **Root diameter** | Exploration | Decrease of root diameter in barley (Heydari et al. 2019) and cotton plants (Zhang et al. 2021) | Increase of the average root diameter of switchgrass roots inoculated with a PGPR[a] strain (Wang et al. 2015) and |

| | | | wheat roots inoculated with PSB[b] (Elhaissoufi et al. 2020) |
|---|---|---|---|
| **Root etiolation (high specific root length)** | Exploration/exploitation | Increase of specific root length in *Trifolium* species (Becquer et al. 2021), three grassland forage species (Chippano et al. 2021) and cotton (Zhang et al. 2021) | Lower specific root length in *Arabidopsis* inoculated with a PGPR[a] strain (Wang et al. 2015); increase of specific root length in sugarcane inoculated with PSB[b] under low available P level (Safirzadeh et al. 2019) |
| **Cortical senescence and aerenchyma** | Exploration | Reduced metabolic cost of root development by increasing the rate of cortical senescence (Schneider and Lynch 2018) and the formation of aerenchyma in the root apex (Lynch and Ho 2005) | No information found |
| **Exudates** | Exploitation | Increase of organic anion exudation (mainly citrate and malate) in many plant species (Wang and Lambers 2020); high activity of acid phosphatase in the rhizosphere of field-grown wheat (Teng et al. 2013). | Contribution to/stimulation of the metabolic processes mobilizing P in the rhizosphere (acidification, organic anion and phosphatase release) (Richardson et al. 2009; Sun et al. 2022); stimulation of the exudation of sugars by wheat roots (Talboys et al. 2014); high turnover rates of exudates in soil due to consumption by microorganisms (Raymond et al. 2020) |
| **Number and activity of ion transporters in roots** | Exploitation | Up-regulation of the expression of high affinity P transporters under P deprivation in roots of rice (Jia et al. 2011), wheat (X. Liu et al. 2013) and *Medicago falcata* (Li et al. 2011) | Reduction of the P uptake rate in inoculated wheat under low P level, associated with a lower expression of P transporters (Talboys et al. 2014); inoculation of wheat with PGPR[a], AMF[c] |

| | | | or both up-regulated the expression of P transporters in a field experiment (Saia et al. 2015); upregulation of the expression P transporters in inoculated *Arabidopsis* (Sun et al. 2022) |
|---|---|---|---|
| **Root-associated microbiome** | Exploitation and exploration | Recruitment of specific microbial communities by the plants and up-regulation of the expression of P-solubilization traits in PSM[d] (Raymond et al. 2020); increase of AMF[c] colonization rate in field-grown wheat (Teng et al. 2013) | Enhancement of P acquisition from rock phosphate in onion crop inoculated with mycorrhiza helper bacteria in combination with an AMF[c] (Sangwan and Prasanna 2022); improvement of plant growth due to interaction between bacteria and an AMF[c] (Muñoz et al. 2021) |
| **Cluster roots** | Exploitation | Increase in the surface of the root system and the release of organic acids, protons and acid phosphatases into the rhizosphere due to the formation of cluster roots (Gerke 2015; Müller et al. 2015) | Indirect enhancement of cluster root production through stimulation of the root system growth by rhizobacteria and direct enhancement of cluster root production through bacterial production of IAA[e] (Lamont et al. 2014) |

[a]PGPR, plant growth-promoting rhizobacteria; [b]PSB, phosphate solubilizing bacteria; [c]AMF, arbuscular mycorrhizal fungi; [d]PSM, phosphate solubilizing microorganism; [e]IAA, indole-3-acetic acid

# 5.   Biostimulants

Rhizospheric traits, considering the root-soil-microorganism tripartite interaction, are not yet integrated into breeding programmes (de la Fuente Cantó et al. 2020; Trivedi et al. 2020). However, they are determinants of improved P acquisition efficiency, one of the highlighted strategies to obtain P efficient genotypes (Cong et al. 2020). The interaction of plants with their microbiome and beneficial rhizospheric microorganisms is gaining more interest (Compant et al. 2019; Wei and Jousset 2017) and should be seen as a way to obtain new phenotypes with increased fitness (Trivedi et al. 2020). The use of 'microbial biostimulants' products may help to reduce the input required to achieve an acceptable yield by increasing the bioavailability of nutrients in the soil and/or improving the plant nutrient use efficiency (du Jardin 2015).

A plant biostimulant is defined in Regulation (EU) 2019/1009 of the European Parliament and of the Council of 5 June 2019 based on claims that it is '*a fertilizing product the function of which is to stimulate plant nutrition processes independently of the product's nutrient content with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere: nutrient use efficiency, tolerance to abiotic stress, quality traits or availability of confined nutrients in the soil or the rhizosphere*' (EU 2019). Biostimulant products are composed of substances or microorganisms: humic and fulvic acids, protein hydrolysates, seaweed and plant extracts, biopolymers (*e.g.*, chitosan), inorganic compounds (*e.g.*, aluminium, cobalt, sodium, selenium and silicon), beneficial fungi and bacteria (*i.e.*, microbial biostimulants, bioinoculants) (du Jardin 2015). Biostimulant products aim to affect the plant's physiology rather than supplying nutrients or protecting the plants against pathogens or pests. They should be considered in the context of 'high-output low-input' agriculture (du Jardin 2015, 2020). Formulation of living organisms assisting the acquisition of nutrients by plants are termed biofertilizers (Schmidt and Gaudin 2018). The market of biofertilizers is expending with an expected annual growth rate of 12.2% from 2021 to 2031. Nitrogen fixing product dominate the biofertilizer market but phosphate mobilizing products also represent an important part of the market. For the forecast period 2021-2031, the demand for biofertilizers in cereals and grains production is anticipated to drive the market (Transparency Market Research, 2022).

As the root system exhibits plasticity in response to its biotic environment, modulation of the plant microbiome is of great interest to optimize plant production systems (Compant et al. 2019). Modulation of the plant microbiome can be achieved by inoculation of single strains or consortia as well as by agricultural management and plant selection (Compant et al. 2019; Hartman et al. 2018). The development of single strain inoculants usually starts with the screening of strain collections for beneficial functions like P solubilization, N fixation, plant hormones and 1-

aminocyclopropane-1-carboxylic acid ("ACC") deaminase production. Promising strains are then tested in (semi-)controlled conditions and finally in the field. Using this bottom-up approach, many performant strains in the lab fail to reproduce this success in the field (Compant et al. 2019). Limited success of inoculants in the field and low reproducibility can be explained by competition between well-adapted microorganisms of the receiving environment and the introduced bacteria. The extent to which such priority effects and their associated mechanisms (niche pre-emption and niche modification) modulate the assembly of soil microbial communities and determine the success of plant inoculation in the field certainly deserves more attention in future research (Debray et al. 2022; Fukami 2015). The ability of the strain to colonize the targeted plant species (rhizocompetence) and to exhibit the desired function in the environment is also important (Compant et al. 2019). The establishment of a lasting relationship between the host and the inoculated bacteria will depend on the ability of the bacteria to persist in the environment, to colonize the host and to be metabolically active (Charron-Lamoureux et al. 2020). Short exposure of plants to bacterial biostimulants might also result in positive outcomes through a priming effect (Cordovez et al. 2018). The assessment of bacterial population dynamics can be challenging, but it is essential to determine how to efficiently use bacterial inoculants in various environmental conditions. The presence of desired taxa and reactions can be assessed by using high-resolution tools (e.g. *in situ* sensors and omics analyses) measuring diagnostic molecules (e.g. exudates and volatiles) or microorganisms (Trivedi et al. 2020, supplementary information). Quantitative PCR can be used with specific primers to assess inoculant survival in the rhizosphere (Renoud et al. 2022), while next-generation sequencing techniques allow an in-depth characterization of the root-associated microbial diversity (Azarbad et al. 2022; Renoud et al. 2022).

By inoculating bacterial consortia, different mechanisms and desired traits can be combined. Strains with the same mode of action but tolerating different environmental conditions can also be co-inoculated (Compant et al. 2019). Synergistic effects were observed on PUE in wheat when rhizosphere and endophytic bacteria were inoculated in consortium (Emami et al. 2020). Based on plant-bacteria binary-association assays, Herrera Paredes et al. (2018) found that functional stacking within a bacterial consortium gives information on the effects of the consortium on the plant phenotypic response. The expression of phosphate starvation responsive genes and immune system-related genes was modulated by the bacterial synthetic communities and the effects of the bacteria were dependent on the nutritional status of the plant (Herrera Paredes et al. 2018). The construction of synthetic microbial communities (through culture and screening for beneficial traits or synthetic biology) and their use to increase plant fitness and productivity can now be translated into practice but have not yet been integrated into crop breeding (Trivedi et al. 2020).

Soil-plant-bacteria interactions are complex and the beneficial properties of the strains may be specific to plant species and soil properties. Therefore, isolating and characterizing native bacterial strains living in the rhizosphere of plants growing in a target environment constitutes an alternative to the use of non-native consortia to obtain competitive strains which are well adapted to local biotic and abiotic conditions (Majeed et al. 2015; Santoro et al. 2015; Zahid et al. 2015).

The success of a microbial biostimulant depends on several factors (Parnell et al. 2016). The most important factor is its ability to increase yield or to maintain it under adverse conditions. The efficacy challenges arise from the variable translation of efficacy in controlled conditions to success in the field (Nicot et al. 2011) and from scaling the production from laboratory to industrial bioreactors, which may bring issues of genetic instability of the strains (Takors 2012). A great versatility of the selected strains also determines the efficacy of the product to variable field conditions, these strains being able to interact with different hosts and adapt to the soil conditions and composition (Parnell et al. 2016). Then, the practicality of the product for the farmer is important. It should be easy to the growers to integrate bioinoculant products into their cropping systems (equipment and practices) (Parnell et al. 2016). The formulation should be appropriate to ensure that the microorganisms are delivered to the target plants and that they are robust enough to survive on roots or leaves. Formulation of bioinoculants is a crucial factor to improve the stability, the shelf life and the field performance of the microorganisms. This is particularly determinant for microbes that do not form spores or that are highly sensitive to humidity and temperature conditions. Targeted delivery with precise site and timing of application also helps to improve the efficacy and cost-effectiveness of bioinoculants (Parnell et al. 2016). The persistence of the microorganisms should be long enough to ensure compatibility to the target environment, a good occupation of the niche space and colonization before competition arises (Dini-Andreote and van Elsas 2013; Verbruggen et al. 2012). Microbial biostimulants facing some difficulties such as genetic instability and variable quality or high space requirements, the production costs are higher than for chemical products. These high production costs and variable return on investment for the farmers are a barrier to the commercial viability of microbial products (Nicot et al. 2011; Parnell et al. 2016).

Biofertilizers can be applied as solid or liquid formulations. They are inoculated to plants by seed treatment (for crops such as pulses, cereals, oilseed rape, grasses), cuttings treatment (flower crops, banana, sugarcane), seedling root treatment (vegetable, rice, flower crops, agro-forestry crops and other crops that must be transplanted in field) or soil application (almost all field crops and perennial crops) (Gautam et al. 2021). PSB are commonly applied as seed treatment or to the soil, in case seeds are treated with pesticides which may impair the bacteria development (Berde et al. 2021).

# 6.  Modulation of root system development by beneficial bacteria

Rhizospheric microorganisms may influence root traits that are determinant for the plant PUE, as described in Table 1-1. Numerous bacterial strains produce phytohormones, including auxins and cytokinins, as well as secondary metabolites that affect the auxin/cytokinin ratio and the ethylene level *in planta*. The auxin/cytokinin ratio is an important regulator of root system development (Vacheron et al. 2013). The stimulation of root development and branching by bacterial auxins increases the available root surface and the carbon supply for colonization by bacteria (Talboys et al. 2014). Bacteria-produced cyclodipeptides were shown to impact the root system architecture of *Arabidopsis thaliana* through modulating auxin-responsive gene expression in roots (Ortiz-Castro et al. 2019). Volatile organic compounds emitted by rhizobacteria were also found to alter root system morphology in different plant species (Delaplace et al. 2015; He et al. 2023; Sharifi and Ryu 2018). Most beneficial rhizobacteria produce the enzyme ACC deaminase, which degrades the precursor of ethylene ACC in plants. By lowering the ethylene level in plants, the bacterial ACC deaminase impacts the root system architecture (Vacheron et al. 2013) as ethylene level in plants modulate the formation and elongation of lateral roots as well as root hairs (Neumann 2016). Root hair density and length was significantly increased in inoculated plants under normal conditions and P deficiency (Sun et al. 2022). The modulation of plant growth rate and phenology by bacteria (Delaplace et al. 2015; Poupin et al. 2013; Zaheer et al. 2019) also impacts the root system development and plant nutrition (Vacheron et al. 2013). Stimulating root growth rate can improve soil exploration through increased root surface area, which can lead to increased acquisition of soil resources (Poupin et al. 2013). Ion uptake kinetics were shown to be modulated by bacteria-released auxin. Despite increased root production, expression of Pi transporters per unit root surface was reduced in inoculated wheat plants under low P conditions, which resulted in lower P uptake per unit of root surface area (Talboys et al. 2014). In contrast, other studies showed an upregulation of the expression level of Pi transporters of the family PHT1  (Saia et al. 2015; Sun et al. 2022), as well as the Pi transporter PHO1 and the microRNA399 in inoculated plants (Sun et al. 2022). These examples show how beneficial bacteria modulate root traits and trigger the plant responses to P limitation. The role of bacteria in the timing of the triggering of plant P responses is a point that could be investigated.

Beneficial bacteria also improve plant growth by impacting plant nutrition. This can be achieved by increasing nutrient availability in the root vicinity (P solubilization and mineralization) or enhancing the plant's nutrient acquisition processes (rhizosphere acidification, changes in root exudation) (Vacheron et al. 2013). Mineralization of organic P forms occurs through enzymatic hydrolysis. Non-specific APases constitute an important group of enzymes which dephosphorylate the

phosphor-ester or phosphoanhydride bonds in organic compounds. Phosphatases can either be acid or alkaline. Phytase is another enzyme involved in organic P mineralization. This enzyme releases P from organic materials in which P is stored in the form of phytate (plant seeds and pollen) (Alori et al. 2017; Richardson and Simpson 2011). Both plants and microorganisms can release phosphates from organic P forms but microorganisms are more efficient in this process (Richardson and Simpson 2011). An increase in root-associated APase activity was shown in plants inoculated with a bacterial strain (Sun et al. 2022). Solubilisation of P by microorganisms is mainly driven by lowering of pH (proton extrusion or production of organic acids releasing protons by dissociation) and/or releasing compounds that release P from soil adsorption sites (ligand exchange, metal chelation). Microorganisms produce organic acids like acetic, formic, lactic, gluconic, glycolic, 2-keto gluconic, oxalic, succinic, malic and citric acids. Gluconic acid is seen as the most important one for phosphate solubilization (Prabhu et al. 2019). 2-keto-gluconic acid, humic acid, and fulvic acid can solubilize inorganic P complexed with calcium, iron, and aluminium through chelation (Prabhu et al. 2019). Inorganic acids like sulfuric acid, nitric acid and carbonic acid, produced by nitrifying and sulfur-oxidizing bacteria, can also solubilize phosphate but less efficiently than organic acids (Prabhu et al. 2019). Indirect mobilisation of P can also occur through microbial respiration ($CO_2$ in soil water forms carbonic acid and reduces pH in the rhizosphere), reduction of P-bound metals resulting in more soluble phosphate, nitrogen assimilation (extruded protons following assimilation of ammonium lower the pH in the rhizosphere), assimilation of P from the media (dissolution of P compounds to maintain the solution P equilibrium, sink theory) (Bashan, Kamnev and de-Bashan 2013b; Owen et al. 2015). Exopolysaccharides (EPS), produced by microorganisms in response to stress, can also solubilize phosphate from metal phosphate forms through their ability to bind metals in soils (Prabhu et al. 2019). Mechanisms of inorganic and organic P solubilization are summarized in Fig. 1-4. Soil bacteria genera reported for their ability to mobilize P through solubilization and mineralisation include *Pseudomonas*, *Agrobacterium*, *Bacillus*, *Azotobacter*, *Azospirillum*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Kushneria*, *Paenibacillus*, *Ralstonia*, *Rhizobium*, *Rhodococcus*, *Serratia*, *Bradyrhizobium*, *Salmonella*, *Sinomonas* and *Thiobacillus* (Alori et al. 2017; Berde et al. 2021).
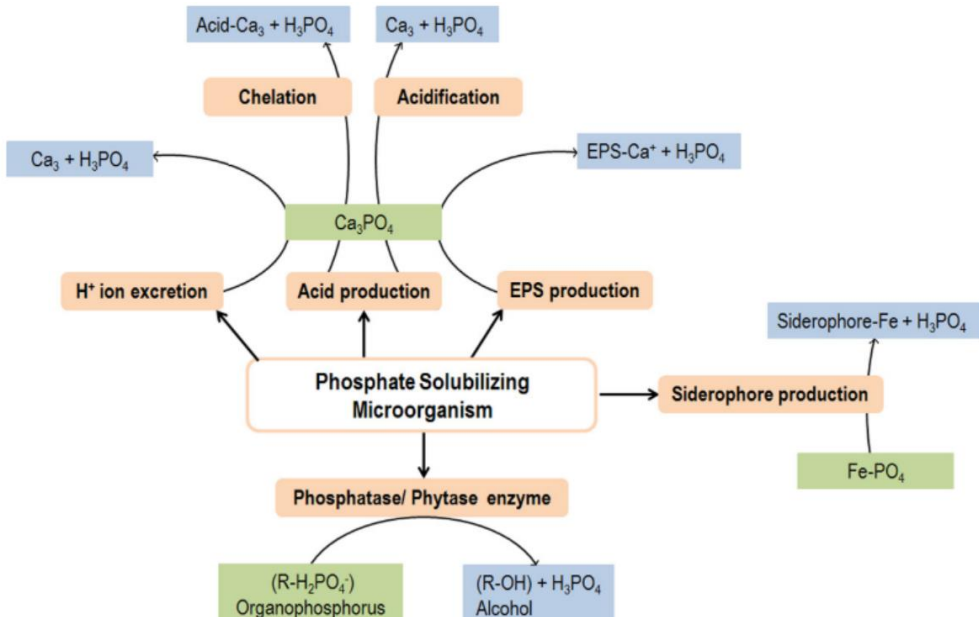
**Figure** 1-4: Mechanisms of organic and inorganic P solubilization by microorganisms (Prabhu et al. 2019).

There is little evidence to suggest that phosphate solubilizing microorganisms (PSM; bacteria and fungi, AMF excluded) solubilize sufficient P to meet plants' needs under field conditions. PSM can however show positive effects on the plant's response to P-limiting conditions through other mechanisms that impact root system development. The turnover of P in microbial biomass is more likely to provide P to plants over a long time (Raymond et al. 2020) yet the recently discovered plant-fungus symbiosis referred to as 'feremycorrhiza' (non-root-colonizing fungi benefitting plant growth through rhizosphere modification and nutrients mobilization) offers promise for more efficient P solubilization (Kariman et al. 2020). Mycorrhizal fungi make an important contribution to plant P nutrition by solubilizing P and enhancing soil exploration through their hyphae (Chippano et al. 2021; Richardson et al. 2011). AMF are obligate symbionts which associate to 80% of land plant species through mutualistic symbioses. The host plant provides carbohydrates to the AMF, which provides water and nutrients in return (Berruti et al. 2016). The mycelium emerging from the host root system explore soil volumes that are inaccessible to the roots (Smith et al. 2000) and can therefore alleviate the plant growth limitation due to nutrients deficiency (Nouri et al. 2014). AMF can improve plant P acquisition in low-P conditions but high inorganic P levels due to fertilization suppresses AMF colonization. Decreasing the applied P doses and cultivation with AMF-friendly techniques (*i.e.* cover cropping and conservation tillage) will allow the AMF community to recover and persist (Berruti et al. 2016). However the importance of

plant association with AMF in the plant strategies to cope with P deficiency is variable. Indeed, if association with AMF is the main strategy of some plant species, others invest more in their ability to explore the soil by developing large root systems and acquire P (Chippano et al. 2021; Han et al. 2022; Wen et al. 2019).

# 7. Characterizing root system plasticity

## 7.1. Environmental conditions

Plant phenotyping can be challenging, especially when focusing on the root system which is not easily accessible. Considering that soil is a complex and heterogeneous matrix where many interactions occur, it is useful to work with simplified systems to improve our understanding of rhizosphere processes (Baudson et al. 2021; Rich and Watt 2013). However, the transposability of results from the lab to the field depends on the realism of the growing conditions used to perform the experiments. Arguments for a reversed lab-to-field pipeline arise as discrepancies between lab and field studies are often reported, as well as poor predictability of the outcome of field studies from greenhouse studies (Schmidt and Gaudin 2018).

Regarding bacteria, an artificial system may lead to a starvation of the bacteria in carbon sources, reducing the P-solubilizing activity (Nico et al. 2012). A closed system can also be subjected to an accumulation of metabolites reaching toxic levels (Rybakova et al. 2016). The substrate structure can also influence the ability of the bacteria to survive and maintain in the root vicinity through impacting the root development (Saleem et al. 2018; Watt et al. 2003). On the plant's side, it is of general knowledge that the structural properties of the substrate shape the root system (Rich and Watt 2013), which in turn impacts the root microbiome (Saleem et al. 2018). Root hairs showed contrasted development profiles under field, rhizoboxes, flooded field and nutrient solution conditions (Nestler et al. 2016). Hydroponic and agar plates systems can be far from the real growing conditions, depending on the species of interest. Any growing system that makes roots enter in contact with a physical barrier (like the border of the growth container) significantly changes the root system architecture and its dynamics (Lynch and Brown 2012). It can therefore also be expected that the plant's plasticity in response to its environment is modulated by the growing substrate. Artificial controlled conditions used in first instance should be part of a multi-tier approach. The first step can be performed in a high-throughput manner, enabling to discard candidates leading to neutral or negative interactions. Testing on further cycles and stages can then be performed in more realistic conditions, ending by field trials (Negin and Moshelion 2017). A similar multi-tier approach was followed by Nguyen et al. (2019), testing three bacterial strains on wheat grown under contrasting N fertilization conditions.

The treatment conditions should ideally represent the environmental conditions the crop is expected to face (Negin and Moshelion 2017). However, in the attempt to

characterizing phenotypic plasticity, using a wide range of environmental conditions allows to avoid the under/overestimation of the trait plasticity. Indeed, in case the reaction norm (*i.e.* the shape or specific form of the phenotypic response to the environment of an individual or genotype (Arnold et al. 2019) is non-linear, using only low- and high-level of environmental condition could lead to the misrepresentation of the plasticity (Fig. 1-5). The whole shape of the reaction norm is better estimated by using at least three levels of condition and ideally as much as it is possible to manage (Arnold et al. 2019).
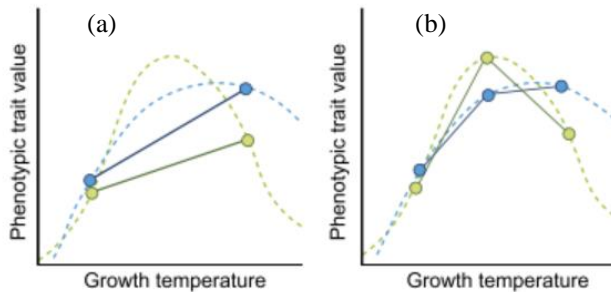


**Figure** 1-5: Examples of nonlinear reaction norms (dashed lines) in response to growth temperature. (a) Using only two levels of environmental conditions to describe reactions norms (solid lines and points) leads to loss of fundamental and biologically meaningful information regarding the underlying reaction norm shape (dashed lines). (b) The underlying reaction norm shape is better captured by adding just one level of environmental condition (adapted from Arnold 2019).

## 7.2. *Quantifying phenotypic plasticity*

The responsiveness to changes in the growing conditions can be studied following different approaches. The plasticity can be addressed using a quantitative estimator of the variation of a single trait between untreated plants and bioinoculant-treated plants both under stressful and optimal conditions. Many estimators exist and were used, all having advantages and weaknesses according to the context (Dalal et al. 2017; Valladares et al. 2006). We can cite, for the most commonly used, the slope of the norm of reaction, phenotypic plasticity index, coefficient of variation, relative distances plasticity index (Valladares et al. 2006). Among them, coefficient of variation (calculated as the standard deviation/mean, Valladares et al. 2006) was and is still widely used (Dalal et al. 2017). One of its advantages is that it is standardized and allows to compare different traits for a same plant genotype (Valladares et al. 2006). Arnold et al. (2019) propose to implement the use of "random regression mixed models" (RRMMs). These models can model any shape of reaction norm in response to a changing environment, at the population and individual level. Linear or non-linear functions can be used (Arnold et al. 2019). A new integrative index to quantify

phenotypic plasticity was proposed by Pennacchi et al. (2021). This multivariate plasticity index allows to characterize the plant phenotypic plasticity as a complex and integrative property of the plant.

For some traits, the quantification of the plasticity may be biased by ontogenetic effects. Indeed, numerous traits show an allometric response to environmental change, their response being related to a change in growth rate (Valladares et al. 2006). For such traits, the measured plasticity may be qualified as "apparent plasticity", as the variation in the trait is mostly due to a change in growth rate, resulting in a change in the plant size (Weiner 2004). Traits exhibiting "true plasticity" show a direct response to environmental variance (De Kroon et al. 2005). To encounter for ontogenetic drift in the analysis of plasticity, it can be necessary to compare individuals of similar size by sampling at several time points. Biomass allocation is acknowledged to be an ontogenetic process. Poorter and Sack (2012) have described different approaches that may be used to study biomass allocation. Allometric analyses, *i.e.* the log-regression of two traits plotted against each other, allow to observe how a trait changes relative to the other and at which trait value the change occurs. Plotting the considered trait against the plant size is quite a direct manner to assess the ontogenetic drift in a plant developmental response. Different statistical methods (standardized major axis, "SMA", and ordinary linear regression, "OLS", for instance) exist to perform allometric studies. Inference analyses about the regression coefficients (slope and intercept in case of a linear relationship) are performed to distinguish between apparent and true plasticity, true plasticity being translated into significant change in the allometric trajectory (Fig. 1-6) (Warton et al. 2006). Allometric analyses can also be applied to root architecture traits versus root biomass *e.g.*, to unravel how the plant invests its below-ground biomass under contrasted conditions (Hanslin et al. 2019).

The classical approaches for studying phenotypic traits of root system architecture consider geometric traits (*e.g.* total root length, number of roots) individually. Combining numerous traits to increase the visibility of the overall morphological variation can be complex and time consuming. Topological analyses (referring to the connectedness of structures) can be applied to plants for assessing their branching of above- and below-ground structures. These analyses are based on a skeletal description of the root system and allow to characterize the shape of the root system independently of the geometric traits that shaped the root system (Bucksch et al. 2017). Li et al. (2017) proposed to apply persistent homology to study the topology of plant branching structures like the root system. Multivariate statistical analyses can then be used to visualize how the root systems are topologically different (Delory et al. 2018). This approach offers the opportunity to detect overall morphological differences among branching root systems, which can be related to differences in soil exploration capability. The root system architecture can then be deeper investigated by considering individual traits to unravel which traits differ among the treatments.
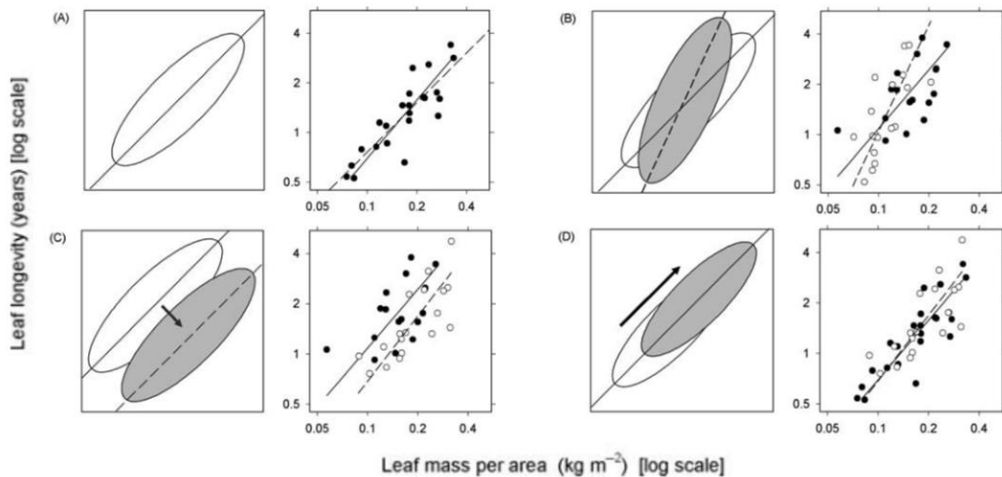
**Figure** 1-6: Inference tests about the regression coefficients to distinguish between apparent and true plasticity. (A) Fitting a line for a single group of observations; comparing lines of two groups of observations and testing (B) if the lines have a common slope, (C) if the lines have a common elevation and (D) testing for no shift along lines of common slope (Warton et al. 2006).

# 8. *Brachypodium distachyon*, a model plant

Phenotyping the adult root system of crop species is challenging due to their large size. The use of model plant species facilitates the phenotyping of root systems thanks to their small size. During the last 30 years, most genetic information about roots came from *Arabidopsis thaliana*, a dicotyledon and member of the Brassicaceae family (Watt et al. 2009). However, humanity relies on grasses for nutrition (directly with cereals and indirectly via animal feed) but also increasingly as a renewable energy source. Given their importance, grasses became a focus point of research to improve agronomy and breeding but the size and complexity of their genomes raise barriers to the analysis of traits at the molecular level. *Brachypodium distachyon* (L.) P. Beauv. was proposed to fill this gap (Draper et al. 2001) and has become a grass model system, counterpart of *Arabidopsis thaliana* (Vain 2011). The genus *Brachypodium* rapidly became a model for understanding grass traits including cell wall biology, vernalization, evolutionary biology, root biology, host-microbe/microbiota interactions and responses to abiotic stresses (Scholthof et al. 2018).

*Brachypodium* is a member of the grass subfamily Pooideae and has an intermediate position between Triticeae (including wheat and barley) and Ehrhartoideae (including rice). It can be used as a functional model for other grasses thanks to the high conservativeness of most gene families between *Brachypodium*, rice, wheat, barley

and sorghum (Vain 2011). *Brachypodium* also shares biological characteristics with energy, turf and forage grasses (Opanowicz et al. 2008).

*Brachypodium distachyon* (common name purple false brome) originates from countries around the Mediterranean basin, the Middle East, south-west Asia and north-east Africa. It is now also naturalized in temperate regions worldwide (Mur et al. 2011, Kellogg 2015). The main interesting characteristics of *B. distachyon* include:

- a small size (20 cm),
- a compact genome (272 Mb),
- simple growth requirements,
- short life cycle (2-3 months),
- self-fertility,
- available resources (complete genome sequence, T-DNA lines, recombinant inbred lines,…) and bioinformatics tools,
- ease of transformation,
- and great natural variation in biological traits (Bevan et al. 2010; Opanowicz et al. 2008; Vain 2011).

The natural variation observed in wild *B. distachyon* accessions is useful to reveal interesting root traits that were lost during the domestication process of cereals. Some of these traits may be helpful to cope with suboptimal growing conditions (Chochois et al. 2012). Natural variability in root morphology and physiology results in different responses to nutrient deficiency among *B. distachyon* accessions (Scholthof et al. 2018). A gene expression atlas, mapping gene expression in different organs and at different growth stages was completed. Another gene expression atlas project is ongoing, which is dedicated to gene expression responses occurring during pathogenic and beneficial *Brachypodium*-microbe interactions (Scholthof et al. 2018).

The root system of *B. distachyon* has many interesting features for research on cereals (Fig. 1-7; root nomenclature according to Freschet et al. 2021). *B. distachyon* produces only one seminal root from the base of its embryo after germination (referred after as the primary seminal root), like rice and maize. On the other hand, wheat and other small grain temperate cereals can produce up to six seminal roots (Watt et al. 2009, Chochois et al. 2012). *B. distachyon* forms then two coleoptile nodal roots developing above the seeds and successive leaf nodal roots (Chochois et al. 2012). Unlike wheat, maize and sorghum, *B. distachyon* does not produce scutellar nodal roots. Regarding the number of seminal roots, the seedling root system of *B. distachyon* appears to be simpler than that of wheat and other small grain temperate cereals. At adult phase, however, the development of leaf nodal roots in *B. distachyon* is similar to that of wheat (Chochois et al. 2012). The coordination between root and shoot development in *B. distachyon* is identical to that in wheat (Watt et al. 2009). *B. distachyon* and wheat roots share a similar anatomy and comparable environmental and nutritional requirements (Watt et al. 2009, Chochois et al. 2012). Root exudates

and rhizosphere microbiota of *B. distachyon* are also comparable to those of wheat (Kawasaki et al. 2016). *B. distachyon* allows to study interaction with AMF, unlike *Arabidopsis* which cannot form association with AMF (Scholthof et al. 2018).
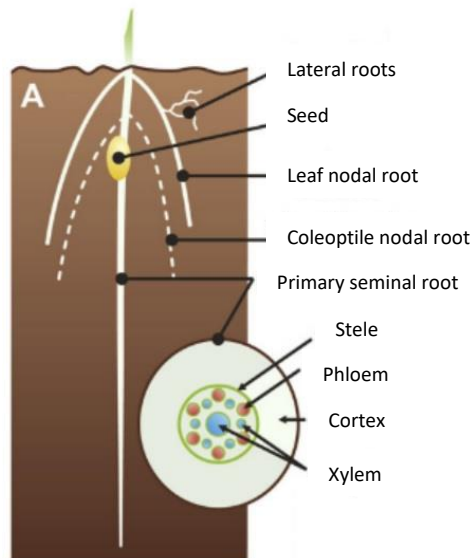


**Figure** 1-7: Main root types and root anatomy of *Brachypodium distachyon* (modified from Chochois et al. 2012; root nomenclature according to Freschet et al. 2021).

Although wheat adult root systems can reach as deep as 1.6 m (Chochois et al. 2012), the small stature of *B. distachyon* makes it possible to work on adult root systems in pots of reasonable size under controlled conditions (Fig. 1-8). Mature root system architecture and function are determinant for yield as they condition water uptake and grain filling (Watt et al. 2009). Containers allowing full growth of *B. distachyon* can be 100 times smaller in volume than containers used to grow wheat. Experiments can be three times shorter. Phenotyping of *B. distachyon* can therefore be less resource and space consuming compared to phenotyping of wheat (Chochois et al. 2012).

**Figure** 1-8: Shoot and root systems of 5-leaf stage wheat (*Triticum aestivum* cv. Janz) (left) and *Brachypodium distachyon* (line Bd21) (right) grown in 50-cm deep tubes with a mixture of sand and soil (Watt et al. 2009). Bar = 10 cm.

# Chapter 2

## Objectives of the thesis

Phosphorus is a major element for plant growth. The most used P fertilizers in agriculture contain inorganic P extracted from phosphate rock, which is a non-renewable resource. P is generally poorly available for plant uptake due to its high reactivity with soil ions and particles, even where it is abundant (Chapter 1, Section 1). To counteract these fixation phenomena in soils, P fertilizers have often been applied excessively, leading to environmental problems in some parts of the world whereas P is lacking in poor countries (Cordell et al. 2009) (Chapter 1, Section 1).

These concerns lead scientists to search for strategies allowing a better PUE in agriculture. This implies to develop ways to exploit the P reserves present in the soils that are not directly available to plants. Plants have developed such strategies in response to P starvation, like expanded root systems, organic anions secretion to solubilize inorganic P and phosphatase secretion to mineralize organic P forms (Chapter 1, Section 4). Nevertheless, such traits are generally not sufficient for crops to face severe P starvation. Interactions with soil microorganisms appear to be interesting in that respect. Microbial associations of the root system are also an important component of the plant plastic response to P deficiency. The root system exhibits plasticity in response to its biotic environment and modulation of the plant microbiome is of great interest to optimize plant production systems (Compant et al. 2019) (Chapter 1, Section 5). The root microbiome can be modulated by inoculating microorganisms. Biofertilizers, *i.e.* microbial products able to improve the nutrient acquisition by plants through several mechanisms (Chapter 1, Section 6), are of particular interest in the context of fertilization management. Bacteria are known to affect plant P nutrition through various mechanisms including improvement of P availability and modulation of plant growth (Pii et al. 2015).

This thesis project followed a research project aiming at studying the response of the root system architecture of *Brachypodium distachyon* Bd21 to volatile compounds emitted by rhizobacteria (Delaplace et al. 2015). Some bacterial strains significantly promoted the root system growth of *Brachypodium* seedlings in an *in vitro* co-cultivation system after 10 days of exposure to the bacterial volatiles. These results initiated a reflexion about the interest of root system growth promotion at seedling establishment for the plant nutrition.

In this context, this research project aimed at studying the effect of inoculation of rhizobacteria on the root system plasticity of the model grass *Brachypodium distachyon* in response to P deficiency. The PhD project was initiated in the end of 2012 and continued until 2019. The experiments presented in Chapters 3 to 5 took place between 2014 and 2019. At the time of the project initiation, published data regarding P nutrition of *Brachypodium* and its interaction with beneficial rhizospheric bacteria were poor. Three questions were defined to meet the general objective of this thesis.

**Question 1. How does *Brachypodium distachyon* Bd21 respond to inorganic P deficiency (Chapter 3)?**

Underlying questions:

- What are the Pi concentrations leading to contrasted plant phenotypes?
- Which Pi concentrations should be used later when plants will be grown in interaction with bacteria?

At the beginning of this thesis project in 2012, few data regarding the response of *Brachypodium* to P deficiency were available. The first report of *Brachypodium* root system response to limited Pi availability was published in 2012 (Ingram et al. 2012). They characterized the root system architecture of two *Brachypodium* accessions (Bd21 and Bd3-1) under contrasted Pi, after 19 days of cultivation in a jellified medium.

For this project, *Brachypodium* was grown during 30 days in PVC tubes filled with sterilized and washed river sand placed in a growth chamber. When studying the root system architecture, it is expected that the chosen growing medium has a great impact on the outcoming results (Rich and Watt 2013). The growing medium should also enable an easy adaptation of the nutritional conditions. For these reasons, washed river sand was chosen as a substrate to impose mechanical resistance to the growing root system and mimic realistic conditions. Plants were watered with modified Hoagland solution (Hoagland and Arnon 1950) containing contrasted soluble Pi concentrations as $KH_2PO_4$. The following parameters were measured:

- biomass production and allocation,
- total P content in shoots and roots,
- intracellular acid phosphatase activity in shoot and root,
- root system architecture (primary seminal root length, number and length of coleoptile nodal roots, number and length of second order lateral roots with repartition along the primary seminal root).

**Question 2. How do the selected bacterial strains solubilize poorly available P sources (Chapter 4)?**

Four bacterial strains were selected based on literature for their potential plant growth promotion and P solubilization properties, to be used in the next step of this thesis: *Bacillus velezensis* GB03, *B. velezensis* FZB42, *Pseudomonas fluorescens* 29ARP, and *Azotobacter vinelandii* F0819. *Escherichia coli* DH5α 99B829, was selected as a negative control for plant growth promotion (Delaplace et al. 2015).

This question was tackled by performing *in vitro* solubilization assays in liquid medium with two poorly soluble inorganic P forms. Two forms of calcium phosphate were selected: tricalcium phosphate (TCP) which is the most used P form in such studies but is easily solubilized, and hydroxyapatite (HA) which is a less soluble calcium phosphate form (Bashan et al. 2013a). The bacteria were inoculated in NBRIP

liquid medium (Nautiyal 1999) containing either TCP or HA as Pi source and incubated for 3 days. Soluble P content and pH of the solutions were measured.

**Question 3. How is the developmental plasticity of *Brachypodium distachyon* Bd21 modulated by inoculation with P-solubilizing bacteria (Chapter 5)?**

The first two questions enabled to choose suitable P conditions and confirmed that the selected strains were able to solubilize TCP and HA in an *in vitro* experiment. The impact of bacterial strains inoculation on *Brachypodium* response to contrasted P conditions was studied in co-cultivation experiments under gnotobiotic conditions (*i.e.* including the plant and selected bacterial strains only, avoiding contaminations). The plants were inoculated with single bacterial strains and cultivated for 4 weeks in Magenta® boxes containing quartz gravel, under contrasted P conditions applied as modified Hoagland solutions (Hoagland and Arnon 1950). The P conditions were:

- low level of soluble Pi,
- low level of soluble Pi complemented with TCP,
- low level of soluble Pi complemented with HA,
- high level of soluble Pi.

The following parameters were studied:

- biomass allocation (allometry analyses and root mass fraction approaches were considered),
- morphology of the root system (geometric and topological descriptors were measured),
- shoot P content,
- P use efficiency (uptake, utilization and physiological P use efficiencies were determined).

Eventually, a broad discussion encompassing the three questions of this thesis is proposed in Chapter 6 and Chapter 7 offers a general conclusion.

# Chapter 3

How does *Brachypodium distachyon* Bd21 respond to inorganic P deficiency?

# 1. Foreword

This chapter aims at studying how *Brachypodium distachyon* Bd21 respond to Pi deficiency. Particularly, concentrations of Pi inducing contrasted phenotypes in *B. distachyon* Bd21 will be determined. The results will allow to select the Pi concentrations to be used for the experiments described in Chapter 5.

# 2. Introduction

Grasses are crucial to Humanity as they are a primary source of human food. The top four agricultural products are sugarcane, maize, rice and wheat, all being grass crops (Bevan et al. 2010). At global scale, P limitation causes a yield gap of 22, 55 and 26% in winter wheat, maize and rice production, respectively. The soil legacy in areas with high historical P fertilization results in potential P supply higher than P demand (Kvakic et al. 2018). However, other parts of the world areas face P limitation and need P input to increase yields, due to high sorption capacity of the soils and past low to negative P balance (Ringeval et al. 2017). With the growing population and the concern of sustainability in crop production, higher crop yields have to be reached with fewer inputs (Bevan et al. 2010). *Arabidopsis thaliana* remained the principal model plant in plant biology for decades (Bevan and Walsh 2005). However, given the importance of grass crops in agricultural production and the differences in development between grasses and dicotyledonous species, a model cereal plant was necessary. *Brachypodium distachyon* has interesting characteristics that raised it as grass model species (Draper et al. 2001).

In order to be able to study the impact of bacterial bioinoculants on *Brachypodium*'s plastic response to P deficiency, adequate P levels leading to contrasted plant phenotypes needed to be defined. At the time of this thesis start, few data regarding *Brachypodium*'s response to P deficiency were available. Ingram et al. (2012) characterized the root system architecture of two *B. distachyon* genotypes (Bd21 and Bd3-1) under nitrogen and phosphorus high- and low-levels. Plants were grown for 19 days in a gellan gum nutrient solution containing 2 mM or 1 µM Pi. They observed that the root system architecture of both accessions was responsive to Pi deficiency but Bd21 showed more modulated traits than Bd3-1 (Ingram et al. 2012). Further investigation of *B. distachyon* Bd21 response to Pi deficiency would be useful to select suitable Pi conditions for the next experiment (described in Chapter 5).

# 3. Materials and methods

## 3.1. Objectives and methodology

This part of the project aimed at characterizing the response of *B. distachyon* Bd21 to Pi deficiency. The decision was made in this project to cultivate *Brachypodium* in solid growing substrate to mimic realistic growing conditions. *Brachypodium* development was characterized by measuring developmental (biomass production and

partitioning, root system architecture) and biochemical (APase activity and P content) traits. The measured traits will allow to define Pi conditions leading to contrasted phenotypes after 30 days of growth. The results will be used to select suitable Pi levels to be applied to *B. distachyon* Bd21 in the experiment described in Chapter 5.

## 3.2. Plant material

*Brachypodium distachyon* (L.) P. Beauv. (Bd21 line) caryopses were kindly provided by Dr Philippe Vain from the John Innes Centre (Norwich, UK) and propagated under greenhouse conditions.

## 3.3. Brachypodium distachyon Bd21 cultivation

*Brachypodium* seeds were surface sterilized (30 s in 70% v/v ethanol, rinsed once with sterile water, 10 min in sodium hypochlorite 5% v/v, rinsed three times with sterile water) and stratified for 2 days at 4°C on Hoagland agar plates (Plant Media™, Dublin, USA). The seeds were then incubated for 24 hours in a growth chamber (23°C, 16h/8h day light, PPFD 140 $\mu mol.m^{-2}.s^{-1}$) for germination.

Homogeneous 24 hour-old plantlets were selected and transferred in sand (2 mm sieved washed and sterilized) in 20 cm-high and 9 cm-diameter PVC pipes. The tubes were place in a growth chamber (23°C, 16h/8h day light, PPFD 140 $\mu mol.m^{-2}.s^{-1}$). Prior to transplantation, the sand was humidified with Hoagland nutrient solutions to reach 10% w/w water content. The Hoagland solution (Table 3-1) was modified to expose plantlets to 7 different Pi concentrations: 0, 50, 100, 150, 200, 600 and 1000 $\mu M$ $KH_2PO_4$. During the first 10 days, the sand was maintained at 10% water content by weighing the tubes and adding the adequate volume of Hoagland solution. After the 10 first days of cultivation, the tubes were watered every 5 days with the modified Hoagland solutions. Sixteen plants were grown for each Pi level (with a total of 112 plants) and 3 independent experiments were carried out. The plants were harvested after 30 days of growth. The different steps of the experiment are presented in Fig. 3-1. The experimental design is presented in Fig. 3-2.

**Table** 3-1. Composition of Hoagland solution (Hoagland and Arnon 1950).

|  | Concentration ($10^{-3}$ mol/l) |
|---|---|
| $KNO_3$ | 5 |
| $NH_4NO_3$ | 1 |
| $Ca(NO_3)_2.4H_2O$ | 4 |
| $KH_2PO_4$ | 1 |
| $MgSO_4.7H_2O$ | 2 |
| Fe-EDDHA | 0.01797 |
| $H_3BO_3$ | 0.04625 |
| $MnCl_2.4H_2O$ | 0.00915 |
| $ZnSO_4.7H_2O$ | 0.00077 |
| $CuSO_4.5H_2O$ | 0.00032 |
| $Na_2MoO_4.2H_2O$ | 0.00011 |
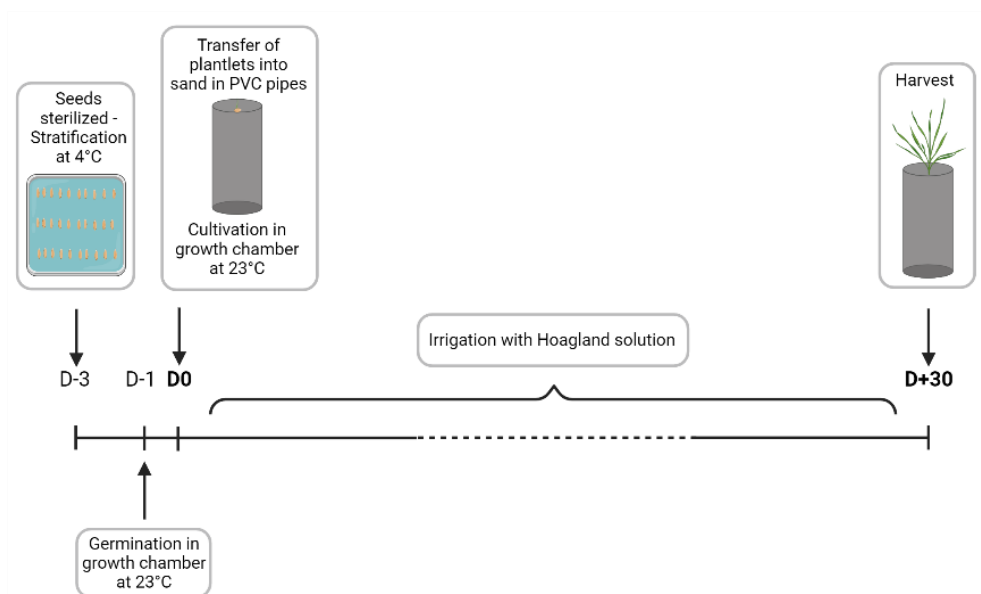


**Figure** 3-1. Steps of *ex vitro B. distachyon* Bd21 cultivation in sand, exposed to different Pi levels. Created with BioRender.com.
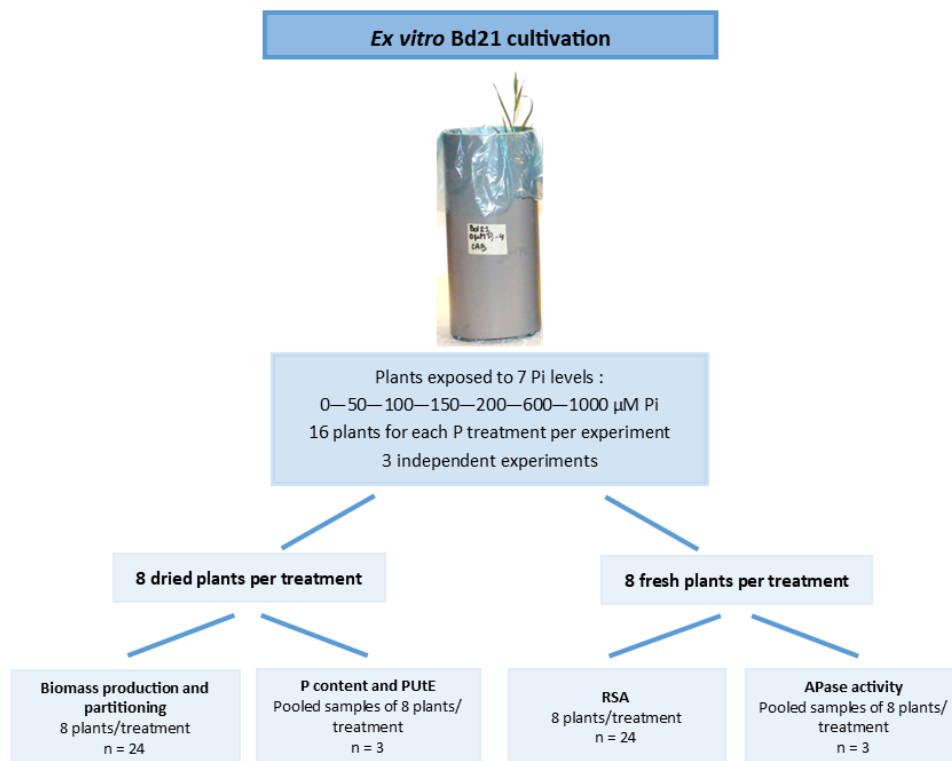
**Figure** 3-2: Description of the experimental design used for the *ex vitro* cultivation of *B. distachyon* Bd21 grown under varying Pi level.

## *3.4. Root system architecture measurement*

At harvest, the root system of 8 plants per treatment (half of the plants) was washed in water. The roots were spread in a transparent tray containing a thin layer of water and scanned using a flatbed scanner (200 dpi, HP Scanjet G4010 A4 scanner (Hewlett-Packard, Palo Alto, CA, USA)) in order to perform root system architecture (RSA) analyses. The images were used to manually vectorize first (primary seminal root, nodal roots) and second order roots (lateral roots) of the root system with DART (Le Bot et al. 2009) and the output files were then analysed with the archiDART package (Delory et al. 2016). The roots were classified according to the "developmental approach" considering the most proximal roots arising from the embryo, coleoptile or shoot as 1st order roots, while most distal roots in the root system are highest order roots (Freschet et al. 2021). The following parameters were measured: primary seminal root length, second-order lateral root number and length, number and length of coleoptile nodal roots, number and length of second-order lateral roots developed along the primary seminal root between 0-5, 5-10, 10-15, 15-20 cm of primary seminal root length.

## 3.5. Biomass production and partitioning

Half of harvested plants were dried in a ventilated oven at 65 °C until constant biomass was reached. Shoot and root systems were weighed. The dry weight of shoot and roots was recorded as measurements of biomass production. Biomass partitioning was estimated by the root mass fraction (hereafter named "RMF", mg root biomass/mg total biomass).

## 3.6. Acid phosphatase activity

The other half of harvested plants were frozen at -80°C for subsequent analysis of APase activity in tissues. Frozen shoots and roots of each independent experiment were pooled and crushed in a mortar (1 sample is made of 8 shoot or root systems). Soluble proteins were extracted in a sodium acetate buffer (50 mM NaOAc, 1 mM DTT, pH 5). 100 mg of plant powder were placed in 1.5 ml Eppendorf tubes and 500 µL of sodium acetate buffer were added. The tubes were vortexed and then centrifuged (15 minutes, 13000 rotations per minute "rpm", 4 °C). The supernatant was kept for further analyses. Soluble proteins content was determined with Bradford assay (dye-binding method, Bio-Rad Protein Assay Dye Reagent Concentrate, Hercules, California, USA) using bovine serum albumin as standard. APase activity was then measured with p-nitrophenyl phosphate assay. Briefly, p-nitrophenyl phosphate is an artificial substrate which is hydrolysed by APases under acidic conditions. The product of the dephosphorylation reaction is p-nitrophenol. Under alkaline conditions, p-nitrophenol colour turns to yellow which can be quantified by spectrophotometric measurement (Neumann 2006, Sigma-Aldrich, Ciereszko, Szczygła, et al. 2011). The optical density at 410 nm was measured after 0-5-10-15 minutes with a standard solution of p-nitrophenol (0-5-10-20-30-40 µg p-nitrophenol/ml).

## 3.7. P concentration in plant tissues and P utilization efficiency

The dried plants were used to measure the total P concentration in plant tissues. The method of Briggs (1924) was applied. Under acidic condition and in contact with ammonium molybdate, phosphate ions form a phosphomolybdate complex. When reduced by hydroquinone and sodium sulphite, the solution colour turns to blue, which can be measured by spectrophotometric measurement.

Shoots and roots of the dried plants were weighed and pooled separately (1 sample is made of 8 shoots or root systems). The samples were placed in 10 ml borosilicate glass beakers. All the glassware used for this analysis was previously washed with 10% nitric acid to remove phosphate which can be adsorbed on the glassware. Samples were calcinated overnight in an oven at 450°C. Ashes were suspended in Milli-Q purified water and 2.5 ml of 0.4 N HCl were added in the beakers. The ashes suspensions were heated and filtered with MN 615 filters (Macherey-Nagel, Dueren,

Germany). The solutions were collected in 25 ml glass flasks. The beakers were washed 3 times by adding 2.5 ml HCl and heating, to recover all the solubilized P. The filters were rinsed with Milli-Q water and the volume of solution was brought to 25 ml with Milli-Q water. Ammonium molybdate reagent was prepared (8 mM $(NH_4)_6Mo_7O_{24}.4H_2O$, 30 mM $H_2SO_4$, 245 mM $CCl_3-COOH$, 159 mM $Na_2SO_3$, 4.5 mM hydroquinone). Four and 8 ml of sample solutions were placed in 20 ml glass flasks for shoot and root samples, respectively. Ten ml of reagent were added, then the volume was brought to 20 ml with Milli-Q water. The optical density at 690 nm was measured after 20 minutes, with a standard solution of $KH_2PO_4$ (0-0.8-2-4-8 µg P/ml).

The results were expressed as total P concentration in shoot and root samples. P utilization efficiency (PUtE), corresponding to the biomass produced by unit of P, was calculated for the whole plant (mg dry weight per µg P in the sample) (Neto et al. 2016).

$$PUtE = \frac{DW}{[P] \text{ x } DW}$$

## *3.8. Statistical analysis*

Two-way analyses of variance (ANOVAs) were performed to study the impact of P supply and independent repetitions on the following response variables: shoot, root and total dry weight parameters; RMF; shoot and root P concentration; PUtE; shoot and root APase activity; primary seminal root length; number and total length of coleoptile nodal roots; number and total length of 2nd order lateral roots; number and length of 2nd order lateral roots between 0-5, 5-10, 10-15 and 15-20 cm along the primary seminal root. A model with crossed fixed factors was applied (lm, glm and anova functions, R 4.2.3, R Core Team 2023; Gamma family distribution with a log-link function was used for GLM models, excepted for count variables for which negative binomial distribution was used). Dunnett's post-hoc tests were performed to compare the treatments to the control situation (1000 µM Pi) (R 4.2.3, R Core Team 2023; multcomp package version 1.4-8, Hothorn et al. 2008).

A principal component analysis (PCA) was conducted on the mean value of independent repetition. A hierarchical clustering analysis was then performed to group Pi levels inducing similar phenotypes. The analyses were performed separately for biomass and biochemical variables, and for root system architecture (RSA) variables because it was expected that the variability would not allow to clearly define clusters if all variables were combined. The package FactoMineR was used to conduct the analyses in R (R 3.5.2, R Core Team 2018; "FactoMineR", Lê et al. 2008). Graphs were generated using the package ggplot2 (R 3.5.2, R Core Team 2018; "ggplot2" version 3.1.0, Wickham 2016).

# 4. Results

## 4.1. Biomass production and partitioning

The shoot dry weight of 30-d old *B. distachyon* Bd21 showed a strong response to Pi level variation ($P < 0.001$, Fig. 3-3). Significant reduction in shoot biomass production was observed in treatments 0 and 50 µM Pi. Plants exposed to 0 µM Pi had the lowest shoot biomass production with a reduction of 48.5% compared to the treatment 1000 µM Pi. Shoot biomass production was reduced by 23.5% in the treatment 50 µM Pi, compared to 1000 µM Pi. Shoot biomass production strongly increased with increasing Pi concentration until 100 µM Pi. Between 100 and 200 µM Pi, shoot biomass production still moderately increased (13.3%, 10.6% and 7.6% reduction in the treatment 100, 150 and 200 µM Pi, respectively). Then shoot biomass production levelled off between 600 and 1000 µM Pi to reach a plateau.

Root biomass production exhibited a weaker and opposite response to Pi level, compared to shoot biomass production ($P < 0.001$, Fig. 3-4). The highest root dry weight was observed in plants exposed to 0 µM Pi with a significant increase of 28.1% compared to plants exposed to 1000 µM Pi. Root dry weight decreased and rapidly levelled off until the highest P concentration.

The total biomass production showed the same trend as the shoot biomass production, with a significant decrease of 37.3% and 19.8% in plants exposed to 0 and 50 µM Pi respectively ($P < 0.001$).

Regarding partitioning of biomass, RMF was the highest at 0 µM Pi with a significant increase of 103% compared to the treatment 1000 µM Pi (Fig. 3-5). Plants exposed to 50 µM Pi also exhibited a significant increase in RMF of 24.0% compared to 1000 µM Pi ($P < 0.001$). RMF rapidly decreased to level off from 100 µM Pi to 1000 µM Pi. The response of root mass fraction is consistent with the strong increase observed in shoot biomass production and the weaker decrease in root biomass production with increasing Pi level. The effects of Pi level on the phenotype of 30-day old *Brachypodium* are summarized in Table 3-2. A table comprising mean values per treatment, standard deviation and coefficients of ANOVAs is available in Appendix 1.
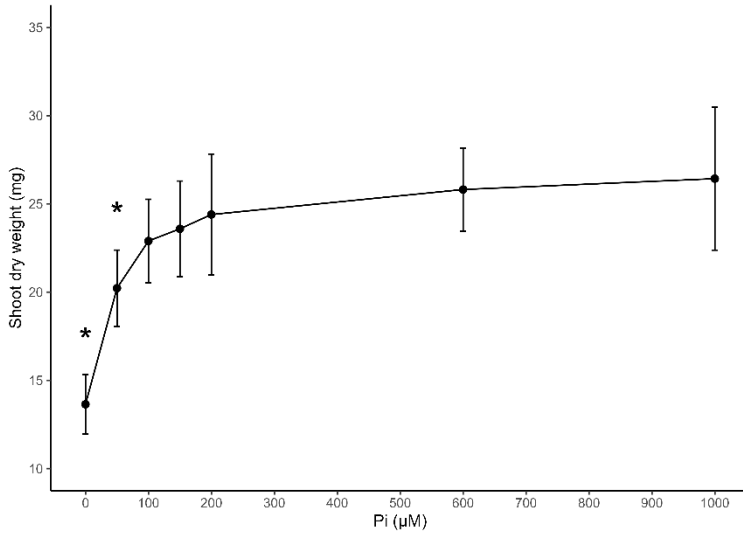
**Figure** 3-3: Shoot dry weight production in 30-day old *B. distachyon* Bd21 grown under varying Pi level (n = 24, mean ± 95% confidence interval). Significant differences among treatments are marked with stars (Dunnett's *post hoc* test; 1000 µM Pi used as reference treatment).
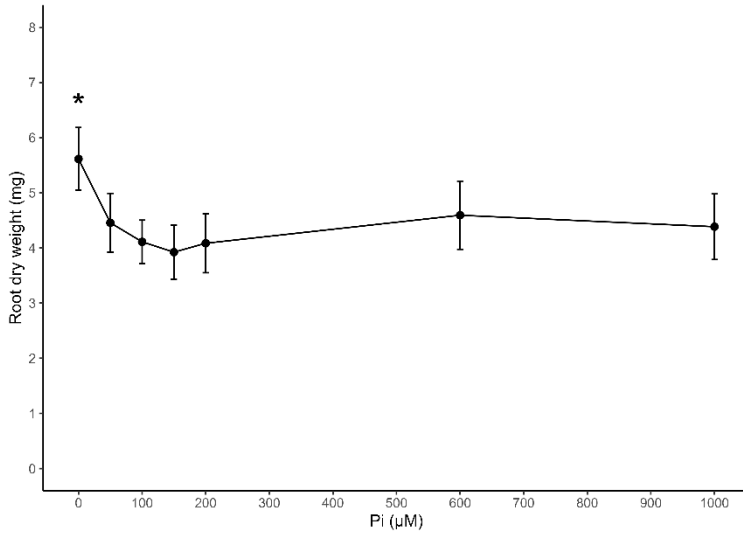


**Figure** 3-4: Root dry weight production in 30-day old *B. distachyon* Bd21 grown under varying Pi level (n = 24, mean ± 95% confidence interval). Significant differences among treatments are marked with stars (Dunnett's *post hoc* test; 1000 µM Pi used as reference treatment).
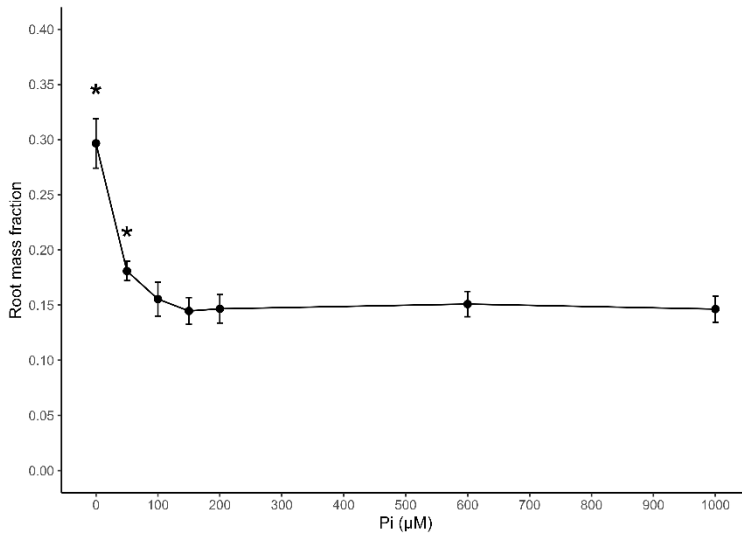
**Figure** 3-5: Root mass fraction of 30-day old *B. distachyon* Bd21 grown under varying Pi level (n = 24, mean ± 95% confidence interval). Significant differences among treatments are marked with stars (Dunnett's *post hoc* test; 1000 µM Pi used as reference treatment).

## *4.2. Biochemical variables*

APase activity in shoot was the highest at 0 µM Pi with a significant increase of 80.6% compared to plants exposed to 1000 µM Pi ($P < 0.001$, Fig. 3-6). APase activity sharply decreased with increasing Pi level and levelled off from 50 µM Pi. APase activity in roots was lower than in shoot and showed a decrease with increasing Pi level. Root APase activity was significantly increased by 52.7% at 0 µM Pi compared to the treatment 1000 µM Pi ($P = 0.005$, Fig. 3-6).
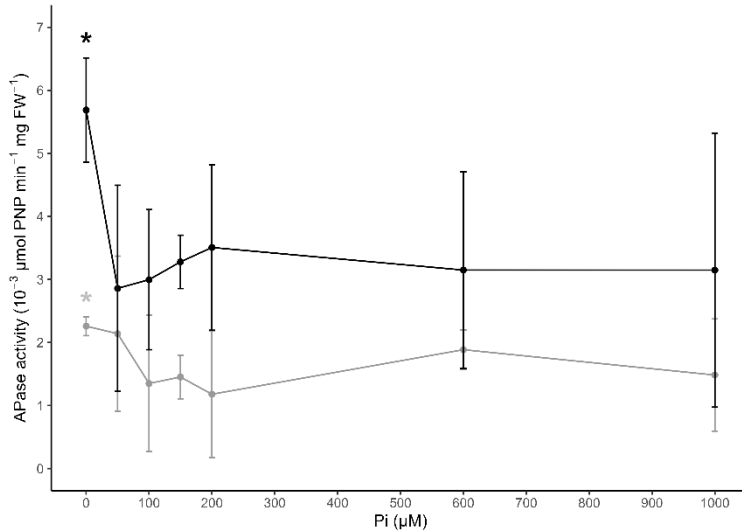
**Figure** 3-6: Acid phosphatase activity in shoot (black) and root (grey) tissue of 30-day old *B. distachyon* Bd21grown under varying Pi level (n = 3, mean ± 95% confidence interval). Significant differences among treatments are marked with stars (Dunnett's *post hoc* test; 1000 µM Pi used as reference treatment).

Regarding P content in plant tissue, the trend was similar in shoot and root (Fig. 3-7). A significant decrease in shoot P content was observed for treatments 0 to 150 µM Pi, with reduction from 76.4% to 14.2% respectively compared to the treatment 1000 µM Pi ($P < 0.001$). In roots, P content was significantly decreased from 0 µM Pi to 200 µM Pi, with effects ranging from 81.6% to 30.9% compared to the treatment 1000 µM Pi ($P < 0.001$). The P content strongly increased from 0 to 150 µM Pi and then increased more moderately until 600 µM Pi. A plateau was reached from 600 to 1000 µM Pi.

PUtE for the whole plants showed a sharp increase with decreasing Pi level between 150 and 0 µM Pi (Fig. 3-8). Effects ranging from 364.7% to 20.6% compared to the treatment 1000 µM Pi were observed at 0 and 150 µM Pi, respectively ($P < 0.001$). The PUtE stabilized from 200 µM Pi to reach a plateau from 600 µM Pi. This trend is consistent with the response observed in biomass production and P content in plant tissue. The plants produced more biomass per unit of P when exposed to low P levels. A table comprising mean values per treatment, standard deviation and coefficients of ANOVAs is available in Appendix 1. The effects of Pi level on the phenotype of 30-day old *B. distachyon* Bd21 are summarized in Table 3-2.
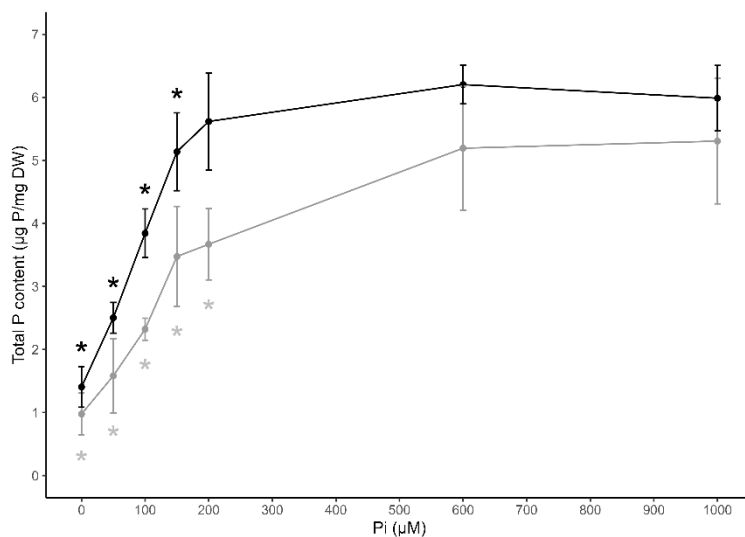
**Figure** 3-7: Total P content in shoot (black) and root (grey) tissue of 30-day old *B. distachyon* Bd21 grown under varying Pi level (n = 3, mean ± 95% confidence interval). Significant differences among treatments are marked with stars (Dunnett's *post hoc* test; 1000 µM Pi used as reference treatment).
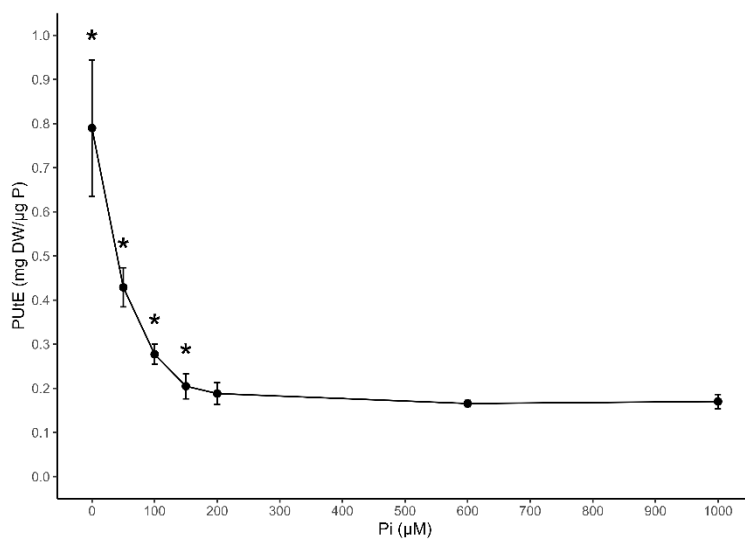


**Figure** 3-8: PUtE of 30-day old *B. distachyon* Bd21 grown under varying Pi level (n = 3, mean ± 95% confidence interval). Significant differences among treatments are marked with stars (Dunnett's *post hoc* test; 1000 µM Pi used as reference treatment).

## *4.3. Multivariate analysis for biomass and biochemical variables*

The PCA and hierarchical clustering for biomass and biochemical variables allowed to visualize how the measured variables are correlated to each other and evolve along with P concentration. Clusters of P concentrations resulting in similar plant phenotypes were identified. The first two principal components (axes) were chosen to visualize the data and explain 81% of the data variation. The PCA map (Fig. 3-9, right side) shows that variation in biochemical variables and RMF is mainly described by the first principal component (Dim 1). Biomass production variables are correlated to the first and the second principal components. While total and shoot dry weight, shoot and root P content evolve in the same direction as Pi level, root dry weight, RMF, shoot and root APase and PUtE evolve in the opposite direction as Pi level.

Based on hierarchical clustering, 4 different clusters of Pi levels were identified (Fig. 3-9, left side). The most distanced cluster includes plants exposed to 0 µM Pi only. Another cluster with plants exposed to 50 µM Pi was identified. The two other clusters contain several Pi concentrations: one cluster is constituted of plants exposed to 100, 150 and 200 µM Pi and the last one contains the plants exposed to 600 and 1000 µM Pi. It appears that most of the variation in the measured variables is observed at the lowest Pi levels, between the cluster 0 µM Pi and the cluster 50 µM Pi.



**Figure** 3-9: On the left: PCA score plot for biomass and biochemical variables (mean of independent experiments (dots) and general mean (triangles) of each Pi level), groups defined by the hierarchical clustering are depicted in different colours. On the right: PCA map of biomass and biochemical variables (SDW: shoot dry weight, RDW: root dry weight, TDW: total dry weight, RMF: root mass fraction, ShootP: shoot P content, RootP: root P content, ShootAPase: acid phosphatase activity in shoot, RootAPase: acid phosphatase activity in root, PUtE: P utilization efficiency, Plevel: Pi concentration).

## *4.4. Root system architecture*

The response of the RSA variables to Pi level was more tenuous than the other measured variables and the variability was higher. The most noticeable changes were an increase in primary seminal root length (Fig. 3-10) and in length of $2^{nd}$ order lateral roots between 10 and 20 cm along the primary seminal root (Fig. 3-16) at 0 µM Pi. The longest primary seminal root was observed at 0 µM Pi with $21.5 \pm 4.7$ cm and at 1000 µM Pi with $19.7 \pm 2.3$ cm (mean $\pm$ SD). Plants grown under 150 µM Pi had the shorter primary root length with $16.9 \pm 3.8$ cm (mean $\pm$ SD). Significant difference in primary seminal root length was highlighted among Pi treatments by the statistical analysis ($P = 0.004$) but Dunnett's analysis did not reveal any difference compared to the treatment 1000 µM Pi.
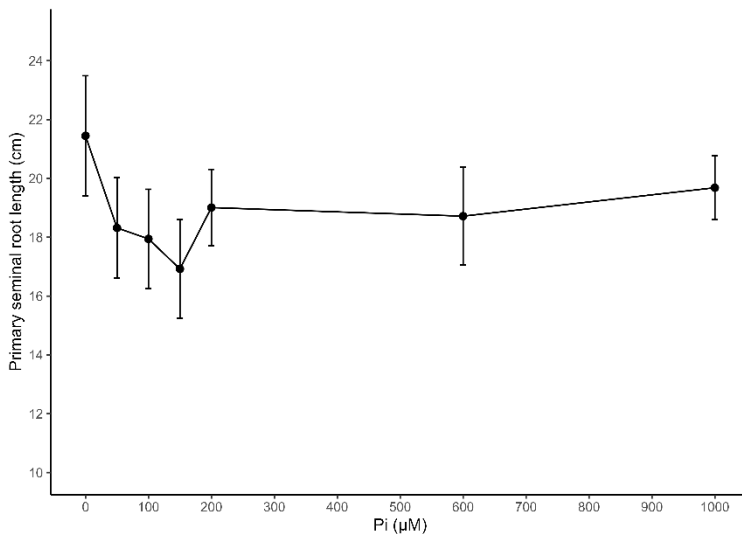


**Figure** 3-10: Length of primary seminal root (cm) in *B. distachyon* Bd21 grown under varying Pi level (n = 24, mean $\pm$ 95% confidence interval).

Regarding coleoptile nodal roots (Fig. 3-11 and 3-12), the high variability observed in the results makes the interpretation unreliable. No conclusion over these variables can be drawn.

The highest number of $2^{nd}$ order lateral roots was observed in plants grown under 1000 µM Pi ($96 \pm 25$, mean $\pm$ SD) (Fig. 3-13). Plants exposed to the treatment 600 µM Pi had a similar development of $2^{nd}$ order lateral roots. Plants exposed to other treatments showed a reduction in the number of $2^{nd}$ order lateral roots compared to the treatment 1000 µM Pi, with the greatest reduction of 22.2% in the treatment 150 µM Pi ($P = 0.015$). The total length of lateral roots (Fig. 3-14) was the greatest in the treatment 1000 µM Pi ($212.7 \pm 60.4$ cm, mean $\pm$ SD), followed by the treatment 0 µM Pi with a total length of $204.5 \pm 66.2$ cm (mean $\pm$ SD). Plants exposed to other

treatments developed reduced total length of 2nd order lateral roots with the greatest reduction of 19.2% observed in the treatment 100 µM Pi ($P = 0.062$). The great length of 2nd order lateral roots observed in the treatment 0 µM Pi is not associated with a high number of 2nd order lateral roots. It can therefore be deducted that the plants grown under 0 µM Pi tended to produce fewer but longer 2nd order lateral roots than plants exposed to 1000 µM Pi.
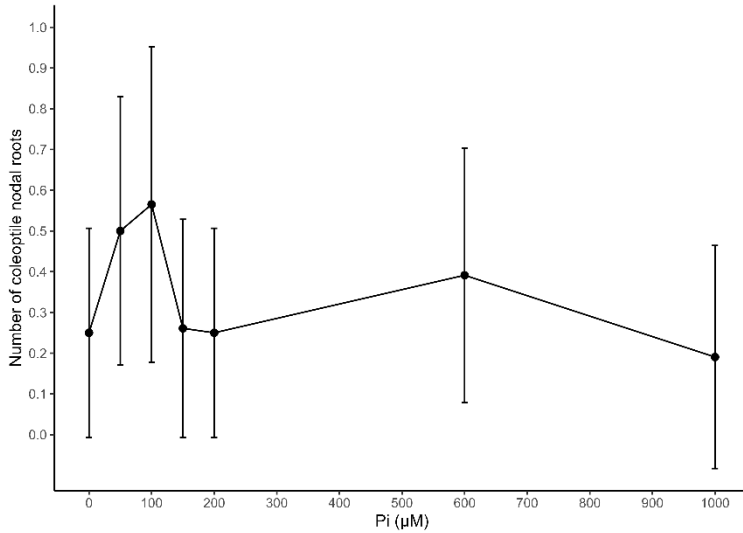


**Figure** 3-11: Number of coleoptile nodal roots in *B. distachyon* Bd21 grown under varying Pi level (n = 24, mean ± 95% confidence interval).
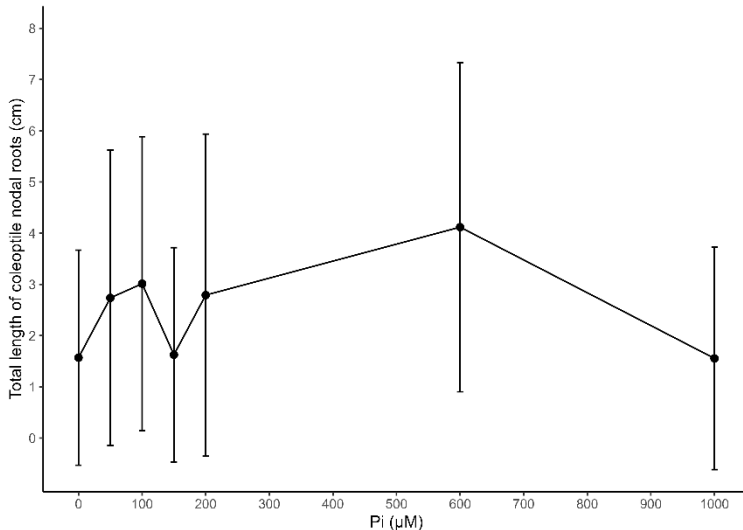


**Figure** 3-12: Total length of coleoptile nodal roots (cm) in *B. distachyon* Bd21 grown under varying Pi level (n = 24, mean ± 95% confidence interval).
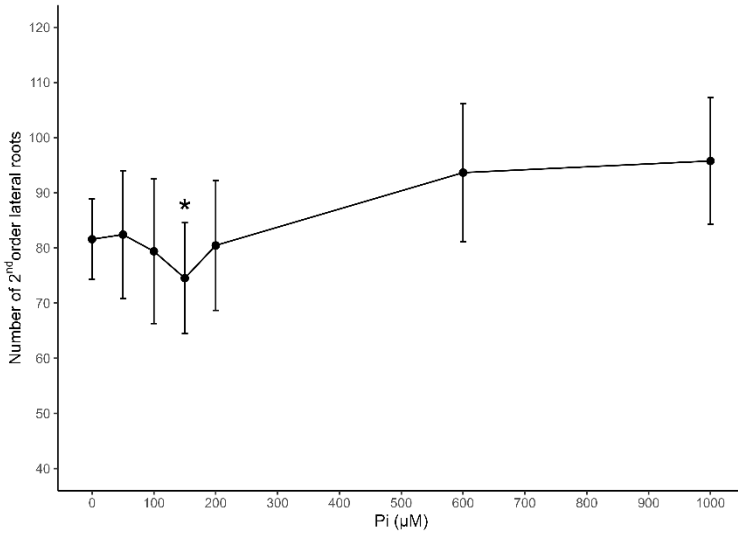
**Figure** 3-13: Number of $2^{nd}$ order lateral roots in *B. distachyon* Bd21 grown under varying Pi level (n = 24, mean ± 95% confidence interval). Significant differences among treatments are marked with stars (Dunnett's *post hoc* test; 1000 µM Pi used as reference treatment).
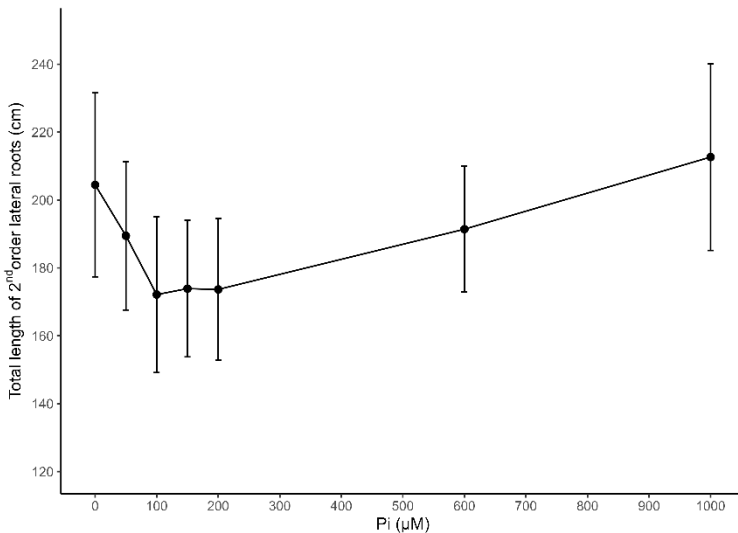


**Figure** 3-14: Total length of $2^{nd}$ order lateral roots (cm) in *B. distachyon* Bd21 grown under varying Pi level (n = 24, mean ± 95% confidence interval).

The repartition of $2^{nd}$ order lateral roots along the primary seminal root (Fig. 3-15) shows that plants developed more roots between 0 and 10 cm of the primary seminal root and fewer deeper. The response of the plants was variable between 0 and 10 cm along the primary seminal root. Between 10 and 20 cm along the primary seminal root, the number of lateral roots diminished with Pi deficiency but plants exposed to

0 µM Pi produced more $2^{nd}$ order lateral roots than in the treatments 50, 100, 150 and 200 µM Pi. Compared to the treatment 1000 µM Pi, lateral root number was significantly reduced in plants exposed to 50 µM Pi between 10 and 15 cm along the primary seminal root ($P = 0.019$).
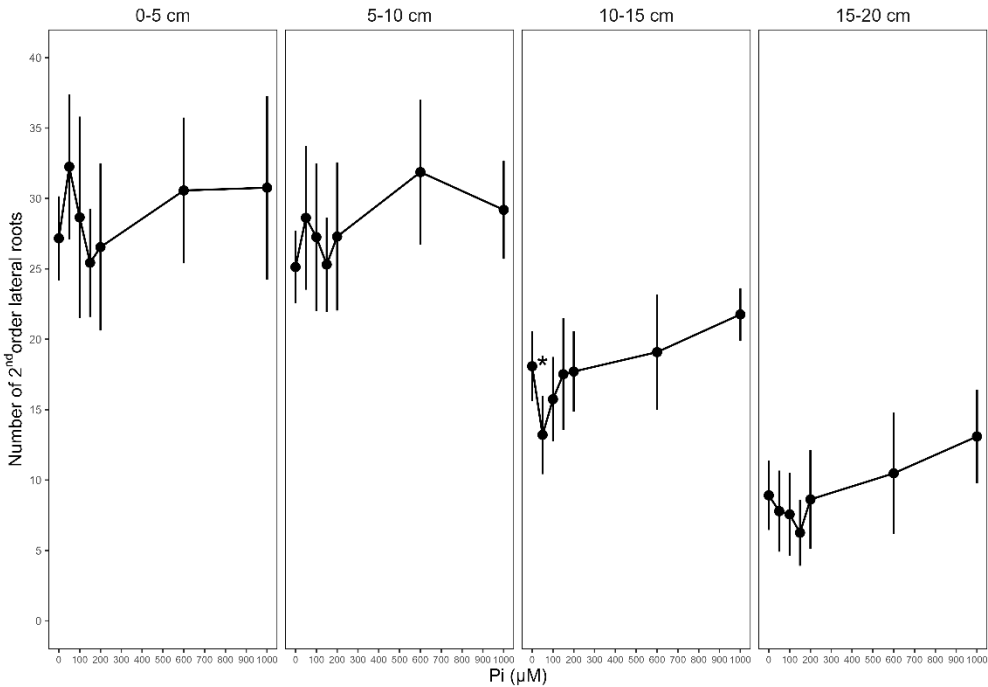


**Figure** 3-15: Number of second order lateral roots of 30-day old *B. distachyon* Bd21 grown under varying Pi level along the primary seminal root (n = 24, mean ± 95% confidence interval). Significant differences among treatments are marked with stars (Dunnett's *post hoc* test; 1000 µM Pi used as reference treatment).

Regarding the general trend in total length of $2^{nd}$ order lateral roots (all treatments combined) (Fig. 3-16), the total length is similar between 0 and 10 cm along the primary seminal root. The length decreases while growing deeper into the substrate. However, plants grown under 0 µM Pi had a higher total length of $2^{nd}$ order lateral roots than other treatments between 10 and 20 cm of the primary seminal root (49.2 ± 25.5 cm between 10-15 cm and 15.6 ± 13.2 cm between 15-20 cm along primary seminal root, mean ± SD). Similar to the primary seminal root length, the treatment 1000 µM Pi lies just behind 0 µM Pi (43.1 ± 23.8 cm between 10-15 cm and 14.9 ± 10.8 cm between 15-20 cm along the primary seminal root, mean ± SD). The length of lateral roots was significantly reduced in treatments 50 and 100 µM Pi between 10-15 cm of the primary seminal root ($P < 0.001$) and in treatments 100 and 150 µM Pi between 15-20 cm of the primary seminal root ($P = 0.0016$) compared to the treatment 1000 µM Pi.
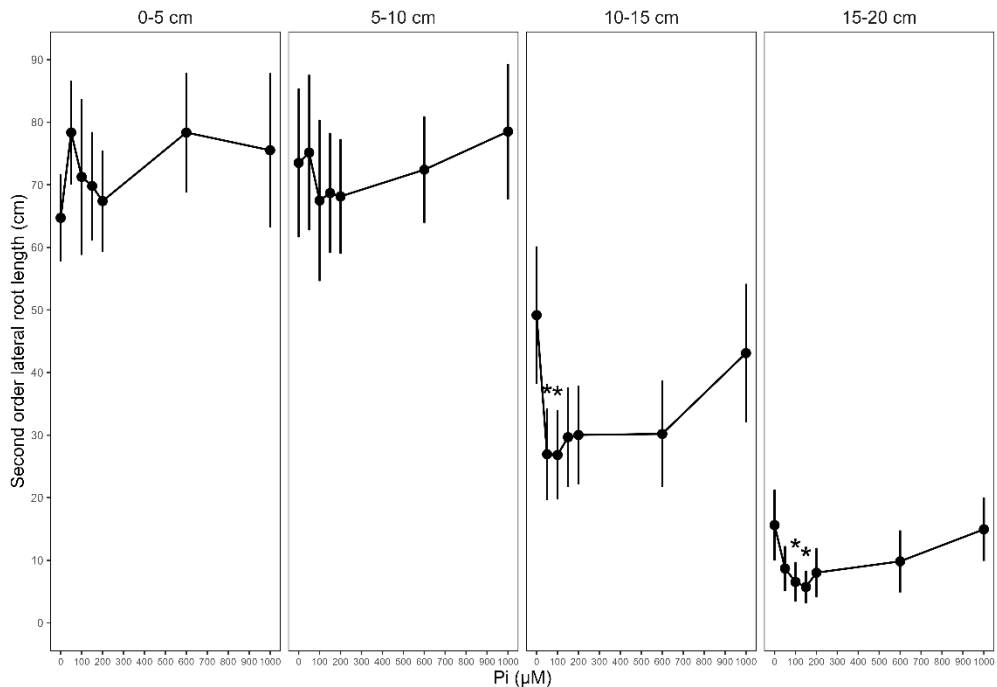
**Figure** 3-16: Total length of second order lateral roots of 30-day old *B. distachyon* Bd21 grown under varying Pi level along the primary seminal root (n = 24, mean ± 95% confidence interval). Significant differences among treatments are marked with stars (Dunnett's *post hoc* test; 1000 µM Pi used as reference treatment).

The results globally show a weak response of the root system architecture to varying Pi level. Regarding the primary seminal root length, the total length of $2^{nd}$ order lateral roots and the length of $2^{nd}$ order lateral roots between 10 and 20 cm along the primary seminal root, plants exposed to the treatments 0 and 1000 µM Pi showed similar root system architecture. This is consistent with the observation made in root biomass production, which was higher in plants grown under 0 µM Pi than in the other treatments. The results of the ANOVAs show a significant effect of the independent repetitions on most of the variables. This can also be visualized by a high variability in the results. Significant effects of the Pi treatment could however be highlighted. The effects of Pi level on the phenotype of 30-day old *Brachypodium* are summarized in Table 3-2. Representative root systems of *Brachypodium* plantlets are illustrated in Fig. 3-18. A table comprising mean values per treatment, standard deviation and coefficients of ANOVAs is available in Appendix 2.

## 4.5. *Multivariate analysis for root system architecture variables*

The first two principal components (axes) were chosen to visualize the data and explain 79% of the data variation. All RSA variables evolve in the same direction as Pi level (Fig. 3-17, right side) along the $2^{nd}$ principal component. The primary seminal root length, the number and length of coleoptile nodal roots are related to the first principal component. The number and total length of second order lateral roots, total length of $2^{nd}$ order lateral roots between 5-10 cm and number of $2^{nd}$ order lateral roots between 10-15 cm along the primary seminal root are correlated to the $2^{nd}$ principal component. Other variables, regarding number and length of $2^{nd}$ order lateral roots along the primary seminal root are correlated to both axes.

The hierarchical clustering did not allow to identify clusters of Pi levels leading to contrasted plant phenotypes for root system architecture. Looking at the general mean of all experiments, the treatments 0 and 1000 µM Pi were closer to each other than to all other treatments. This is consistent with the observations made about the primary seminal root length and the total length of $2^{nd}$ order lateral roots between 10 and 20 cm along the primary seminal root, which were highest at 0 µM Pi and followed by the treatment 1000 µM Pi (Fig. 3-17, left side).



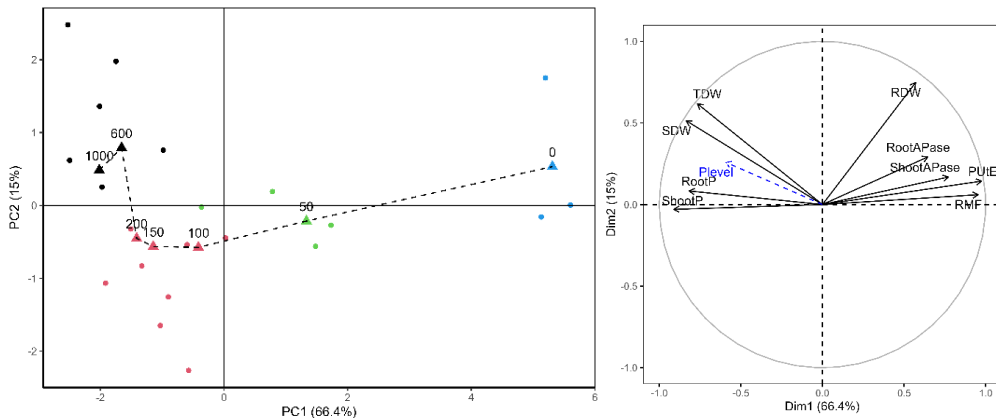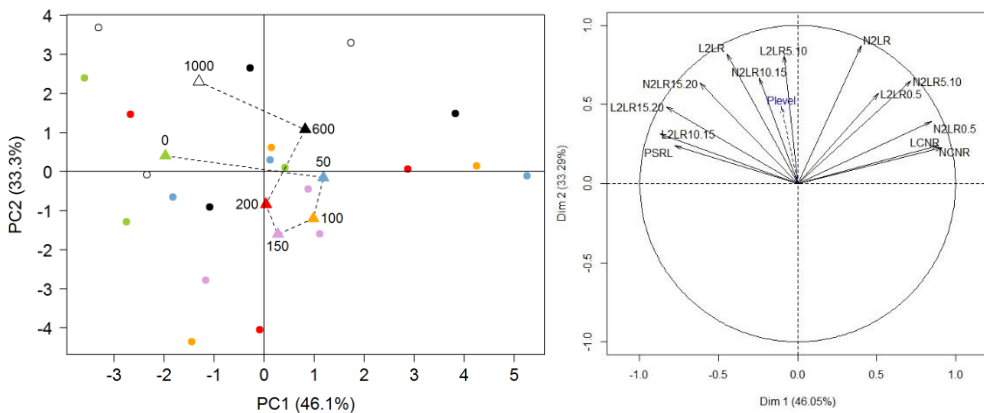**Figure** 3-17: On the left: PCA score plot for RSA variables (mean of independent experiments (dots) and general mean (triangles) of each Pi level; Pi levels are depicted in different colours). On the right: PCA map of RSA variables (PSRL: primary seminal root length, NCNR: number of coleoptile nodal roots, LCNR: total length of coleoptile nodal roots, N2RL: number of $2^{nd}$ order lateral roots, L2RL: total length of $2^{nd}$ order lateral roots, N2RL0.5 -5.10 -10.15 -15.20: number of $2^{nd}$ order lateral roots between 0 and 5 cm – 5 and 10 cm – 10 and 15 cm – 15 and 20 cm along the primary seminal root, L2RL0.5 -5.10 -10.15 -15.20: total length of $2^{nd}$ order lateral roots between 0 and 5 cm – 5 and 10 cm – 10 and 15 cm – 15 and 20 cm along the primary seminal root, Plevel: Pi concentration)

**Table** 3-2. Effects observed in 30-day old *B. distachyon* Bd21 grown under varying Pi level.

| | SDW | RDW | RMF | Shoot Apase | Root Apase | Shoot P | Root P | PutE | PSRL | N2LR | L2LR | N2LR 0-5 | N2LR 5-10 | N2LR 10-15 | N2LR 15-20 | L2LR 0-5 | L2LR 5-10 | L2LR 10-15 | L2LR 15-20 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 µM | ↘↘* | ↗↗* | ↗↗↗* | ↗↗↗* | ↗↗↗* | ↘↘↘* | ↘↘↘* | ↗↗↗* | ↗ | ↘ | → | ↘ | ↘ | ↘ | ↘↘ | ↘ | ↘ | ↗ | → |
| 50 µM | ↘↘* | → | ↗↗* | ↘ | ↗↗ | ↘↘↘* | ↘↘↘* | ↗↗↗* | ↘ | ↘ | ↘ | → | → | ↘↘* | ↘↘ | → | → | ↘↘* | ↘↘ |
| 100 µM | ↘ | ↘ | ↗ | → | ↘ | ↘↘* | ↘↘↘* | ↗↗↗* | ↘ | ↘ | ↘ | ↘ | ↘ | ↘↘ | ↘↘ | ↘ | ↘ | ↘↘* | ↘↘↘* |
| 150 µM | ↘ | ↘ | → | → | → | ↘* | ↘↘* | ↗↗* | ↘ | ↘↘* | ↘ | ↘ | ↘ | ↘ | ↘↘↘ | ↘ | ↘ | ↘↘ | ↘↘↘* |
| 200 µM | ↘ | ↘ | → | ↗ | ↘↘ | ↘ | ↘↘* | ↗ | → | ↘ | ↘ | ↘ | ↘ | ↘ | ↘↘ | ↘ | ↘ | ↘↘ | ↘↘ |
| 600 µM | → | → | → | → | ↗↗ | → | → | → | ↘ | ↗ | ↘ | → | ↗ | ↘ | ↘ | → | ↘ | ↘↘ | ↘↘ |
| 1000 µM | Reference treatment | | | | | | | | | | | | | | | | | | |

↗↗↗ or ↘↘↘ : effects ≥ 50%

↗↗ or ↘↘ : 20% ≤ effects < 50%

↗ or ↘ : 5% ≤ effects < 20%

→ : effects < 5%

Shades of orange: decrease; shades of green: increase.

Significant differences highlighted with Dunnett's *post hoc* tests are annotated with stars.
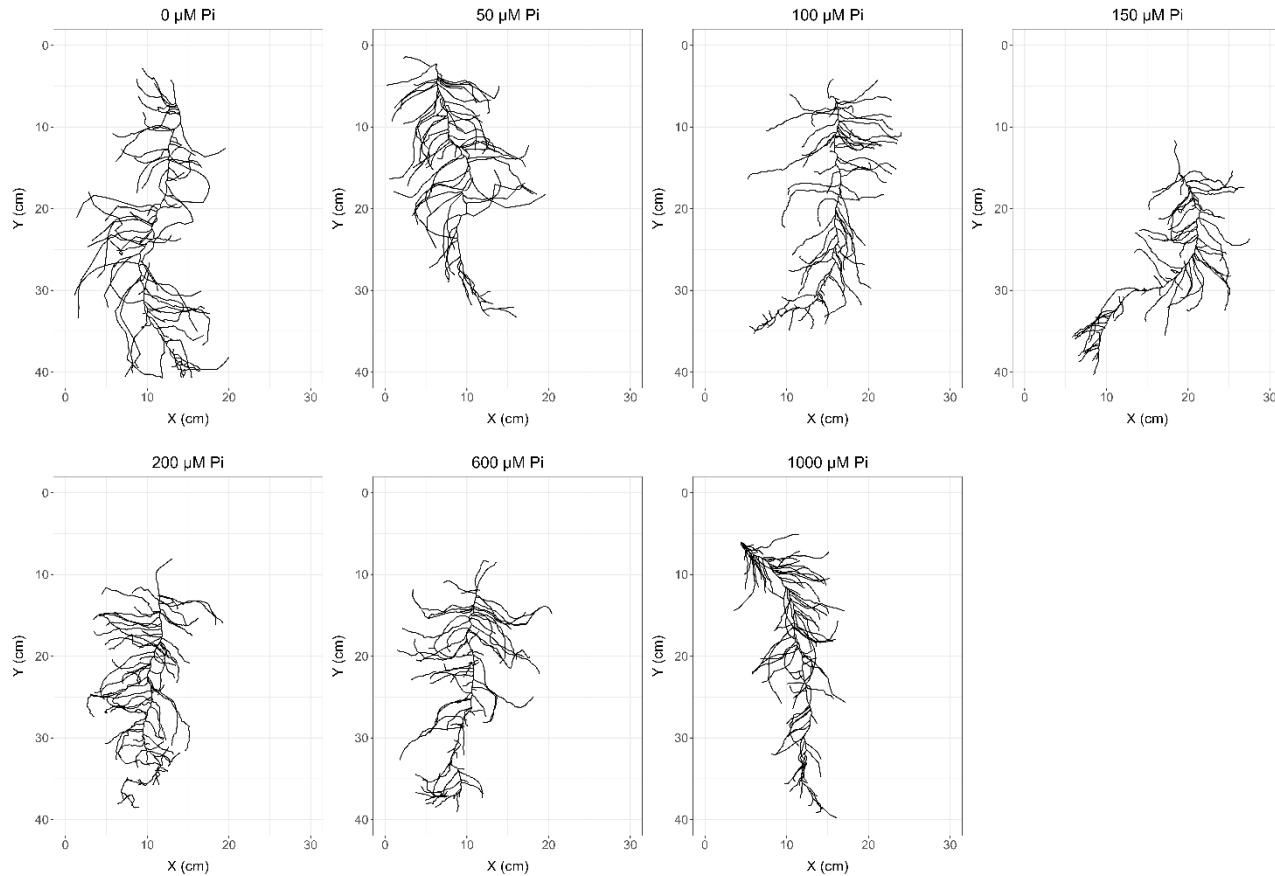
**Figure** 3-18. Representative root systems of 30-day old *B. distachyon* Bd21 plants grown under varying Pi level, selected among all the vectorized root systems based on their proximity to the mean value of each measured variable.

# 5.   Discussion

The biomass production and partitioning were highly responsive to Pi deficiency. Shoot biomass production decreased while root biomass production was maintained and slightly increased with Pi deficiency. While the response in shoot biomass production is consistent with observations made on *B. distachyon* by other researchers, this is not the case for root biomass production. Indeed, Poiré et al. (2014) observed a similar trend in shoot and root biomass production of 5-week old *B. distachyon* Bd21-3 with an increase in biomass from 0 to 600 µM Pi and a plateau above 600 µM Pi. Bd21-3 plants grown in the study of Poiré et al. (2014) reached higher biomass than Bd21 plants grown in this experiment. These results could be explained by the younger growth stage obtained in our experimental system. Indeed, plants allocate more biomass to the roots when they are small and develop more the aboveground system as they become larger (Weiner 2004). Similarly to our results, Giles et al. (2017) showed a decrease in shoot dry weight and an increase in root dry weight in hydroponically grown barley under Pi deficiency. The stimulation of root development under low P conditions is a common reported response, facilitating plant exploration of the substrate and P uptake (Lynch et al., 2012). Nonetheless, it seems that both the growing conditions and the plant growth stage are important factors affecting biomass accumulation and partitioning in response to Pi deficiency.

Biochemical parameters (P content, PUtE and APase activity) also show a strong response to Pi level. The observed decrease in P content and increase in APase activity in shoot and root tissue is consistent with observations made in wheat and barley (Ciereszko, Szczygła, et al. 2011; Ciereszko, Żebrowska, et al. 2011). The increase in PUtE observed at low level of Pi suggests that plants exposed to Pi deficiency built more biomass per unit of P than plants exposed to higher Pi level. This could be explained by an adaptation of the plant metabolism to low P requirements, as described in Chapter 1, Section 4 (*e.g.* replacement of phospholipids in membranes by sulfo- and/or galactolipids, synthesis of P-free polysaccharides as cell wall constituents, replacement of triose-P by starch and sucrose). Four groups of Pi concentrations, inducing contrasted plant phenotypes, were identified with hierarchical clustering including these variables. A stress level was associated to each cluster (Table 3-3).

**Table** 3-3 : Groups of P concentration inducing contrasted plant phenotypes and stress level, identified with hierarchical clustering on biomass and biochemical variables.

| Cluster 1 | 0 µM | Severe stress |
|---|---|---|
| Cluster 2 | 50 µM | Mild stress |
| Cluster 3 | 100 µM<br>150 µM<br>200 µM | Sub-optimal growth |
| Cluster 4 | 600 µM<br>1000 µM | Optimal growth |

Regarding RSA variables, plants exposed to 0 µM Pi tended to develop a primary seminal root length and length of $2^{nd}$ order lateral roots between 10 and 20 cm along the primary seminal root similar to those of plants exposed to 1000 µM Pi. This is not consistent with the observations made by Poiré et al. (2014), who observed a reduction in primary seminal root length in plants grown under Pi deficiency. In their study, Ingram et al. (2012) also observed a reduction of the root system depth in *B. distachyon* Bd21 exposed to Pi deficiency. Contrasted response in root system architecture are observed among species and cultivars, but the most commonly observed response to P deficiency is a shallow root system with high root length density in upper soil layers, allowing topsoil foraging (Richardson et al. 2011). This is in contradiction with the results of this study. A reason could be the experimental system, with sand allowing the nutrient solution to percolate through the substrate and accumulating in the bottom of the tubes due to gravity. The hierarchical clustering based on root system architecture variables did not allow to define clear groups of Pi concentrations inducing contrasted phenotypes.

The increase in root biomass production observed under Pi deficiency in our experimental conditions suggests that *Brachypodium* maintained the development of its root system under low Pi condition at the expense of shoot biomass production. This increase in root biomass under low Pi level could be explained by an increase in root diameter and/or greater root tissue density. Plants exposed to the treatments 0 and 1000 µM Pi showed similar root system architecture, regarding the primary seminal root length, the total length of $2^{nd}$ order lateral roots and the length of $2^{nd}$ order lateral roots between 10 and 20 cm along the primary seminal root. This is also consistent with the higher root biomass production in plants grown under 0 µM Pi compared to other treatments. Combined to the increase in APase activity and PUtE, these observations show that *Brachypodium* exhibited developmental and physiological plasticity in response to Pi level and developed strategies to improve P acquisition and use efficiency. As presented in Chapter 1 Sections 1 and 4, P bioavailability is greater in the topsoil. Due to the poor mobility of P in soil and the high spatial variability in P bioavailability, plasticity in soil exploration ability and local adaptability of the root

system remain important to grow beyond the depletion zone in the rhizosphere, locate P solubilization processes and uptake in P-rich domains.

For the co-cultivation experiments that will be presented in Chapter 5, *B. distachyon* Bd21 should ideally be exposed to two different inorganic soluble P concentrations inducing contrasted phenotypes. A low Pi concentration inducing severe to mild stress condition in the plant will be selected. The concentration 0 µM Pi being unrealistic, a concentration greater than 0 but inferior to 50 µM Pi will be preferred. A reference treatment allowing optimal growth of the plant will also be selected. The treatment 1000 µM Pi appears to be appropriate as a reference treatment. This Pi concentration is in the plateau stage of the reaction curve for most variables and shows no sign of toxicity to the plant.

# Chapter 4

**How do the selected bacterial strains solubilize poorly available P sources?**

The material presented in this chapter is adapted from Baudson C, Delory BM, Spaepen S, et al. (2021) Developmental plasticity of *Brachypodium distachyon* in response to P deficiency: Modulation by inoculation with phosphate-solubilizing bacteria. Plant Direct 5(1):1–17

# 1.  Foreword

In the former chapter (Chapter 3), Pi concentrations inducing contrasted phenotypes in *Brachypodium* were determined, based on the analyses of biomass, biochemical and root system architecture variables. In this chapter, bacterial strains selected based on literature data were tested for their ability to solubilize poorly available inorganic P forms, tricalcium phosphate and hydroxyapatite. The P-solubilizing strains will be further tested in Chapter 5, in co-cultivation experiments including *Brachypodium*.

# 2.  Introduction

Microorganisms are the key players in the global cycling of organic and inorganic P forms in soil (Prabhu et al. 2019). Among them, bacteria play a role in the mobilization of sparingly available P forms in soil to the benefit of plants. Many genera of bacteria are reported for their ability to solubilize P. They are cited in Chapter 1 Section 5, with the mechanisms of P solubilization.

The screening of bacteria for their ability to solubilize P is important for the development of biofertilizers and for quality control (Mehta and Nautiyal 2001). The first developed screening method was agar plate cultivation with Pikovskaya medium. Other media, eventually containing a coloured indicator, have also been developed. In these tests, a clear halo appears around the colonies able to solubilize the tested insoluble P form (Nautiyal 1999; Prabhu et al. 2019). This method is however not always reliable as strains that did not show a clear zone on agar plates solubilized phosphate in liquid medium. Testing in liquid medium is therefore preferred (Mehta and Nautiyal 2001; Nautiyal 1999).

The form of insoluble P to be used in the screening test is also important. Tricalcium phosphate (TCP) became the universal factor for assessing phosphate solubilizing bacteria (Bashan et al. 2013a). However, TCP is easily solubilized by numerous bacteria through release of protons and organic acids. TCP could therefore be not selective enough and the use of less soluble forms is recommended. Chelating or complexing compounds are involved in the solubilization of more stable P forms (Bashan et al. 2013b). A universal selection factor cannot be defined but it is advised to use other P forms than TCP (in addition to TCP or not) in solubilization assays. The choice of insoluble P forms can be based on the type of soil where the PSB will be applied. Finally, the successful strains should be tested on plants to confirm their potential to solubilize P (Bashan et al. 2013a).

In this chapter, bacterial strains selected for their potential plant growth promotion ability based on literature, were tested for P solubilization. The strains for which the P solubilizing ability will be confirmed will be used in co-cultivation in the experiment described in Chapter 5.

# 3. Materials and methods

## 3.1. Objectives and methodology

The ability of selected bacterial strains to solubilize poorly available inorganic P sources was characterized by performing *in vitro* P solubilization assays in liquid medium. Calcium phosphates are stable inorganic P forms found in alkaline soils. Tricalcium phosphate (TCP) is the most used P form in studies aiming at demonstrating P solubilizing ability. We selected hydroxyapatite (HA), a less soluble calcium phosphate form than TCP (Bashan et al. 2013b), in addition to TCP to quantify the ability of bacterial strains to solubilize P. After incubation, the solubilized P was quantified by spectrophotometric measurement.

## 3.2. Bacterial material

Four bacterial strains were selected based on literature for their potential plant growth promotion and P solubilization capacities: *Bacillus velezensis* GB03 (BveGB03), *Bacillus velezensis* FZB42 (BveFZB42), *Pseudomonas fluorescens* 29ARP (Pfl29ARP) and *Azotobacter vinelandii* F0819 (AviF0819). The *Bacillus* strains were selected for their plant growth promotion activities on *Poaceae* (Delaplace et al. 2015; Myresiotis et al. 2015; Zhang et al. 2014), as well as their ability to solubilize different forms of P (Giles et al. 2014; Idris et al. 2007; Idriss et al. 2002; Liu et al. 2015). *Pseudomonas fluorescens* also exhibited P solubilizing activities and promoted wheat (Shaharoona et al. 2008) and maize growth (Li et al. 2017). *Azotobacter vinelandii*, a free diazotrophic bacteria, exhibited P solubilization activity (Nosrati et al. 2014) and plant growth-promoting traits (Taller and Wong 1989) but did not exhibit significant plant growth-promotion effect on *B. distachyon* through emission of volatiles (Delaplace et al. 2015). *Escherichia coli* DH5α 99B829 (Eco99B829), was selected as a negative control for plant growth promotion (Delaplace et al. 2015; Wu et al. 2016; Zhou et al. 2016) but is expected to solubilize easily-soluble P forms like tricalcium phosphate (TCP) through the production of organic acids in the metabolism of sugars (Bashan et al. 2013b). This strain selection is made of strains with different plant growth-promoting and P solubilization abilities, with some exhibiting both effects. *Pseudomonas* and *Bacillus* genera were identified in the root microbiome of *B. distachyon* (Kawasaki et al. 2016). However, the interaction between a host plant and bacterial strains showed specificity at the strain level (Drogue et al. 2012). Considering the strain level specificity in plant-bacteria interaction and that the nutritional status of the plant can affect its interaction with microorganisms (Paries and Gutjahr 2023), the outcome of the interaction in a particular context is difficult to predict and testing is necessary.

The strains BveGB03 and Eco99B829 were kindly provided by Dr Paul W. Paré and Dr John McInroy (Texas Tech University, Lubbock, TX, USA), Pfl29ARP by Dr

Alain Sarniguet (Institut National de la Recherche Agronomique, Rennes, France), AviF0819 by the Katholieke Universiteit Leuven (Leuven, Belgium), and BveFZB42 by Pr Rainer Borriss (Nord Reet UG, Greifswald, Germany). The bacterial strains were stored at -80°C in Luria-Bertani ("LB") medium containing 20% v/v glycerol before plating.

### 3.3. *In vitro P solubilization assay*

One week before the experiment, the bacteria were plated on LB agar plates (2.5% w/v LB broth, Prod. No. L3152; 1.5% w/v agar, Prod. No. 05039, Sigma-Aldrich Co., St. Louis, USA) and incubated at 28°C. The day before the experiment, the bacteria were suspended in 40 ml of LB (2.5% w/v LB broth) and incubated overnight at 150 rpm and 30°C (Innova 4340, New Brunswick Scientific Co. Inc., Edison, USA). The concentration of the bacterial suspensions was derived from the optical density based on internal laboratory calibration, measured at 540 nm. The tubes were centrifuged (20 min at 4000 rpm) and the LB medium was removed. The bacterial pellets were rinsed with 25 ml of 10 mM $MgSO_4$ in order to avoid P contaminations. The tubes were centrifuged again (20 min at 4000 rpm) and the $MgSO_4$ solution was removed. The bacteria were suspended in an adequate volume of NBRIP medium (National Botanical Research Institute's phosphate growth medium, Nautiyal 1999) (Table 4-1) containing TCP ($Ca_3(PO_4)_2$, Prod. No. C0506.1000, Duchefa Biochemie, Haarlem, The Netherlands) or HA ($Ca_5(PO_4)_3OH$, Prod. No. 8450.1, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) at a concentration of 5 g/l (pH 7) in order to obtain a bacterial concentration of $10^7$ colony-forming unit ("CFU")/ml. Bottles containing 90 ml of NBRIP medium were successively inoculated with 10 ml of the prepared suspensions to obtain a final concentration of $10^6$ CFU/ml, and incubated for 3 days at 30°C and 150 rpm.

10 ml were sampled daily for subsequent analysis. 1 ml was subsampled for serial dilution and plating on LB agar plates in order to monitor bacterial growth. The remaining samples were centrifuged and the supernatant was filter-sterilized (pore size 0.2 µm) for pH and soluble Pi content measurements. The Pi content in the solution was measured according to the phosphomolybdate blue colorimetric method (Murphy and Riley 1962) (Prod. No. 69888, molybdate reagent solution, Fluka Sigma-Aldrich Co., St. Louis, USA). The different steps of the P solubilization assay are summarized in Fig 4-1.

**Table** 4-1. Composition of NBRIP medium (Nautiyal 1999).

|  | Concentration (g/l) |
|---|---|
| Glucose | 10 |
| $Ca_3(PO_4)_2$ or $Ca_5(PO_4)_3OH$ | 5 |
| $MgCl_2.6H_2O$ | 5 |
| $MgSO_4.7H_2O$ | 0.25 |
| KCl | 0.2 |
| $(NH_4)_2SO_4$ | 0.1 |
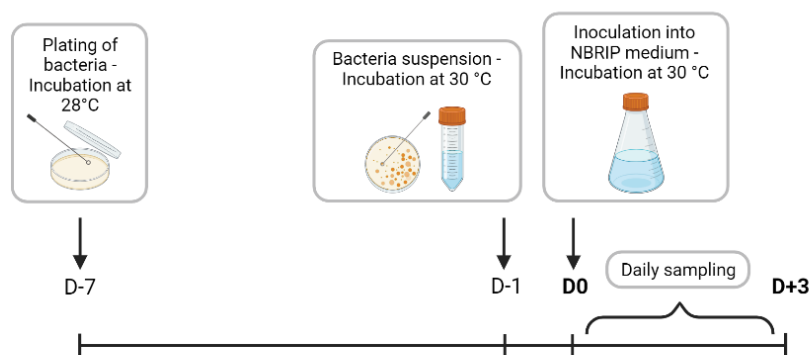


**Figure** 4-1. Steps of *in vitro* P solubilization assay. Created with BioRender.com.

## 3.4. Statistical analyses

The relationship between P solubilization and pH variation in the NBRIP medium was studied by performing regression analyses (lm function, R 3.5.2, R Core Team 2018). The model order was increased until there was not significant difference with the higher order model (anova function, R 3.5.2, R Core Team 2018).

# 4. Results

The bacteria's ability to solubilize poorly available forms of P was assessed using TCP and HA in a modified NBRIP medium. After three days, all the selected strains were able to solubilize both forms of P to some extent (Fig. 4-2a) compared to the non-inoculated control treatment. For both forms of P, the bacterial strains showing the highest solubilization ability were Eco99B829 and Pfl29ARP. The solubilization of TCP and HA were similar for all bacterial strains with the exception of Eco99B829, which exhibited a stronger solubilization ability for HA despite a greater variability between independent replicates. All the strains were able to maintain stable population size during the duration of the experiment (data not shown). BveGB03, AviF0819,

Pfl29ARP and Eco99B829 generated a pH drop during the experiment for both forms of P (Fig. 4-2a). Eco99B829 and Pfl29ARP induced the strongest acidification.

Regarding HA solubilization, the relationship between the soluble P concentration and $\Delta$pH in the growing medium was best fitted by a 4th order polynomial model (Fig. 4-2b). The HA solubilization activity clearly intensified as the acidification became stronger. The regression between soluble Pi concentration and $\Delta$pH for TCP solubilization was best fitted by a 2nd order polynomial model (Fig. 4-2b). As for HA, the TCP solubilization activity intensified with increasing pH variation, but to a lesser extent.
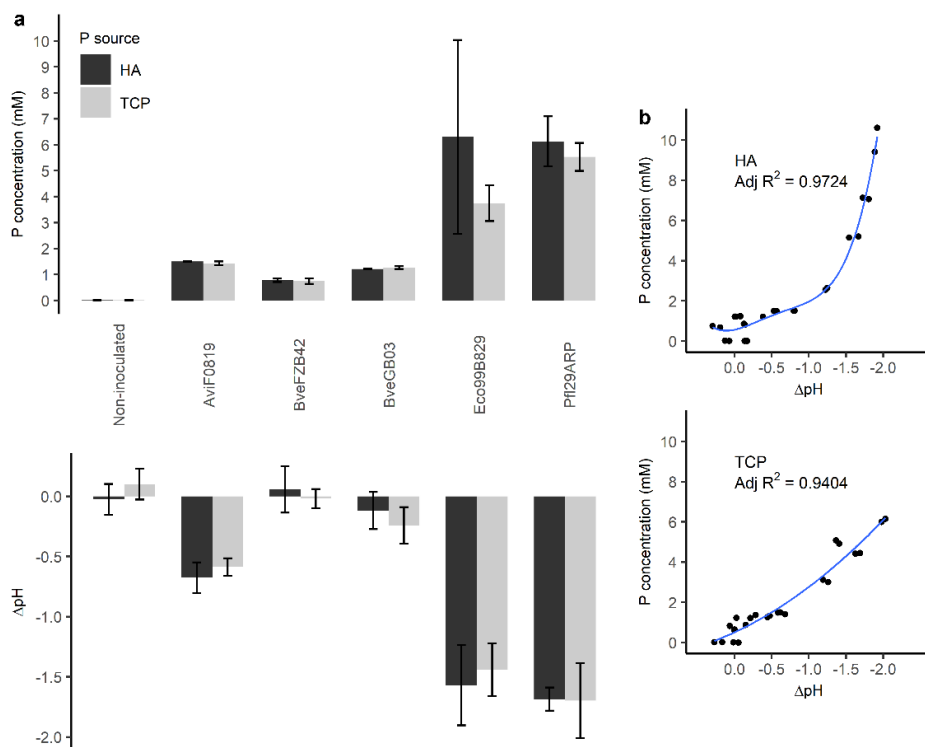


**Figure** 4-2: (a) Soluble Pi concentration and pH variation in NBRIP medium after three days of bacteria cultivation in the presence of either TCP or HA as poorly soluble forms of P (n = 4 replicates, mean ± SD). (b) Regression curves linking the observed Pi concentration and the $\Delta$pH in the growing medium after three days of incubation. For each regression model, adjusted R² values are displayed on the graphs. Regression coefficients for HA solubilization: $y = 0.571 - 0.814x + 2.7675x^2 + 4.219x^3 + 2.039x^4$; regression coefficients for TCP solubilization: $y = 0.513 - 1.727x + 0.530x^2$.

# 5. Discussion

All the selected bacteria were able to solubilize the poorly available forms of P (TCP and HA) in NBRIP medium (Nautiyal 1999). HA, despite being reported as less soluble than TCP (Bashan et al. 2013b), was as easily solubilized as TCP. Some acidification of the medium was observed, with the best solubilizer strains acidifying the most. Medium acidification by proton release is the most straightforward P solubilization process (Bashan et al. 2013b) and numerous studies have reported an acidification-associated P solubilization (Collavino et al. 2010; Fernández et al. 2012; Pereira and Castro 2014; Yu et al. 2011). The relationship between soluble P concentration and pH variation tended towards an intensification of P solubilization activity as the pH variation became stronger. This was more pronounced for HA than for TCP solubilization. This raises the hypothesis that HA solubilization mechanisms other than acidification are involved, such as complexing or chelating reactions (Bashan et al. 2013b). All the tested bacterial strains, having demonstrated ability to solubilize TCP and HA, were selected to be used in the co-cultivation experiment described in Chapter 5.

# Chapter 5

**How is the developmental plasticity of *Brachypodium distachyon* Bd21 modulated by inoculation with phosphate solubilizing bacteria?**

# 1.  Foreword

The experimental design presented in this chapter is based on the results presented in Chapters 3 and 4. Chapter 3 allowed to define adequate Pi concentrations to obtain contrasted plant phenotypes. The concentration 0 µM Pi was considered as unrealistic. The concentration 25 µM Pi was chosen as treatment inducing a severe to mild stress to the plant. The concentration 1000 µM Pi was selected as reference treatment inducing optimal growth of the plant. The cultivation system used for the co-cultivation experiments being closed, this concentration allows to ensure a supply of P to the organisms during all the cultivation duration. In Chapter 4, bacterial strains were tested for their ability to solubilize two forms of poorly soluble inorganic P, tricalcium phosphate and hydroxyapatite. All the strains being able to solubilize these two P forms, they were selected for the co-cultivation experiments presented in this chapter.

# 2.  Introduction

Soil P reserve is mostly unavailable to plants due to the capacity of many soils to fix P (White et al. 2013). Technologies to mobilize this soil P reserve must be developed in order to reduce the use of inorganic P fertilisers (Macdonald et al. 2011). Plants have developed strategies to cope with P deficiency and enhance their PUE, including alteration of the root morphology and architecture, as well as exudation of carboxylates and hydrolytic enzymes for P solubilization. Micro-organisms can also be useful in mobilizing soil P reserves, by directly increasing the P availability in soils through solubilization (by releasing protons, organic anions and chelating compounds) and mineralization (by releasing hydrolytic enzymes), or enhancing plant P nutrition processes through hormonal stimulation of root growth, for example (Richardson et al. 2011). The use of bioinoculants in addition to fertilisers, with the aim of optimising the efficiency of those fertilisers and reducing nutrient application rates (European Parliament and Council of the EU 2019) is a promising strategy.

Phenotypic plasticity gives the plant a great potential to respond to fluctuating environments (Nicotra et al. 2010; Schmid 1992). Breeding programs have traditionally opted for stable high yielding phenotypes in constant agricultural systems with high inputs. However, the uncertainty of the future environment and climate requires us to reconsider the place of phenotypic plasticity in breeding strategies. Indeed, phenotypic plasticity can be an advantage to plants living in changing or heterogeneous environments by increasing plant fitness (Lobet et al. 2019; Nicotra et al. 2010). Plasticity in root traits among contrasting environments was associated with stable yield in rice genotypes (Sandhu et al. 2016; Topp 2016). The plastic response of plants to abiotic factors and to the presence of microorganisms still needs clarification. Additionally, the microbial-triggered change in plants' plastic response

to P deficiency deserves greater attention in order to optimize plant P nutrition and reduce the use of fertilisers (Goh et al. 2013).

This study aimed to characterize *Brachypodium distachyon*'s response to contrasted P supplies (soluble and poorly soluble forms of inorganic P), as well as the impact of plant inoculation with single strains of PSB on this response in terms of developmental plasticity. The following hypotheses were tested: (i) biomass allocation and root system development in *Brachypodium* show plasticity in response to contrasted P conditions; (ii) inoculation with PSB modulates the plant's plastic response to contrasted P supplies; and (iii) this modulation induces changes in plant PUE. Biomass accumulation and allocation, shoot P concentration and PUE, as well as root architectural traits, were considered. *Brachypodium*'s developmental plasticity was assessed using tools including allometry analysis for the biomass allocation and persistent homology analysis for the RSA. It is the first time, to our knowledge, that these tools have been used to precisely evaluate the impact of biostimulants on a plant's response to nutrient limitation.

# 3. Materials and methods

## 3.1. Objectives and methodology

In order to evaluate the impact of single bacterial strains on *Brachypodium* development, a closed system allowing gnotobiotic conditions (*i.e,*. including the selected plant and bacterial organisms only, avoiding contaminations) was used for co-cultivation experiments. Plants inoculated with single strains were individually cultivated for 4 weeks under contrasted P conditions (low level of soluble P, low level of soluble P supplemented with a poorly soluble P form TCP or HA and high level of soluble P). The way in which the plants allocated their biomass between shoot and roots was unravelled using allometric analyses, making it possible to detect plasticity in biomass allocation. In complement, the root mass fraction (an indicator of the fraction of the total biomass allocated to the roots) was also analysed. Biomass allocation in plants is an allometric process that varies with plant size. Allometry analyses, performed by plotting the size of two organs against one another, are a good way to visualize and test how the growth of a given organ changes relative to the others (Poorter and Sack 2012). A major advantage of allometry analyses is that it highlights the adaptability of the biomass allocation pattern in response to the environment (true plasticity). By comparison, the analysis of biomass ratios and fractions (e.g., root: shoot ratio and root mass fraction) independently from plant size does not only reflect the adaptability of a plant to environmental conditions, but also integrates the global productivity (dependent on growth rate) of the plant in those conditions (apparent plasticity) (Weiner 2004). The effect of our experimental treatments on the morphology of plant root systems was investigated using two complementary approaches. First, root systems were compared based on their total

root length (geometric descriptor). Second, we applied persistent homology, a mathematical framework developed for the quantification of plant morphologies at different scales (Li et al. 2018; Li et al. 2017). The main goal of this method is to produce a persistence barcode encapsulating geometric (*e.g.*, total root length and number of root tips), but also topological (*i.e.*, how individual roots are connected to each other through branching) information for each root system (see Figures 1 and 3 in Delory et al. 2018). The degree of dissimilarity between root systems can then be assessed by comparing persistence barcodes against each other (Li et al, 2017). Finally, we measured the shoot P content and were interested in *Brachypodium*'s PUE (considering different components of it) as it can be potentially impacted by any alteration of the plant morphology.

## 3.2. *Plant and bacterial materials*

Plant and bacterial materials are identical to those described in Chapter 3 Section 2.2 and Chapter 4 Section 2.2, respectively.

## 3.3. *In vitro Brachypodium-bacteria co-cultivation*

One week before the experiment, the bacteria were plated on LB agar plates and incubated at 28°C. The day before the experiment, the bacteria were suspended in 40 ml of LB and incubated overnight at 150 rpm and 30°C. The tubes were centrifuged (20 min at 4000 rpm) and the LB medium was removed. Inoculums at $10^8$ CFU/ml were finally prepared in 10 mM $MgSO_4$ for subsequent inoculation of the plantlets.

*Brachypodium distachyon* (L.) P. Beauv. Bd21 seeds were surface sterilized (30 s in 70% v/v ethanol, rinsed once with sterile water, 10 min in sodium hypochlorite 5% v/v, rinsed three times with sterile water) and stratified for 2 days at 4°C on Hoagland agar plates (0.125% w/v Hoagland, Prod. No. DU1201, Duchefa Biochemie, Haarlem, The Netherlands; 0.094% w/v $Ca(NO_3)_2.4H_2O$; 0.8% w/v Plant agar, Prod. No. P1001, Duchefa Biochemie, Haarlem, The Netherlands). The seeds were then incubated for 24 hours in a growth chamber (23°C, 16h/8h day light, PPFD 140 µmol.m$^{-2}$.s$^{-1}$) for germination.

Homogeneous 24 hour-old plantlets were selected and inoculated with bacteria by dipping them into 10 mM $MgSO_4$ containing a bacterial strain at $10^8$ CFU/ml for 10 minutes (control plantlets were dipped into 10 mM $MgSO_4$). The plantlets were then transferred into Magenta® boxes (GA-7 Magenta vessel, Magenta LLC, Lockport, USA) filled with 180 g of sterilized black gravel (rinsed three times with tap water and autoclaved; 1-3 mm quartz gravel, prod. no. 400723, Flamingo, Geel, Belgium ) and 50 ml of sterile nutrient solution. One plantlet was placed into each Magenta® box. Three modified Hoagland nutrient solutions and a reference solution, corresponding to the contrasting P treatments, were used: a P-limiting supply containing 25 µM of $KH_2PO_4$ (soluble inorganic P form, "P-"), a P-limiting supply

supplemented with 1 g/l TCP (poorly soluble inorganic P form, "P-/TCP") or 1 g/l HA (poorly soluble inorganic P form, "P-/HA"), and a P-sufficient supply containing 1 mM $KH_2PO_4$ (soluble inorganic P form, "P+") (see original composition of Hoagland solution in Table 3-1). The quantity of TCP and HA (1 g/l) allows to potentially raise non-limiting Pi concentration in case they are completely solubilized (the maximum soluble Pi concentration is 6.4 mM in P-/TCP treatment and 6.0 mM in P-/HA treatment). The boxes were sealed with Leukopor® tape (prod.no. 02454-00, BSN medical GmbH, Hamburg, Germany) and incubated in the growth chamber for four weeks (23°C, 16h/8h day light, PPFD 140 µmol.m$^{-2}$.s$^{-1}$). The steps of the *in vitro* co-cultivation are summarized in Fig. 5-1. Six independent experiments were carried out (three with P-, P-/TCP and P+ treatments; three with P-, P-/HA and P+ treatments) and five plants were cultivated as replicates for each treatment combination (factorial design, 6 different bacterial treatments*3 P treatments*5 replicates = 90 plants per experiment) (Fig. 5-2).
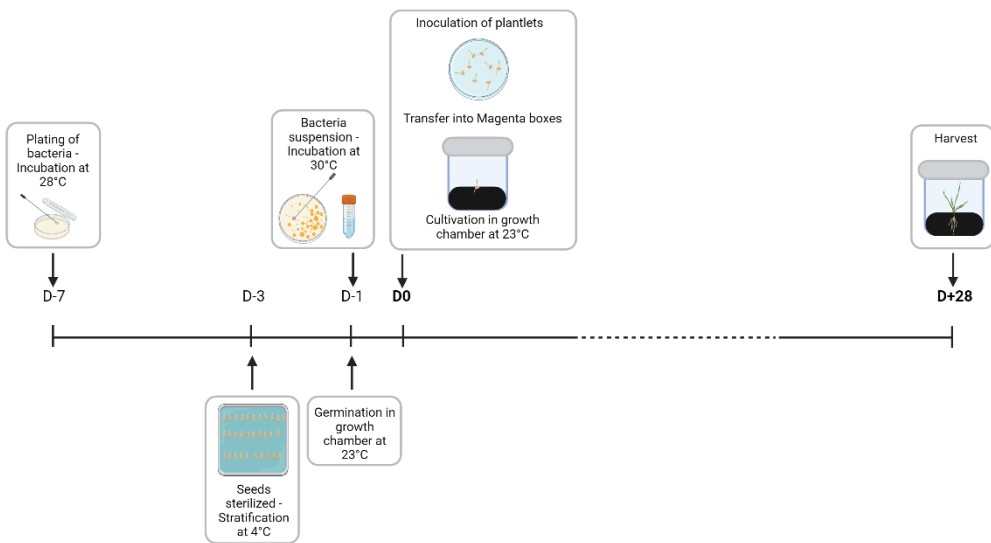


**Figure** 5-1. Steps of *in vitro* co-cultivation of *B. distachyon* Bd21 and PSB exposed to contrasted P supplies. Created with BioRender.com.
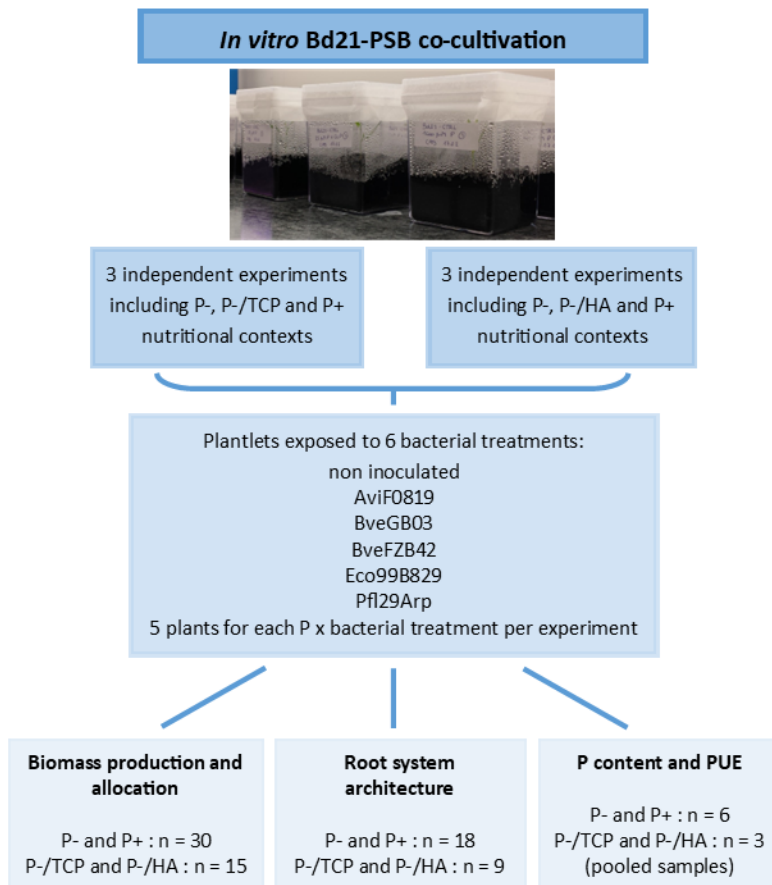
**Figure** 5-2: Description of the experimental design used for the *in vitro* co-cultivation experiments exposing PSB-inoculated *Brachypodium* plantlets to contrasted P supplies.

Four week-old plants were harvested and cut to measure fresh biomass accumulation in shoot and roots. Sticking gravel was removed by gently shaking the plants in water and removing it by hand. The presence of bacteria was assessed by scratching agar plates with the root system. The root system was scanned for three plants per treatment. The roots were spread in a transparent tray containing a thin layer of water and scanned using a flatbed scanner equipped with a transparency unit (backlight) (8-bit grayscale images saved as jpg files, 1200 dpi, Epson Perfection V800 Photo, Epson America Inc., Long Beach, USA) in order to perform RSA analyses. Shoots were stored at -80°C before P content measurements. Total biomass and root mass fraction ("RMF", mg root biomass/mg total biomass) were computed from the measured biomasses. RMF was recorded in order to analyse biomass allocation in *Brachypodium*, considering allocation as a partitioning process. According to this perspective, plants divide a given amount of resources among

structures according to their developmental priorities (Weiner 2004). A description of the experimental design used for the co-cultivation experiments is provided in Fig. 5-2.

## 3.4. P concentration in plant tissues

P content in *Brachypodium* shoots was measured by ICP-OES on frozen samples (C.A.R.A.H. ASBL, Ath, Belgium). The samples were calcinated overnight at 450°C. The ashes were then suspended in nitric acid for digestion. The P concentration was measured by ICP-OES (Thermo Fisher iCAP 7600, Thermo Fisher Scientific, Waltham, USA). The five replicates of each treatment in each independent experiment were pooled. Three pooled samples were analysed for the P-/TCP and P-/HA treatments. Six pooled samples were analysed for the P- and P+ treatments. The results were expressed as total shoot P concentration (µg P/ mg fresh weight).

## 3.5. Root system architecture measurement

An automated evaluation of the total root length ("TRL") was performed for all scanned root systems using the ImageJ macro IJ_Rhizo (Pierret et al. 2013; Schneider et al. 2012). For each image, the TRL was estimated using the Kimura method as it provides more accurate length estimates than the other methods available in IJ_Rhizo (Delory et al. 2017).

In addition, more detailed root system architecture analyses were performed using SmartRoot (Lobet et al. 2011). Only the $1^{st}$ and $2^{nd}$ order roots were analysed because the thinner, higher order, roots easily break at harvest. These manual analyses were performed for the control treatment and for the two strains mostly impacting plant development. We used persistent homology, a mathematical framework for quantifying plant morphology (Li et al. 2018), to analyse the topology of plant root systems. Persistent homology-based methods are complementary to multivariate approaches relying on the quantification of a number of geometric traits (*e.g.*, total root length) (Li et al. 2018; Delory et al, 2018) and are therefore well suited to accurately characterize and compare root systems treated with different bacterial strains or biostimulants. All RSML (Root System Markup Language) files exported by SmartRoot were processed with persistent homology-related functions of the R package archiDART (R 3.5.2, R core Team 2018; archiDART package version 3.3, Delory et al., 2016; Delory et al. 2018). The root system can be represented as a tree graph made of a succession of nodes connected by straight lines (referred to as a zero-order homology group in mathematics, $H_0$). The persistent homology analysis studies how the lines in the root system persist by applying a geodesic distance function, measuring the distance between the birth and the death of each branch of the root system. The geodesic distance measures the distance along the roots between the root system base and any point on the root system and was used to compute a persistence

barcode for each root system, depicting birth and death of each branch (Fig. 5-3). This method is not sensitive to transformation and deformation. This means that the computed persistence barcode is not affected by root washing and 2D scanning (Delory et al. 2018). The degree of dissimilarity between barcodes (*i.e.,* root systems) was assessed by computing a pairwise distance matrix containing dissimilarities calculated using a bottleneck distance method. Morphological differences between root systems were then visualized using multidimensional scaling (R 3.5.2, R Core Team 2018).
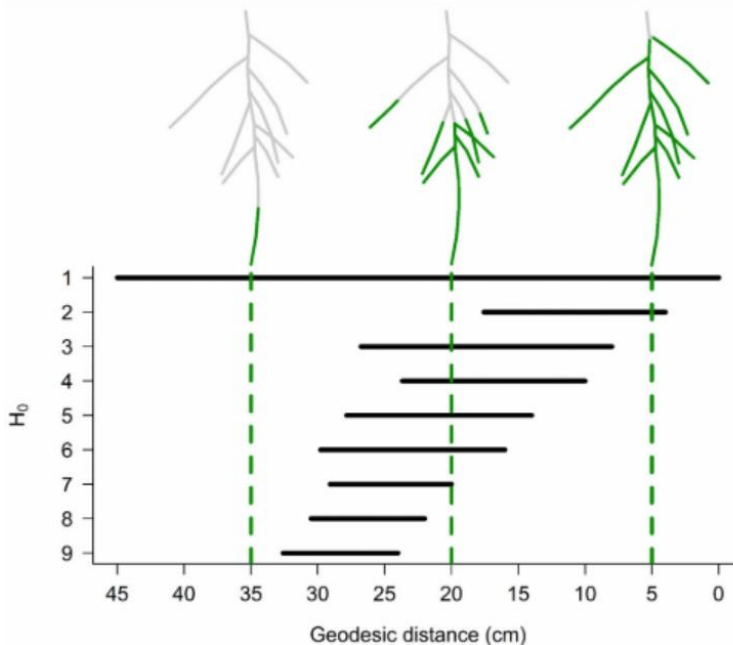


**Figure** 5-3. Persistence barcode of the topology of a plant root system computed using a geodesic distance function. $H_0$ numbers correspond to each branch of the root system. Vertical lines indicate the position along the geodesic distance function (from left to right) (Delory et al. 2018).

## 3.6. P use efficiency

The PUE analysis was performed by considering three different parameters: (i) the P uptake efficiency, (ii) the P utilization efficiency and (iii) the physiological P use efficiency, as described in Chapter 1 Section 3.

## 3.7. Statistical analyses

Three-way ANOVAs were performed to study the impact of P supply, bacteria inoculation, independent repetitions and the interaction between P supply and bacteria inoculation on the following response variables: shoot, root and total biomass parameters; RMF; TRL; shoot P concentration; and the three components of PUE. A

model with crossed fixed factors was applied (lm, glm and anova functions, R 3.5.2, R Core Team 2018; Gamma family distribution with a log-link function was used for GLM models). Dunnett's post-hoc tests were performed to compare the treatments to the control situation (non-inoculated plants for inoculation treatment, P+ for P treatment) (R 3.5.2, R Core Team 2018; multcomp package version 1.4-8, Hothorn et al. 2008).

Allometry analyses were performed on shoot and root biomass in order to study the biomass allocation pattern. The "smatr" package (R 3.5.2, R Core Team 2018; "smatr" version 3.4-8) was used for estimation, inference and plotting of allometric lines as well as for checking assumptions (Warton et al. 2012). The standardized major axis ("SMA") analysis was used and all variables were log-transformed. In brief, this analysis consists of a model II regression, estimating how one variable scales against another. The obtained allometric trajectories depict the relative development of the shoot and root compartments, *i.e.* how the root system growth impacts the shoot development. Inference statistics compare coefficients of the regression lines (slope and intercept) between the populations (Warton et al. 2006). Firstly, differences in slope between groups were tested. If there was no difference in slope between groups, differences in elevation were tested using a common slope for all groups. When significant differences between groups were highlighted, pairwise multiple comparisons were performed in order to identify which populations differed from each other. Differences in slope (*i.e.,* investment in shoot biomass per additional unit of root biomass) or elevation (*i.e.,* shoot productivity for similar root biomass) among treatments led to different allometric trajectories. Change in allometric trajectory due to different treatments revealed plasticity in the biomass allocation process (Weiner 2004; Xie et al. 2015). The analysis of allometric trajectories is complementary to the analysis of RMF for the study of biomass allocation plasticity.

Differences in root system architecture were investigated using permutational multivariate analysis of variance (PERMANOVA) (R 3.5.2, R Core Team 2018; vegan package version 2.5-4, Oksanen et al. 2019). The dissimilarity matrix used in the model formula was the pairwise distance matrix returned by the persistent homology analysis of plant root systems. Bacterial strain, P treatment and their interaction were used as independent variables in the model. For each fixed factor, a post-hoc test was performed by running a separate PERMANOVA for each pairwise comparison. *P* values were adjusted for multiple comparisons using the Bonferroni method.

All figures shown in this study were generated using the "ggplot2" package (R 3.5.2, "ggplot2" version 3.1.0, Wickham 2016).

Data and R scripts are accessible on Zenodo repository at https://doi.org/10.5281/zenodo.3555566

# 4. Results

## *4.1. Biomass accumulation in Brachypodium was altered by soluble P deficiency and inoculation with P solubilizing bacteria*

Shoot biomass production was lower in plants grown under P-, P-/TCP and P-/HA conditions, with a diminution of 43.1%, 35.2% and 33.4% compared to the P+ treatment, respectively ($P \geq 0.001$, Fig. 5-4a-d). Plant inoculation with PSB strains had either no impact or induced a lower shoot biomass accumulation (Fig. 5-4e). Inoculation with BveFZB42 and Pfl29ARP led to a lower shoot biomass, with up to 13.2% reduction in plants inoculated with BveFZB42 under the P+ treatment and 30.3% reduction in plants inoculated with Pfl29ARP under the P-/TCP treatment ($P < 0.001$). The impact of P conditions on the accumulation of biomass in roots was more limited, with only plants grown under the P- treatment having a greater root biomass (+13.3%) compared to the plants exposed to P+ conditions ($P < 0.001$, Fig. 5-4f-i). Inoculation had either no impact or a negative impact on the accumulation of biomass in roots ($P = 0.003$, Fig. 5-4j). Indeed, plants inoculated with BveFZB42 exhibited a significant reduction of the root biomass of up to 14.5% under the P- treatment. The total biomass decreased by 27.8%, 25.2% and 24.1% under the P-, P-/TCP and P-/HA treatments respectively ($P < 0.001$, Fig. 5-4k-n). Plant inoculation with PSB strains led to a repression of the biomass accumulation at the whole plant level ($P < 0.001$, Fig. 5-4o). Inoculation with BveFZB42 and Pfl29ARP induced a significantly lower total biomass in comparison with the non-inoculated control. The growth reduction reached 12.2% with BveFZB42 under P-/HA conditions and 21.1% with Pfl29ARP under P- conditions. A table comprising mean values per treatment, standard deviation and coefficients of ANOVAs is available in Appendix 3.
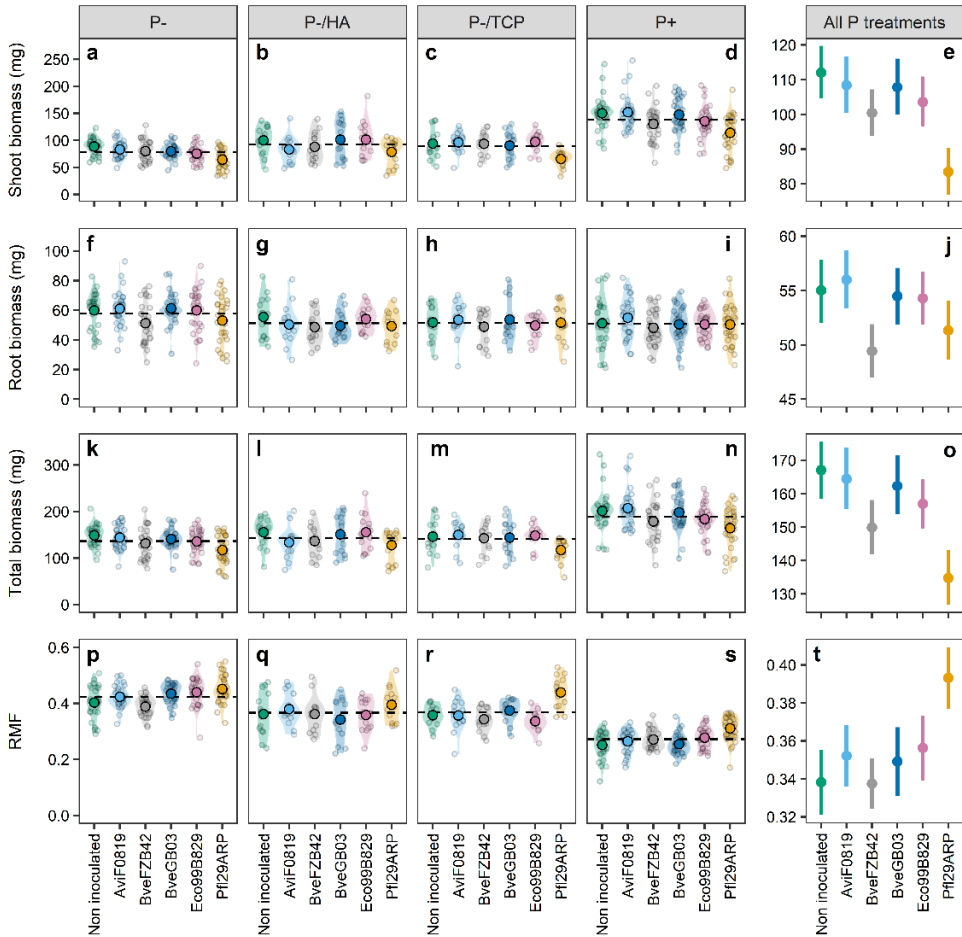
**Figure** 5-4: Average shoot biomass (a-e), root biomass (f-j), total biomass (k-o) and root mass fraction (p-t) of four-week-old *Brachypodium* plantlets exposed to contrasted P supplies and either inoculated or not inoculated with bacteria. n = 30 for the P- and P+ treatments, n = 15 for the P-/HA and P-/TCP treatments. For each P treatment, the grand mean is shown by a dashed horizontal line. For each inoculation treatment, large black-circled dots represent mean values, and shaded areas show the density distribution of each population. Individuals are displayed as small grey-circled dots in the graphs. In panels e, j, o and t, values are means +/- 95% confidence intervals calculated across P treatments.

## 4.2. Shifts in biomass partitioning and allometric trajectories of *Brachypodium* were observed when exposed to contrasted P supplies and inoculated with P solubilizing bacteria

Exposure of *Brachypodium* to soluble P limitation (P-, P-/HA and P-/TCP) increased RMF by 55.8%, 34.9% and 35.7% compared to the P+ treatment,

respectively ($P < 0.001$, Fig. 5-4p-s). The impact of *Brachypodium* inoculation with PSB was dependent on the P environment, as there was an interaction between these two variables ($P < 0.001$). Plants inoculated with Pfl29ARP exhibited the greatest RMF under all treatments (Fig. 5-4t) and this effect was significant for the P-, P-/TCP and P+ treatments (12.1%, 22.7% and 23.4% increase, respectively). Under P- conditions, plants inoculated with BveGB03 and Eco99B829 also had a significantly greater RMF compared to non-inoculated plants (Fig. 5-4p-t). Mean values per treatment, standard deviation and coefficients of ANOVAs are available in Appendix 3.

The allocation pattern between shoots and roots was further analysed using SMA regression models (Fig. 5-5). In non-inoculated plants grown under P-/TCP and P-/HA conditions, the shoot biomass increase per unit of root biomass was greater than that of non-inoculated plants grown under P- and P+ conditions (slopes: 1.15, 1.19, 0.81 and 0.60, respectively; $P = 0.021$; Fig. 5-5a). Non-inoculated plants grown under P+ conditions exhibited the greatest shoot productivity, but invested the lowest amount of biomass into the shoot per unit of root production. Inoculation of plants grown under P- conditions did not induce a significant difference in slope ($P = 0.757$, Fig. 5-5b). Differences in elevation were observed ($P < 0.001$), with non-inoculated plants and plants inoculated with BveFZB42 showing the greatest shoot productivity and plants inoculated with Pfl29ARP the lowest, for similar root biomass. Under the P-/HA treatment, slope and elevation did not vary among groups ($P = 0.174$ and $0.433$, respectively; Fig. 5-5c), even if plants inoculated with BveGB03 and Eco99B829 showed greater shoot biomass increase per unit of root biomass, when considering the graphical trends. When plants were grown in the presence of TCP, the inoculation with PSB did not affect the slope ($P = 0.835$, Fig. 5-5d). Elevation was altered when plants were inoculated with Pfl29ARP, leading to the lowest shoot productivity for similar root biomass (lowest elevation, $P < 0.001$). Differences in slope were observed under the P+ treatment ($P = 0.008$), with the greatest production of shoot biomass per unit of root production in plants inoculated with Pfl29ARP, Eco99B829 and BveFZB42 (Fig. 5-5e). SMA coefficients and results of covariance analysis are available in Appendix 4.
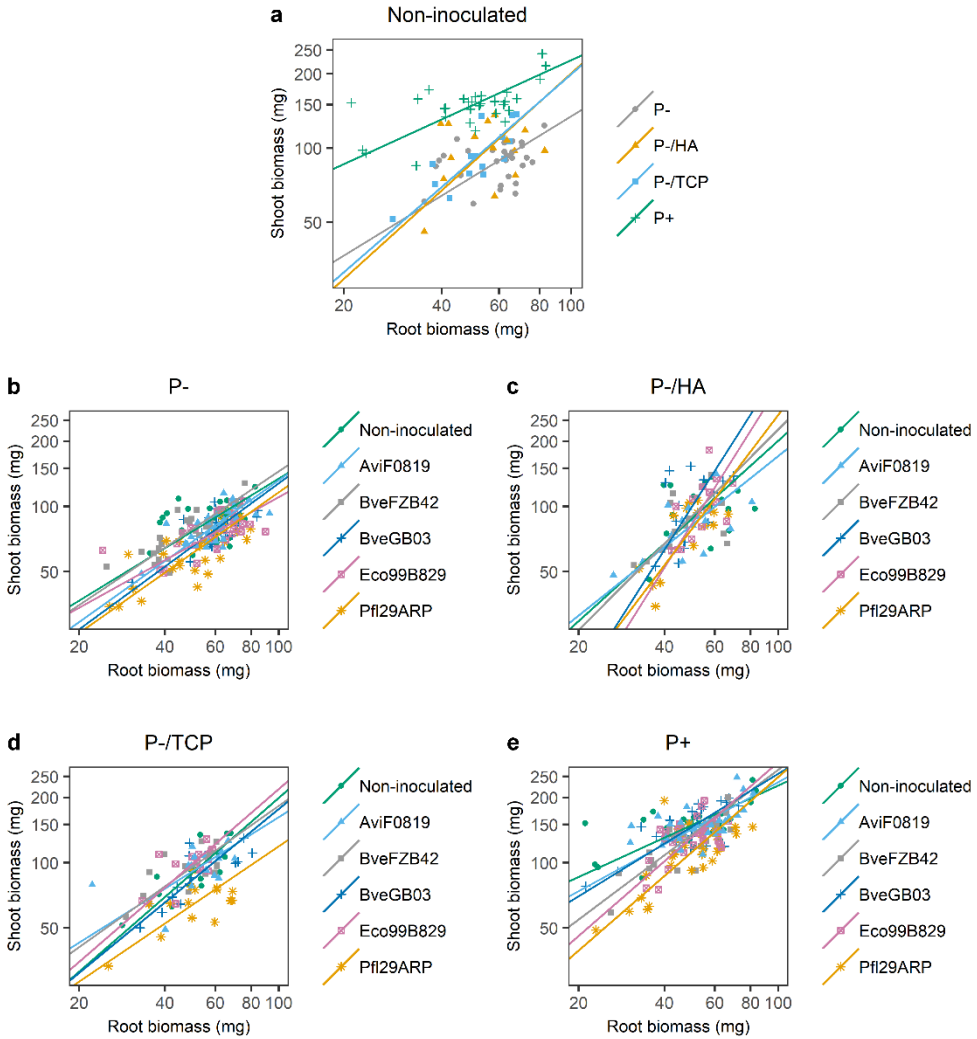
**Figure** 5-5: Allometric relationship between shoot biomass and root biomass of four-week-old *Brachypodium* plantlets exposed to contrasted P supplies and grown with or without bacterial inoculation. X and Y axes are log-scaled. Symbols represent individuals. Lines represent SMA regression lines. (a) Non-inoculated plants exposed to contrasted P supplies. Inoculated and non-inoculated plants grown under (b) P-, (c) P-/HA, (d) P-/TCP and (e) P+ conditions.

## *4.3. Brachypodium's root system morphology was impacted by P supply and inoculation with P solubilizing bacteria*

*Brachypodium* TRL increased by 8.97% when plants were exposed to the P-/HA treatment compared to the P+ treatment ($P = 0.023$, Fig. 5-6 a-d). In comparison with the TRL measured in non-inoculated plants, the TRL of plants inoculated with

BveFZB42, Eco99B829 or Pfl29ARP decreased by 9.64%, 11.61% and 16.67% respectively, whatever the nutritional context ($P < 0.001$, Fig. 5-6 e). Mean values per treatment, standard deviation and coefficients of ANOVAs are available in Appendix 5.
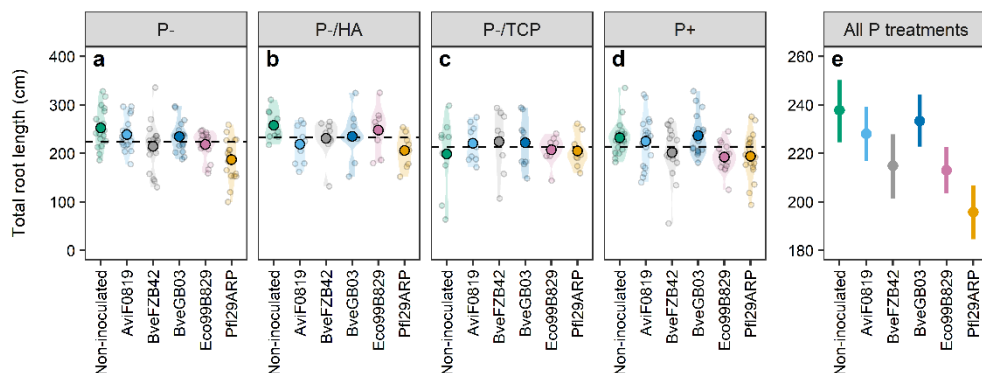


**Figure** 5-6: Average total root length of four-week-old *Brachypodium* plantlets exposed to contrasted P supplies and either inoculated or not inoculated with bacteria. n = 18 for the P- and P+ treatments, n = 9 for the P-/HA and P-/TCP treatments. For each P treatment, the grand mean is shown by a dashed horizontal line. For each inoculation treatment, large black-circled dots represent mean values, and shaded areas show the density distribution of each population. Individuals are displayed as small grey-circled dots in the graphs. In panel e, values are means +/- 95% confidence intervals calculated across P treatments.

The persistent homology analysis of the root systems was performed on 1st and 2nd order roots of non-inoculated plants and plants inoculated with Pfl29ARP or BveFZB42, as those strains showed a strong impact on root biomass accumulation (Fig. 5-7). Both PSB inoculation and P treatment had an impact on root system morphology ($P < 0.001$ and $= 0.006$ respectively). Pairwise comparisons revealed that, on average, the morphology of plant root systems inoculated with Pfl29ARP was different from those of non-inoculated plants and plants inoculated with BveFZB42. Despite a significant impact of P treatment on root system morphology, pairwise comparisons did not highlight the P treatments that differed from one another. The coefficients of the statistical analysis are available in Appendix 6.
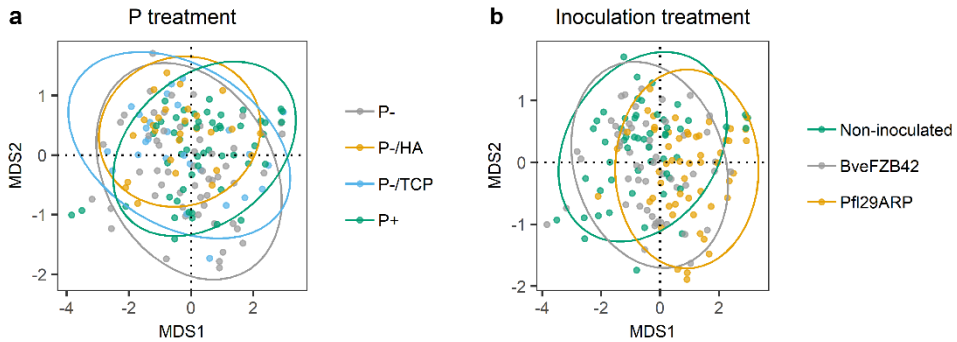
**Figure** 5-7: Multidimensional scaling plots displaying morphological differences between root systems, induced by P (a) and inoculation (b) treatments. The Euclidean distance separating two branching structures (dots) on the plot is a close representation of the true dissimilarity between these structures. 95% confidence ellipses for the centroids are plotted for each treatment.

## 4.4. Low P availability induced lower shoot P concentration, even in the presence of P solubilizing bacteria

P concentration in the shoot of plants exposed to the P-, P-/HA and P-/TCP treatments was lower than in plants exposed to P+ (-68.9%, -56.2% and -63.2% respectively; $P < 0.001$; Fig. 5-8 a-d). Plants grown under these three treatments showed P deficiency symptoms, such as necrosis starting from the apex of mature leaves (Arvalis, Institut du végétal). Inoculation with bacteria did not help the plants to increase the shoot P concentration, even in the presence of the potentially mobilizable P sources TCP or HA (Fig. 5-8 e). Mean values per treatment, standard deviation and coefficients of ANOVAs are available in Appendix 7.
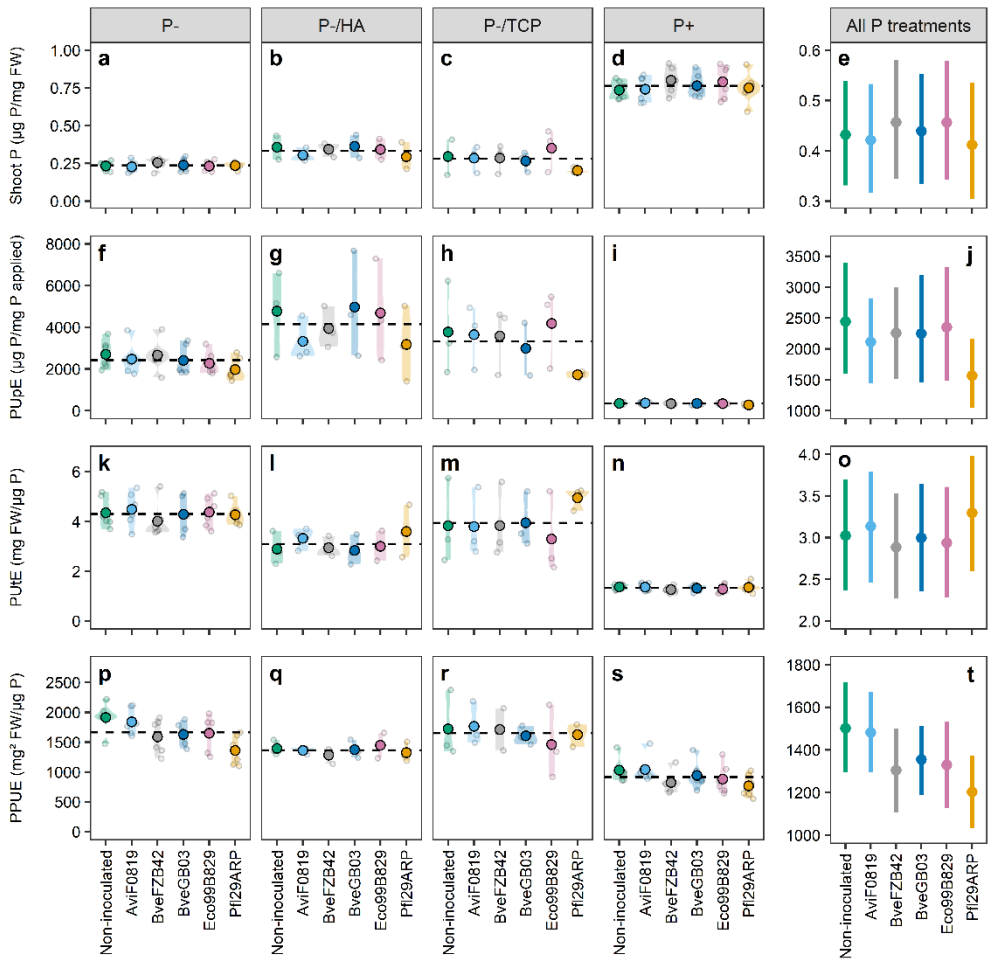
**Figure** 5-8: Average shoot P concentration (a-e), P uptake efficiency "PUpE" (f-j), P utilization efficiency "PUtE" (k-o) and physiological P use efficiency "PPUE" (p-t), of four-week-old *Brachypodium* plants grown under contrasted P supplies and either inoculated or not inoculated with bacterial strains. n = 6 for the P- and P+ treatments, n = 3 for the P-/HA and P-/TCP treatments. For each P treatment, the grand mean is shown by a dashed horizontal line. For each inoculation treatment, large black-circled dots represent mean values, and shaded areas show the density distribution of each population. Individual data points (pool of 5 plantlets) are displayed as small grey-circled dots in the graphs. In panels e, j, o and t, values are means +/- 95% confidence intervals calculated across P treatments.

## 4.5. P supply and inoculation with Pfl29ARP impacted P use efficiency components in Brachypodium

Regarding the PUpE (*i.e.* the ratio between shoot P content and applied soluble P), plants exposed to soluble P deficiency had a greater uptake efficiency ($P < 0.001$), with the greatest values measured on plants grown in the presence of TCP and HA (883.5% and 1128.8% increase, respectively, compared to plants exposed to P+; Fig. 5-8 f-i). *Brachypodium* acquired and accumulated a greater amount of P in shoots when TCP or HA were added to the nutrient solution, in comparison with the P-treatment and regardless of the bacterial treatment. Plants inoculation with Pfl29ARP led to lower PUpE values under all P treatments compared to non-inoculated plants (average decrease of 35.8% across all P treatments, $P = 0.011$, Fig. 5-8 j).

Plants grown under soluble P deficiency were more efficient at utilizing P for biomass accumulation (PUtE, biomass produced by unit of plant P content; $P < 0.001$; Fig. 5-8 k-n). These plants accumulated more shoot biomass per unit of shoot P content compared to plants exposed to P+ condition. Plants grown under the P-treatment were globally the most efficient. Inoculation of *Brachypodium* by any of the bacterial strains had no impact on PUtE ($P = 0.436$, Fig. 5-8 o).

The PPUE (*i.e.* shoot biomass divided by shoot P concentration), was higher in plants grown under P-, P-/HA and P-/TCP conditions compared to plants exposed to sufficient P supply (81.8%, 49.1% and 80.1% increase respectively compared to P+ condition, $P < 0.001$, Fig. 5-8 p-s). Plants exposed to a deficiency in soluble P produced shoot biomass more efficiently at lower shoot P concentration. The inoculation with Pfl29ARP induced a 19.9% reduction in PPUE compared to non-inoculated plants ($P = 0.008$, Fig. 5-8 t). Mean values per treatment, standard deviation and coefficients of ANOVAs are available in Appendix 7.

# 5. Discussion

This study aimed to explore the impact of PSB inoculation on the response of *Brachypodium distachyon* Bd21 to contrasted P conditions. *Brachypodium* and the PSB were co-cultivated over four weeks in an *in vitro* gnotobiotic system and exposed to four different nutritional conditions: a low level of soluble P (P-); a low level of soluble P supplemented with poorly soluble forms of P (P-/TCP and P-/HA); and a high level of soluble P (P+). The plant biomass production and allocation, the root system architecture and the P use efficiency were studied.

## 5.1. Brachypodium shows developmental plasticity in response to contrasted P conditions

Our study demonstrated that *Brachypodium* biomass accumulation is highly responsive to P supply, with lower shoot biomass but stable or greater root biomass

accumulation under soluble P deficiency compared to high soluble P levels. This is consistent with the observations made in Chapter 3. This has been discussed in Chapter 3 Section 4.

*Brachypodium* displayed different allometric trajectories under contrasted P conditions, showing responsiveness of the allocation pattern to the P supply. Plants grown in the presence of TCP or HA exhibited a higher shoot development per unit of root biomass than plants grown under the P- and P+ treatments. From this we can infer that the presence of unavailable but potentially mobilizable P sources induced a reduction of investment into the development of the root system, in comparison with plants grown under P- conditions. Nevertheless, for similar root biomass, the shoot biomass was the highest in plants supplied with the P+ treatment compared to the three other treatments. We can hypothesize that stressed plants (P- conditions) maintained root development at the expense of the shoot. This is confirmed by the greater RMF observed under soluble P limitation. On the contrary, when there was no nutrient limitation, there was no need for the plants to prioritize extension of their root systems and the plants maintained the biomass accumulation into the above-ground compartment.

Regarding the root system, plants exposed to the P-/HA treatment exhibited a greater TRL. The observed root system lengthening was associated with greater root biomass and RMF for plants grown under P- conditions. These results are consistent with those of a hydroponics experiment on several barley varieties, which revealed a general trend towards root lengthening in response to P deficiency (Giles et al. 2017).

## 5.2. Despite their ability to solubilize tricalcium phosphate and hydroxyapatite, the bacterial inoculants did not alleviate P deficiency stress in Brachypodium under the experimental growing conditions

All the selected bacteria were able to solubilize the poorly available forms of P (TCP and HA) in NBRIP medium, as described in the results of Chapter 4.

The use of PSB as bioinoculants is increasingly reported in the literature, with interesting effects of microbial P mobilization on plant development and yield (Bakhshandeh et al. 2015; Li et al. 2017; Oteino et al. 2015; Pereira and Castro 2014), but few results have reported the inefficiency of *in vitro*-selected PSB to promote plant growth in the presence of poorly soluble forms of P (Collavino et al. 2010; Yu et al. 2011). In our study, the biomass accumulated in shoots and roots was reduced when plants were grown in the presence of bacteria. The strains Pfl29ARP and BveFZB42 had the strongest impact on plant development. Despite their ability to solubilize TCP and HA in NBRIP medium, the selected strains were not able to mobilize these poorly soluble forms of P to the benefit of plants under co-cultivation conditions and by this

way alleviate P-starvation stress in *Brachypodium*. The soluble P concentration in the Hoagland solution at the end of the cultivation was below the detection limit of our analytical method for the P-, P-/TCP and P-/HA treatments (data not shown). A slight acidification of the nutrient solution was observed at the end of the co-cultivation in the presence of bacteria, but the pH remained within an acceptable range for plant development (data not shown).

The absence of P stress alleviation in the plant by bacteria during the co-cultivation experiments can be explained by different processes. C supply through root exudates may be too low to sustain bacterial activity, as observed by Nico et al. (2012) in *in vitro* gnotobiotic conditions and Bradácová et al. (2019) in soils with low organic matter content. Some studies have revealed a deleterious impact of inoculation with bacterial strains on plant growth under gnotobiotic conditions due to the accumulation of bacterial toxic metabolites (Rybakova et al. 2016; Timmusk et al. 2015). Competition for P between plant and bacteria could also occur. Microorganisms may limit the availability of P to the plant by immobilizing P in the microbial biomass, decomposing root exudates mobilizing P, inhibiting root growth and counter-acting the rhizosphere acidification induced by the roots (Marschner et al. 2011). It was also observed that the transcription factor PHR1, which is involved in the plant phosphate starvation response, contributes to the transcriptional regulation of the plant immunity in *Arabidopsis* by negatively regulating defense-related genes under Pi limiting conditions (Castrillo et al. 2017). This repression of plant defense may alter the colonization processes and induce shifts from beneficial to detrimental interaction between plant and bacteria (Finkel et al. 2017). Plants may also interfer with bacterial quorum sensing, altering root colonization by the bacteria and the bacterial behaviors leading to changes in symbiosis or pathogenesis (Goh et al. 2013). *Bacillus* spp. and *Pseudomonas* spp. are reported as biocontrol agents inducing systemic resistance in plants. However, the activation of inducible defenses in plants may induce fitness costs impacting plant growth and yield (Walters and Heil 2007; Van Wees et al. 2008). These costs are expected to be higher in plants growing under nutrient deficiency than in plants growing under optimal nutritional conditions (Walters and Heil 2007). The efficacy of a system to test for PSB activity in the presence of a host plant appears to be highly dependent on the considered organisms, but also on the co-cultivation conditions.

## 5.3. *The plastic response of Brachypodium to P deficiency was modulated by inoculation with phosphate solubilizing bacteria*

Regarding the biomass allocation pattern, inoculation with PSB revealed an alteration of the plant's response to P conditions, except in the presence of HA. Under P- conditions, inoculation with PSB (except with BveFZB42) led to a reduced shoot productivity for similar root biomass. The same observation was made under the P-

/TCP treatment, mainly with Pfl29ARP. The depletion in shoot growth benefited the root system, the development of which was either unaffected or less impacted than the shoot. This resulted in an increase in RMF. Under the P+ treatment, investment into the root system was reduced in inoculated plants, except with AviF0819 and BveGB03. The RMF was still increased for the same reason as before: a repression of shoot biomass but a steady root biomass accumulation. As the root system is the place where the interaction with the bacteria occurs, it appears that the plant modulated the development of this interface of interaction depending on the nutritional context. These contrasted behaviours in *Brachypodium* should be explored more deeply.

The total root length of *Brachypodium* was significantly impacted by the P supply and inoculation with PSB. Regardless the P treatment, inoculation with BveFZB42, Eco99B829 and Pfl29ARP led to a reduction in TRL. These results contrast with others reported in the literature. Indeed, Talboys et al. (2014) demonstrated a root elongation promotion effect of BveFZB42 inoculation on wheat (through auxin production), in both low and high P-level soils. In a soil experiment, *Pseudomonas fluorescens* strains also exhibited a positive impact on wheat root elongation under contrasted P fertilisation (Zabihi et al. 2011). The persistent homology analysis performed in our study revealed that inoculation with Pfl29ARP impacted the morphology of the plant root system (considering 1$^{st}$ and 2$^{nd}$ order roots) in comparison with non-inoculated plants and plants inoculated with BveFZB42. The P conditions also induced changes in root system morphology, but these were less easily characterized. According to our results, *Brachypodium* showed a modification of root development, triggered by contrasted P supply and inoculation with bacteria. The measured variables (total root length and root system topology) are related to soil exploration by the root system. This study did not consider root hairs or other soil exploitation strategies, yet they constitute an important strategy for P nutrition (Lynch 2011) and should be further investigated.

## 5.4. Inoculation with P solubilizing bacteria did not improve Brachypodium P use efficiency under the experimental growth conditions

The shoot P concentration and PUE in *Brachypodium* were mainly affected by the P supply, but also by PSB inoculation to some extent. The shoot P concentration was the lowest in plants grown under P- conditions, confirming the P-deficient status of those plants. Despite the demonstrated ability of the bacterial strains to solubilize TCP and HA, they did not alleviate P deficiency in the plants. The soluble P concentration in the Hoagland solution at the end of the cultivation was null for the P-, P-/TCP and P-/HA treatments (data not shown). This result reinforces the above-mentioned hypothesis that the PSB did not sufficiently mobilize TCP and HA in our gnotobiotic

conditions to meet the plant needs. On the other hand, the P+ solution contained enough soluble P after four weeks for avoiding nutritional stress in the plants (data not shown). Considering the slightly higher shoot P concentration in the presence of TCP and HA regardless the inoculation treatment, we assume that *Brachypodium* was able to partly solubilize those poorly soluble forms of P. Indeed, plants are able to acidify the rhizosphere and release organic anions, mobilizing poorly available P sources (Hinsinger et al. 2003; Wang and Lambers 2020). The PUpE was significantly higher in plants exposed to soluble P deficiency compared to plants grown under the P+ treatment, as the stressed plants took up all the available soluble P and partly used it to build their shoots. The highest PUpE values were obtained in the presence of TCP and HA. This observation is consistent with the higher shoot P concentration observed under these treatments and reinforces the above-mentioned hypothesis of partial P solubilization by *Brachypodium*. The PUpE reduction in plants inoculated with Pfl29ARP is consistent with the observed decrease in shoot biomass accumulation, which impairs their P accumulation ability. The PUtE was significantly higher under soluble P deficiency than under the P+ treatment, with the highest efficiency under the P- treatment. Therefore, stressed plants produced the largest biomass per unit of accumulated P. The inoculation of *Brachypodium* with bacteria did not impact the PUtE, as expected from their poor P solubilization activity during the co-cultivation experiment. As observed for PUpE and PUtE, the PPUE values were higher under soluble P deficiency, meaning that for similar shoot P concentration the stressed plants produced more shoot biomass. The inoculation with Pfl29ARP induced a reduction in PPUE. Indeed, shoot P concentration was similar in non-inoculated plants and in plants inoculated with Pfl29ARP, but shoot biomass accumulation was reduced in inoculated plants.

## 5.5. *Allometry and persistent homology analyses are convenient tools for unravelling the impact of bioinoculants on plant plasticity in response to a variable environment*

In this study, biomass allocation was explored considering RMF and allometry analyses. Our results are in accordance with the "functional equilibrium model", which states that a plant shifts allocation towards the organ involved in the acquisition of the most limiting resources (Brouwer 1963), and reveal a true plasticity in response to P supply as well as a real modulation of this plastic response when plants are inoculated with bacteria. Contrasted results were found in previous studies about the allocation pattern in response to P nutrition. Some of these conclude in a "conservative response" of the plants adjusting their size rather than their allocation pattern (apparent plasticity; Müller et al. 2000). Others described an impact on the allocation pattern, but only under severe P stress (Rubio et al. 2013) or in interaction with

nitrogen fertilisation (Sims et al. 2012). Plasticity of biomass allocation was also demonstrated, with a strong impact from the nutritional context (Poorter et al. 2012; Poorter and Nagel 2000; Shipley and Meziane 2002). The complementarity between biomass partitioning (RMF) and allometric trajectories appears clearly here for the analysis of biomass allocation patterns under environmental variation. Both approaches should be considered when studying the impact of biostimulants on plant biomass allocation in response to environmental constraints.

Morphological plasticity and local adaptation are important processes allowing plant roots to take up soil resources that are heterogeneously distributed in space and time (Koevoets et al. 2016). The use of persistent homology to quantify differences in root system morphology (based on $1^{st}$ and $2^{nd}$ order roots) showed that the strain Pfl29Arp had a strong impact on *Brachypodium*'s root system development. As this analysis gathers geometrical and topological information, we can infer that the root system morphology is modulated in at least one of these components. The reduction in total root length ($1^{st}$ and $2^{nd}$ order roots) observed on plants inoculated with Pfl29Arp is partly responsible for this modulation of root system morphology.

All the methodological approaches used in this study appear to be suitable tools for the accurate characterization of above- and belowground plant responses to various P sources and inoculation with PSB. As plant plasticity is getting more and more attention in breeding programs and agricultural systems, we believe that the complementary methodological approaches used in this paper will be useful in helping us unravel the mechanisms by which bioinoculants modulate plant plasticity.

# Chapter 6

**Discussion and perspectives**

# 1. Objectives and key results of the research project

Bacterial inoculants are considered to develop sustainable agricultural practice using limited input of fertilizer. Their ability to improve nutrients availability and to modulate plant growth have been investigated for long but the effectiveness of microbial products under realistic field conditions is still unreliable. Our knowledge of the complex tripartite interaction soil-plant-microbes within a specific soil matrix needs to be deepened to develop efficient products (Chapter 1, Sections 5 and 6). While impact of bacteria on P availability and plant growth is reported, the modulation of plant plastic response to P nutritional stress by bacteria still deserves attention. The objective of this thesis was to study the impact of phosphate solubilizing bacteria on the plant plastic response to P condition. The key results from this work are listed hereafter.

- *Brachypodium* plantlets exhibited developmental and physiological plasticity in response to varying Pi condition. The root biomass production was maintained at the expense of shoot biomass production, which decreased under low Pi condition. This could be explained by an investment in root development under low P conditions to facilitate plant exploration of the substrate and P uptake. APase activity in shoot and root tissue increased while tissue P content decreased, with decreasing Pi level. Plants exposed to Pi deficiency built more biomass per unit of P than plants exposed to higher Pi level. This could be explained by an adaptation of the plant metabolism to low P requirements. The response of the root system architecture to low Pi level was more tenuous but the plant exposed to 0 µM Pi tend to develop similar length of primary seminal root and length of $2^{nd}$ order lateral roots similar to plants exposed to 1000 µM Pi.
- Despite their ability to solubilize TCP and HA in NBRIP medium, the selected strains were not able to alleviate P deficiency in plants exposed to poor soluble Pi concentration. Possible reasons are a competition for P between the plant and the bacteria, a limited bacteria activity due to poor C supply through root exudates, an accumulation of bacterial toxic metabolites into the system, a depressed plant immunity inducing a shift from beneficial to detrimental interaction, a fitness cost in the plant due to systemic resistance induced by the bacteria.
- The biomass allocation pattern of *Brachypodium* in response to P condition was modulated by inoculation with PSB. Accumulation of biomass in shoot and root was reduced in plants grown in the presence of bacteria. Regarding the root system architecture, the total root length of *Brachypodium* was significantly impacted by the P supply and inoculation with PSB. Inoculation with BveFZB42, Eco99B829 and Pfl29ARP led to a reduction in total root length. The presence of unavailable but potentially mobilizable P sources induced a reduction of investment into the development of the root system, in comparison with plants grown under P- condition.

- It is assumed that *Brachypodium* was able to partly solubilize the poorly available P forms TCP and HA.
- Different methodological approaches were used in this project to characterize the plant plastic response to P condition and its modulation by inoculation with PSB: single RSA traits analysed independently, allometric analyses to highlight "true" plasticity in allocation pattern and persistent homology analyses integrating geometrical and topological components of the root system morphology. These methods appear to be suitable tools for an accurate characterization of above- and belowground plant responses to various P sources and inoculation with PSB.

In the light of the results summarized above, the methods selected in the project are discussed in the following sections. Areas subjected for improvement and perspectives of research are also described.

# 2. General discussion

## 2.1. *Focus on single bacterial strains vs. consortia*

In this research project, we chose to study plant-bacteria interaction but fungi also play an important role in plant nutrition (Richardson et al. 2011). The focus on bacteria was partly due to the heritage from former research in the laboratory but is also justified by the objective to study the plant developmental plasticity. Indeed, mycorrhizal fungi extend the root system to increase the soil exploration and therefore P acquisition. By this strategy, plants seem to invest resources to construct and maintain the association with the mycorrhizal fungi but develop to a lesser extent their own strategies (Han et al. 2022; Wen et al. 2019). Han et al. (2022) studied the role of root phosphatase activity within the root economics space of subtropical tree species. They qualified root phosphatase activity as a "do it yourself" strategy which is positively correlated with specific root length and specific root area but negatively correlated with root diameter and root mycorrhizal colonization. Wen et al. (2019) also showed different strategies in herbaceous species with trade-offs among 3 groups of functional traits: root morphology, root exudation and mycorrhizal symbioses. Therefore, it is likely that plants with traits enabling exploration of large soil volumes and P solubilization, can develop "do it yourself" strategies and higher plasticity in response to P conditions than plants which rely on symbiosis with mycorrhizal fungi for P uptake.

Nevertheless, we should keep in mind that under agricultural conditions, plants will develop in the presence of both bacteria and fungi and in a more complex substrate. It should therefore be interesting to study how the plant respond to varying nutritional conditions in presence of a more complex biotic environment (*e.g.*, with synthetic communities) and a more realistic soil matrix. Considering that trade-offs among P-responsive traits resulting in different P acquisition strategies were recently highlighted (Chapter 1, Sections 2 and 4), the main strategy of the plant species of

interest should be investigated to orientate the study towards the most appropriate plant-microbe associations.

Regarding the choice of the bacterial strains, the selection was based on literature evidence of plant-growth promotion properties. At the time of this project initiation, the rhizosphere microbiome of *B. distachyon* had not been characterized. This work was initiated by Kawasaki et al. (2016) who studied the rhizosphere microbiota and root exudate profiles of *B. distachyon*. They found that these profiles were similar to those of wheat. Soil type is the major driver of the microbial community composition of the rhizosphere, but there are common patterns in the development of core microbial communities for a plant species, which form independently of the soil and environmental conditions (Compant et al. 2019; Richardson et al. 2021). The core microbiome of a plant species is tightly associated to this species and contains function genes that are essential for the plant fitness. On the other hand, some microbial taxa that are present in lower abundance and in specific habitats form the satellite microbiome. Satellite taxa provide key functions in ecosystems, that might be disproportionate to their limited abundance (Compant et al. 2019). The conditions of the origin environment of the bacterial strains should ideally be considered. PSB are more common under P-deficient conditions and the selection of bacteria thriving in P-poor environment may increase the success of the interaction with the plant (Compant et al. 2019). Bacteria from *B. distachyon* core and satellite microbiome would be an interesting target for future research, in the aim to promote interaction with species beneficial to plant P nutrition. *Azotobacter* sp. were not identified in the root microbiome of *B. distachyon* (Kawasaki et al. 2016). Therefore, the rhizocompetence of this strain in *B. distachyon* rhizosphere should be confirmed before further testing. *Pseudomonas* and *Bacillus* genera were identified in the root microbiome of *B. distachyon* (Kawasaki et al. 2016). However, the interaction between a host plant and bacterial strains showed specificity at the strain level (Drogue et al. 2012) and is impacted by the nutritional status of the plant (Finkel et al. 2017) so the outcome of an interaction remains difficult to predict.

Finally, it was chosen to inoculate *B. distachyon* with single bacteria in order to study their impact on the plant plasticity individually. If it is interesting to characterize the effectiveness of the strains individually in a fundamental approach, the use of consortia could be more efficient and present advantages. Different mechanisms and desired traits or strains efficient under different environmental conditions can be combined in bacterial consortia (Chapter 1, Section 6). The inoculation of wheat in field with a consortium of 13 *Bacillus* species benefitted plant growth and nutrient uptake when applied in combination with an organic fertilizer (Saia et al. 2015). An enhanced effect of P-fertilization was shown on ryegrass inoculated with a consortium of 5 bacterial strains, with greater development, nutrition and alleviation of oxidative stress in plant (Barra et al. 2019). The combination of bacterial and AMF inoculants in consortia also

showed promising results to increase crop productivity in maize under field conditions (Pacheco et al. 2021). However, developing consortia needs to study the compatibility of the microbial candidates. Strains can produce antagonistic effects that inhibit each other's development and impede the plant growth-promoting potential of individual strains. As a result, consortia may show reduced plant growt-promoting effects compared to the single inoculants (Compant et al. 2019; Díaz et al. 2023). The absence of antagonistic effects on microbial growth should be tested *in vitro*. Tests *in vivo* can also be performed to check if the host colonization capacity of the strains is affected when applied in a consortium (Díaz et al. 2023).

## 2.2. *Choice of P sources*

As described in Chapter 1 Section 1, P exists in many forms in soil with different availabilities for plant nutrition. Tricalcium phosphate (TCP) was imposed in solubilization assays by microorganisms as the most used P source. Other P compounds are also tested but at smaller scale (Bashan et al. 2013a). Considering that soils vary in their chemical properties, a universal P-compound for selection of phosphate solubilizing microorganisms can not be defined. Moreover TCP, despite its theoretical insolubility, dissolves relatively easily compared to other insoluble P compounds. In studies using several P compounds as selection factors for demonstrating P solubilization capacity, TCP was solubilized more intensively and the highest number of P-solubilizers were obtained with TCP (Bashan et al. 2013b). It appears therefore that the use of a combination of two or three P compounds (including TCP or not) is necessary to select P-solubilizing candidates (Bashan et al. 2013b). The combination of aluminium or iron phosphates (as present in acidic soils), calcium phosphates (present in alkaline soils) and organic P forms (ubiquitous in agricultural soils) should enable to identify versatile P-solubilizing candidates.

In this research project, we focused on two different calcium phosphate forms, tricalcium phosphate and hydroxyapatite. Both can potentially be solubilized through acidification of the medium and metal complexing, but hydroxyapatite is reported to be more stable than TCP. The results of Chapter 4 show that hydroxyapatite was as efficiently solubilized as TCP in our experimental conditions. Looking at the relationship between solubilization and pH variation in the medium, we hypothesized that acidification was the main solubilization process for both compounds, but other processes could also be involved in solubilization of hydroxyapatite, such as metal complexing or chelating. In order to test this hypothesis, organic acid production in the medium should be measured (Khourchi et al. 2022). In light of the literature, it appears that testing two different calcium P forms including TCP is the minimum to identify PSB candidates. It also restricts their use to neutral to alkaline soils. The media for the bacterial solubilization assay and the co-cultivation experiments in Chapters 4 and 5 were prepared at pH = 7. We limited our experiments to TCP and hydroxyapatite to

keep a manageable number of P treatments in the co-cultivation experiments described in Chapter 5.

## 2.3. *Assessment of plastic response*

The response of *B. distachyon* Bd21 to P condition was first studied in Chapter 3 considering 7 different soluble Pi concentrations ranging from 0 to 1000 µM Pi. *B. distachyon* Bd21 exhibited developmental (biomass production and partitioning, root system morphology) and physiological (acid phosphatase activity, tissue P content) plasticity in response to Pi condition. By selecting multiple Pi levels, the shape of the reaction curve could be determined and Pi conditions leading to contrasted plant phenotypes were determined. Based on these results, adequate levels of soluble Pi were selected for subsequent experiment in Chapter 5. The response of *Brachypodium* to Pi level was confirmed by the results in Chapter 5 and a plastic response to P sources was showed. These experiments also highlighted that the P-responsive plasticity was modulated in plants inoculated with PSB. However, this modulation of *Brachypodium* response to P condition by PSB was not associated to an enhanced PUE.

In our research (Chapter 3 and Chapter 5), plant traits were analysed separately from each other, and the responsiveness of the traits was highlighted by univariate statistical analyses. The plasticity of a trait can also be characterized using quantitative indicators (*e.g.*, coefficient of variation, slope of the reaction norm, plasticity index; Chapter 1 Section 7.2). They are usually analysed with univariate statistical methods to compare the phenotypic plasticity of different genotypes (Pennacchi et al. 2021; Valladares et al. 2006). However, the currently used indicators show limitations in their interpretation, mainly due to statistical issues to compare phenotypic plasticity between different genotypes and under multiple environments, as well as due to non-normal distribution of the data (Valladares et al. 2006). Another limitation in considering single traits independently, is that the phenotypic plasticity is not characterized as a complex and integrative property of the plant (Pennacchi et al. 2021). Therefore, using indices that integrate diverse variables appear to be more appropriate to characterize phenotypic plasticity. In Chapter 5, the plasticity of the root system morphology of *B. distachyon* was also assessed using persistent homology. This analysis integrates geometrical and topological information of the root system morphology. It constitutes an interesting approach to get into the complexity of plant phenotypic plasticity. Pennacchi et al. (2021) propose a new multivariate plasticity index combining multiple traits, which was correlated to traits commonly reported in plants under water stress. Further use and evaluation of this index is needed, but it appears as a promising tool in characterizing phenotypic plasticity.

For traits exhibiting an allometric response to environmental changes, the ontogenetic effects should be considered to ensure that the observed response is independent from change in growth rate ("true plasticity"). This approach was

followed to characterize biomass partitioning between shoot and root systems (Chapter 5). The results confirmed that the mass fractions and allometric analyses are complementary and should be both considered to characterize developmental plasticity.

The methods used in this project appeared to be appropriate to study the plastic response of *B. distachyon* to P condition and inoculation with bacteria.

## 2.4. From in vitro to field conditions

Different cultivation systems were used in the course of this project. First, *B. distachyon* Bd21 response to P deficiency was characterized with a sand substrate under *ex vitro* conditions. Then, the bacteria ability to solubilize poorly available P forms was characterized in a liquid medium. Finally, plants and bacteria were cultivated in an *in vitro* system using quartz gravel as substrate.

Phosphorus being highly reactive with the soil components and particles, the choice of the growing substrate was determinant to control the amount of P available to plants and microbes. The substrate needed to be washed carefully to get rid of organic matter and other particles that could bring P contaminations. The substrate should also be easily sterilized without generating additional inorganic P through unintentional mineralization. For these reasons, an inert substrate amended with nutrient solution was preferred. In addition to these conditions, the substrate must also allow to easily extract the root system without breaking roots. Sand was selected for the first experiment because it was easily available in great quantities and cheap. For the co-cultivation experiment, commercial quartz gravel was chosen, providing a greater porosity for root development and a more constant quality among different batches.

The characterization of *B. distachyon* response to Pi deficiency did not require working in closed *in vitro* systems. The sand was nonetheless sterilized to ensure similar conditions at start among the independent replicates. The co-cultivation experiment however was conducted under gnotobiotic conditions to control specific plant-bacteria interaction. The artificial substrate (gravel and nutrient solution) allowed to control the type and quantity of P available to the plants and the bacteria. A simplified system like this one is convenient to measure many plant traits and to avoid the constraints of P reactivity in soils. As already discussed in Chapters 3 and 5, the growing conditions impacted the growth stage of the plants and therefore the response of the plant to P deficiency. Poor photosynthetic efficiency of plantlets grown *in vitro* (Hazarika 2006) potentially affected the plant root exudation process. Low carbon source level for the bacteria may have impaired their development and their P solubilization activity. The negative outcome of the interaction may also be explained by accumulation of bacterial toxic metabolites into the system, competition between *B. distachyon* and the bacteria, root growth inhibition induced by the bacteria, repression of the plant defense system or a fitness cost of induced systemic resistance

in the plant (Chapter 5, Section 5.2). The composition of the microbial community of the rhizosphere was also shown to vary in time with the plant development (Richardson et al. 2021). It is therefore likely that the cultivation system, by conditioning the plant growth dynamics, also impacted the interaction between *B. distachyon* and the inoculated bacterial strains. Sasse et al. (2020) studied the root system morphology of *B. distachyon* in substrates with different particle size and showed that the root system morphology was significantly impacted by the substrate. The exudation pattern of roots being a complex process varying along the roots and a major element in shaping the root microbiome (Sasse et al. 2018), the choice of the substrate is also determinant for microbial interaction.

The presence of the bacteria in the co-cultivation system was checked by scratching agar plates with the root systems at harvest. However, the capacity of the bacteria to colonize the root system and maintain their population could have been further tested. Indeed, positive effects of the inoculation are unlikely if the bacteria did not significantly colonize the root system. When bioinoculant products are applied, rhizocompetence traits such as biofilm formation, siderophore production, antagonism, ability to utilize root exudates, motility and protease activity are important elements that can condition their success (Kaur et al. 2017). The composition of exudates differs according to the physiological status of the plant and impacts its capacity to attract various rhizobacteria (Beauregard 2015). It was shown that quorum sensing signalling among bacteria population can be modulated by plants. By interfering with bacterial quorum sensing, the plant my alter root colonization by the bacteria and the bacterial behaviors leading to changes in symbiosis or pathogenesis (Goh et al. 2013). A repression of the plant defense system under P deficiency may also alter the colonization processes and the interaction between plant and bacteria (Finkel et al. 2017). In our *in vitro* growing system, testing the capacity of the strains to form biofilms on the root system and their ability to utilize *B. distachyon* Bd21 root exudates could deliver valuable information regarding the plant-bacteria interaction.

It is also noteworthy that the growing system used to evaluate the ability of the bacterial strains to solubilize TCP and HA was different than the co-cultivation system. The growing conditions of the bacteria were different and testing the ability of the bacteria to solubilize TCP and HA in the co-cultivation system without the plant would be interesting to confirm their PSB potential. However, such test in the *in vitro* co-cultivation system would require adding a carbon source to the medium. *Brachypodium* root exudates could be used to this purpose, but they potentially contain P-solubilizing compounds. Another exogenous carbon source could alternatively be used but would expose the bacteria to different conditions from the ones of the co-cultivation.

The inoculation technique is another element that could be investigated. It can be expected that the inoculation technique can impact the colonization process and the

outcome of the interaction (Zhu et al. 2017). For field uses, biofertilizers can be inoculated as seed treatment, seedling root treatment or soil application (Chapter 1, Section 5). Cereals are generally inoculated via seed treatment. In the experiment presented in Chapter 5, 24-h old plantlets of *B. distachyon* were inoculated by dipping into a bacterial suspension. Seed treatment or application of bacteria to the substrate could also be possible, but seedling treatment was preferred to apply the bacteria directly in contact with the root of pre-selected 24-h old homogeneous plantlets. The presence of the bacteria at the end of the co-cultivation experiment was confirmed. However, it would be interesting to investigate the effects of the inoculation technique on the colonization of the root system by the bacteria.

Combining all these elements, it is obvious that the growing conditions are determinant in the expression of plant and microbe phenotypes, as well as their interaction. This makes difficult the comparison between results obtained from different projects using different growing systems. In 2018, a new growing system enabling the use of different substrate types under controlled gnotobiotic conditions, easy sampling and imaging of root morphology has been proposed: the EcoFAB (Ecosystem Fabrication) system (Gao et al. 2018). The size of the system limits its use to young growth stage (*e.g*., 20-day old *B. distachyon* as illustrated in Gao et al. 2018) but it is easily accessible to most laboratories. Its use in plant-bacteria interaction would make the results of different studies more easily comparable. However, the extrapolation of results obtained with such simplified cultivation systems to field conditions is not straightforward. This is confirmed by reports of discrepancies between lab and field studies and poor predictability of the outcome of field studies from greenhouse studies (Schmidt and Gaudin 2018). *In vitro* experiments should be considered as a preliminary step in the characterization of plant plasticity in response to nutritional conditions and interaction with microorganisms, but such simplified system are a necessary step considering the high complexity of realistic field conditions. We should also keep in mind that the high biological variability (plant and bacteria growth, behaviour of the soil biotic component and their interaction) and soil variability will always modulate the fate and behaviour of bioinoculants in the field. The use of growing systems enabling to approach more realistic conditions while keeping easy access to the root system for various analysis (*e.g.* rhizoboxes; Alonso-Crespo et al. 2022, Nassal et al. 2018) is an interesting option.

# 3. Perspectives of research

Testing in natural soils with contrasted P reserves would expose plant and bacteria to conditions closer to agronomic ones. Such experiment could be conducted with *Brachypodium distachyon* in rhizoboxes, enabling to reach late developmental stages and deeper investigate its root system morphology. Only the number and length of 1st and 2nd order lateral roots were considered in this project. Other traits such as root

hairs, root diameter and root P transporter activity also are determinant for P acquisition and could be the focus of further research. As mentioned before, analysing the traits of interest in a multivariate approach (*e.g.*, with a multivariate plasticity index) would constitute a major step in the study of plant phenotypic plasticity by considering plasticity as an integrative property of the plant.

It was hypothesized in Chapter 5 that *B. distachyon* Bd21 was able to solubilize TCP and HA. Characterizing the exudation profile of *B. distachyon* exposed to different P sources and how bacteria modulate the plant exudation would be an important step. Quantitative and qualitative characterization of the root exudation profile can be conducted under *in vitro* conditions with a hydroponic growing system, but also under *ex vitro* conditions in rhizoboxes as the soil solution can be sampled *in situ* in a non-destructive manner (*e.g.* using Rhizons samplers). Other non-destructive rhizosphere imaging technique such as zymography and pH-optodes (Blossfeld et al. 2013; Ma et al. 2021) could also be applied to investigate rhizosphere processes.

Regarding the microbial compartment, native bacteria rather than selected exogenous ones could be selected as candidates for biofertilizers. Indeed, bacteria originating from the microbiome of the plant species of interest are more likely to establish an efficient and long-lasting interaction with their host when they are inoculated. Bacteria that are shared by the microbiome of *B. distachyon* and cultivated cereals could be good candidates to facilitate the application of the biofertilizers to the field. Monitoring the bacterial populations is necessary to interpret the outcome of the interaction and the impact of the plant stress level on bacterial growth, since it is known that the plant P status and its PSR system modulate the bacteria development in the rhizosphere (Paries and Gutjahr 2023). The colonization pattern of the selected bacteria could be studied using bacterial strains modified to express green fluorescent protein (Fan et al. 2011). To go even further, a consortium combining PSB and AMF could be inoculated to *B. distachyon* to investigate if the plant's strategies to mobilize P change according to its microbial environment. Hydroponics growing systems with a solid substrate, like the Magenta boxes used in the co-cultivation experiment (Chapter 5), are also suitable to conduct experiments with AMF (Das et al. 2020).

Finally, inoculation of microorganisms could be combined to application of a P fertilizer at different levels to determine the most efficient combination in an agronomic point of view. Polyphosphates, a recently described source of P fertilizer, appear to be a promising P source when applied in combination with PSB (Khourchi et al. 2022).

# Chapter 7

**Conclusion**

Phosphorus is an essential element to crop production but its high immobility in soil and reactivity with soil components result in a poor availability to plants. Moreover, the reserves of the main source of P for fertilizers, phosphate rock, are depleting. Strategies are being developed to reduce the dependence to phosphate rock in agriculture. The use of microbial biostimulants, able to increase the bioavailability of nutrients in the soil and/or to improve the plant nutrient efficiency, is one of these strategies and was the focus of this PhD project.

In this context, this research project studied the impact of PSB strains on the response of the model plant *Brachypodium distachyon* to P deficiency. The first part was dedicated to the study of the response of *B. distachyon* Bd21 to Pi deficiency. *B. distachyon* Bd21 showed developmental and biochemical plasticity in response to varying Pi level and Pi concentrations leading to contrasted plant phenotypes were defined. In the second part, the ability of the selected bacterial strains to solubilize poorly available inorganic P forms, tricalcium phosphate and hydroxyapatite, was demonstrated. The third part investigated how the developmental plasticity of *B. distachyon* Bd21 is modulated by inoculation with PSB. The results showed a modulation of the plant plastic response to P condition when inoculated with the bacteria, even though the bacterial strains could not alleviate P stress level and increase PUE in *B. distachyon* Bd21 in our experimental conditions.

The selected growing system did not allow the selected bacterial strains to alleviate P deficiency stress in *B. distachyon* Bd21 in presence of the poorly available P forms tricalcium phosphate and hydroxyapatite. However, the methods chosen to study the plastic response of the plant to P conditions and inoculation with bacteria appeared to be appropriate to answer the research questions. To our knowledge, it was the first time that such techniques of analysis were applied to characterize the plastic response of a plant in this context. This opens the way to further research using cultivation systems placing the plants and the bioinoculants under more realistic conditions.

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# Appendix 1

Biomass accumulation (SDW, RDW, TDW) and allocation (RMF), tissue P concentration (ShootP, RootP), PUtE and APase activity in shoot and root of 30-day old *Brachypodium* plantlets, exposed to different soluble P levels (n = 24 for biomass parameters, n = 3 for P concentration, PUtE and APase activity). Results of 2-way ANOVAs (degree of freedom "df", $P$ and $F$ values) and Dunnett's *post hoc* tests (annotated with stars; 1000 µM Pi used as a reference treatment).

| | SDW | | RDW | | TDW | | RMF | | ShootP | | RootP | | PUtE | | ShootAPase | | RootAPase | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | mean | sd | mean | sd | mean | sd | mean | sd | mean | sd | mean | sd | mean | sd | mean | sd | mean | sd |
| *P* treatment | | | | | | | | | | | | | | | | | | |
| 0 | 13.6* | 4.00 | 5.61* | 1.35 | 19.3* | 5.00 | 0.297* | 0.053 | 1.41* | 0.129 | 0.977* | 0.134 | 0.790* | 0.062 | 5.69* | 0.333 | 2.26* | 0.061 |
| 50 | 20.2* | 5.11 | 4.46 | 1.26 | 24.7* | 6.24 | 0.181* | 0.021 | 2.50* | 0.098 | 1.58* | 0.238 | 0.429* | 0.018 | 2.86 | 0.658 | 2.14 | 0.495 |
| 100 | 22.9 | 5.59 | 4.11 | 0.929 | 27.0 | 6.07 | 0.155 | 0.036 | 3.84* | 0.155 | 2.32* | 0.070 | 0.277* | 0.009 | 3.00 | 0.447 | 1.35 | 0.435 |
| 150 | 23.6 | 6.41 | 3.92 | 1.17 | 27.5 | 7.25 | 0.145 | 0.029 | 5.14* | 0.249 | 3.48* | 0.320 | 0.205* | 0.012 | 3.28 | 0.170 | 1.45 | 0.139 |
| 200 | 24.4 | 8.12 | 4.08 | 1.26 | 28.5 | 9.03 | 0.147 | 0.030 | 5.62 | 0.309 | 3.67* | 0.228 | 0.188 | 0.010 | 3.51 | 0.529 | 1.18 | 0.406 |
| 600 | 25.8 | 5.57 | 4.59 | 1.46 | 30.4 | 6.61 | 0.151 | 0.027 | 6.20 | 0.123 | 5.19 | 0.396 | 0.166 | 0.003 | 3.15 | 0.629 | 1.89 | 0.127 |
| 1000 | 26.4 | 9.59 | 4.38 | 1.41 | 30.8 | 10.7 | 0.146 | 0.028 | 5.99 | 0.209 | 5.31 | 0.400 | 0.170 | 0.006 | 3.15 | 0.875 | 1.48 | 0.358 |
| ANOVA | df=6, *P*=1.29e-12, *F*=13.83 | | df=6, *P*=5.76e-4, *F*=4.22 | | df=6, *P*=1.72e-7, *F*=7.93 | | df=6 , *P*<2.2e-16, *F*=55.69 | | df=6, *P*=5.30e-13, *F*=282 | | df=6, *P*=1.40e-10, *F*=118 | | df=6, *P*=2.14e-14, *F*=464 | | df=6, *P*=8.60e-5, *F*=12.79 | | df=6, *P*=0.005, *F*=5.51 | |
| *Repetition* | | | | | | | | | | | | | | | | | | |
| ANOVA | df=1, *P*=0.009, *F*=7.03 | | df=1, *P*=0.466, *F*=0.532 | | df=1, *P*=0.016, *F*=5.96 | | df=1, *P*=0.003, *F*=9.07 | | df=1, *P*=0.42, *F*=0.71 | | df=1, *P*=0.22, *F*=1.66 | | df=1, *P*=0.56, *F*=0.36 | | df=1, *P*=5e-4, *F*=20.85 | | df=1, *P*=0.09, *F*=3.26 | |

# Appendix 2

RSA parameters of 30-day old *Brachypodium* plantlets, exposed to different soluble P levels (n = 24). Results of 2-way ANOVAs (degree of freedom "df", *P* and *F* values) and Dunnett's *post hoc* tests (annotated with stars; 1000 µM Pi used as a reference treatment).

| | PSRL | | NCNR | | LCNR | | N2LR | | L2LR | | N2LR0-5 | | N2LR5-10 | | N2LR10-15 | | N2LR15-20 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | mean | sd | mean | sd | mean | sd | mean | sd | mean | sd | mean | sd | mean | sd | mean | sd | mean | sd |
| *P treatment* | | | | | | | | | | | | | | | | | | |
| 0 | 21.4 | 4.8 | 0.25 | 0.61 | 1.6 | 5.0 | 81.6 | 17.3 | 204 | 64.2 | 27.2 | 7.1 | 25.1 | 6.1 | 18.1 | 5.9 | 8.9 | 5.8 |
| 50 | 18.3 | 4.0 | 0.50 | 0.78 | 2.7 | 6.8 | 82.4 | 27.5 | 189 | 51.8 | 32.2 | 12.2 | 28.6 | 12.1 | 13.2* | 6.6 | 7.8 | 6.8 |
| 100 | 17.9 | 3.9 | 0.57 | 0.90 | 3.0 | 6.6 | 79.4 | 30.3 | 172 | 53.2 | 28.7 | 16.6 | 27.3 | 12.1 | 15.7 | 7.0 | 7.6 | 6.8 |
| 150 | 16.9 | 3.9 | 0.26 | 0.62 | 1.6 | 4.8 | 74.5* | 23.2 | 174 | 46.5 | 25.4 | 8.9 | 25.3 | 7.7 | 17.5 | 9.2 | 6.3 | 5.4 |
| 200 | 19.0 | 3.1 | 0.25 | 0.61 | 2.8 | 7.5 | 80.5 | 27.9 | 174 | 49.5 | 26.5 | 14.0 | 27.3 | 12.4 | 17.7 | 6.8 | 8.6 | 8.3 |
| 600 | 18.7 | 3.8 | 0.39 | 0.72 | 4.1 | 7.4 | 93.7 | 29.0 | 191 | 42.8 | 30.6 | 12.0 | 31.9 | 11.9 | 19.1 | 9.5 | 10.5 | 10.0 |
| 1000 | 19.7 | 2.4 | 0.19 | 0.60 | 1.6 | 4.8 | 95.8 | 25.2 | 213 | 60.4 | 30.8 | 14.3 | 29.2 | 7.6 | 21.8 | 4.1 | 13.1 | 7.3 |
| ANOVA | df=6, P=0.004, F=3.30 | | df=6, P=0.291, F=1.22 | | df=6, P=0.731, F=0.599 | | df=6 , P=0.015, F=2.62 | | df=6, P=0.062, F=2.05 | | df=6, P=0.137, F=1.619 | | df=6, P=0.076, F=1.905 | | df=6, P=0.019, F=2.52 | | df=6, P=0.484, F=0.913 | |
| *Repetition* | | | | | | | | | | | | | | | | | | |
| ANOVA | df=1, P=0.004, F=8.53 | | df=1, P=1.91e-11, F=45.06 | | df=1, P=8.67e-6, F=21.20 | | df=1, P=6.10e-8, F=29.33 | | df=1, P=0.503, F=0.45 | | df=1, P=2.04e-9, F=35.93 | | df=1, P=6.07e-13, F=51.82 | | df=6, P=0.293, F=1.11 | | df=6, P=0.321, F=0.985 | |

| | L2LR0-5 | | L2LR5-10 | | L2LR10-15 | | L2LR15-20 | |
|---|---|---|---|---|---|---|---|---|
| | mean | sd | mean | sd | mean | sd | mean | sd |
| *P treatment* | | | | | | | | |
| 0 | 64.7 | 16.5 | 73.5 | 28.2 | 49.2 | 26.0 | 15.6 | 13.4 |
| 50 | 78.4 | 19.7 | 75.1 | 29.4 | 26.9* | 17.3 | 8.7 | 8.5 |
| 100 | 71.3 | 28.9 | 67.5 | 29.8 | 26.8* | 16.5 | 6.6* | 7.2 |
| 150 | 69.8 | 20.0 | 68.7 | 22.1 | 29.7 | 18.4 | 5.7* | 5.9 |
| 200 | 67.4 | 19.2 | 68.1 | 21.7 | 30.0 | 18.7 | 8.0 | 9.2 |
| 600 | 78.4 | 22.1 | 72.4 | 19.6 | 30.2 | 19.7 | 9.8 | 11.5 |
| 1000 | 75.5 | 27.1 | 78.5 | 23.7 | 43.1 | 24.4 | 15.0 | 11.1 |
| ANOVA | df=6, P=0.199, F=1.45 | | df=6, P=0.728, F=0.603 | | df=6, P=0.0003, F=4.51 | | df=6, P=0.0016, F=3.77 | |
| *Repetition* | | | | | | | | |
| ANOVA | df=1, P=0.003, F=9.37 | | df=1, P=0.04, F=4.11 | | df=1, P=0.005, F=8.10 | | df=1, P=0.006, F=7.66 | |

# Appendix 3

Biomass accumulation and RMF of four-week-old *Brachypodium* plantlets grown in Magenta® boxes, exposed to contrasted P supplies and either inoculated or not inoculated with bacterial strains (n = 30 for the P- and P+ treatments, n = 15 for the P-/TCP and P-/HA treatments). Results of 3-way ANOVAs (degree of freedom "df", *P* and *F* values) and Dunnett's *post hoc* tests (annotated with stars; P+ and non-inoculated treatments used as references).

| | Shoot biomass (mg) | | Root biomass (mg) | | Total biomass (mg) | | RMF | |
|---|---|---|---|---|---|---|---|---|
| | mean | sd | mean | sd | mean | sd | mean | sd |
| *P treatment* | | | | | | | | |
| P- | 78.65 * | 17.53 | 57.78 * | 13.43 | 136.43 * | 27.98 | 0.42 | 0.05 |
| P-/HA | 92.09 * | 29.20 | 51.25 | 11.08 | 143.34 * | 35.52 | 0.37 | 0.06 |
| P-/TCP | 89.56 * | 23.15 | 51.59 | 11.75 | 141.25 * | 31.30 | 0.37 | 0.06 |
| P+ | 138.25 | 33.14 | 51.00 | 12.81 | 188.90 | 41.99 | 0.27 | 0.04 |
| ANOVA | df=3, *P*<2.2e-16, *F*=188.9276 | | df=3, *P*=4.278e-07, *F*=11.1453 | | df=3, *P*<2.2e-16, *F*=84.2410 | | df= 3, *P*<2.2e-16, *F*=313.3280 | |
| *Inoculation treatment* | | | | | | | | |
| Non-inoculated | 112.05 | 36.78 | 55.02 | 14.04 | 167.07 | 41.82 | 0.34 | 0.08 |
| AviF0819 | 108.42 | 39.08 | 56.00 | 13.06 | 164.42 | 44.44 | 0.35 | 0.08 |
| BveFZB42 | 100.46 * | 32.72 | 49.42 * | 12.10 | 149.88 * | 39.84 | 0.34 | 0.06 |
| BveGB03 | 107.84 | 38.83 | 54.49 | 12.45 | 162.33 | 42.21 | 0.35 | 0.09 |
| Eco99B829 | 103.56 | 34.44 | 54.28 | 11.58 | 156.97 | 35.83 | 0.36 | 0.08 |
| Pfl29ARP | 83.48 * | 32.72 | 51.33 | 13.27 | 134.81 * | 39.88 | 0.39 | 0.08 |
| ANOVA | df=5, *P*=8.956e-16, *F*=17.2350 | | df=5, *P*=0.002956, *F*=3.6519 | | df=5, *P*=3.476e-11, *F*=12.2019 | | df=5, *P*=2.356e-15, *F*=16.7725 | |
| *Repetition* | | | | | | | | |
| ANOVA | df=1, *P*=0.2585, *F*=1.2795 | | df=1, *P*=0.013298, *F*=6.1721 | | df=1, *P*=0.1091, *F*=2.5765 | | df=1, *P*=0.2085544, *F*=1.5855 | |
| *Interaction* | | | | | | | | |
| P- non-inoculated | 88.84 | 15.04 | 60.01 | 11.97 | 148.86 | 22.02 | 0.40 | 0.06 |
| P- AviF0819 | 83.13 | 15.56 | 61.05 | 12.06 | 144.18 | 25.07 | 0.42 | 0.04 |
| P- BveFZB42 | 80.21 | 19.12 | 51.28 | 13.53 | 131.49 | 31.51 | 0.39 | 0.03 |
| P- BveGB03 | 79.81 | 13.45 | 61.35 | 10.44 | 141.16 | 21.41 | 0.43 * | 0.04 |
| P- Eco99B829 | 75.62 | 14.65 | 59.99 | 14.23 | 135.61 | 26.00 | 0.44 * | 0.05 |
| P- Pfl29ARP | 64.34 | 17.60 | 53.11 | 15.08 | 117.44 | 30.64 | 0.45 * | 0.05 |
| P-/HA non-inoculated | 100.09 | 25.94 | 55.54 | 13.70 | 155.62 | 31.15 | 0.36 | 0.08 |
| P-/HA AviF0819 | 83.17 | 23.63 | 50.40 | 13.22 | 133.57 | 32.78 | 0.38 | 0.06 |
| P-/HA BveFZB42 | 87.99 | 26.38 | 48.61 | 10.86 | 136.61 | 32.44 | 0.36 | 0.06 |
| P-/HA BveGB03 | 101.44 | 35.68 | 49.55 | 9.45 | 150.99 | 40.80 | 0.34 | 0.07 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| P-/HA Eco99B829 | 101.27 | 32.89 | 54.15 | 8.14 | 155.43 | 37.93 | 0.36 | 0.06 |
| P-/HA Pfl29ARP | 78.59 | 24.21 | 49.23 | 9.98 | 127.83 | 32.08 | 0.40 | 0.06 |
| P-/TCP non-inoculated | 94.26 | 26.26 | 51.83 | 11.63 | 146.09 | 36.12 | 0.36 | 0.04 |
| P-/TCP AviF0819 | 96.39 | 19.21 | 53.53 | 11.80 | 149.92 | 27.51 | 0.36 | 0.06 |
| P-/TCP BveFZB42 | 93.72 | 20.98 | 48.94 | 11.43 | 142.65 | 29.81 | 0.34 | 0.04 |
| P-/TCP BveGB03 | 90.40 | 24.71 | 53.77 | 14.86 | 144.16 | 37.03 | 0.37 | 0.04 |
| P-/TCP Eco99B829 | 97.48 | 17.70 | 49.70 | 7.81 | 148.43 | 23.06 | 0.34 | 0.04 |
| P-/TCP Pfl29ARP | 65.7 | 14.42 | 51.70 | 12.92 | 117.40 | 23.81 | 0.44 * | 0.05 |
| P+ non-inoculated | 150.13 | 31.20 | 51.36 | 16.15 | 201.49 | 43.82 | 0.25 | 0.05 |
| P+ AviF0819 | 152.36 | 30.56 | 54.98 | 13.41 | 207.34 | 40.21 | 0.26 | 0.04 |
| P+ BveFZB42 | 130.30 | 30.35 | 48.21 | 11.87 | 178.51 | 40.32 | 0.27 | 0.03 |
| P+ BveGB03 | 147.60 | 30.07 | 50.53 | 11.90 | 198.13 | 38.98 | 0.25 | 0.04 |
| P+ Eco99B829 | 135.48 | 28.99 | 50.50 | 9.14 | 183.69 | 32.12 | 0.28 | 0.04 |
| P+ Pfl29ARP | 113.97 | 32.82 | 50.40 | 13.33 | 164.36 | 42.14 | 0.31 * | 0.05 |
| ANOVA | df=15, $P$=0.1269, $F$=1.4330 | | df=15, $P$=0.735267, $F$=0.7482 | | df=15, $P$=0.6874, $F$=0.7917 | | df=15, $P$=0.0004245, $F$=2.7517 | |

# Appendix 4

Coefficients, R² and *P* value of SMA lines (n = 30 for the P- and P+ treatments, n = 15 for the P-/HA and P-/TCP treatments), results of covariance analysis for differences among SMA lines coefficients (degree of freedom "df", *P* and likelihood ratio test "*LR*" values). If no significant difference was noticed between slopes, a common slope was used to test for difference in elevation. Treatments without any common letter are significantly different from each other (pairwise comparison).

| | elevation | slope | R² | *P* |
|---|---|---|---|---|
| *Non-inoculated* | | | | |
| P- | 0.51 | 0.81 *ab* | 0.090 | 0.10762 |
| P-/HA | -0.08 | 1.19 *a* | 0.072 | 0.33277 |
| P-/TCP | 0.00 | 1.15 *a* | 0.668 | 0.00019 |
| P+ | 1.15 | 0.60 *b* | 0.399 | 0.00018 |
| Covariance analysis | / | df= 3, *P*=0.020606, *LR*=9.772 | | |
| *P-* | | | | |
| Non-inoculated | 0.38 *ab* | | 0.090 | 0.10762 |
| AviF0819 | 0.34 *bc* | | 0.468 | 3.0804e-5 |
| BveFZB42 | 0.39 *a* | 0.88 | 0.718 | 3.5339e-9 |
| BveGB03 | 0.32 *cd* | | 0.460 | 5.3114e-5 |
| Eco99B829 | 0.31 *cd* | | 0.411 | 0.00013 |
| Pfl29ARP | 0.28 *d* | | 0.604 | 4.3258e-7 |
| Covariance analysis | df=5, *P*=2.4697e-9, *LR*=48.77 | df=5, *P*=0.75661, *LR*=2.631 | | |
| *P-/HA* | | | | |
| Non-inoculated | -0.71 | | 0.072 | 0.33277 |
| AviF0819 | -0.73 | | 0.394 | 0.01228 |
| BveFZB42 | -0.69 | 1.56 | 0.264 | 0.05028 |
| BveGB03 | -0.65 | | 0.227 | 0.07242 |
| Eco99B829 | -0.71 | | 0.384 | 0.01371 |
| Pfl29ARP | -0.75 | | 0.582 | 0.00093 |
| Covariance analysis | df=5, *P*=0.43264, *LR*=4.865 | df=5, *P*=0.17355, *LR*=7.7 | | |
| *P-/TCP* | | | | |
| Non-inoculated | 0.23 *ab* | | 0.668 | 0.00019 |
| AviF0819 | 0.23 *ab* | | 0.249 | 0.05839 |
| BveFZB42 | 0.25 *ab* | 1.02 | 0.513 | 0.00269 |
| BveGB03 | 0.19 *b* | | 0.630 | 0.00069 |
| Eco99B829 | 0.27 *a* | | 0.339 | 0.03689 |
| Pfl29ARP | 0.08 *c* | | 0.419 | 0.00905 |
| Covariance analysis | df=5, *P*=4.4342e-7, *LR*=37.65 | df=5, *P*=0.83515, *LR*=2.1 | | |
| *P+* | | | | |
| Non-inoculated | 1.15 | 0.60 *c* | 0.399 | 0.00018 |
| AviF0819 | 0.93 | 0.72 *bc* | 0.312 | 0.00132 |
| BveFZB42 | 0.45 | 0.99 *ab* | 0.621 | 2.3175e-7 |
| BveGB03 | 0.79 | 0.81 *bc* | 0.512 | 1.2825e-5 |
| Eco99B829 | 0.17 | 1.15 *a* | 0.312 | 0.00165 |
| Pfl29ARP | 0.10 | 1.15 *a* | 0.529 | 5.3439e-6 |
| Covariance analysis | / | df=5, *P*=0.00842, *LR*=15.5 | | |

# Appendix 5

TRL of four-week-old *Brachypodium* plantlets grown in Magenta® boxes, exposed to contrasted P supplies and either inoculated or not inoculated with bacterial strains (n = 18 for the P- and P+ treatments, n = 9 for the P-/HA and P-/TCP treatments). Results of 3-way ANOVAs (degree of freedom "df", *P* and *F* values) and Dunnett's *post hoc* tests (annotated with stars; P+ and non-inoculated treatments used as references).

| | TRL (cm) | |
|---|---|---|
| | mean | sd |
| *P treatment* | | |
| P- | 224.58 | 42.78 |
| P-/HA | 233.06* | 42.37 |
| P-/TCP | 213.26 | 49.83 |
| P+ | 213.87 | 46.39 |
| ANOVA | df=3, *P*=0.02126, *F*=3.2821 | |
| *Inoculation treatment* | | |
| Non-inoculated | 237.85 | 48.83 |
| AviF0819 | 228.11 | 42.30 |
| BveFZB42 | 214.92* | 50.62 |
| BveGB03 | 233.41 | 40.92 |
| Eco99B829 | 213.11* | 35.64 |
| Pfl29ARP | 195.82* | 41.59 |
| ANOVA | df=5, *P*=1.99e-06, *F*=7.2513 | |
| *Repetition* | | |
| ANOVA | df=1, *P*=0.07758, *F*=3.1363 | |
| *Interaction* | | |
| P- non-inoculated | 252.83 | 40.23 |
| P- AviF0819 | 239.12 | 33.89 |
| P- BveFZB42 | 214.76 | 49.38 |
| P- BveGB03 | 234.62 | 31.18 |
| P- Eco99B829 | 218.86 | 26.65 |
| P- Pfl29ARP | 187.32 | 42.97 |
| P-/HA non-inoculated | 257.96 | 31.15 |
| P-/HA AviF0819 | 219.12 | 39.79 |
| P-/HA BveFZB42 | 231.37 | 40.44 |
| P-/HA BveGB03 | 235.36 | 49.72 |
| P-/HA Eco99B829 | 248.26 | 45.64 |
| P-/HA Pfl29ARP | 206.27 | 33.97 |
| P-/TCP non-inoculated | 198.82 | 76.65 |
| P-/TCP AviF0819 | 221.07 | 38.39 |
| P-/TCP BveFZB42 | 224.41 | 61.96 |
| P-/TCP BveGB03 | 222.32 | 51.99 |
| P-/TCP Eco99B829 | 207.66 | 28.38 |
| P-/TCP Pfl29ARP | 205.30 | 33.29 |
| P+ non-inoculated | 232.32 | 36.08 |
| P+ AviF0819 | 225.11 | 52.78 |

| | | |
|---|---|---|
| P+ BveFZB42 | 202.13 | 51.09 |
| P+ BveGB03 | 236.76 | 41.63 |
| P+ Eco99B829 | 192.52 | 27.13 |
| P+ Pfl29ARP | 194.36 | 47.95 |
| ANOVA | df=15, $P$=0.15092, $F$=1.3891 | |

# Appendix 6

Results of PERMANOVA performed on the persistent homology analysis output of plant root systems. n = 18 for the P- and P+ treatments, n = 9 for the P-/HA and P-/TCP treatments. Post-hoc tests were performed by running a PERMANOVA for each pairwise comparison and $P$ values were adjusted for multiple comparisons using the Benferroni method.

| | Df | $F$ model | $P$ |
|---|---|---|---|
| Inoculation treatment | 2 | 11.1650 | 0.000999 |
| P treatment | 3 | 2.8237 | 0.005994 |
| Interaction | 6 | 1.2461 | 0.217782 |
| Residuals | 150 | | |

Post-hoc tests:

| | $F$ model | $P$ |
|---|---|---|
| P- vs P-/TCP | 1.8911 | 0.68931 |
| P- vs P+ | 2.1396 | 0.60539 |
| P- vs P-/HA | 2.5906 | 0.28771 |
| P-/TCP vs P+ | 3.1768 | 0.19780 |
| P-/TCP vs P-/HA | 1.4693 | 1.00000 |
| P+ vs P-/HA | 3.5882 | 0.17982 |
| Pfl29ARP vs non-inoculated | 18.7287 | 0.00099 |
| Pfl29ARP vs BveFZB42 | 13.9209 | 0.00099 |
| Non-inoculated vs BveFZB42 | 1.0035 | 0.38462 |

# Appendix 7

Shoot P concentration and PUE parameters of four-week-old *Brachypodium* plantlets grown in Magenta® boxes, exposed to contrasted P supplies and either inoculated or not inoculated with bacterial strains (n = 6 for the P- and P+ treatments, n = 3 for the P-/HA and P-/TCP treatments). Results of 3-way ANOVAs (degree of freedom "df", *P* and *F* values) and Dunnett's *post hoc* tests (annotated with stars; P+ and non-inoculated treatments used as references).

| | Shoot P concentration (µg P/mg FW) | | PUpE | | PUtE | | PPUE | |
|---|---|---|---|---|---|---|---|---|
| | mean | sd | mean | sd | Mean | Sd | mean | sd |
| *P treatment* | | | | | | | | |
| P- | 0.237* | 0.032 | 2418.692* | 662.318 | 4.296* | 0.603 | 1664.549* | 294.805 |
| P-/HA | 0.335* | 0.064 | 4146.142* | 1768.202 | 3.099* | 0.626 | 1365.267* | 135.433 |
| P-/TCP | 0.281* | 0.092 | 3318.446* | 1565.915 | 3.940* | 1.267 | 1649.241* | 361.100 |
| P+ | 0.764 | 0.086 | 337.427 | 60.022 | 1.325 | 0.151 | 915.617 | 222.696 |
| ANOVA | df=3, P<2e-16, F=292.0433 | | df=3, P<2e-16, F=306.9495 | | df=3, P<2e-16, F=217.6723 | | df=3, P<2.2e-16, F=60.9819 | |
| *Inoculation* | | | | | | | | |
| Non- | 0.432 | 0.233 | 2444.236 | 2012.003 | 3.026 | 1.488 | 1501.401 | 467.108 |
| AviF0819 | 0.421 | 0.243 | 2110.551 | 1542.716 | 3.136 | 1.493 | 1482.431 | 419.206 |
| BveFZB42 | 0.457 | 0.260 | 2255.018 | 1662.922 | 2.886 | 1.410 | 1304.184 | 436.268 |
| BveGB03 | 0.440 | 0.248 | 2247.915 | 1942.801 | 2.997 | 1.443 | 1354.915 | 360.533 |
| Eco99B829 | 0.457 | 0.261 | 2350.842 | 2048.284 | 2.937 | 1.485 | 1329.075 | 453.353 |
| Pfl29ARP | 0.412 | 0.256 | 1568.265* | 1258.735 | 3.298 | 1.543 | 1202.833* | 381.678 |
| ANOVA | df=5, P=0.2825, F=1.2751 | | df=5, P=0.01069, F=3.2079 | | df=5, P=0.4357, F=0.9788 | | df=5, P=0.007738, F=3.3921 | |
| *Repetition* | | | | | | | | |
| ANOVA | df=1, P=0.6384, F=0.2225 | | df=1, P=0.83696, F=0.0426 | | df=1, P=0.4312, F=0.6257 | | df=1, P=0.648586, F=0.2092 | |
| *Interaction* | | | | | | | | |
| P- non- | 0.234 | 0.031 | 2704.936 | 682.528 | 4.350 | 0.608 | 1913.742 | 245.850 |
| P- | 0.228 | 0.037 | 2476.670 | 739.706 | 4.487 | 0.686 | 1841.606 | 221.698 |
| P- | 0.255 | 0.037 | 2664.388 | 746.187 | 4.009 | 0.710 | 1588.721 | 289.616 |
| P- | 0.239 | 0.040 | 2412.522 | 692.725 | 4.284 | 0.701 | 1631.159 | 186.833 |
| P- | 0.232 | 0.033 | 2275.638 | 540.169 | 4.378 | 0.607 | 1649.714 | 296.266 |
| P- | 0.236 | 0.022 | 1977.999 | 544.750 | 4.268 | 0.430 | 1362.351 | 248.987 |
| P-/HA non- | 0.357 | 0.079 | 4775.731 | 2043.682 | 2.896 | 0.664 | 1395.929 | 126.322 |
| P-/HA | 0.305 | 0.044 | 3327.955 | 1067.802 | 3.323 | 0.456 | 1361.300 | 58.025 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| P-/HA | 0.344 | 0.046 | 3942.647 | 989.521 | 2.944 | 0.416 | 1283.964 | 129.002 |
| P-/HA | 0.363 | 0.076 | 4969.660 | 2542.434 | 2.836 | 0.608 | 1374.750 | 154.550 |
| P-/HA | 0.343 | 0.069 | 4684.583 | 2454.629 | 2.998 | 0.602 | 1449.523 | 212.164 |
| P-/HA | 0.295 | 0.089 | 3176.275 | 1807.334 | 3.594 | 1.055 | 1326.138 | 163.137 |
| P-/TCP | 0.296 | 0.117 | 3770.126 | 2229.912 | 3.826 | 1.715 | 1722.268 | 566.133 |
| P-/TCP | 0.285 | 0.089 | 3654.221 | 1536.643 | 3.792 | 1.388 | 1765.498 | 368.753 |
| P-/TCP | 0.286 | 0.095 | 3586.993 | 1625.492 | 3.835 | 1.530 | 1714.052 | 354.350 |
| P-/TCP | 0.266 | 0.067 | 2989.873 | 1264.261 | 3.944 | 1.112 | 1606.530 | 157.781 |
| P-/TCP | 0.351 | 0.141 | 4186.080 | 1884.872 | 3.296 | 1.666 | 1460.423 | 611.666 |
| P-/TCP | 0.203 | 0.020 | 1723.384 | 157.770 | 4.947 | 0.453 | 1626.672 | 188.967 |
| P+ non- | 0.736 | 0.062 | 354.844 | 41.169 | 1.368 | 0.114 | 1031.362 | 204.255 |
| P+ | 0.741 | 0.088 | 363.894 | 57.738 | 1.365 | 0.163 | 1042.288 | 220.108 |
| P+ | 0.802 | 0.086 | 335.845 | 48.824 | 1.260 | 0.138 | 824.824 | 180.071 |
| P+ | 0.765 | 0.078 | 351.455 | 70.629 | 1.318 | 0.129 | 942.947 | 232.530 |
| P+ | 0.791 | 0.111 | 341.557 | 38.802 | 1.286 | 0.186 | 882.537 | 248.321 |
| P+ | 0.751 | 0.102 | 276.967 | 73.901 | 1.354 | 0.193 | 769.744 | 189.371 |
| ANOVA | df=15, $P$=0.5820, $F$=0.8858 | | df=15, $P$=0.72824, $F$=0.7489 | | df=15, $P$=0.9355, $F$=0.4976 | | df=15, $P$=0.937471, $F$=0.4938 | |

# Publications and scientific communications

## Publications

Delaplace P, Delory BM, **Baudson C**, et al. (2015) Influence of rhizobacterial volatiles on the root system architecture and the production and allocation of biomass in the model grass Brachypodium distachyon (L.) P. Beauv. BMC Plant Biol. 15:195.

Delory BM, **Baudson C**, Brostaux Y, et al. (2016) archiDART: an R package for the automated computation of plant root architectural traits. Plant Soil 398:351–365.

**Baudson C**, Delory BM, Spaepen S, et al. (2021) Developmental plasticity of Brachypodium distachyon in response to P deficiency: Modulation by inoculation with phosphate-solubilizing bacteria. Plant Direct 5(1):1–17.

**Baudson C**, Delory BM, du Jardin P, et al. (2023) Triggering root system plasticity in a changing environment with bacterial bioinoculants – Focus on plant P nutrition. Plant Soil 484:49–63.

## Scientific communications

**Baudson C**, Bouché F, Saunier de Cazenave M, et al. (2013) Implication of microRNAs in the response of Brachypodium distachyon (L.) Beauv. root system architecture to rhizobacterial volatiles. *18$^{th}$ National Symposium on Applied Biological Sciences*. Ghent, Belgium, February 8$^{th}$ (Poster)

Mendaluk M, Varin S, **Baudson C**, et al. (2013) Influence of rhizobacterial volatile compounds on growth and root system architecture of Brachypodium distachyon (L.) Beauv. *18$^{th}$ National Symposium on Applied Biological Sciences*. Ghent, Belgium, February 8$^{th}$ (Poster)

**Baudson C**, Saunier de Cazenave-Mendaluk M, Blondiaux A, et al. (2013) Impact of rhizobacterial volatiles on Brachypodium distachyon (L.) Beauv. Growth and response to abiotic stresses. *1$^{st}$ International Brachypodium Conference*. Modena, Italy, June 19-21 (Oral presentation)

**Baudson C**, Saunier de Cazenave-Mendaluk M, du Jardin P, et al. (2014) Rhizobacterial volatile organic compounds implication in Brachypodium distachyon response to phosphorus deficiency. *19$^{th}$ National Symposium on Applied Biological Sciences*. Gembloux, Belgium, February 7 (Poster)

Mendaluk M, **Baudson C**, Delory BM, et al. (2014) Development of an ex-vitro system allowing plant-bacteria interactions through VOCs in the context of water stress. *19$^{th}$ National Symposium on Applied Biological Sciences*. Gembloux, Belgium, February 7 (Poster)

Delory BM, **Baudson C**, Brostaux Y, et al. (2014) archiDART : a R package allowing root system architecture analysis using Data Analysis of Root Tracings (DART) output files. *19$^{th}$ National Symposium on Applied Biological Sciences*. Gembloux, Belgium, February 7 (Poster)

**Baudson C**, Saunier de Cazenave-Mendaluk M, Delory BM, et al. (2014) Impact of

rhizobacterial volatiles on the response of the model grass Brachypodium distachyon to phosphorus deficiency. *5th International Symposium Phosphorus in Soils and Plants.* Montepellier, France, October 26-29 (Poster)

**Baudson C**, Delory BM, du Jardin P, et al. (2015) Can plant growth-promoting rhizobacteria mitigate P-starvation stress in Brachypodium distachyon? *Rhizosphere 4*. Maastricht, The Netherlands, June 21-25 (Poster)

Delory BM, **Baudson C**, Brostaux Y, et al. (2015) archiDART : an R package for the automated 2D computation of root architectural traits. *Rhizosphere 4*. Maastricht, The Netherlands, June 21-25 (Oral presentation)

Delaplace P, Ormeño – Lafuente E, Delory BM, et al. (2015) Rhizobacterial volatiles influence root system architecture, biomass production and allocation of the model grass Brachypodium distachyon (L.) Beauv. *10th International PGPR Workshop*. Liège, Belgium, June 16-19 (Oral presentation)

Delaplace P, Ormeño – Lafuente E, Nguyen M, et al. (2016) How do rhizobacterial volatiles influence root system architecture, biomass production and allocation of the model grass Brachypodium distachyon? 24th Plant and Animal Genome Conference, Brachypodium Genomics Workshop. San Diego, USA, January 9-13 (Oral presentation)

**Baudson C**, Delaplace P, du Jardin P (2017) Impact of plant growth promoting rhizobacteria on phosphorus use efficiency in the model grass Brachypodium distachyon. PGPR… The Good, the Bad or the Ugly? *International Symposium Micrope. Microbe-assisted crop production – opportunities, challenges and needs.* Vienna, Austria, December 4-7 (Poster talk)