UNIVERSIDAD DE LA FRONTERA

Facultad de Ingeniería y Ciencias

Doctorado en Ciencias de Recursos Naturales

"ASSESSMENT OF THE PHOSPHORUS ACQUISITION RELATED ROOT TRAITS OF TWO WHEAT CULTIVARS DIFFERING ON EFFICIENCY: TOWARDS PHOSPHORUS SUSTAINABILITY"

DOCTORAL THESIS IN FULFILLMENT OF THE REQUERIMENTS FOR THE DEGREE DOCTOR OF SCIENCES IN NATURAL RESOURCES

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TEMUCO-CHILE

2020

UNIVERSITY OF GRANADA

CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

DOCTORAL PROGRAM IN FUNDAMENTAL AND SISTEMS BIOLOGY

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Esta tesis fue realizada bajo la supervisión del Dr. Fernando Ricardo Borie Borie perteneciente al Departamento de Ciencias Químicas y Recursos Naturales de la Universidad de La Frontera y ha sido aprobada por los miembros de la comisión examinadora.

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Dedico esta tesis a mi esposa e hijo

Agradecimientos

Quisiera agradecer a mi tutor de Tesis, Dr. Fernando Borie, por el gran apoyo en el desarrollo de mi tesis doctoral. A los Drs. Alex Seguel y Pablo Cornejo por sus constantes aportes en la discusión de los resultados y manuscritos desarrollados, y al Dr. Álvaro García-López por su apoyo en el desarrollo del segundo objetivo de tesis. También quisiera agradecer a todo mi equipo de laboratorio por su ayuda y amistad, en especial a Natalia Aguilera, Christian Santander, Alan Bizarro y Humberto Aponte. También agradezco a Gladys y Mariela por su paciencia y gestiones a lo largo del camino.

Agradezco enormemente a mi otro tutor de Tesis, Dr. Juan Antonio López Ráez quien me brindó la oportunidad de realizar mi pasantía de doble graduación por la Universidad de Granada. Gracias por todo su apoyo durante mi estadía en España y por ayudarme durante mi proceso de formación. También agradezco a la Dra. María José Pozo y a mis compañeros de laboratorio en el extranjero por todo el apoyo durante esta etapa.

Agradezco al Fondo Nacional de Desarrollo Científico Tecnológico, a través del Proyecto FONDECYT 11160385, en cuyo marco se inserta el desarrollo de esta investigación. De forma individual, agradezco al Programa de Formación de Capital Humano Avanzado de la Comisión Nacional de Investigación Científica y Tecnológica (CONICYT), por la beca CONICYT Nº 21161474 y al Programa de Atracción e Inserción de Capital Humano Avanzado (PAI-CONICYT) a través del Concurso Nacional Tesis de Doctorado en el Sector Productivo por el Proyecto Nº T7817120011, que en conjunto me permitieron financiar esta tesis. Relacionado a lo anterior, también agradezco a la empresa Campex Baer por patrocinarme y asesorarme durante la postulación y ejecución del Proyecto Nº T7817120011.

A mi familia y amigos, en especial mi esposa Maca e hijo Joaquim, gracias por amarme siempre, ustedes son todo para mi. Pai, Mãe, Lu, Vó Glorinha, Vó Zezé, Vô Romero e Vô Pedro, obrigado por tudo.

Summary and thesis outline

Phosphorus (P) scarcity is going to become a serious problem for agricultural productivity and sustainability as high-quality phosphate (Pi) rock reserves are getting quickly depleted. Therefore, there is an urgent need for a more efficient use of P-fertilizers to feed the growing population as sub-optimal fertilization would affect crop productivity and over-fertilization can cause environmental problems. In this context, breeding for P-efficient plants has been advocated as an environmental-social-economical friendly alternative to improve the sustainability of both high- and low-input systems. In general, two main P-efficiency categories can be distinguished: P use efficiency (PUE) and P acquisition efficiency (PAE) – being the last one the focus of this Doctoral Thesis.

Plants have evolved a series of mechanisms to adapt themselves to low P availability in the soil. In this way, a complex regulation of phytohormones such as auxin, gibberellins, ethylene, and strigolactones (SL) – with special emphasis on the latter - triggers a series of root modifications to increase P acquisition under limiting conditions, which includes: i) modifications in shoot and root morphology, growth and development; ii) exudation of low molecular weight organic acids anions and P-releasing enzymes; iii) modulation of the expression/activity of phosphate transporters; and iv) association with soil microorganisms, especially arbuscular mycorrhizal (AM) symbiosis - the most widespread symbiosis in terrestrial ecosystems, recognized for their ability to increase P acquisition through their fungal mycelium among other benefits.

Remarkably, SLs have been shown to be involved in all of the processes mentioned above, both as regulator of plant physiology and as signaling molecules in the rhizosphere, stimulating AM spore's germination and initial hyphal branching. However, more studies are necessary to fully elucidate the role of SL on P starvation responses, especially at plant intra-specific level. Therefore, the study of the PAE mechanisms of P

efficient plants colonized by AM fungi (including AM community composition) and their relationship with SL signaling is necessary to generate a trait-based background for breeders oriented to the development of more efficient genotypes to reduce P fertilization.

This Doctoral Thesis was focused on the study of the root morphology, organic acid and phosphatase exudation, expression of phosphate transporters, and root mycorrhizal colonization as the main mechanisms involved in the PAE of wheat genotypes growing under P-limiting conditions, and the underlying SL modulation behind those traits. In chapter I, a general overview on the topic was presented, describing the state of the art and showing the hypothesis and aims of this study. In chapter II, plant adaptations related to P acquisition root traits, with emphasis on AM symbiosis and its possible effects on plant' P-related root traits were deeply reviewed. A summary of results from 1980-2018 regarding AM growth responses and P uptake in wheat plants was presented to discuss the generally accepted lack of positive responses in this plant species. The importance of considering AM functional diversity on future studies and the need to improve PAE definition considering carbon trade between all the directly related PAE traits and its benefits to the host plant were also discussed.

In chapter III, the effects of two indigenous AM fungal isolates (*Claroideoglomus claroideum* and *Rhizophagus intraradices*) on nutrient uptake and root traits of two commercial Chilean wheat genotypes with contrasting P-acquisition efficiency were evaluated in order to determine if the responses were dependent on the interaction between host plant genotype and AM ecotype and if the symbiosis can effectively enhance P acquisition on these cultivars (specific objective 1). The results showed that biomass production and root morphological responses to AM colonization significantly varied between genotypes and AM isolate, being the most P-efficient genotype – *cv.* Crac

– not affected by the symbiosis in these parameters, while the less efficient – *cv.* Tukan – showed a higher mycorrhizal dependency. Moreover, P and Ca accumulation were increased in both cultivars when colonized by *C. claroideum* and *R. intraradices*, respectively. It was demonstrated that AM growth responses vary at intra-specific level, depending on the basal efficiency of each cultivar and that the nutritional benefits were specific to each AM isolate. Therefore, AM symbiosis could contribute to increase agricultural sustainability; however, genotype and environmental-specific combinations should be considered to maximize the benefits from the symbiosis.

The chapter IV is related to the specific objective 2, where the P acquisition-related root traits (root morphology, organic acid and phosphatase exudation, and root mycorrhizal colonization) of the wheat genotypes under study were evaluated to determine those that mainly contributed to P accumulation. To that, a pot and rhizobox experiments were performed using a high P-fixing volcanic soil, either fertilized or not with P. Crac plants showed higher P accumulation regardless of P treatment and experiment, being root morphological traits the most correlated with P acquisition, both in the presence and in absence of Pi fertilization. Also, the results suggest that AM symbiosis and oxalate exudation could be other important mechanisms to enhance P acquisition under P-limiting conditions. Finally, the traits evaluated in this study can become important targets for future breeding programs oriented to generate cultivars adapted to both high- and low-P input systems.

In chapter V, the SL production among the studied wheat cultivars and its effects on Pi-starvation responses (PSRs) and P acquisition (specific objective 3) were assessed. To accomplish this goal, plants were grown hydroponically, and P-starvation was applied to evaluate the main PSRs at the transcriptional level – especially related to the modulation of the P signaling and homeostasis pathway (*IPS1*-mir399-*PHO2*) and Pi

transporters expressions. Here, a higher expression of the Pi transporters *TaPht1;2* and *TaPht1;10* and a faster and higher modulation of the *IPS1*–miR399–*PHO2* pathway was observed in Crac plants. Remarkably, Crac presented higher levels of SLs, suggesting a direct relationship with the responses mentioned above. Finally, an improved model for the regulation of the P homeostasis module was proposed. Taking together, the higher PAE in Crac was associated with a faster and improved P signaling through a fine-tuning regulation of *PHO2*, which seems to be regulated by SLs. This knowledge could help to develop new strategies for improved plant performance under P stress limitation. All the results obtained here are discussed comprehensively in chapter VI of this Thesis.

TABLE OF CONTENTS

CHAPTER I

General Introduction

1.1 General Introduction

Agriculture practices and technology have been greatly improved over the last decades; however, agricultural productivity showed slightly increases since the 1980s (Grassini et al., 2013). Meanwhile, world population is increasing at high rates, being expected to be up to 9 billion people by the year 2050 (https://population.un.org/wpp/, Last access: August 2019). This claims for a more efficient use of resources to enhance food productivity in order to feed future generations. In this scenario, cereal crops play an important role, as more than 50% of the daily caloric intake corresponds to cereal consumption, with values exceeding 80% in the poorest countries (Awika, 2011).

Wheat (*Triticum aestivum* L.) cropping is responsible for 26% of worldwide total cereal production, only after maize (with 38%; http://www.fao.org/faostat/es, Last access: July 2019). Nevertheless, maize high productivity is restricted to few countries (i.e. USA and China accounts for more than 50% of total maize production), while wheat production is more evenly distributed globally. Therefore, increasing wheat productivity would provide benefits to almost every country in the world. In Chile, wheat is also the most cultivated crop, with more than 35% of the total area sowed with annual crops (http://historico.ine.cl, Last access: July 2019), being La Araucanía Region responsible for more than 40% of the national production in the 2018/2019 season (https://odepa.gov.cl), representing one of the main economic activities from Southern Chile.

Despite its great nutritional importance, wheat production is highly dependent on phosphorus (P) fertilizers, configuring it as the crop with highest consumption of this resource (Heffer, 2013). The latter is further aggravated considering that the majority of

the soils worldwide possess relatively low P availability, thus, requiring continuous P fertilization to maintain high productivity (Lynch, 2011). In this context, Southern Chile is mainly covered by Andisols and Ultisols, which are characterized by the great abundance of clay particles with highly reactive surfaces (*e.g.,* allophanes, imogolite and/or 2:1 layer silicates; Dahlgren et al., 2004). Thus, the high reactivity of Andisols generates an accumulation of humic materials, a high P-fixing capacity, and high activities of H^+ and phytotoxic Al^{3+} (Borie et al., 2019). Accordingly, the high wheat yields achieved in La Araucanía Region compared to national averages are only possible by applying high doses of P fertilizers and lime. The latter raises soil pH, alleviating phytotoxicities problems, and at the same time enhancing P acquisition, as it is absorbed by plants from the soil solution as phosphate (Pi), having maximal uptake rates in a pH between 5-6 (Holford, 1997; Marschner and Rengel, 2012). Nevertheless, the abuse of P-fertilizers increases production costs and can lead to environmental problems related to eutrophication of groundwater (Bennett et al., 2001).

The primary source of P fertilizer is from phosphate rock mining, only found in few places in the world. As every non-renewable resource, its extraction is going to decrease along time (Mogollón et al., 2018). Accordingly, high-quality Pi rock reserves are decreasing, and although peak in P production estimates are variable, farmers have already seen the effects of global P insecurity, as from 2007 to 2008 the price of phosphate rock increased by 700% (Cordell et al., 2009). Impacts in emerging economies such as Chile, must be considered, especially based on the low abundance of natural high-quality phosphate rock sources in the country.

Increasing P fertilizer availability to crops using liquid fertilizers (Holloway et al., 2001) or strategic fertilizer placements, are alternatives to improve P fertilization efficiency (Ma et al., 2009). However, both strategies require technology and high investments. Alternatively, breeding for P-efficient crop cultivars has been advocated as an environmental-social-economical friendly alternative for improving the P efficiency of cropping systems for its relatively low cost and because it can provide benefits to both high and low input systems (Rose et al., 2010). Nevertheless, it is important to consider that plant breeding is a complex process, often exceeding ten years from its initial development to their final use (Bailey-serres et al., 2019). Moreover, a better integration between research oriented to trait selection for one side and applied breeding for the other is required, as until now, few genes successfully characterized to increase plant efficiency has been utilized in the development of new varieties (Wissuwa et al., 2016). Meanwhile, it is imperative to increase our knowledge regarding P efficiency for providing a faster transition of science to field when time has come.

According to Wang et al. (2010), two overall basic mechanisms of P efficiency in plants can be distinguished: i) Phosphorus use efficiency (PUE), which is referred to the internal efficiency of allocation/mobilization of P (*i.e.* producing biomass with lower P "cost"), and ii) plants' ability to actively acquire P from the soil through their root system, also known as phosphorus acquisition efficiency (PAE). Improving PUE would be the best scenario as less P would be needed to produce biomass. However, traits related to PUE are complex, difficult to assess and it still remains unknown whether exploitable genetic variation exists for the genes associated with those traits (Rose and Wissuwa, 2012; van de Wiel et al., 2016). In addition, to date, no crop species or genotypes within species are

known to be capable of reducing its net P uptake if the demand is reduced (Rose and Wissuwa, 2012). The latter is highly important, as increased P uptake under optimal conditions is related to higher uptake of other nutrients, as nitrogen (Sadras, 2006), reducing overall efficiency. On the other hand, PUE and PAE have been shown to correlate under P-limiting conditions, including in Chilean Andisols (McDonald et al., 2015; Sandaña and Pinochet, 2014; Seguel et al., 2017). Therefore, improving wheat PAE would configure an important strategy in order to reduce P fertilizer input and increase agricultural sustainability.

Phosphate acquisition by plants is largely based on the physiological, biochemical and morphological characteristics of the root system (Haling et al., 2016; López-Arredondo et al., 2014). However, plants first need to sense the P status both locally and systemically in order to orchestrate the appropriate responses (Lan et al., 2017; Scheible and Rojas-Triana, 2015). In this context, differential regulation of phytohormones such as auxin, cytokinin, abscisic acid, ethylene, and in particular strigolactones (SLs) is promoted when plants are subjected to P limitation, modulating gene expression and root functioning (Chien et al., 2018; Waters et al., 2017). SLs are the latest class of phytohormones described and have been shown to function as regulators of plant development/architecture under P limitation by reducing shoot apical growth while increasing lateral root formation, root hair number and elongation, among others (López-Ráez et al., 2017; Waters et al., 2017). Indeed, SLs biosynthesis is highly promoted under this stress condition (López-Ráez et al., 2008; Yoneyama et al., 2007, 2012). In addition to their role as phytohormones, they also function as signaling molecules in the rhizosphere, stimulating arbuscular mycorrhizal spore germination and initial hyphal branching (López-Ráez et al., 2017;

Waters et al., 2017). Despite the key role of SLs under Pi starvation, how they modulate plant responses and whether they are also involved in Pi signaling remain unclear.

As a consequence of the initial reprogramming of the root systems to cope and overcome P limitation, a series of adaptive strategies can take part, including: i) changes in root morphology and architecture that improve soil volume exploration (Lambers et al., 2006); ii) root exudation of protons and/or low molecular weight organic acids anions, such as malate, citrate and oxalate, that chelate (or precipitate) metals and solubilize P bound to Al and Fe complexes (Richardson et al., 2009), or displace P from the soil matrix by ligand exchange (Jones, 1998); iii) exudation of phosphatases, especially in soils with high amounts of phytates and other organic P forms (Nannipieri et al., 2011); iv) increasing root efficiency in acquiring P through regulation of expression and activity of high-affinity Pi transporters (Huang et al., 2011); and v) root association with microorganisms, especially mycorrhizal symbiosis.

Arbuscular mycorrhizal (AM) symbioses are associations between plant roots or rhizoids with some fungal species from Glomeromycota phylum, found in *ca.* 80% of plant species among all major plants' lineages and in most of agricultural plants (Brundrett and Tedersoo, 2018). It is generally accepted that colonization by AM fungi improve plants' water and nutrients acquisition, particularly P, through the mycelium network developed by the fungi, increasing soil exploration capacity by the symbiosis (Smith and Read, 2010). Nevertheless, plant responses related to P acquisition are not always correlated to mycorrhizal hyphal production (Lendenmann et al., 2011; Thonar et al., 2011). Indeed, mycorrhizal symbiosis was shown to impact plant' own P acquisition traits (e.g. root morphology, exudation pattern and metabolisms; reviewed in detail on Chapter II),

although little is known about the former and its relationship with PAE. Moreover, even though some mycorrhizal responses have been widely studied, its effects cannot be generalized, as a single host plant species colonized by different AM fungi can have contrasting responses, and in the same way, colonization by the same AM fungus can result in different responses in different plant species, or genotypes within the same species (Feddermann et al., 2010; Smith et al., 2011). In addition, AM effects are also environment-dependent, and plants are usually colonized by more the one fungal taxa at field conditions (Neuenkamp et al., 2018; Santander et al., 2019; Smith et al., 2011).

Wheat has a high variability of responses to AM colonization, being considered to have low to negative responses (Grace et al., 2009; Hetrick et al., 1996). However, positive responses are often found, indicating that AM symbiosis can be an effective agronomic practice under the appropriated circumstances (Pellegrino et al., 2015; Suri et al., 2011). It is uncertain whether AM symbiosis can contribute to significantly enhance P acquisition in wheat cropping. Many factors involved in this large growth response variation as well as in the processes affecting AM function are still unknown. Thereby, it is crucial to develop studies to assess the responsiveness of local wheat genotypes, and the PAE mechanisms of P efficient genotypes colonized by indigenous AM fungi (including AM community composition) and their relation with SL signaling, in order to generate a trait-based background for local breeders oriented to the development of more efficient genotypes and for the integrated nutrient management strategies to reduce P fertilization.

1.2 Hypothesis

Considering that: i) Chilean volcanic soils (as Andisols) present high P-fixing capacity, reducing the efficiency of Pi fertilizer application; ii) that Chilean wheat cultivars show high variability in their P acquisition efficiency (PAE; assessed on a screening experiment analyzing P-responses of eighteen local wheat cultivars (data not published) and further confirmed at field; Seguel et al., 2017); iii) that P concentration is higher in the firsts developmental stages and decreases along with plant development (Elliott et al., 1997; Greenwood et al., 2001) and iv) that the main mechanisms of PAE in plants are related to root traits, the present proposal **hypothesizes** that:

1. The growth and nutritional responses - especially on P - of wheat cultivars colonized by arbuscular mycorrhizal (AM) fungi will depend on species-specifics interactions between the host plant genotype and the AM ecotype.

2. Higher P acquisition under P-deficient condition will be related either to an enhanced root growth, higher organic acid and phosphatase exudation, or increased root mycorrhizal colonization.

3. Strigolactone production will be higher in the P-efficient cultivar, affecting Pi-starvation responses and improving P acquisition.

1.3 *Research Objectives*:

1.3.1 *General Objective*: To study the root morphology, organic acid and phosphatase exudation, expression of phosphate transporters, and root mycorrhizal colonization as the

main mechanisms involved in the PAE of wheat genotypes growing under P-limiting conditions, and the strigolactone modulation underlying those traits.

1.3.2 *Specific Objectives*:

1. To study the physiological and biochemical traits of wheat cultivars colonized by different ecotypes of indigenous AM fungi in relation to root morphology, and organic acid and phosphatase exudation.

2. To determine the mechanisms (root morphology, organic acid and phosphatase exudation, or root mycorrhizal colonization) that mainly contribute to P acquisition of wheat cultivars growing in a high P-fixing volcanic soil.

3. To analyze the relationship between strigolactone production and P-starvation responses in wheat cultivars with contrasting PAE.

CHAPTER II

"Phosphorus Acquisition Efficiency Related to Root Traits: Is Mycorrhizal Symbiosis a Key Factor to Wheat Cropping?"

Article published in *Frontiers on Plant Science* (2018) 9:752

doi: 10.3389/fpls.2018.00752

Phosphorus acquisition efficiency related to root traits: is mycorrhizal symbiosis a key factor to wheat cropping?

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ABSTRACT

Wheat (*Triticum aestivum* L.) is a major crop cultivated around the world, thus playing a crucial role on human diet. Remarkably, the growing human population requires a significant increase in agricultural production in order to feed everybody. In this context, phosphorus (P) management is a key factor as it is component of organic molecules such as nucleic acids, ATP and phospholipids, and it is the most abundant macronutrient in biomass after nitrogen (N), although being one of the scarcest elements in the lithosphere. In general, P fertilization has low efficiency, as only a fraction of the applied P is acquired by roots, leaving a substantial amount to be accumulated in soil as not readily available P. Breeding for P-efficient cultivars is a relatively low cost alternative and can be done through two mechanisms: i) improving P use efficiency (PUE), and/or ii) P acquisition efficiency (PAE). PUE is related to the internal allocation/mobilization of P and is usually represented by the amount of P accumulated per biomass. PAE relies on roots ability to acquire P from the soil and is commonly expressed as the relative difference of P acquired under low and high P availability conditions. In this review, plant adaptations related to improved PAE are described, with emphasis on arbuscular mycorrhizal (AM) symbiosis, which is generally accepted to enhance plant P acquisition. A state of the art (1980 to 2018) of AM growth responses and P uptake in wheat plants is made to discuss about the commonly accepted growth promoting effect and P increased uptake by AM fungi and the contrasting evidence about the generally accepted lack of positive responses in this plant species. Finally, the mechanisms by which AM symbiosis can affect wheat PAE are discussed, highlighting the importance of considering AM functional diversity on future

studies and the necessity to improve PAE definition by considering the carbon trading between all the directly related PAE traits and its return to the host plant.

2.1 Introduction

Cereals have been cultivated for more than 10,000 years, playing a crucial role in the development of human civilization. Today, cereals are still important, being the principal crops harvested in the world with more than 2.8 Gt of combined grain production (http://www.fao.org/faostat/, Last access: July 2019). Among major cereals, wheat (*Triticum aestivum* L.) cropping represent almost 30% of global grain yield (http://www.fao.org/faostat/, Last access: July 2019). Cereals are also the major component of human diet worldwide with more than 50% of daily caloric intake, with values exceeding 80% in the poorest countries (Awika, 2011). Agricultural practices and technology have greatly improved over the last decades to reduce problems associated with food scarcity and to provide cereals for the daily diet. However, risks and unprecedented challenges still remain considering that global food, and grain production must increase a 70% by the year 2050 as world population is expected to be reach 9 billion people (https://population.un.org/wpp/, Last access: August 2019). Meanwhile, the slight increase in crop yields observed since the 1980s and the scarcity of available land suitable for production make the focus on reducing crop losses empirical due to various kinds of biotic and abiotic stresses factors, such as pathogen attack, cold, heat, drought, salt, deficiency of nutrients as phosphorous (P), and phytotoxicity by heavy metal stresses (Bhardwaj et al., 2014; Ray et al., 2012).

P fertilizers are manufactured from rock phosphate found only in a few places in the world, being Morocco the owner of 85% of the known active mining reserves. As a non-renewable resource, rock phosphate, as well as other non-renewable resources such as oil and coal is expected to become scarce near the 2030s (Cordell et al., 2009), or more

optimistically within two to three centuries (Sattari et al., 2012). The market and countries are already responding to this scenario, which is reflected in the fact that both USA and China (the biggest P producer in the world) have stopped exporting this resource (van de Wiel et al., 2016). In addition, P fertilizers may cause environmental problems associated with eutrophication (Gaxiola et al., 2011) and can contain heavy metals such as cadmium that may accumulate in arable soils as a result of the addition of rock phosphate (van de Wiel et al., 2016). Remarkably, usually only about 10 to 30% of the P fertilizer applied in the first year is taken up by the roots, with a substantial part accumulated in the soil as residual P not readily available for plants (Syers et al., 2008). In alkaline soils, P can be bound to calcium, and in acidic soils it can be readily complexed to charged Al and Fe oxides and groups hydroxyls on clay surfaces (Kochian et al., 2004; Seguel et al., 2013), limitations that can occur in *ca*. 30% of arable soils worldwide (Kochian, 2012). Moreover, organic material present in the soil (e.g. from manure or crop residues) can also bind phosphate ions as well as phytate (inositol compounds).

Ideally, P taken up by agricultural products should represent the same amount of applied P fertilizer, achieving a neutral balance (Helyar, 1998; Syers et al., 2008). However, this situation is often only achieved in low input, low production farming systems (Burkitt et al., 2007; McIvor et al., 2011), on intrinsically low P-buffering capacity soils in productive agriculture (e.g. sands), or where P-buffering capacity is low because sorption sites for P are close to saturation and soil P availability is relatively high (e.g. Syers et al., 2008). Elsewhere, P-balance is relatively low, which contributes to an inefficient P use (Richardson et al., 2011). Thus, P management must be improved in order

to enhance plant uptake in soils, as well as using the less available and bound-P through a better understanding of the processes happening in the soil-plant systems.

2.2 Phosphorus in the soil-plant continuum

In general, P is present in plants either as organic phosphate esters or as free inorganic orthophosphate forms, representing up to *ca*. 0.2% of plants dry weight, making it the most abundant macronutrient in plants after nitrogen (N). However, unlike N, the amount of P available for agriculture is finite (Bovill et al., 2013). When compared to other essential macronutrients, P is one of the less-abundant elements in the lithosphere (0.1% of the total). P is an important component of organic molecules such as nucleic acids, ATP and phospholipids; thus playing a crucial role in energy metabolism of both plants and animals (Abel et al., 2002; Vance et al., 2003). Phosphate is also involved in signal-transduction pathways via phosphorylation/dephosphorylation, hence regulating key enzyme reactions in general cellular metabolism, including N fixation on N-fixing plants (Marschner and Rengel, 2012; Schachtman et al., 1998; Theodorou and Plaxton, 1996).

Plants acquire P from the soil solution predominately as inorganic phosphate (Pi) ($H_2PO_4^-/HPO_4^{-2}$), having maximal uptake rates at pH 5-6 (Holford, 1997; Marschner and Rengel, 2012; Rae et al., 2003). It acquisition occurs by a plasma membrane-localized phosphate transporter-mediated process, which has been suggested to operate as a H^+ co-transporter (Rae et al., 2003; Raghothama and Karthikeyan, 2005). Phosphate transporters are classified into four families: Pht1, Pht2, Pht3 and Pht4, which are located on the plasma membrane, plastidial membrane, mitochondrial membrane, and Golgi-compartment, respectively (López-Arredondo et al., 2014). Two different uptake systems have been

proposed: one with high-affinity, which is regulated by Pi availability and activated mainly under P deficiency, with a Km of 3-7 μM; and the other is a low-affinity system constitutively expressed system with Km of 50-300 μM (Bucher et al., 2001; Liu et al., 2011; Preuss et al., 2010; Tian et al., 2017). Despite of having a high-affinity acquisition system, P has a low availability and poor mobility in the soil, being one of the most inaccessible elements for plants (Holford, 1997). Concentrations of available P in soil solution are extremely low, being generally lower than 10 μM (Do Nascimento et al., 2016; Holford, 1997), whereas in wheat leaves and stems concentrations of over 100 mM can be achieved (Bauer et al., 1987; Seguel et al., 2017). Therefore, as plants normally take up P faster than it is supplied by diffusion a depletion zone around the root system is quickly created, inducing P deprivation (Fig. 1.1) (Hinsinger, 2001).

The rhizosphere encompasses the first millimeters of the soil surrounding plant roots, where biological and ecological complex processes occur. This is the critical zone for P dynamics as plants roots are capable of modifying this environment through their physiological activities, especially by exudation of organic acid anions, enzymes, secondary metabolites and sugars (Bais et al., 2006; Giles et al., 2017). These processes not only determine solubilization/mineralization, acquisition of soil nutrients and microbial dynamics, but also control the efficiency of nutrient use by plants and crops, therefore influencing productivity and sustainability of the agroecosystems (Hinsinger et al., 2009; Zhang et al., 2010).

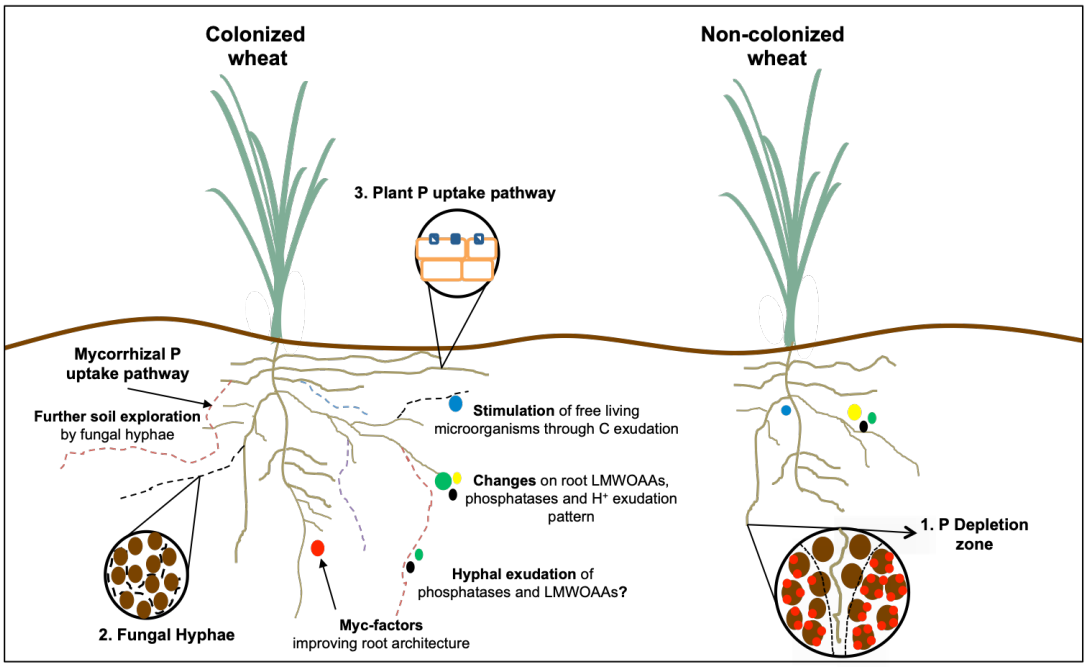

Figure 1. Phosphorus acquisition efficiency related traits of wheat roots affected by arbuscular mycorrhizal symbiosis in comparison to a non-colonized counterpart. (1) Representation of P depletion zone around the rhizosphere; (2) Access to smaller soil pores by AM fungal hyphae; and (3) Modulation of plant P transporters following colonization.

2.3 Phosphorus efficiency

Great efforts have been made in the last decade concerning P efficiency. In this sense, agronomic strategies for increasing P fertilizer availability to crops has been developed, for example, by applying liquid fertilizers (Holloway et al., 2001) or by localized fertilizer placement (Ma et al., 2009). However, those techniques require modern technologies and increase operational costs. On the other hand, breeding for P-efficient crop cultivars has been advocated due to its relatively low cost, providing benefits to both high and low-input systems (Rose et al., 2010).

Despite the growing knowledge in the field, there is still controversy in the concept and measurement of efficiency, as it has many definitions, and even different terms are

often used although they are calculated in the same way (Bovill et al., 2013). Nowadays, P efficiency is understood as two different mechanisms: i) the internal efficiency of allocation/mobilization of P in order to produce higher biomass with lower input, and ii) plant ability to acquire P from the soil, also known as P acquisition efficiency (Rose and Wissuwa, 2012; Sandaña and Pinochet, 2016; Wang et al., 2010).

The internal use efficiency or P use efficiency (PUE) is here defined as the amount of biomass unit per P accumulated in the tissue (shoot and/or root) or grain produced (Rose and Wissuwa, 2012). It is related to a range of metabolic modifications that can occur for reducing P demand during plant development (Hammond et al., 2009; Veneklaas et al., 2012). Improving internal PUE will lead to a more resource-efficient use of P rather than increasing uptake of potentially scarce P forms, as in theory less P will be acquired by crops, minimizing P fertilizer requirement and removal from fields. However, to date no crop species or genotypes within species are known to be capable of reducing its net P uptake if the demand is reduced (Rose and Wissuwa, 2012). This is operating in sandy or low P-sorption capacity soils. On the contrary, in soils rich in sorbed P, which are observed in the majority of acid soils, breeding programs focused on the optimization of P scavenging mechanisms would be a key role to improve P efficiency. Consequently, this review has been mainly focused on P acquisition efficiency.

2.3.1 Phosphorus acquisition efficiency

While PUE aims to produce more biomass with lesser P costs, P acquisition efficiency (PAE) is related to enhancing its acquisition from soil, especially from unavailable forms, and for this purpose root traits are a key factor. PAE is commonly expressed in the literature

as the relative difference of P taken up in low and high P availability conditions (Vandamme et al., 2013; Seguel et al., 2015, 2017). However, this definition does not take into account the root traits involved. In this sense, Liao et al. (2008) made a more realistic approximation by integrating root length and root biomass. Nevertheless, other traits related to root architecture and physiology must be integrated in the PAE definition due to their key role in uptake as discussed below (Fig. 2).

2.3.1.1 Root architecture

P status is a major factor modulating root architecture, being a higher root: shoot ratio the most evident change in the majority of plants experiencing P deprivation (Gruber et al., 2013; Wissuwa et al., 2005). Phosphate, the available form of P, presents a heterogeneous distribution (patches) given its high affinity for the soil matrix. Root P gathering implies a continuous root growth due to the quick depletion of rhizosphere P and the need of looking for new hotspots in soils (Fig. 1.1).

The upper soil layer (0-10 cm) - known as topsoil - is the zone where P availability for plants and microorganisms is generally higher, mainly due fertilizers input in the surface and its poor mobility through soil profile. Important adaptations of plants to access this richer environment are the development of axial roots with shallower angle, enhancing adventitious rooting, and greater density and dispersion of lateral roots and root hairs (Lynch, 2007; Wang et al., 2004). These traits, together with root length, diameter and surface area comprise the most important inter- and intra-specifically functional variations of plant root adaptations for PAE for most plant species. During their screening for traits directly related to PAE, Manske et al. (2000) found that higher root length density in top

soil of wheat crops was the most important root trait for P uptake, which was positively correlated with enhanced recovery of fertilized P. Basal roots in some legumes (as bean and soybean) appear in distinct nodes or "whorls", which affect root growth angles and therefore top soil exploration. Differences of up to 100% of improved P acquisition can be found in common bean cultivars as basal root whorl number varies among genotypes (Lynch, 2007). However, a certain tradeoff occurs between P and water uptake since plants with higher density of roots in top soil and shallower angles have lower water use efficiency, as water is usually more abundant in deeper layers under drought conditions (Ho et al., 2005). Another obstacle in improving root density is the associated carbon cost of producing root hairs, that have to be compensated by producing either smaller or thinner hairs and/or increased proportion of aerenchyma in the cortex and less secondary growth of the stele (Lynch and Ho, 2005; Zhu et al., 2010). Plants would otherwise spare carbon allocated in developing 'productive' parts.

Modeling root traits are clearly advantageous strategy for enhancing PAE. However, screening and phenotyping for these traits remain a complex challenge as soil-based study systems are high technology based, and hydroponic/aeroponic systems cannot totally emulate the complexity of the processes occurring in the soil. Therefore, genotypes selected in this way do not always show their superiority in field trials (van de Wiel et al., 2016).

2.3.1.2 Root exudates

If P is present on fixed sources and/or unavailable forms, plants having larger and/or more branched root architecture do not significantly improve P acquisition. In this case, root

physiology and biochemical responses play a major role on accessing P from sparingly available pools in soil. Hence, the exudation of low molecular weight organic acids (LMWOAAs), proton extrusion, phosphatase exudation and/or association with symbiotic and non-symbiotic microorganisms present in the rhizosphere are the most important adaptations developed by plants (Fig. 1).

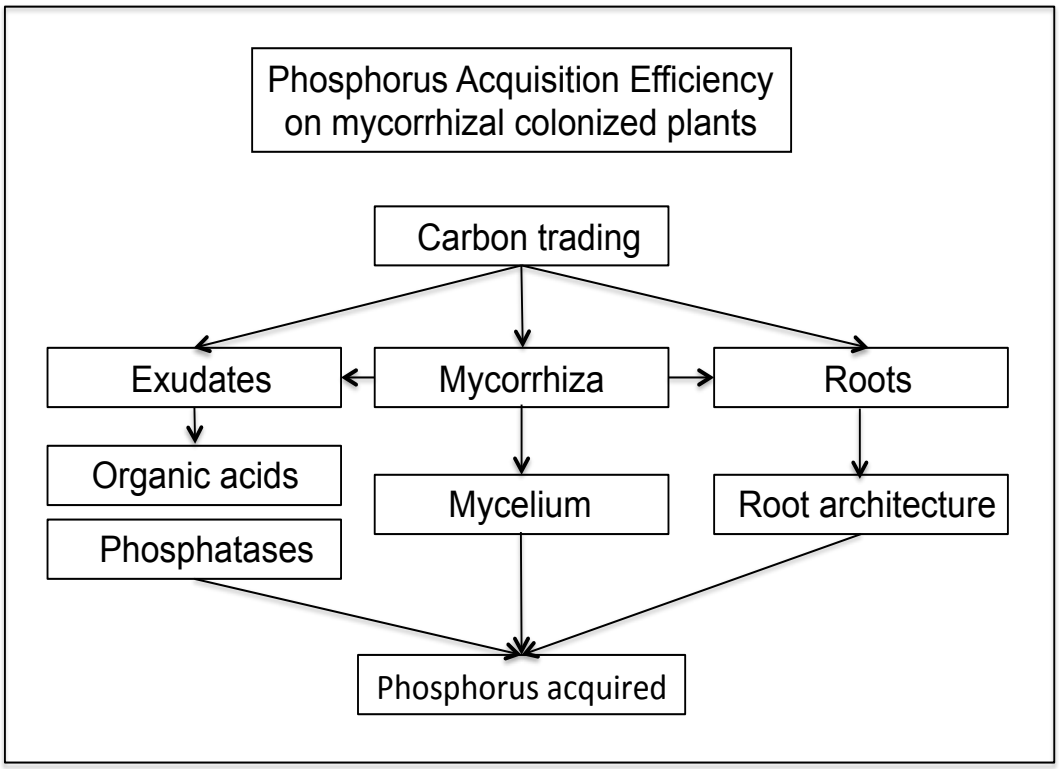

Figure 2. General scheme showing the proposed PAE determination based on carbon trading between all directly related P acquisition traits in AM colonized plants.

As inorganic P forms availability and enzymatic activity are strongly affected by soil pH (Hinsinger, 2001), P solubility can be increased by root-induced acidification in alkaline soils or by pH increase of the rhizosphere in acidic and deeply weathered soils (Gahoonia et al., 1992). This process occurs mainly because changes in pH in the rhizosphere can influence surface charges on soil particles and therefore Pi availability (Geelhoed et al., 1999). Plants have the ability to either increase or decrease rhizospheric

pH up to 2-3 pH units, mainly by absorption or release of protons in order to equilibrate cation/ anion balance (Hinsinger et al., 2003). In the specific case of the cereals wheat and barley, Gahoonia and Nielsen (1996) observed that when rhizospheric pH was invariable, the plants displayed significant genotypic variation in terms of PAE, indicating that other mechanisms should also be involved in causing variation on P acquisition.

Carboxylates and the corresponding carboxylic acids, also known as LMWOAAs, constitute the major fraction of root exudates during P deficiency (Fig. 1). Usually, the most common organic acid anions found in rhizosphere are lactate, acetate, oxalate, succinate, fumarate, malate, citrate, isocitrate and aconitate (Jones, 1998). They have distinct functions on energetic cell metabolism, maintaining charge balance or osmotic potential. It has been widely suggested that LMWOAAs can improve P availability by mobilizing sparingly available P forms in the soil solution. This occurs by chelating metals ions like Al, Fe or Ca involved in P sorption and occupying sorption sites on minerals (Jones, 1998). P mobilizing activity through LMWOAAs is based on their variable negative charge, which would allow the complexation of metal cations and the displacement of anions from the soil matrix. The above is supported by several studies reporting an increase of organic acids exudation by roots in response to P deprivation, especially in plants from Proteacea family that possess cluster roots (Delgado et al., 2013; Jones, 1998; Vance et al., 2003). In addition, the presence of LMWOAAs in solution has been seen to increased P availability compared to water treatments (Gerke, 1992; Khademi et al., 2009, 2010). The efficiency in mobilizing P differs across LMWOAAs as follows: citrate > oxalate > malate > acetate. However, organic acid anion-induced P release depends on many factors, such as pH, soil mineralogy and anion concentration (> 100 mM

for citrate, >1 mM for oxalate, malate and tartrate) (Bolan et al., 1994; Jones and Darrah, 1994; Lan et al., 1995). Indeed, the rates to which Pi and organic anions are replaced in soil solution make predictions of the real effect difficult. Organic acid anions have a fast turnover as they can be quickly adsorbed in acidic soils and rapidly degraded in alkaline counterparts, with half-lives of several hours (Wang et al., 2010). Contrasting evidence found that, despite exuding citrate, pea genotypes were not capable of mobilizing P from Al-P and Fe-P complexes (Pearse et al., 2007). Nevertheless, organic acid production constitutes an important carbon cost in plant metabolism, with 5-25% of total fixed carbon by photosynthesis being used to sustain exudation. However, this does not seem to significantly affect net biomass production as P deficiency can reduce growth to an even greater extent (Johnson et al., 1996; Keerthisinghe et al., 1998).

Sparingly available organic P forms represent between 30% and 90% of total P in some soils (Borie et al., 1989, 2019; George et al., 2017). Substantial flows of P occur between inorganic and organic P pools in soil through immobilization and mineralization, being both processes mediated predominantly by soil microorganisms (Richardson and Simpson, 2011). In order to utilize this P source, organic compounds have to be mineralized; that is, organic P substrates must be hydrolyzed by enzymatic activity of phosphatases to release Pi. This activity seems to be more pronounced in the rhizosphere and it is associated with a depletion of soil organic P (Chen et al., 2002; Gahoonia et al., 1992; Spohn and Kuzyakov, 2013b). Phosphatases are enzymes responsible for catalyzing the hydrolysis of phosphoric acid anhydrides and esters (Schmidt and Laskowski, 1961). These are classified by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology into 5 groups: phosphomonoesterases (EC 3.1.3),

phosphodiesterases (EC 3.1.4), triphosphoric monoester hydrolases (EC 3.1.5), enzymes acting on phosphoryl-containing anhydrides (EC 3.6.1) and on P–N bonds (EC 3.9) (Nannipieri et al., 2011). Phosphomonoesterases are the most abundant enzymes in soils and include acid and alkaline forms and phytases, among others. To date, there is no evidence that any plants produce alkaline phosphomonoesterases.

There is an increasing interest on phytases due to the fact that they hydrolyze inositol phosphates (isomers and lower order derivatives of inositol hexakisphosphate) which generally constitute a major component of soil total organic P. Ranging from 4 to 40% of total P in soils (Borie et al., 1989; Smernik and Dougherty, 2007; Turner, 2007), inositol phosphates are readily adsorbed to soil particles and can react with cations (Fe and Al in acidic soils and Ca in alkaline ones) depending on pH to form poorly soluble precipitates (Shang et al., 1992). However, in most plant species phytase activity has limited capability to mineralize inositol phosphate due to its low production and exudation from roots and the poor availability of the substrate in solution (George et al., 2007; Richardson et al., 2001). Attempts to creating transgenic plants overexpressing phytases and/or other phosphatases have been achieved (Lung et al., 2005; Wasaki et al., 2009) with little successes under natural soil conditions, where substrate availability is restricted (Lung and Lim, 2006; Wang et al., 2009). Interestingly, phosphatase activities are higher near the rhizosphere, with maximum activities found from 2 to 3.1 mm to the root surface for acid and 1.2 to 1.6 mm for alkaline phosphomonoesterases, showing a negative correlation with rhizospheric organic P content in wheat plants (Nannipieri et al., 2011). Phosphatase activity is also regulated by other factors, such as soil mineralogy, organic matter content,

P availability and bacterial communities present in the rhizosphere (Joner and Jakobsen, 1995; Šnajdr et al., 2008; Štursová and Baldrian, 2011).

2.3.1.3 Microorganisms

Non-symbiotic soil microorganisms play a key role on organic P ecosystem dynamics (Fig. 1) (Harvey et al., 2009; Khan et al., 2010). It has been proposed that all alkaline phosphomonoesterases found in soil have a microbial origin, mainly bacterial (Tabatabai, 1994; Yadav and Tarafdar, 2003). Additionally, the majority of Pi mineralized from phytase activity is mediated by free-living bacteria and fungi (Richardson and Simpson, 2011; Unno et al., 2005). Spohn et al. (2013) using the ^{33}P isotopic approach found that the release of root exudates could be a plant strategy to increase P mineralization by enhancing microbial activity.

Free-living soil microorganisms are believed to be more efficient than plants in absorbing and incorporating P into their biomass. Therefore, microbial P represents an important soil sink (Xu et al., 2013) and a potential source of available P for most plants as microbial P is located in more labile intracellular compounds with a fast turnover (Bünemann et al., 2013; Hinsinger et al., 2015). Despite having an important role in organic P dynamics, most research related to free-living soil microorganisms to enhance PAE has been focused on microorganisms capable of solubilizing sparingly available P (Wakelin et al., 2004). Microorganisms can release protons, LMWOAAs, and other secondary organic metabolites that may contribute to P solubilization from minerals (Jones and Oburger, 2011). Indeed, between 1-50% of soil bacteria and about 0.5-0.1% of soil fungi can be classified as P-solubilizing microorganisms (Gyaneshwar et al., 2002; Kucey et al., 1989).

Fungal isolates (particularly the Genus *Penicillium*) have been largely studied due to their great capacity for solubilizing Pi in both solid and liquid media (Gyaneshwar et al., 2002; Morales et al., 2011). A group of bacteria, usually denominated as plant-growth-promoting rhizobacteria (PGPR), are widely found in the rhizosphere of cropping and wild species and have the potential of enhancing PAE mainly through influencing nutrient availability, such as P, or via the indirect production of phytohormones, or plant growth regulators (Richardson et al., 2009). Among the latter, classical phytohormones as auxin, cytokinin, ethylene, gibberellin, and abscisic acid are included. These regulators influence root architecture and other features related to plant development (Peleg and Blumwald, 2011; Vacheron et al., 2013). Although the benefits of using PAE enhancing microorganisms have been evidenced in laboratory and glasshouse conditions, inconsistent results have been observed in field trials (Goos et al., 1994; Karamanos et al., 2010), with the exception of arbuscular mycorrhizal symbiosis established with certain soil fungi.

2.4 Arbuscular mycorrhizal symbiosis

Mycorrhizal symbiosis is an association between plant and some fungal species that generally colonize root or rhizoids, and is beneficial to both partners, at least under some circumstances (Jansa et al., 2011). Arbuscular mycorrhizal (AM) is the most common and widespread type of mycorrhizal symbiosis (Smith and Read, 2010; Wang and Qiu, 2006), found in *ca.* 80% of plant species among all major plants lineages (Brundrett, 2009; Wang and Qiu, 2006) and in most of agricultural species (exceptions include *Brassica* spp., and *Lupinus* spp.). Although AM symbiosis is facultative for many plant species, fossil evidence indicates that the symbiosis matches with the first appearance of land plants, more than 400 million years ago, playing a crucial role in the development of terrestrial plants

(Bonfante and Genre, 2008; Brundrett, 2009). AM fungi (subphylum Glomeromycotina) are obligate biotrophs that when associated with plant roots can provide an enhanced foraging system in order to improve acquisition of soil water and nutrients, particularly P, and to improve resistance to biotic and abiotic stresses in exchange of energy (using carbohydrates as trade) for fungal growth and reproduction (Armada et al., 2016; Jansa et al., 2003a; Jung et al., 2012; Pozo et al., 2015; Santander et al., 2017; Smith and Read, 2010). P appears to be one of major regulators of AM symbiosis establishment and efficiency, as root colonization, P uptake through fungal pathway (Fig. 1) (See section 2.4.1.3) and growth responses diminish with increasing soil P availability (Smith and Read, 2010; Richardson et al., 2011; Smith et al., 2011). However, plants can also modulate the symbiosis, by stimulating fungal metabolic activity and hyphal branching among other effects (Besserer et al., 2006; Bücking and Shachar-Hill, 2005), through the exudation of strigolactones (Akiyama et al., 2005; López-Ráez et al., 2017; Parniske, 2005). Accordingly, the production of these strigolactones is promoted by P deprivation, although in wheat can be also promoted in a small fraction by N deficiency (López-Ráez et al., 2008; Yoneyama et al., 2012).

Despite its broad host range and that its cosmopolitan distribution, AM diversity involves only ~250 morphologically and 350 to 1000 molecularly defined AM fungi (Kivlin et al., 2011; Öpik et al., 2013), with low endemism patterns at global scale (Davison et al., 2015). The absence of AM fungal colonization is rare in natural conditions in plants able to perform the symbiosis, only being achieved in soils lacking AM fungal propagules or in non-mycorrhizal (NM) plant species (Smith et al., 2011). Commonly, the difference in plant growth in presence and absence of mycorrhizal fungal partners is defined as

mycorrhizal growth responses (MGR) and vary widely from positive to negative depending on plant/fungi species and growth conditions (Johnson et al., 1997; Klironomos, 2003). When compared to MGR observed from other cereal crops [positives responses in maize (Karasawa et al., 2001; Sylvia et al., 1993) and rye (Baon et al., 1994a); and excluding rice, which is often not colonized or poorly colonized under continuous submersion (Vallino et al., 2009)], wheat present a high variable response to AM colonization, being generally considered as low and sometimes showing even negative effects in plant growth (Grace et al., 2009; Hetrick et al., 1996). However, positive responses can also be found when applying different experimental conditions or analyzing at different growth stages, which indicates that AM fungal inoculation under appropriated circumstances can be an effective agronomic practice also in these crops (Borie and Rubio, 1999; Seguel et al., 2016a, 2016b, 2017).

Interestingly, a recent meta-analysis by Pellegrino et al. (2015) looking at wheat responses to AM symbiosis inoculation under field conditions found out that although straw biomass was weakly correlated with root AM fungal colonization rate, grain yield and P accumulation correlated positively. A review of the main mycorrhizal growth responses and P uptake from mycorrhizal and NM treatments in wheat are presented below highlighting the idea that growth responses associated to AM symbiosis are not directly related to P acquisition (Table 1). Growth depletions upon AM fungal colonization are normally attributed to an excess of photosynthates shared with the fungal partner, which are estimated to be up to 20% of the C fixed by the host plant (Li et al., 2005; Morgan et al., 2005; Ortas et al., 2002). However, some studies indicated that growth depletion resulting from C drain to the fungal symbiont do not apply in all cases. Hetrick et al. (1992)

and Grace et al. (2009) reported that growth reductions in wheat and barley did not vary when associated with two different AM fungal partners with contrasting capacity to colonize their roots (e.g. 61% and 5%, respectively), and therefore, hypothetically different C demand from the host plant (Hetrick et al., 1992). Even so, plants do benefit from AM symbiosis despite growth and/or nutritional benefits (such as net P uptake) are not apparent. Special techniques such as isotopic labeling are necessary to demonstrate symbiosis functioning (nutrient, water and carbohydrate exchange) in these cases (Grace et al., 2009; Smith et al., 2004, 2009).

2.4.1 Mycorrhizal influence on PAE traits of wheat plants

2.4.1.1 Root architecture and surface area

The root systems of grain cereals as wheat consist of two types of roots. The first type is known as primary or seminal roots and comprises between three to seven roots growing from the seedling. They have 0.2-0.4 mm diameter, occupying 5-10% of total root volume in mature plants. The second type is the secondary roots, also called nodal, crown, or adventitious roots. These roots emerge from nodes at the base of main stem and tillers one to three months after germination, having a larger diameter (0.3-0.7 mm) than primary roots (Hoad et al., 2001). Significant genetic variation for root architectural traits has been found among cereal cultivars (Kujira et al., 1994; Marschener, 1998). Interestingly, it was found out that the number of tillers positively correlated with root length density and grain yield of semi-dwarf bread wheat cultivars grown under P deficiency (Manske et al., 2000). In addition, Gahoonia et al. (1997) showed that the presence of root hairs increased the total root surface of winter wheat from 95 to 341%.

Perhaps the main mycorrhizal-associated mechanism enhancing plant PAE is the increase of explored soil volume by the AM fungal hyphae, which can extend plant access from millimeters to centimeters from root surface. Fungal hyphae can also access soil pores that root hairs cannot due to their smaller diameter (20 to 50 um) (Fig. 1.2). Moreover, AM roots can improve water and nutrients uptake efficiency compared to non-colonized roots due to a lower C cost per unit of hyphal surface related to the root surface (Jakobsen et al., 2005; Jansa et al., 2003a; Schnepf et al., 2008).

There is a complex interplay between root architecture and AM fungi and, as expected, root traits can influence how plants respond to mycorrhizal colonization (Newsham et al., 1995; Smith and Read, 2010). It is suggested that species with root systems characterized by low root hair length and density, and roots with relatively large diameters would display the greatest growth benefits from the symbiosis (Brundrett, 2002; Fitter, 2004; Smith and Read, 2010), especially under P-limiting conditions. Several studies have corroborated this assumption by making this comparison between wild and agricultural species, reporting associations between root traits and MGR (Baon et al., 1994a; Declerck et al., 1995; Schweiger et al., 1995). However, a recent meta-analysis carried out by Maherali (2014) does not support this hypothesis.

Usually, root system architecture is also frequently modified before and following the establishment AM symbiosis (Hodge et al., 2009; Scannerini et al., 2001), especially through some fungal exudates, known as Myc-factors (Fig. 1) (Maillet et al., 2011; Mukherjee and Ané, 2011). These signal molecules are exuded even in the absence of a host plant and are involved not only in symbiotic signaling stimulating colonization, but also acting as plant growth regulators by modifying root development in some plant species

(Maillet et al., 2011; Mukherjee and Ané, 2011). The formation of lateral roots has been found to be the most affected trait, making roots progressively more branched, probably to increase the number of suitable sites for colonization (Harrison, 2005). However, mycorrhizal-induced modifications on root traits are still poorly understood and seem to vary according to specific plant-fungal combinations, (Berta et al., 2005; Fusconi, 2014; Schellenbaum et al., 1991). In the case of wheat, evidences are controversial as well. Behl et al. (2003) found a significant increase of total root length in wheat colonized by *G. fasciculatum*, being up to 90% higher than control plants when co-inoculated with *Azotobacter*. The same pattern was found by Al-Karaki and Al-Raddad (1997), who studied the response of two durum wheat genotypes to AM colonization, detecting an increase of 25% and 20% in root length. On the other hand, AM fungal inoculation decreased wheat root length and surface area under high rates of P application in a calcareous soil (Mohammad and Malkawi, 2004).

2.4.1.2 Organic acid anion and phosphatase exudation

It has been suggested that AM fungi may have biochemical and physiological capacities to increase plant PAE through the uptake of P from sparingly available forms in soil, being the exudation of protons, phosphatases and LMWOAAs the suggested mechanisms involved in these processes (Fig. 1) (Klugh and Cumming, 2007; Koide and Kabir, 2000; Tarafdar and Marschner, 1994).

AM fungi possess many genes encoding acid phosphatases (EC 3.1.3.2, ACP) in their genomes, with at least seven genes expressed in *Rhizophagus clarus* (Sato et al., 2015). However, exudation of phosphatases was mostly associated with the cell wall

(Olsson et al., 2002) and their presence in the rhizosphere has been demonstrated only in limited cases (Koide and Kabir, 2000; Tarafdar and Marschner, 1994). The magnitude of these processes is questioned as it is difficult to isolate the effects of plants, fungi and other microorganisms present in the experiments under unsterile conditions (Joner and Jakobsen, 1995). However, Sato et al. (2015) in an experiment with separated compartments for hyphal growth, collected exudates from soil solution, sand culture and *in vitro* monoxenic culture, providing strong evidence that the corresponding acid phosphatase activity was originated from *R. clarus*. Little information is available about the relationship between AM symbiosis and changes in enzymatic exudation and activity patterns in wheat plants. Rubio et al. (1990) found out a positive correlation between wheat colonization by AM fungi and acid phosphatase activity in roots and soil, mainly under P-limiting conditions. Using a different experimental approach with separated compartments for hyphal growth, (Tarafdar et al., 1994; Tarafdar and Marschner, 1994) observed depletion in organic P content with a concomitant increase of phosphatase activity when wheat was colonized by *Glomus mosseae* (Nicol & Gerd) Gerd & Trappe.

The phosphate-solubilizing activities of AM fungi are still controversial although AM plants have generally been shown to increase the uptake of insoluble Pi (Klugh and Cumming, 2009; Tawaraya et al., 2006; Yao et al., 2001b). In many studies, mycorrhizal inoculants proved to alter the composition and/or amount of total LMWOAAs exuded by *Liriodendron tulipifera* and *Andropogon virginicus*, respectively (Fig. 1) (Klugh and Cumming, 2007, 2009). However, direct evidence for solubilization of P by AM fungi has not been obtained so far. Despite that AM fungi might not exude LMWOAAs by themselves, they can, however, improve P solubilization and/or mineralization indirectly

by stimulating the surrounding soil microbes via the exudation of labile C, thus increasing local nutrient availability in the hyphosphere and in soil patches beyond the root hairs (Cheng et al., 2012; Hodge et al., 2010; Jansa et al., 2013). Recently, Kaiser et al. (2015) using nanoscale secondary ion mass spectrometry imaging and ^{13}C-phospho and neutral lipid fatty acids, traced the flow of recently photoassimilated C and found out that a significant and exclusive proportion of photosynthates was delivered through AM pathway and used by different microbial groups compared to C directly released by the roots.

The interaction between phosphate-solubilizing microorganisms with AM wheat plants has been assessed by some researchers, with positive responses on growth and P uptake. Omar (1998) observed that the interaction between *Funneliformis constrictum* and the rock-phosphate-solubilizing *Aspergillus niger* and *Penicillium citrinum* fungi significantly increased biomass production of wheat plants under all experimental conditions tested. The effect was more evident in non-sterilized conditions. Bacteria from the *Azotobacter* and *Pseudomonas* genera also improved AM wheat growth under field and pot conditions, with positive correlation between AM colonization and *Azotobacter* survival in the rhizosphere (Behl et al., 2003; Kucey, 1987; Yousefi et al., 2011; Zaidi and Khan, 2005). Singh and Kapoor (1999) analyzed the effect of *Bacillus circulans*, *Cladosporium herbarum* and an isolated AM fungus in wheat where larger populations of phosphate-solubilizing microorganisms in the rhizosphere of mycorrhizal roots and an enhanced P acquisition in combined inoculation were found. Similarly, the inoculation with *Penicillium variable* alone negatively affected the biomass production of wheat. However, when applied in combination with *Azotobacter chroococcum*, *Pseudomonas*

striata and the AM fungus *G. fasciculatum*, grain yield significantly increased compared with the other treatments (Zaidi and Khan, 2005).

In another study, wheat grain yield was enhanced by 92,8% in the presence of the rhizobacteria *Pseudomonas fluorescens* and *Burkholderia cepacia* and the AM fungus *Claroideoglomus etunicatum* (Saxena et al., 2013). The synergistic effect of combined inoculation with plant growth-promoting rhizobacteria and AM fungi on wheat was also proved to be effective under field conditions. It was shown that the combination of *A. chroococcum* and *Bacillus sp.* with *G. fasciculatum* significantly increased the dry matter by 2.6-fold and grain yield by 2-fold when compared to the control (Khan and Zaidi, 2007). In another field study, Mehrvarz et al. (2008) found that although bacterial inoculation alone achievied the maximum biological yield, its application combined with AM fungi produced grains with higher weight.

2.4.1.3 Changes in P transporters

In general, AM plants have two different pathways for P uptake from the soil (Fig. 1.3) with different P transporters involved in both of them. The direct P uptake is the plant endogenous pathway, which occurs via root epidermis and root hairs, while in the AM pathway the external hyphae is the responsible for acquiring P from the medium and transport to intracellular symbiotic interfaces where it finally goes to the plant (Grace et al., 2009; Smith et al., 2011). According to their function, plant transporters involved in the direct pathway are expressed mostly in the root apex and root hairs (Gordon-Weeks et al., 2003) and down-regulated in more mature regions. However, up-regulation of genes encoding phosphate transporters proved to have little influence on P acquisition. Park et al.

(2007) studying transgenic barley plants over-expressing a gene encoding for a phosphate transporter found no improvement on P uptake under any of the tested conditions, suggesting that post-transcriptional mechanisms could be involved affecting the activity of these transporters. AM transporters are less known due to their obligate biotrophic nature, coupled with the fact that they are multinuclear and heterocaryotic organisms (Sanders, 1999), which make the use of traditional genetic approaches difficult (Maldonado-Mendoza et al., 2001). These authors observed that the expression of a phosphate transporter gene from the extra-radical mycelium of *Rhizophagus intraradices* was regulated in response to P concentrations in the environment surrounding the extra-radical hyphae and that it was modulated by the overall phosphate status of the AM fungus rather than the host plant (Maldonado-Mendoza et al., 2001). Another important aspect of the AM pathway is the presence of AM-inducible plant P transporters, which are generally present at much higher levels in AM roots than other P transporters (Javot et al., 2007). These transporters are responsible for the exchange of P between the fungal hyphae and plant cell. They have been found in all AM plants investigated, regardless their growth response to colonization, and are mainly expressed in the colonized cortical cells, specifically in the arbusculated cells which is the place where the nutrient exchange takes place (Bucher, 2007; Javot et al., 2007). Genes encoding for AM-inducible transporters have been described in cereals and include the *TaPHT1.8*, *TaPHT1.11*, *TaPHT1.12* and *TaPHT1.14* in wheat (Teng et al., 2017).

The two P pathways were believed to be additive in their contribution to plant nutrient uptake, and it was assumed that direct pathway made a constant contribution to the total P uptake, while the AM pathway participated as an extra contribution in plants

with positive growth responses (Pearson and Jakobsen, 1993). However, further investigations proved that AM colonization could reduce the direct uptake pathway in some species (even in plants that respond positively to the symbiosis as in *Medicago truncatula*), and deactivate completely in others (Liu et al., 1998; Smith et al., 2004). Therefore, in order to not become P deficient AM pathway should compensate the reduced contribution of direct pathway (Smith et al., 2011). Recent studies using radioactive P isotopes has shown that AM pathway contributed significantly to total P uptake on wheat plants. In this sense, Smith et al. (2015) clearly demonstrated that indigenous AM fungi contribute to wheat P uptake in 6.5 to 21% of total plant P in field conditions and 3 to 40% when grown in pots. However, mycorrhizal wheat plants acquired less P and produced less biomass when compared to their non-mycorrhizal counterpart (Grace et al., 2009; Li et al., 2006). It was suggested that negative growth responses could be generated by suppression of the direct pathway in these species, especially in the plants with very low colonization. Conversely, Grace et al. (2009) found out that the magnitude of the negative responses was independent of contrasting colonization by two AM fungal species (*R. intraradices* and *F. geosporum*). In addition, the expression of P transporters belonging to direct pathway was not affected by the symbiosis as expected. Again, this indicated that possible post-translational modifications of regulatory components could be involved in the plant response.

2.5 AM functional diversity

It is a general consensus that there is little specificity between AM fungal and host plant species, and that AM plants can be colonized by several AM fungal species at the same time (Jansa et al., 2003b; Merryweather and Fitter, 1998; Smith et al., 2011). However, the

existence of different colonization patterns could imply certain preferences for specific AM fungal species, functional groups or the co-evolution strategies between specific plant-fungus associations (Chagnon et al., 2013; López-García et al., 2017; Smith et al., 2009). For instance, Mao et al. (2014) showed that these preferences can exist even across wheat cultivars as they found a variation in AM fungal community composition, displaying a complex pattern of cultivar-AM fungal interaction under experimental field conditions. Despite of the projection of this work, the study of the AM fungal diversity associated to wheat plants is overall scarce. Considering the wide distribution and economic importance of this species, only 131 AM fungal sequences in MaarjAM database, the most complete sequence database of Glomeromycota (Öpik et al., 2010), are associated to wheat (*Triticum* sp.), out of 5,296 sequences belonging to Poaceae in the database. The few studies covering molecular diversity in roots of wheat have shown differences between in community composition associated to wheat and N-fixing crops (Bainard et al., 2014; Higo et al., 2016). Communities associated to wheat have also been found to vary during the growing season and depend on P fluxes and degree of fertilization (Bainard et al., 2014; Qin et al., 2015; Wu et al., 2011). The diversity of AM fungal communities associated directly with roots of wheat is overall high, including members of different taxonomic families (e.g. Manoharan et al., 2017), but being predominantly associated with *Funneliformis* spp. in conventional cropping, and *Claroideoglomus* spp. in organically managed systems (Dai et al., 2014).

The lack of information on molecular diversity has been in some manner compensated with morphological studies of spore communities. In this context, a high taxonomic diversity has been found. Aguilera et al. (2014; 2017) analyzing spore

morphology on acidic soils under continuous wheat cropping, found 24 AM fungal species, being *Acaulospora* and *Scutellospora* the dominant genera. In another study under similar conditions in acidic soils, Castillo et al., (2016b) described 26 fungal species with a prevalence of *Acaulospora* and *Claroideoglomus*. This dominance of *Acaulospora* spores in soils cropped with wheat was also observed by Hu et al. (2015) in North China and by Nadji et al. (2017) in Algeria, however in the last study Glomeraceae species was also detected as highly abundant.

The mycorrhizal growth response of a single host plant species can differ across AM fungal species, and in the same way, colonization by the same AM fungal isolated can result in different growth responses in different plant species or genotypes (Feddermann et al., 2010; Smith et al., 2011; Castillo et al., 2016a). Indeed, previous studies have demonstrated a high variability in the symbiotic response of different combinations of host plant and AM fungi (e.g.; Avio et al., 2006; Jansa et al., 2008; Smith et al., 2004). Variations in MGR have also been revealed across wheat cultivars, which can range from -2% to 107% in different genotypes (Azcón and Ocampo, 1981). On the other hand, Graham and Abbott (2000) showed a huge variation in MGR when testing several AM fungal isolates in symbiosis with wheat, being *Scutellospora calospora* the only one promoting higher plant biomass. In a study in wheat showed that MGR by different AM fungal species and their combination or with *F. mosseae* alone resulted in negative growth responses, while positive responses were reported when inoculated with *R. clarum* (Talukdar and Germida, 1994). This variability in mycorrhizal response comes from the fact that AM fungi are functionally diverse both inter- and intraspecifically (see for example Antunes et al., 2011; Koch et al., 2004, 2017). Differences among AM fungal

species have been suggested to exist in the colonization rates in roots and soils depending on the AM fungal colonization pattern (Hart and Reader, 2002; Powell et al., 2009). Perhaps, although morphological traits seem to be well-conserved across AM fungal phylogeny, i.e. morphological traits into the same species and related clades are similar, most of variation in plant growth promotion and P uptake occurs indeed intraspecifically (Munkvold et al., 2004; Koch et al., 2017). In general, it had been assumed that morphological traits, such as the hyphal length in soil, could be good predictors of P uptake. However, the above-mentioned results on huge variabilities in plant P uptake on morphological and phylogenetically similar fungal isolates redirects the question towards which fungal functional trait have to be measured to understand soil-plant P dynamics in agricultural systems. Therefore, functional diversity among AM fungal species and genotypes need to be considered.

2.6 Future perspectives

Despite displaying negative responses in some studies and being considered as non-responsive by many authors, wheat plants presented positive growth and P responses by performing AM symbiosis (Table 1A, 1B respectively). There could be factors involved in this large PAE variation and the processes affecting both AM function and its benefits are still unknown. The question is complex due to the many factors are involved: plant genotype and fungal functional diversity, as well as their mutual compatibility, soil variable conditions or agricultural management needs to be studied. Indeed, the fact is that a major part of the research carried out in the interaction between crop cereals and AM fungi has only involved a handful of AM fungal isolates. In addition, there is little information available regarding the effect of different -or combined- AM fungal taxa colonization and

different genotypes of wheat on root morphology, development, exudation pattern, interaction with PGPR and/or P-solubilizing fungi, and the interplay between the two pathways of P uptake.

It is widely accepted that AM plants access to poorly available sources more effectively than non-colonized plants, but the mechanisms by which they are operating at field are not well understood (Smith et al., 2015). Studies using more than one crop cultivar and multiple AM species and genotypes should be carried out in order to analyze the effect of fungal diversity on PAE related traits as root length, root hair angles, changes on root-mycorrhiza exudation patterns and degree of inhibition (or not) of plant P transporters. In addition, these studies should be traced along different stages of development, until grain production, as it was found that although mycorrhization could hamper biomass production, it enhanced P acquisition and final grain production (Pellegrino et al., 2015). Isotopic, spectroscopic and molecular techniques coupled to new experimental designs could help identify some of the mechanisms mentioned above and the genetic background behind the different responses. In this sense, we suggest an inclusion of the Carbon costs related to all P acquisition traits (not only root architecture), especially those involved and altered by mycorrhizal colonization, in order to support accurate phenotyping for breeding programs focused on lowering P fertilizer inputs (Fig. 2).

Table 1. Mycorrhizal growth responses (MGR) and P uptake on mycorrhizal (+AM) and non-colonized (-AM) wheat (*T. aestivum* L.) cultivars under greenhouse or field conditions and at different days after sowing (DAS)

Wheat Cultivar	AM specie	MGR (%)	P uptake (mg/g)		Exp. conditions	Harvest (DAS)	Observation	References
			+AM	-AM				
TAM-105	*G. etunicatum*	22	5.20	4.63	Field	175		Al-Karaki et al., 2004
Steardy	*G. etunicatum*	19	5.43	4.67	Field	175		Al-Karaki et al., 2004
Tam-105	*G. mossae*	6	4.73	4.63	Field	175		Al-Karaki et al., 2004
Steardy	*G. mossae*	6	5.20	4.67	Field	70		Al-Karaki et al., 2004
Tormes	*G. mossae*	36	1.6	1.2	Pot	70		Azcón and Ocampo, 1981
Anza	*G. mossae*	27	1.3	0.9	Pot	70		Azcón and Ocampo, 1981
Negrillo	*G. mossae*	-2	0.7	0.8	Pot	70		Azcón and Ocampo, 1981
7 Cerros	*G. mossae*	107	1.5	0.8	Pot	70		Azcón and Ocampo, 1981
Bastion	*G. mossae*	35	1.3	1.1	Pot	70		Azcón and Ocampo, 1981
Pane 247	*G. mossae*	15	2.0	1.3	Pot	70		Azcón and Ocampo, 1981
Lozano	*G. mossae*	28	1.9	1.6	Pot	70		Azcón and Ocampo, 1981
Cocorit	*G. mossae*	87	1.3	1.0	Pot	70		Azcón and Ocampo, 1981
Champlein	*G. mossae*	4	0.9	0.9	Pot	70		Azcón and Ocampo, 1981
Castan	*G. mossae*	3	1.9	1.8	Pot	70		Azcón and Ocampo, 1981
Tajo	*G. mossae*	4	1.8	1.6	Pot	70		Azcón and Ocampo, 1981
Boulmiche	*G. mossae*	3	1.1	1.0	Pot	70		Azcón and Ocampo, 1981
Jupateco	*G. mossae*	0	1.5	1.2	Pot	70		Azcón and Ocampo, 1981
Neepawa	*G. intraradices*	-27	1.5	1.1	Pot	42		Goh et al., 1997
Neepawa	*G. intraradices*	-29	2.6	2.9	Pot	42	50 mg P/kg	Goh et al., 1997
Neepawa	*G. intraradices*	-11	3.8	4.1	Pot	42	100 mg P/kg P	Goh et al., 1997
Neepawa	*G. intraradices*	-24	5.0	6.1	Pot	42	300 mg P/kg	Goh et al., 1997
Newton	*G. etunicatum* + *G. mosseae* + *G. intraradices*	-27	2.7	0.8	Pot	98		Hetrick et al., 1996
Turkey	*G. etunicatum* + *G. mosseae* + *G. intraradices*	160	1.4	0.8	Pot	98		Hetrick et al., 1996
Lewjain	*G. intraradices*	-7	1.56	1.33	Field	Tillering		Mohammad et al., 1998
Lewjain	*G. intraradices*	10	1.17	1.06	Field	Anthesis		Mohammad et al., 1998
Lewjain	*G. intraradices*	19	0.82	0.70	Field	Harvest		Mohammad et al., 1998
Lewjain	*G. intraradices*	5	1.76	1.80	Field	Tillering	30 kg P/ha	Mohammad et al., 1998
Lewjain	*G. intraradices*	-4	1.26	1.28	Field	Anthesis	30 kg P/ha	Mohammad et al., 1998
Lewjain	*G. intraradices*	11	0.93	0.71	Field	Harvest	30 kg P/ha	Mohammad et al., 1998

Diamondbird	*G. intraradices*	8	1.8	1.5	Field	122		Ryan and Angus, 2003
Diamondbird	*G. intraradices*	-9	2.3	2.3	Field	122	20 kg P/ha	Ryan and Angus, 2003
Diamondbird	*Scutellospora calospora*	8	2.1	1.5	Field	122		Ryan and Angus, 2003
Diamondbird	*Scutellospora calospora*	-5	2.1	2.3	Field	122	20 kg P/ha	Ryan and Angus, 2003
HPW-89	*G. mosseae* (local)	15	2.69	2.42	Field	150		Suri et al., 2011
HPW-89	*G. intraradices*	14	2.78	2.42	Field	150		Suri et al., 2011
HPW-89	*G. mosseae* (IARI)	13	2.79	2.42	Field	150		Suri et al., 2011
HPW-89	*G. mosseae* (local)	94	3.14	2.42	Field	150	50% P2O5 based on STCR	Suri et al., 2011
HPW-89	*G. intraradices*	103	3.36	2.42	Field	150	50% P2O5 based on STCR	Suri et al., 2011
HPW-89	*G. mosseae* (IARI)	95	3.34	2.42	Field	150	50% P2O5 based on STCR	Suri et al., 2011
HPW-89	*G. mosseae* (local)	154	3.67	2.42	Field	150	75% P2O5 based on STCR	Suri et al., 2011
HPW-89	*G. intraradices*	153	3.82	2.42	Field	150	75% P2O5 based on STCR	Suri et al., 2011
HPW-89	*G. mosseae* (IARI)	151	3.65	2.42	Field	150	75% P2O5 based on STCR	Suri et al., 2011
Laura	*G. clarum*	-10	1.42	1.10	Pot	95	0 mg P/kg	Xavier and Germida, 1997
Laura	*G. clarum*	-19	2.16	2.77	Pot	95	5 mg P/kg	Xavier and Germida, 1997
Laura	*G. clarum*	12	2.76	2.22	Pot	95	10 mg P/kg	Xavier and Germida, 1997
Laura	*G. clarum*	-7	2.43	2.67	Pot	95	20 mg P/kg	Xavier and Germida, 1997
Neepawa	*G. clarum*	17	0.42	0.57	Pot	95	0 mg P/kg	Xavier and Germida, 1997
Neepawa	*G. clarum*	-8	0.68	0.55	Pot	95	5 mg P/kg	Xavier and Germida, 1997
Neepawa	*G. clarum*	4	1.03	1.07	Pot	95	10 mg P/kg	Xavier and Germida, 1997
Neepawa	*G. clarum*	12	1.00	1.72	Pot	95	20 mg P/kg	Xavier and Germida, 1997
81(85)	*G. versiforme*	3	1.03	0.77	Pot	56		Yao et al., 2001a
Fengxiao 8	*G. versiforme*	39	0.98	0.70	Pot	56		Yao et al., 2001a
NC37	*G. versiforme*	21	1.06	0.91	Pot	56		Yao et al., 2001a
HD 2204	*G. fasciculatum*	78	1.10	1.02	Field	135		Khan and Zaidi, 2007
HD 2204	*G. fasciculatum*	146	1.15	1.02	Field	135	A. chrococum	Khan and Zaidi, 2007
HD 2204	*G. fasciculatum*	155	1.89	1.02	Field	135	Bacillus	Khan and Zaidi, 2007
HD 2204	*G. fasciculatum*	295	1.76	1.02	Field	135	A. chrococum + Bacillus	Khan and Zaidi, 2007
HD 2204	*G. fasciculatum*	178	1.56	1.02	Field	135	A. chrococum + P. variable	Khan and Zaidi, 2007
HD 2204	*G. fasciculatum*	193	1.57	1.02	Field	135	A. chrococum + Bacillus + P. variable	Khan and Zaidi, 2007
WH 283	*Glomus* sp. 88	15	0.17	0.18	Pot	55		Singh and Kapoor, 1999

WH 283	*Glomus* sp. 88	42	0.20	0.18	Pot	55	B. circulans	Singh and Kapoor, 1999
WH 283	*Glomus* sp. 88	51	0.20	0.18	Pot	55	C. herbarum	Singh and Kapoor, 1999
WH 283	*Glomus* sp. 88	97	0.19	0.18	Pot	55	B. circulans + C. herbarum	Singh and Kapoor, 1999
Star	*G. mosseae*	17	2.5	2.2	Pot	60	Bavendorf soil, 200 mg P/kg	Tarafdar and Marschner, 1994
Star	*G. mosseae*	16	1.4	0.8	Pot	60	Bavendorf soil, 200 mg organicP/kg	Tarafdar and Marschner, 1994
Star	*G. mosseae*	28	2.3	2.0	Pot	60	Niger soil, 200 mg P/kg	Tarafdar and Marschner, 1994
Star	*G. mosseae*	22	1.5	0.7	Pot	60	Niger soil, 200 mg organicP/kg	Tarafdar and Marschner, 1994
UP 2003	*G. fasciculatum*	6	2.63	0.42	Pot	80		Zaidi and Khan, 2005
UP 2003	*G. fasciculatum*	136	1.0	0.42	Pot	80	A. chroococum	Zaidi and Khan, 2005
UP 2003	*G. fasciculatum*	142	1.61	0.42	Pot	80	P. striata	Zaidi and Khan, 2005
UP 2003	*G. fasciculatum*	236	1.10	0.42	Pot	80	A. chroococum + P. striata	Zaidi and Khan, 2005
UP 2003	*G. fasciculatum*	108	1.31	0.42	Pot	80	A. chroococum + P. variable	Zaidi and Khan, 2005
UP 2003	*G. fasciculatum*	122	1.5	0.42	Pot	80	A. chroococum + P. variable + P. striata	Zaidi and Khan, 2005

Acknowledgments

We fully acknowledge the financial support of the FONDECYT 11160385 (A. Seguel) and FONDECYT 1170264 (P. Cornejo) grants from Comisión Nacional de Investigación Científica y Tecnológica (CONICYT-Chile) and the support granted by CONICYT scholarship 21161474 (P. Campos). J.A. López-Ráez is supported by the grant AGL2015-64990-C2-1R (MINECO-Spain). A. López-Garcia is supported by the grant 708530 – DISPMIC (European Union's Horizon 2020 Marie Curie Individual Fellowship).

CHAPTER III

"Effects of two arbuscular mycorrhiza fungal isolates on nutrient uptake and root traits of two wheat genotypes contrasting in P-acquisition efficiency"

Article submitted to Plant and Soil (December 2019)

Effects of two arbuscular mycorrhiza fungal isolates on nutrient uptake and root traits of two wheat genotypes contrasting in P-acquisition efficiency

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ABSTRACT

It well established that interactions between plants and soil microorganisms influence nutrients dynamics and agroecosystems processes. However, few studies have assessed the role of plant-microbe interactions on modifying root traits and their consequences on the latter. The objective of this study was to examine how intra-specific variation (both at plant and microbe level) affected the root traits and their consequences in nutrient acquisition. To that, we performed an experiment using two wheat cultivars with contrasting strategies of phosphorus (P) acquisition (*cv.* Crac and Tukan) and two arbuscular mycorrhizal (AM) isolates from different phylogenetic families (*Rhizophagus intraradices* and *Claroideoglomus claroideum*), and the response of root traits and nutrient acquisition among cultivars and mycorrhizal treatment was compared. Results showed that variation on growth responses and root traits were dependent on both wheat cultivar and AM isolate, being almost absent in Crac plants. Conversely, growth responses and morphological root traits were highly dependent on the symbiotic partner in the case of Tukan. Colonization by both fungi reduced acid phosphatase activity in both cultivars, while symbiosis of Tukan plants with *R. intraradices* showed higher organic acids exudation. Interestingly, the two AM fungal isolates differently affected nutrient acquisition, independent of the wheat genotype and the acquisition mechanism. A higher P accumulation was observed when inoculated with *C. claroideum*, while *R. intraradices* highly promoted calcium acquisition. However, Cu and Mg, which are acquired by the same mechanisms as P and Ca respectively, were not affected by AM symbiosis. We hypothesize that higher uptake of P and Ca was attributed to differences in hyphal functioning between AM species and/or induction of specific transporters by the symbiosis. This study showed that the outcomes

in plant-microbe interactions related to nutrient dynamics are highly influenced by intra-specific variations at the microbe level, while growth responses related to improved nutrition depends on plant basal efficiency.

3.1 Introduction

Modern agriculture is facing unprecedented risks and challenges, as food production must increase at least by 70% in order to properly feed the 9 billion people expected to be living in the world by 2050 (https://population.un.org/wpp/, Last access: August 2019). To reach this goal, it is imperative to reduce productivity losses due to various types of biotic and abiotic stressors, such as pathogen attack, cold, heat, drought, salinity, phytotoxicity by heavy metals, and nutrient deficiency (Bhardwaj et al., 2014; Ray et al., 2012). In this context, special attention has been put on P-limitation, which reduces plant growth in over 70% of the soils worldwide (Lynch, 2011).

Plant traits involved in nutrient acquisition are diverse, indeed plants have developed an array of regulatory mechanisms to adapt themselves to low nutrient availability in the soil (Ham et al., 2018; Puga et al., 2017). These responses include modifications in shoot and root morphology, growth and development; exudation of low molecular weight organic acids anions and nutrient-releasing enzymes (as phosphatases in the case of P); modifications in central metabolism; regulation of expression and activity of high-affinity transporters, as well as association with soil microorganisms, with emphasis on arbuscular mycorrhizal symbiosis (Campos et al., 2018; Karamanos et al., 2010; Lambers et al., 2006; Richardson et al., 2011).

Understanding how root traits are related to nutrient acquisition – especially P - is a key question to allow the improvement of agricultural productivity and to increase current knowledge about ecosystem functioning (Bardgett and Van Der Putten, 2014). However, root traits variation and their consequences for plant nutrient status are not well understood

(Norby and Iversen, 2017; Preece and Peñuelas, 2019). Root traits are much more plastic than above-ground ones (Bardgett et al., 2014). In this way, this huge variation in root traits is mainly explained by plant functional types and differences in climate conditions (Freschet et al., 2017). Moreover, high root trait plasticity was also observed at the local scale, which has been traditionally attributed to the high soil heterogeneity, even at very short spatial scales (Warren et al., 2015) . However, few studies have addressed the role of the plant-microbe interactions on modifying root traits. Accordingly, due to its major role on root functioning and development, recent research has raised the necessity to include mycorrhizal symbioses as important drivers of plant-environment processes (Bardgett and Van Der Putten, 2014; Laliberté, 2017; McCormack et al., 2017; Wurzburger et al., 2017).

Mycorrhizal symbiosis is an association between plant and some fungal species that colonize roots and increase plant access to water and nutrients - especially P and other nutrients with low mobility in soil - as well as tolerance to biotic and abiotic stress (Delavaux et al., 2017; Pozo et al., 2015). Arbuscular mycorrhiza (AM) is the most common and widespread type of mycorrhizal symbiosis (Smith and Read, 2010), found in *ca.* 80% of plant species among all major plants lineages - including most of agricultural plants (Brundrett and Tedersoo, 2018). Nevertheless, mycorrhizal growth response of a single host plant can differ across AM fungal species, and in the same way, colonization by the same AM fungal isolated can result in different growth responses in different plant species or genotypes (Castillo et al., 2016b; Feddermann et al., 2010). In the same way, nutrient acquisition responses to AM symbiosis have been widely documented, with great variations depending on the plant/fungi species analyzed (Khalil et al., 1994; Porras-Soriano et al., 2009; Taylor and Harrier, 2001).

Although both effects of mycorrhizas (growth and nutrient acquisition) must be related to changes in the plant root functioning, studies addressing their impact on root traits such as exudation or root morphology are scarce [but see Hetrick et al. (1991), Klugh and Cumming (2007), and Yao et al. (2009)]. To fill this gap of knowledge, we aimed to investigate the role of mycorrhizal interactions in determining root traits and their consequences for wheat nutrition and growth. For that, we designed a pot experiment to compare the outcome of the inoculation with two arbuscular mycorrhizal fungal species on the root traits of wheat (*Triticum aestivum* L.) plants, one of the most important crops worldwide (http://www.fao.org/faostat/es, Last access: July 2019). Wheat production is, furthermore, highly dependent on P levels (Heffer, 2013), making the presence and effects of mycorrhizal fungi crucial for a more sustainable agricultural production. In order to understand the patterns of interactions between the AM fungi and the wheat root traits, we included the study of two wheat cultivars with contrasting strategies of P uptake and two AM isolates from different phylogenetic families, namely *Claroideoglomeraceae* and *Glomeraceae* (Redecker et al., 2013), previously shown to differently affect plant responses (Jansa et al., 2005; Munkvold et al., 2004).

3.2 Material and methods

3.2.1 Experimental setup and plant growth conditions

A soilless pot experiment was performed on 0.5 L plastic pots using as substrate a mixture of autoclaved sand and vermiculite (1:1). Seeds of wheat cultivars differing in their P efficiency: cv. "Crac" and cv. "Tukan", were surface-sterilized and pre-germinated for three days prior to be transferred to final pots. Mycorrhizal inoculants of *Claroideoglomus*

claroideum (CC) and *Rhizophagus intraradices* (RI) were provided by the Universidad de La Frontera collection of local AM fungi and were mixed with the substrate (20% v/v) before planting. Pots without mycorrhizal inoculation were set as control for each genotype. At the beginning of the experiment, 200 mL soil-water filtrate without AM fungal spores of each inoculum were added to each non-mycorrhizal pot to re-establish the free-living microflora. Plants were grown under greenhouse conditions for 33 days with temperatures ranging from 16-23ºC during the day and 10-18ºC at night. A photosynthetic photon flux density of 300 mmol m^{-2} s^{-1} was applied as supplementary light. Plants were irrigated manually once a week in the first two weeks, and then twice a week for rest of the experiment with a Taylor and Foy nutrient solution low in P (10 µM). Plants were sown in December 2017 and harvested at first signs of tillering (Zadoks 20; Zadoks et al. 1974).

3.2.2 Biomass and nutrient acquisition

At harvest, plants were separated into roots and shoot, and the substrate firmly attached to the root system (rhizospheric substrate) were gently removed and store at -20ºC until further determinations. Plant material were dried at 65ºC in a forced-air oven for 72 h and weighed to determine the biomass. After drying, an aliquot of roots was separated for mycorrhizal colonization quantification (see next section). The remaining dry samples were crushed, ground, ashed in a furnace at 550ºC, and digested using a H_2O:HCl:HNO_3 mixture (8:1:1, v:v:v). After digestion, P concentration was determined by blue molybdenum method (Murphy and Riley, 1962) on a Helios gamma spectrophotometer (Thermo electro corporation), and Ca, Mg and Cu concentration were determined by atomic absorption spectroscopy (Unicam SOLAAR, mod. 969). In order to assess the

effects of AM symbiosis in nutrient dynamics, we calculated the molar ratio between P and other nutrients analyzed in this study.

3.2.3 Mycorrhizal colonization and hyphal length density

Arbuscular mycorrhizal colonization was quantified according to the gridline intersection method as described by Giovannetti and Mosse (1980), following clearing the roots with 10% KOH solution and staining in 0.05% trypan blue. Hyphal length density in the substrate was measured as in Rubio et al. (2003) and quantified by using the gridline-intersect method (Giovannetti and Mosse, 1980).

3.2.4 Root morphology

Before drying the detached root systems, they were further cleaned thoroughly with distilled water, placed in an A3-sized Perspex tray filled with water, arranged to minimize overlaps, and scanned in both grey-scale (transparency adapter, 310 dpi) and color (reflective, white background, 310 dpi) in an Epson Expression 11000XL calibrated for Image Analysis. The images were then subjected to software analysis (WinRhizo; Regent Instruments, Quebec), and total root length, root surface area, number of forks and average diameter of the root systems were assessed.

3.2.5 Low molecular weight organic acid anions exudation (LMWOAA)

After harvest, 0.5 g of rhizospheric substrate was collected in a 50 mL tube containing 5 mL of $CaCl_2$ (0.2 M), shaken, centrifuged, filtered through a 0.22 µm pore, and stored at -20ºC until determination. The identification of LMWOAA was performed by comparing retention times and absorption spectra with known standards (oxalate, citrate, succinate,

and malate) by high-performance liquid chromatography (HPLC) in a Shimadzu Prominence LC-20A (Shimadzu Corp, Japan). The analysis was carried out using a Symmetry ® C18 column (25 x 4.6 mm, 5 µm) with a Novapak, Waters C18 pre-column (22 × 3.9 mm, 4 µm) at 40ºC using a mobile phase of ortho-Phosphoric acid (0.2 M, pH 2.1; 85% purity, Merck, Germany). The flow rate was of 1 mL min^{-1} and quantification was carried out at 210 nm by external calibration.

3.2.6 Acid phosphatase activity in substrate was quickly determined by incubating one g of rhizospheric substrate in Modified Universal Buffer (pH 6.5), with 25 mM p-nitrophenyl phosphate as substrate at 35ºC for one hour as described by Tabatabai and Bremner (1969).

3.2.7 Calculations and statistical analysis

Mycorrhizal responses (MR%) were calculated for each plant respectively, for all variables under study, using the formula described by Baon et al. (1993):

$$\mathrm{MR\%} = \frac{(value(inoculated) - mean(non - inoculated))}{mean(non - inoculated)} * 100$$

Root efficiency for each nutrient was calculated by dividing the nutrient acquisition by its root surface area.

A Principal Component Analysis (PCA) was carried out to visualize general patterns of root traits and their relationship with mycorrhizal inoculation and Pearson correlation analysis were performed between the main variables under study.

The effects of mycorrhizal inoculation and wheat cultivar were tested by means of ANOVA. A two-way ANOVA was used to test the effect of the inoculum on mycorrhizal

colonization and hyphal length density, and another two-way ANOVA was applied to assess the interaction between wheat cultivar and mycorrhizal treatments (this time including non-inoculated plants) for the rest of the variables analyzed in this experiment. Prior to undertaking data analysis, the Shapiro–Wilk and Levene tests were used to test the normality and homoscedasticity of the data, respectively, and data was square root transformed when necessary. Significant differences between means were analysed by Fisher's LSD test. All statistical analyses were carried out with R software (Team, 2018).

3.3 Results

Both *R. intraradices* (RI) and *C. claroideum* (CC) successfully colonized the roots of the wheat cultivars after 5 weeks. Root colonization ranged from 7 to 14% (Table 1). Yet, no significant differences were observed between isolates and cultivars. Hyphal length density (HLD) was in average of 35 $cm.g^{-1}$ of dry substrate, and again, no significant differences were found between the treatments (Table 1).

The PCA reflected the formation of highly homogeneous groups driven by experimental variables (Fig. 1). A trade-off between root architectural traits (length and surface area), plant biomass and Cu and Mg acquisition with P-ase activity was seen along PC1 (41.6% explained variance), and was, as well, related to main differences between wheat cultivars. A second trade-off could also be seen along PC2 (22.3% variance explained) where P efficiency, and the consequent P accumulation, were faced with Ca efficiency and exudation of organic acids, and also split the type of inoculum applied.. In general, PCA showed a clear separation between Crac and Tukan when inoculated by *R.*

intraradices and when non-inoculated. However, samples of Crac and Tukan overlapped when cultivars were colonized by *C. claroideum* (Fig. 1).

Table 1. Mycorrhizal root colonization (%) and hyphal length density of (cm g^{-1}) of two wheat cultivars, Crac and Tukan, inoculated with *R. intraradices* (RI) and *C. claroideum* (CC) or non-inoculated (NI).

Cultivar	Mycorrhiza	Colonization (%)	Hyphal length density (cm g^{-1})
Crac	NI	-	-
	RI	8.4±2	35±6
	CC	12.1±2	31±4
Tukan	NI	-	-
	RI	13.8±3	32±4
	CC	7.2±2	40±7

Data representing mean ± SE, n = 4.

3.3.1 Effects of mycorrhizal colonization on plant growth

The effects of AM fungal inoculation varied within cultivars and between above- and below-ground biomass production (Fig. 2a). In this context, Tukan showed a wider plasticity than Crac. In the Tukan case, while both fungi increased the shoot growth, only the association with *C. claroideum* stimulated root biomass production (Table 2, Fig. 2a), with 54% more root biomass production compared to the non-inoculated plants (NM). Regarding the total biomass, Tukan plants colonized by *C. claroideum* showed 44% increase on plant growth, presenting no significant differences with Crac plants (Fig. 1). By other hand, the root: shoot ratio of Tukan colonized by *R. intraradices* were significantly lower than the other treatments (Fig. 2b). Contrary, Crac plants lacked any signal of plasticity when inoculated with different AM fungi, as far as there was no

significant growth response (either in roots and shoots) to mycorrhizal inoculation, regardless of mycorrhizal specie (Fig. 2a,b, Table 2).

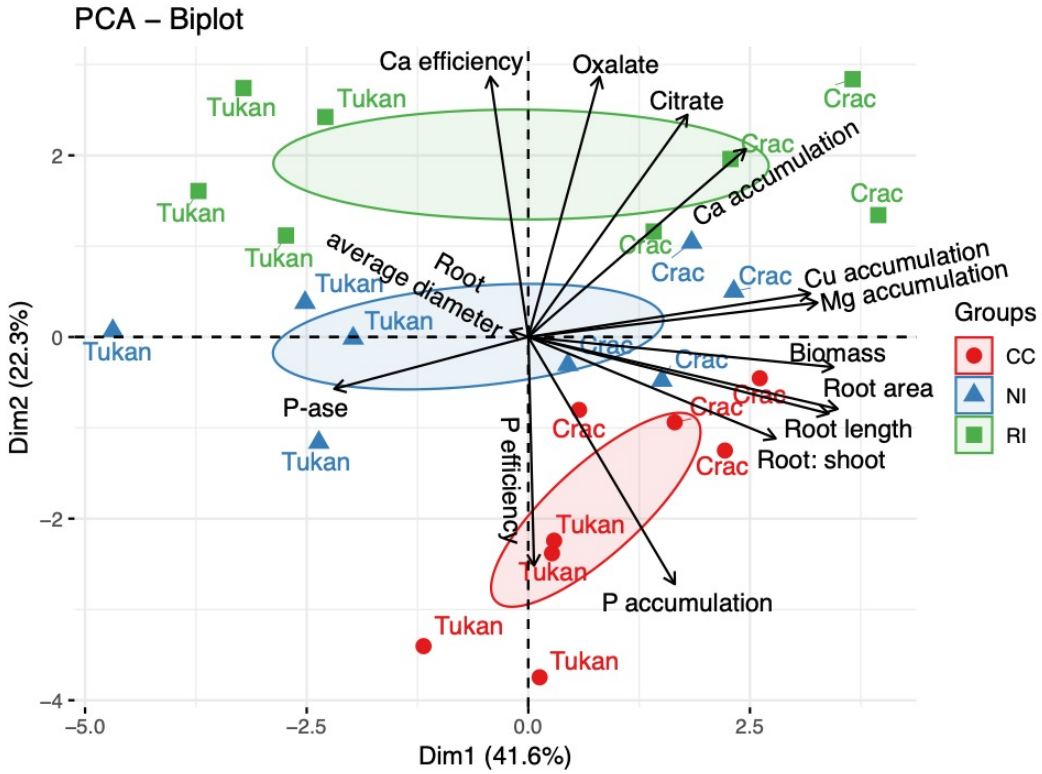

Figure 1. PCA scores for the respective combinations between wheat cultivars and mycorrhizal treatments: inoculation with *C. claroideum* (CC), *R. intraradices* (RI), and non-inoculated (NI). Percentage values in parenthesis indicate the variation explained by each PC.

3.3.2 Effects of mycorrhizal colonization on nutrient acquisition

In contrast with the differential response found for biomass production across cultivars, mycorrhizal inoculation significantly affected Ca and P acquisition for both cultivars (Table 2,3). Accordingly, Ca accumulation increased in both cultivars when plants were inoculated with *R. intraradices* (mycorrhizal response of 95 and 60% in Crac and Tukan, respectively; Fig 3a; Table 2). On the other hand, colonization by *C. claroideum* showed no significant effect on Ca acquisition (regardless of wheat cultivar) when compared to

their respective NM controls. Conversely, *C. claroideum*-colonized plants were the ones showing higher mycorrhizal response in P accumulation (by 78 and 266% in Crac and Tukan compared to their non-inoculated controls, respectively; Table 2), while colonization by *R. intraradices* showed little effects (Fig. 3a; Table 2). The Cu and Mg acquisition was mainly affected by plant genotype (Table 3), in which Crac plants showed overall greater accumulation (Fig. 3a).

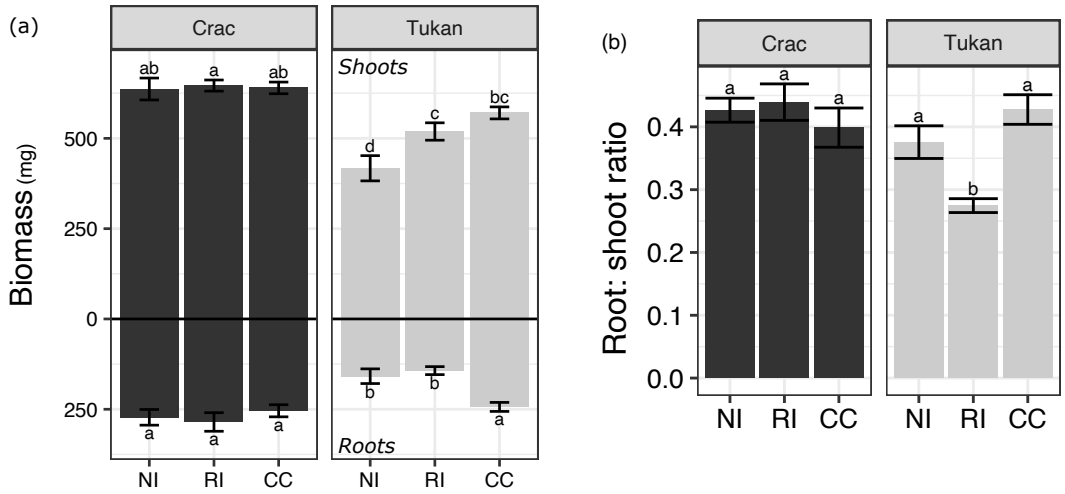

Figure 2. Shoot and root dry biomass (a) and root: shoot ratio (b) of two wheat cultivars, Crac (dark bars) and Tukan (light bars), inoculated with *R. intraradices* (RI) or *C. claroideum* (CC) and non-inoculated (NI). Data representing mean ± SE, n = 4. Means for each response followed by the same letter do not differ significantly by Fisher's LSD test (P<0.05).

Colonization by *C. claroideum* increased the relationship between P and the other nutrients under study, being the highest increased observed in Tukan plants (Table 2). On the other hand, colonization by *R. intraradices* significantly reduced P:Ca ratio in both plant cultivars (Fig. 3b; Table 2).

Table 2. Mycorrhizal responses (%) of Crac and Tukan plants colonized by *R. intraradices* and *C. claroideum* regarding their respective non-inoculated controls.

	Crac		Tukan	
	RI	CC	RI	CC
Shoot biomass	1.5±2.4^{c}	0.5±2.5^{c}	**24.4±5.8^{b}**	**36.7±3.9^{a}**
Root biomass	4.7±9.4^{b}	-6.6±6.1^{b}	-9.8±6.9^{b}	**53.6±7.9^{a}**
Plant biomass	2.4±4.5^{c}	-1.7±2.1^{c}	15.0±6.0^{b}	**41.4±3.9^{a}**
Root: shoot	3.0±6.8^{b}	-6.5±7.3^{b}	**-26.9±2.9^{c}**	13.8±6.2^{a}
P accumulation	12.1±6.8^{c}	**78.5±21.9^{b}**	18.2±7.4^{bc}	**265.7±34.2^{a}**
Ca accumulation	**94.7±11.9^{a}**	4.8±11.5^{b}	**60.2±14.3^{a}**	23.5±8.4^{b}
Mg accumulation	10.5±12.5^{ab}	-1.7±12.9^{ab}	**-17.1±3.2^{b}**	15.3±9.4^{a}
Cu accumulation	10.5±12.4^{b}	**-13.6±2.4^{b}**	15.0±18.2^{b}	**53.6±8.2^{a}**
P efficiency	11.6±12.0^{c}	**81.3±24.2^{b}**	**66.8±7.9^{bc}**	**179.2±26.2^{a}**
Ca efficiency	**91.7±9.4^{a}**	5.2±8.0^{b}	**130.3±22.8^{a}**	-4.9±4.9^{b}
Mg efficiency	11.0±12.3^{ab}	0.7±11.7^{ab}	19.3±8.5^{a}	-11.5±4.9^{b}
Cu efficiency	11.4±16.4^{b}	-11.2±4.1^{b}	**62.8±18.2^{a}**	20.1±8.2^{b}
P: Ca	**-42.4±5.7^{c}**	**74.1±16.7^{b}**	**-33.7±4.8^{c}**	**189.4±28.1^{a}**
P: Cu	4.3±17.4^{b}	**99.6±20.2^{a}**	7.1±14.9^{b}	**135.11±26.3^{a}**
P: Mg	-3.4±7.3^{c}	**73.6±18.8^{b}**	43.3±14.7^{bc}	**220.3±33.6^{a}**
Root length	-8.1±3.2^{b}	2.1±3.1^{b}	**-27.1±3.8^{c}**	**27.3±5.8^{a}**
Root area	1.1±7.8^{b}	-2.3±3.1^{b}	**-30.7±3.3^{c}**	**28.2±4.6^{a}**
Root forks	0.5±9.7^{b}	-6.7±3.3^{b}	**-45.2±3.2^{c}**	**28.8±6.7^{a}**
Root avg. diameter	10.2±6.7^{a}	-3.9±2.1^{b}	-0.8±1.3^{ab}	**5.1±1.1^{ab}**
Oxalate	-1.2±4.6^{b}	-4.5±3.8^{bc}	**12.4±3.0^{a}**	**-16.2±3.1^{c}**
Citrate	9.6±7.1^{ab}	-2.9±3.2^{b}	**18.6±4.1^{a}**	-4.4±9.9^{b}
P-ase	**-37.6±8.2^{a}**	**-38.7±4.1^{a}**	**-24.6±4.6^{a}**	**-25.3±3.7^{a}**

Values are mean ± SE, n = 4. Means for each response followed by the same letter do not differ significantly by Fisher`s LSD test (P<0.05). In black values significantly different from zero at the 95% confidence interval.

3.3.3 Effects of mycorrhizal colonization on root morphology and physiology

Root morphology followed a similar pattern as the one revealed by plant biomass, *i.e.* Crac plants root system were generally larger, wider, more branched and thinner (see root length, surface area, number of forks, and average diameter at Table 3) and less variable AM fungal inoculation, *i.e.* less plastic, than Tukan plants (Fig. 4). Accordingly, root parameters showed high correlation with nutrient acquisition, especially Mg and Cu (Fig. S1).

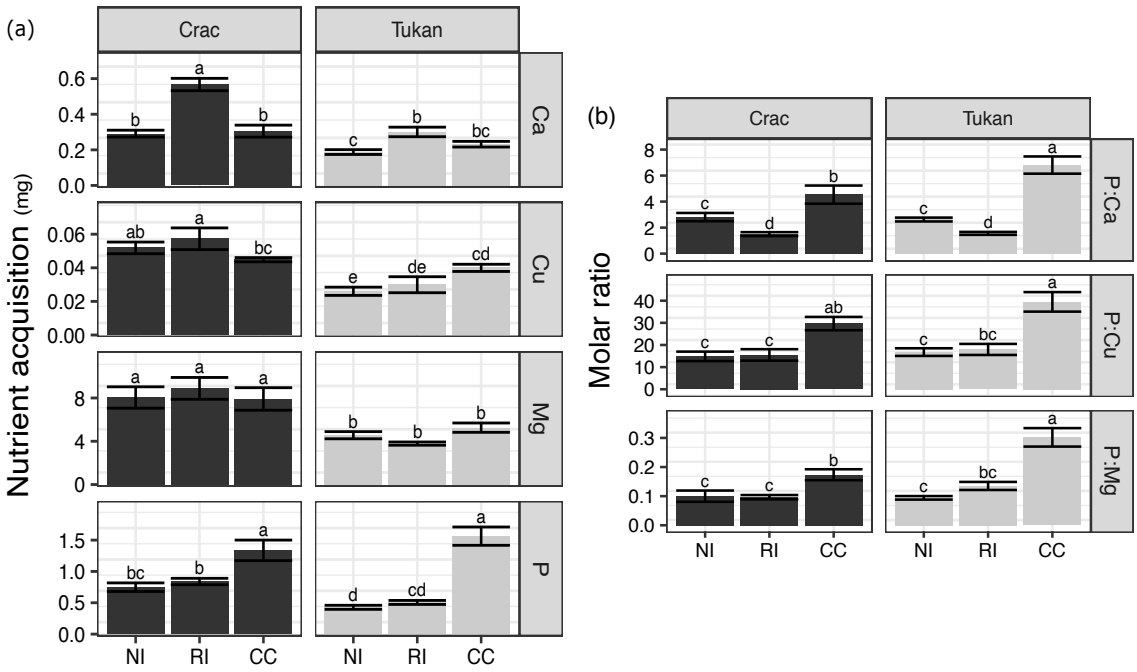

Figure 3. Nutrient accumulation (a) and molar ratio between P and the other nutrients (b) of two wheat cultivars, Crac (dark bars) and Tukan (light bars), inoculated with *R. intraradices* (RI) or *C. claroideum* (CC) and non-inoculated (NI). Data representing mean ± SE, n = 4. For statistics see legend in Fig. 2.

On the other hand, Tukan plants showed a negative response to inoculation with *R. intraradices* regarding root total length, surface area and numbers of forks compared to the un-inoculated control (mycorrhizal response -27, -30 and -45% in, respectively, Fig. 4;

Table 3. Main results of the two-way ANOVA performed in this study.

Parameters	Mycorrhiza		Cultivar		Interaction (Mycorrhiza x cultivar)	
	F-value	*P-value*	*F-value*	*P-value*	*F-value*	*P-value*
Shoot biomass	5.568	0.013*	49.646	<0.001***	4.941	0.019*
Root biomass	2.212	0.138	33.818	<0.001***	6.822	0.006**
Plant biomass	4.062	0.035*	40.697	<0.001***	5.663	0.012*
Root: shoot	2.967	0.076	9.856	0.006**	8.018	0.003**
P accumulation	43.366	<0.001***	2.400	0.139	4.762	0.021*
Ca accumulation	34.067	<0.001***	50.754	<0.001***	8.345	0.002**
Mg accumulation	0.084	0.920	37.303	<0.001***	1.377	0.278
Cu accumulation	0.794	0.467	38.000	<0.001***	5.353	0.015*
P efficiency	28.905	<0.001***	3.915	0.063	2.683	0.095
Ca efficiency	60.139	<0.001***	0.178	0.678	1.989	0.166
Mg efficiency	2.335	0.125	4.623	0.045*	0.431	0.656
Cu efficiency	6.000	0.010*	0.301	0.589	1.919	0.175
P: Ca	52.263	<0.001***	3.984	0.061	5.256	0.015*
P: Cu	27.347	<0.001***	4.120	0.057	1.185	0.328
P: Mg	34.764	<0.001***	12.897	0.002**	6.952	0.005**
Root length	15.354	<0.001***	136.079	<0.001***	4.356	0.028*
Root area	6.376	0.008**	63.513	<0.001***	8.976	0.001**
Root forks	5.240	0.016*	71.055	<0.001***	9.555	0.001**
Root avg. diameter	1.322	0.291	5.129	0.036*	5.400	0.014*
Oxalate	6.191	0.008**	10.324	0.004**	3.554	0.050
Citrate	3.708	0.044*	7.129	0.015*	0.189	0.829
P-ase	17.234	<0.001***	20.494	<0.001***	0.263	0.771

***P<0.001, **P<0.01, *P<0.05

Table 2). And, conversely, a positive response to inoculation with *C. claroideum* (mycorrhizal responses of 27, 28 and 29% in the same parameters, respectively, Fig 4; Table 2).

Root efficiency was found to similarly vary for both cultivars when inoculated with different AM fungi (Table 3). In this context, colonization by *R. intraradices* increased root efficiency in acquiring Ca by 91 and 130% in Crac and Tukan, respectively, and efficiency in acquiring Cu by 63% in Tukan plants (Table 2). On the other hand, *C. claroideum* significantly improved root efficiency in acquiring P in both cultivars, being the highest increase in Tukan plants (179%, Table 2).

Mycorrhizal symbiosis decreased P-ase activity in the rhizosphere of the wheat cultivars, independently of mycorrhizal specie; being Crac levels lower than the ones observed in Tukan plants (Fig. 5a). Oxalate and citrate were found to be present in root exudates of both cultivars in all samples (Fig. 5b, c), being in overall higher on Crac plants (Table 3). In general, slightly changes on rhizospheric OA concentration induced by mycorrhizal colonization were detected in this experiment. Although Crac plants showed almost no plasticity regarding OA exudation, Tukan plants colonized *R. intraradices* presented a positive mycorrhizal response on exudation of both acids (Table 2), while *C. claroideum*-colonized plants presented significantly less amount of rhizospheric OA compared to the plants in symbiosis with the other isolate (Fig. 5b,c).

3.4 Discussion

This work focused on the variability of plant traits due to variations in the microbial taxa associated. Particularly, the effects of different AM fungal taxa on plant growth, root

morphology and physiology, and the consequent nutrient acquisition patterns on two wheat cultivars were assessed. Interestingly, we found that the variation of plant traits was dependent on both the plant cultivar (*i.e.*, genotype) and the AM fungal symbiont. The trait plasticity of root morphology, due to variation in the symbiotic partner identity, was almost absent for the Crac cultivar. By contrast, morphological root traits were highly dependent on the symbiotic partner in the case of Tukan wheat. Although, it is widely known that the mere establishment of the symbiosis can have an impact on root traits, exemplified by, *e.g.*, the reduction in root length and in root surface area of two soybean cultivars when inoculated with *Glomus mosseae* found by Wagg et al. (2011), mycorrhizal effects of different AM fungal species on root morphology have been poorly studied, with few studies analyzing plant intra-specific responses.

Plant exudates play a major role in mobilizing sparingly available nutrients (Mommer et al., 2016; Preece and Peñuelas, 2019; Richardson et al., 2011). Here, colonization by both fungi reduced P-ase activity in the rhizosphere of the wheat cultivars under study (Fig. 5). In general, there is a limited set of observations analyzing the effects of AM symbiosis on soil P-ase activity (including the use of non-inoculated plants), where mycorrhizal responses vary from negative to positive effects (Campos et al., 2018). In this context, these differences can be due to differences in plant-fungal genotypic-genotypic interactions (Marschner and Baumann, 2003). In agreement with our data, Joner and Jakobsen (1995) and Marschner and Baumann (2003) also reported a decrease in acid P-ase activity in cucumber and maize plants when colonized by different AM fungal species. It can be due to that AM colonized plants are often more well P-nourished and less prone to P-limitation, thus not activating P-starvation-induced high P-ase exudation, equilibrating

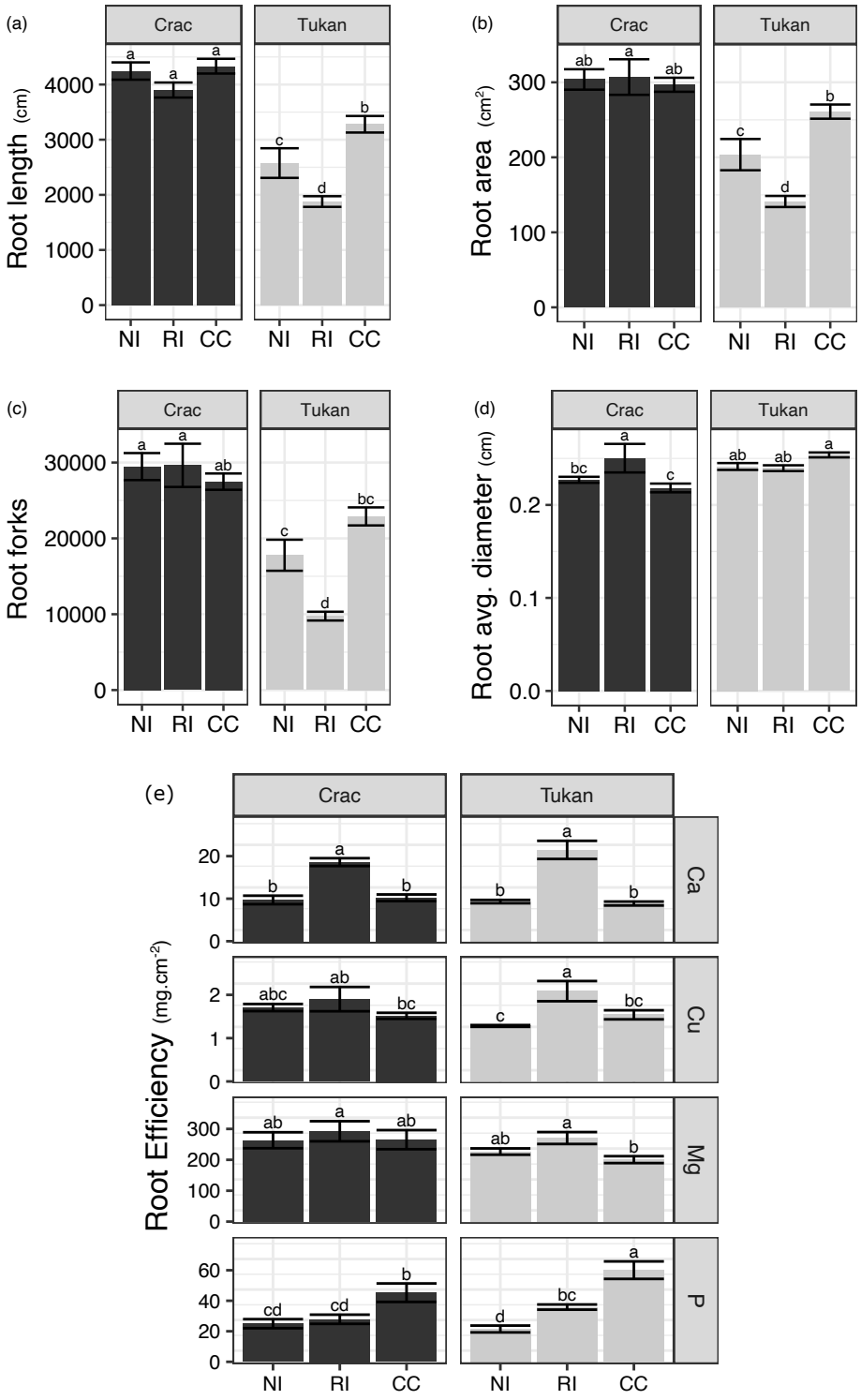

Figure 4. Total root length (a), surface area (b), number of forks (c), average diameter (d) and root efficiency in acquiring nutrients (e) of two wheat cultivars, Crac (dark bars) and Tukan (light bars), inoculated with *R. intraradices* (RI) and *C. claroideum* (CC) or non-inoculated (NI). Data representing mean ± SE, n = 4. For statistics see legend in Fig. 2.

carbon balance in the plant-mycorrhizal system. Indeed, here we observed negative and significant correlations between all measured root exudates and P accumulation, except for citrate concentration in the rhizospheric substrate (Fig. S1).

The observed plasticity of Tukan plants was also revealed when analyzing the effect of mycorrhizal treatments on LMWOAA exudation. Overall, colonization by C. claroideum tended to reduce LMWOAA exudation, while slightly increases were observed when in symbiosis with R. intraradices. Even though exudation of LMWOAA showed weak correlations with plant growth and nutrient acquisition under our experimental condition (compared to root architectural traits), this differential exudation rate, even when seemed low, could affect P acquisition – and other highly sorbed nutrients - in high nutrient-fixing soils, by stimulating microbial activity and their associated P mineralization and solubilization processes (Khademi et al., 2010; Xu et al., 2019), compensating the lower production of roots.

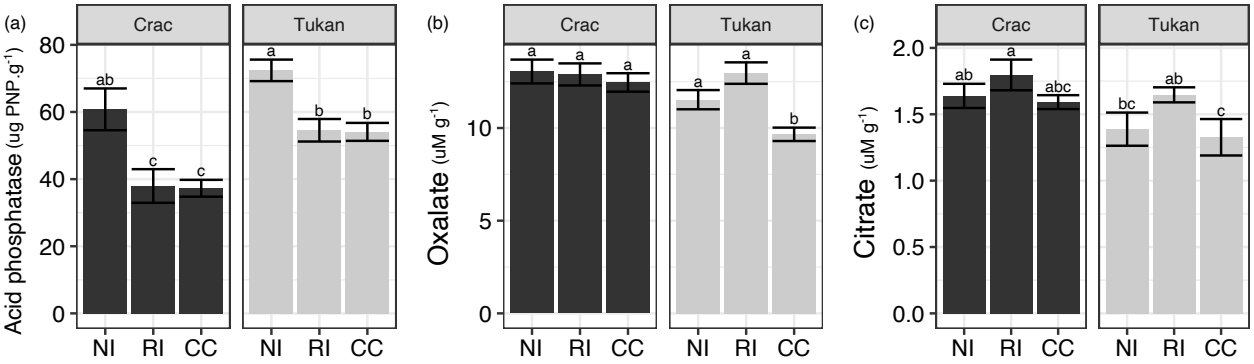

Figure 5. Acid phosphatase activity (a), oxalate (b) and citrate concentration (e) in the rhizosphere soil of two wheat cultivars, Crac (dark bars) and Tukan (light bars), inoculated with *R. intraradices* (RI) and *C. claroideum* (CC) or non-inoculated (NI). Data representing mean ± SE, n = 4. For statistics see legend in Fig. 2.

The found differences in the trait plasticity between wheat cultivars could be attributed to the higher basal P efficiency of Crac. Indeed, Crac plants showed no mycorrhizal response in biomass production and root morphological traits, perhaps due to that P was the only limiting nutrient in the solution (de Souza Campos et al., 2019). The extra P acquisition when associated with *C. claroideum* was probably stored in the vacuole to be used in later periods of plant development rather than immediately used to promote growth (Veneklaas et al., 2012). On the other hand, Tukan plants have intrinsic low P efficiency (de Souza Campos et al., 2019) and seems to rely in the mycorrhizal symbiosis in a wider extent. Indeed, the higher P-acquisition promoted by the symbiosis with *C. claroideum* allowed this cultivar to maintain a higher growth rate and an increased total root length, surface area and number of forks compared to the NI control, equaling levels to Crac (see Fig. 1). Noteworthy, wheat has exhibited a wide range of responses to AM symbiosis, being generally considered to show low to negative growth responses to AM colonization (Hetrick et al., 1996; Zhu et al., 2001). However, positive responses as the ones found in this experiment are often reported when intra-specific variability were considered (*i.e.* using more than one plant or fungi genotypes; Yao et al., 2001; Suri et al., 2011), suggesting that the magnitude and direction of the mycorrhizal responses depend on the identity of both partners involved in the symbiosis (Klironomos, 2003; Smith et al., 2011; Xavier and Germida, 1997).

Nutrient acquisition by roots is comprised under three main mechanisms: mass flow, diffusion, and root interception (Barber, 1995; Marschner and Rengel, 2012). The importance of each mechanism is mainly determined by the nature of each nutrient (electrical charge, atomic size, concentration, and others; Sollins et al. 1988) and its

interaction with soil factors. Nutrients like N, Ca and Mg are usually less bound to soil components, therefore are mainly delivery to roots by mass flow (Barber, 1995). On the other hand, P, K and Cu - among others – are mainly acquired by diffusion, as they present high affinity to clays and other soil constituents (Barber, 1995). In general, a small fraction of the total nutrient acquisition is due to direct root interception, as root volume occupy in average only 1% of the topsoil (Marschner and Rengel, 2012). However, nutrients tend to be distributed in "patches" in agronomic soils due to fertilization practices. In this case, root interception can be an important factor for plant nutrition.

We analyzed if AM symbiosis affected nutrient acquisition of nutrients related to mass flow (Ca and Mg), and diffusion (P and Cu) mechanisms. Interestingly, the two AM fungal isolates affected differently nutrient acquisition, independently of the wheat genotype and the acquisition mechanism (Fig. 4):

Colonization by *R. intraradices*, which tended to increase the production of LMWOAA per root length, increased acquisition and root efficiency in acquiring Ca, which was not the case of Mg – also acquired by mass flow. In this context, Mg accumulation showed higher correlation to root morphological traits compared to Ca accumulation (Fig. S1). On the other hand, symbiosis with *C. claroideum* increased P acquisition, but did not affected Cu acquisition. Again, Cu acquisition showed a higher correlation to morphological root traits than P accumulation (Fig. S1). Therefore, the increase on P and Ca acquisition was independent of the increase in mass flow and diffusion mechanisms regarding root morphology *per se*.

In general, Mg and Cu accumulation were higher in Crac plants, regardless of mycorrhizal colonization, which was mainly related to its root morphology (Fig. 1). In addition, *C. claroideum*-colonized Tukan plants – which showed higher root growth – also showed a higher Cu and Mg accumulation, although not significant for the latter (Fig. 3). These patterns were well visualized in the PCA ordination, where P accumulation and root P efficiency appeared highly related to *C. claroideum*-colonized plants (Fig. 1). In agreement with our data, Santander et al. (2019) working with the same *C. claroideum* isolate, found that this fungi also increased P accumulation in lettuce plants, while Ca, Mg, N and K accumulation were even lower than the non-inoculated control. On the other hand, Ca accumulation was more related to *R. intraradices*-colonized plants (Fig. 1). This fungi has been seen previously to increase Ca accumulation, e.g. *Retama sphaerocarpa* plants (Armada et al., 2016).

At the view of these results, *i.e.* the lack of strong relationship between high P and Ca accumulation with the root traits analyzed in this study, we hypothesize that these effects could be related to differences in the hyphal efficiency in acquiring both nutrients. Indeed, high hyphal plasticity in P accumulation among AM species has been previously reported, even at an intra-specific level (Jansa et al., 2005; Munkvold et al., 2004; Thonar et al., 2011). Also, mycorrhizal symbiosis has been shown to affect plants own Pi transporters expression. In agreement, Poulsen et al. (2005) previously found that induction of Pi transporters in tomato plants were independent of the mycorrhizal colonization extent and differed within the AM species inoculated.

Finally, our data suggest that mycorrhizal response on nutrient acquisition is dependent on the mycorrhizal species, but that growth responses related to higher nutrient

acquisition is dependent of the plant genotype basal efficiency, where plants with low P efficiency would show higher growth responses to mycorrhiza, which is the case of Tukan. The changes in carbon allocation (between shoot growth, root morphology and exudation) observed in this experiment - especially in Tukan plants - could be explained by a differential maintenance cost/physiology requirement of the different AM fungal species used. It is well established that sink strength is dependent on genotypic-genotypic interactions between plant host and the fungi, and that is also influenced by environmental conditions, *e.g.* P availability (Lendenmann et al., 2011; Walder and Van Der Heijden, 2015). Lendenmann et al. (2011), analyzing the carbon cost of three AM species on *Medicago truncatula,* found that *R. intraradices* was the one with highest costs to the plant, leading to similar plant growth benefits compared with less efficient AM species in delivering P but with lower carbon costs. Plant invests up-to 30% of their photosynthetic products in their symbionts (Kaschuk et al., 2009; Walder and Van Der Heijden, 2015). Therefore, in order to have a positive outcome (at least in growth), the benefits provided by the fungi should be higher than carbon shared by the plant.

3.5 Conclusions

In the current context of the study of plant root traits, the heterogeneity of soil abiotic variables had been proposed as a way to explain the huge variability of root traits (Weemstra et al., 2016). The revealed influence of different AM fungal species on root traits at intraspecific level can be easily extrapolated to the effect of other microbial groups, *i.e.* microbial communities in natural soils are known to greatly vary in short distances adding another level of complexity to the understanding of the root trait spectra (Bardgett et al., 2014). By other hand, the main result, showing that the effects on plant root traits,

and finally nutrient acquisition, were mycorrhizal-specific, will help to maximize the benefits that can be obtained from this symbiosis in agricultural systems and could guide breeding programs oriented to increase agricultural sustainability. Indeed, AM symbiosis has been advocated as an environmental-friendly management opportunity to increase sustainability in agricultural systems due to its ability to increase plant fitness under different stress conditions, including nutrient deficiency. However, the factors that regulate responses to mycorrhizal colonization are still poorly understood (Hoeksema et al., 2010; Tylianakis et al., 2008). Here, it was also demonstrated that genotypes within the same specie displays different response, via the study of their root traits, to AM symbiosis depending on their internal efficiency and on mycorrhizal species. In conclusion, the found results add a new piece of complexity to the understanding of the effects of symbiotic interactions on plant and ecosystem functioning that will require further attention in the comings decades.

Supplementary data

Fig. S1. Pearson correlation coefficients between the main variables under study.

Acknowledgments

This work was supported by funding from FONDECYT 11160385 grant from Comisión Nacional de Investigación Científica y Tecnológica (CONICYT-Chile), CONICYT DOCTORADO NACIONAL 2016 (21161474), CONICYT + PAI/ CONCURSO NACIONAL TESIS DE DOCTORADO EN EL SECTOR PRODUCTIVO 2017 (T7817120011), and AGL2015-64990-C2-1R (MINECO-Spain). We also acknowledge

Dr. Gustavo Curaqueo for the analysis on the root scanning system and Paula Aguilera for providing the inoculants.

CHAPTER IV

"Phosphate acquisition root traits: determining the most important ones to wheat plants"

Under co-authors revision prior submitting to Environmental and Experimental Botany

Phosphate acquisition root traits: determining the most important ones to wheat plants

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ABSTRACT

Inorganic phosphorus (P) fertilizers are expected to become scarce in the near future; so, breeding for improved P acquisition related root traits would decrease the need for fertilizers application. In this context, root morphology, exudation of P-releasing compounds (as organic acids and acid phosphatases), and mycorrhizal symbiosis are among the major mechanisms developed by some plants in order to increase P acquisition under limiting conditions. This work aimed to analyze the early P acquisition root traits (including AM colonization and community composition) of two wheat cultivars contrasting in P acquisition efficiency (PAE) in order assess those with higher contribution to the latter. For that, two wheat cultivars – Crac and Tukan – were grown for 9 weeks (Zadoks 23) in an acidic high-P fixing Andisol amended or not with 44 kg P ha^{-1}, and their P acquisition related root traits were assessed. In general, Crac plants presented significantly higher P acquisition compared to Tukan ones. P fertilization increased P acquisition in both cultivars, being the higher increase observed in Crac plants. Plant growth was highly correlated to P acquisition under limiting conditions, where Crac acquired more P and produced more biomass compared to Tukan. Even though fertilized Crac plants did not presented higher growth at this stage, its higher P acquisition could comprehend an important source to maintain a higher growth rate over larger periods of P limitation. Overall, P acquisition showed the highest correlations with root morphological parameters (length, area and number of forks), regardless P conditions. On the other hand, mycorrhizal colonization and oxalate exudation only showed partial effects on P acquisition under P-limited conditions. Finally, understanding the physiological mechanisms of improved PAE and the genetic basis therein would generate valuable

information for breeders to select more P-efficient cultivars. This knowledge would help to diminish the use of P fertilizers in agriculture and to increase the efficiency of its implementation. In this sense, the root traits analyzed in study, especially the morphological ones, could comprehend a useful target for increasing agricultural sustainability.

4.1 Introduction

Wheat (*Triticum aestivum* L.) is one of the most important crops in the world. According to the data from the Food and Agriculture Organization, the area of wheat cultivation in 2017 was approximately of 218 million hectares, making it the crop with highest cultivation area in the world (http://www.fao.org/faostat/es). In Chile, wheat is also the most cultivated crop, with more than 35% of the total area sowed with annual crops (http://historico.ine.cl). However, its production is highly dependent on phosphorus (P) fertilizers, leading to a higher consumption per area when compared to other major crops, such as maize and rice (Heffer, 2013). In addition, volcanic soils, as those present in Southern Chile - and where the bulk of wheat is produced – are characterized by the high amount of clay particles with highly reactive surfaces (*e.g.,* allophanes, imogolite and/or 2:1 layer silicates; Dahlgren et al., 2004), thus, displaying a high P-fixing capacity and greatly limiting the P available to plants, even using large amount of fertilizers (Borie et al., 2019; Redel et al., 2011).

Phosphorus is essential to plant growth and development. Nevertheless, P is the second most limiting nutrient in plants biomass, constraining growth in over 70% of the soils worldwide (Lynch, 2011). Therefore, inorganic P (Pi) fertilization is an important practice nowadays in order to sustain a high agricultural production. Indeed, P fertilization and P accumulation has been shown to be significantly correlated with wheat yields, especially under low P fertilization (Deng et al., 2018; Sandaña and Pinochet, 2014; Seguel et al., 2017). Pi fertilizers are made from a non-renewable resource, which is getting depleted rapidly (Cordell et al., 2009). According to estimations, high grade phosphate rock extraction tends to decrease and Pi fertilizer demand is likely to double within the next 30

years (Mogollón et al., 2018). In this sense, a better knowledge of the traits related to higher P acquisition efficiency (PAE) and the genetic background therein would generate valuable information that could be used by breeders to generate more efficient cultivars and a more sustainable agricultural production nationally, but also worldwide. Nevertheless, plant breeding is a complex process, often exceeding ten years from its initial development to their broad use (Bailey-serres et al., 2019). In addition, breeding programs have used few genes discovered by research oriented to traits selection so far, evidencing that a better integration between the parts are needed for the development of new varieties oriented for more sustainable use of natural resources (Wissuwa et al., 2016).

Plant P nutrition is a complicated subject itself because P availability and acquisition depends on the interplay between soil, plant and microbial processes. At the soil level, P availability is strongly dependent on the pH - having maximal availability rates at pH 5-6 (Marschner and Rengel, 2012), and on soil texture and its organomineral components (Do Nascimento et al., 2016; Vistoso et al., 2012). At the plant level, P acquisition can be increased by: i) modifications on root growth/morphology, as increasing total root length, producing shallower roots, increasing branching pattern, and other morphological and anatomical adaptations (Lambers et al., 2006); ii) exudation of low molecular weight organic acids anions (LMWOAA) and P-releasing enzymes (Péret et al., 2011); and iii) increasing root efficiency through regulation of expression and activity of high-affinity Pi transporters (Huang et al., 2011). Finally, certain soil microorganisms can increase P availability through exudation of LMWOAA, P-releasing enzymes, and other secondary metabolites. Soil microorganisms can also stimulate root growth, and consequently P acquisition, through exudation of plant growth promoting compounds

(Martínez-Viveros et al., 2010). Although the benefits of using PAE-enhancing microorganisms has been evidenced at laboratory level, inconsistent results have been observed in field trials, with the exception of arbuscular mycorrhizal symbiosis (Karamanos et al., 2010; Pellegrino et al., 2015).

Arbuscular mycorrhiza (AM) is the most common and widespread type of mycorrhizal symbiosis (Smith and Read, 2010), found in *ca.* 80% of plant species among all major plants lineages and in most of agricultural plants (Brundrett and Tedersoo, 2018). It is generally accepted that AM symbiosis increases PAE through a higher soil exploration by the fungal mycelium (Jansa et al., 2005; Pearson and Jakobsen, 1993). Nevertheless, plants PAE responses to AM colonization not always correlate to mycelium extent (and therefore acquisition area), suggesting a more complex interplay between the symbiosis and P acquisition than just mycelium production (Campos et al., 2018). Indeed, major changes on plant physiology are produced after mycorrhizal establishment, affecting the plant P nutrition mechanisms explained above (*i.e*, root morphology, exudation, and metabolism; Klugh and Cumming 2007; Fusconi 2014; Mateus et al. 2019). Therefore, since mycorrhization is the common condition in the field, it is necessary to study the PAE mechanisms of AM colonized plants (Smith et al., 2015).

This work aimed to analyze early P acquisition root traits (including AM colonization and community composition) of two local wheat cultivars with contrasting PAE (selected based on their performance on a previous screening and on a field experiment; Seguel et al., 2017), in order to generate a trait-based background that could be used in breeding programs focused on a more sustainable use of P fertilizers.

4.2 Material and methods

4.2.1 Plant material and growth conditions. Seeds from the wheat (*Triticum aestivum* L.) cultivars Tukan and Crac were surface sterilized in 4% sodium hypochlorite, rinsed thoroughly with sterile distilled water and pre-germinated for 48 h on moistened filter paper at 25°C in darkness. Seedlings were then sown into 2,5 L plastic pots filled with a mixture of a high P-fixing soil (Andisol Gorbea series, medial, mesic, Typic Hapludand obtained in Gorbea, La Araucanía Region; Table 1) and fine quartz rocks. Plants were subjected to two P conditions: i) optimal condition (+P) - 44 kg P ha^{-1} was applied as triple superphosphate; or ii) deficient condition (-P) - remained unfertilized [see Acevedo et al., (2011) for P requirements of wheat cropping]. In addition, another experiment with the same conditions as mentioned above was performed, but using rhizoboxes (30 cm height, 20 cm width, and 0.7 cm depth) instead of pots. This system was used to assess root exudation over two physiologically different root zones: i) Top roots: 25% fraction adjacent to plant crown; and ii) Bottom roots: 25% fraction of root near to apex. Before harvest, root exudates from the two root zones in the rhizobox experiment were collected with 4 mm adsorption disks during 1 h (Fig. 1; Neumann and Römheld, 1999). Plants were grown under greenhouse conditions with temperatures ranging from 15-20ºC during the day and 10-17ºC at night, watered with a modified Hewitt solution without P (de Souza Campos et al., 2019), and harvested after 9 weeks (Zadoks 23; Zadoks et al., 1974). A photosynthetic photon flux density of 300 µmol m^{-2} s^{-1} was applied as supplementary light.

4.2.2 Arbuscular mycorrhizal colonization was quantified according to the gridline intersection method as described by Giovannetti and Mosse (1980), following clearing the

roots with 10% KOH solution and staining in 0.05% trypan blue (Phillips and Hayman, 1970).

Figure 1. Rhizoboxes used in this study and the average location of the extraction disks during exudate collection.

4.2.3 Hyphal length density in soil was measured as described in Rubio et al., (2003) and quantified by using the gridline-intersect method (Giovannetti and Mosse, 1980).

Table 1. Selected chemical properties of the Gorbea series soils used in this experiment

N (mg kg^{-1})	22.5±0.3
P (mg kg^{-1})[A]	12.4±0.3
K (mg kg^{-1})	138±2.3
pH[B]	4.92±0.02
Organic matter (%)[C]	11.4±0.3
Exchangeable cations (cmol(+) kg^{-1}):	
K^{D}	0.35±0.006
Na^{D}	0.06±0.003
Ca^{D}	0.52±0.03
Mg^{D}	0.12±0.003

All analytical techniques were carried out according to the Normalisation and Accreditation Commission of the Chilean Soil Science Society (Sadzawka et al., 2006). [A] Extractable by Olsen method. [B] Measured in H_2O. [C] Determined by soil oxidation with sodium hypobromite. [D] Walkley–Black method. Data represent the means of four independent replicates (±SE).

4.2.4 Determining the AM fungal community richness colonized wheat roots. First, a clone library was generated as a "molecular fingerprint" based on denaturing gels for monitoring the presence of AM fungal ecotypes inside roots. Briefly, DNA was extracted from roots, and purified through a silica column using the Wizard Genomic DNA purification kit (Promega). Samples were pooled and subjected to PCRs using the AMF specific primers NS31/AML2 (Table 2), which allows the selective amplification of Glomeromycota phylum (Öpik et al., 2010). The products were then purified, cloned into a pGEM-T vector (Promega) and transformed into competent *E. coli* DH5α cells. Petri dishes with LB medium containing X-gal and ampicillin were used to select the clones effectively transformed and carrying the insert. One hundred clones were randomly selected and subjected to enzymatic digestion by *HinfI* and *AvaII* (Promega), and their fragment pattern analyzed by capillary electrophoresis (Fragment Analyzer, Advanced Analytical). These enzymes were selected since they were previously shown to generate a wide diversity of digestion profiles in AM (López-García et al., 2014). A fraction of the clones that demonstrated identical fragment patterns were sequenced and the observed patterns compared to theoretical ones predicted by *in silico* RFLP analysis using Codon Code Aligner software (Codon Code Corporation, Dedham, MA, USA). In addition, phylogenetic analysis was performed in order to generate Operational Taxonomic Units (OTUs) and assign them to the restriction patterns by similarity analysis and bootstrapping using MEGA7 software (Kumar et al., 2016). Then, DNA extracted from roots of each wheat plant was processed as mentioned above, and their fragment pattern was analyzed by capillary electrophoresis (Fragment Analyzer, Advanced Analytical) and compared to the clone library in order to assign the correspondent OTUs.

Table 2. Primer sequences used in the PCR analysis.

Primer	Secuence (5' → 3')
NS31	TTGGAGGGCAAGTCTGGTGCC
AML2	GAACCCAAACACTTTGGTTTCC

4.2.5 Root morphology. Root systems were cleaned after harvest, placed in an A3-sized Perspex tray filled with water, arranged to minimize overlaps, and scanned in both grey-scale (transparency adapter, 310 dpi) and color (reflective, white background, 310 dpi) in an Epson Expression 11000XL calibrated for Image Analysis. The images were then subjected to software analysis (WinRhizo; Regent Instruments, Quebec) and root length, surface area, average diameter and number of forks were assessed.

4.2.6 Phosphorus uptake. At harvest, plants were separated into roots and shoot and dried at 70ºC in a forced-air oven for 48 h. The dry samples were weighted, crushed, ground, ashed in a furnace at 550ºC and digested using a H_2O:HCl:HNO_3 mixture (8:1:1, v:v:v). After digestion, P contents were determined by the blue molybdenum method (Murphy and Riley, 1962).

4.2.7 Acid Phosphatase (P-ase) activity in soils was determined as described by Tabatabai and Bremner (1969) using Modified Universal Buffer (pH 6.5), with 25 mM p-nitrophenyl phosphate (PNP) as substrate at 35ºC for 1 h. The same procedure was used to determine its activity in the adsorption disks. Here, the reaction volumes were downscaled, and clean disks used as controls. Results obtained in pots are expressed as μg PNP g^{-1} soil as well as ng PNP cm^{-1} h^{-1} of root system.

4.2.8 Exudation of low molecular weight organic acid anions (LMWOAA). Exudates collected with adsorption disks were extracted in Eppendorf reaction vials by adding 500 µL of H_2O (milli-Q grade) with subsequent shaking, centrifugation and filtering through 0.22 µm pore syringe-filter. The supernatant was ready for determination and were kept at -20ºC until analysis. After harvest, 0.5 g of rhizospheric soil was collected in a 50 mL tube containing 5 mL of $CaCl_2$ (0.2 M), shaken, centrifuged, filtered through a 0.22 µm, and stored at -20ºC until determination. The identification of LMWOAA was performed by comparing retention times and absorption spectra with known standards (oxalate, citrate and malate) by high-performance liquid chromatography (HPLC) in a Shimadzu Prominence LC-20A (Shimadzu Corp, Japan). The analysis was carried out using a Symmetry ® C18 column (25 x 4.6 mm, 5 µm) with a Novapak, Waters C18 pre-column (22 × 3.9 mm, 4µm) at 40°C using a mobile phase of ortho-Phosphoric acid (0.2 M, pH 2.1; 85% purity, Merck, Germany). The flow rate was of 1 mL min^{-1}, and quantification was carried out at 210 nm by external calibration. The same procedure was used to determine the nature of the anions in the adsorption disks.

4.2.9 Phosphorus in soil. Olsen-P was measured in 2.5 g of soil. The soil was extracted with $NaHCO_3$ (0.5 M, pH 8.5), shaken for 30 min, filtered, and determined by the blue molybdenum method as described by Sadzawka et al. (2006).

4.2.10 Statistical analysis. Means were obtained from the results of five independent replicates. Data were assessed for normality and homoscedasticity of variance, and significant differences between means were analysed by ANOVA followed by Fisher's LSD test. A Principal Component Analysis (PCA) was carried out to visualize general patterns of the rhizobox experiment and regression analysis were performed between the

main variables under study. All statistical analyses were carried out with R software (Team, 2018).

4.3 Results

4.3.1 Plant growth and P acquisition in Crac and Tukan cultivars

In general, no significant differences in biomass production (shoots and roots) were detected when extra P was applied, suggesting that P levels in the soil were sufficient to maintain optimal growth and development in both cultivars until this phenological stage (Fig. 2a). On the other hand, even though plant growth was greatly limited in the absence of the fertilizer (P<0.001), the impacts of P-deprivation were lower in Crac plants – especially regarding root biomass production (26 and 42% reduction of root growth in Crac and Tukan respectively).

One of the main symptoms of P deficiency in plants is the increased root: shoot ratio; either by a reduction in shoot growth, an increase in root biomass or by both (Chien et al., 2018; Ericsson, 1995). Accordingly, both genotypes showed an increase in root: shoot ratio in the absence of P fertilizer (Fig. 2b). Nevertheless, no significant differences were observed between the cultivars under the same condition.

After 9 weeks, Crac plants presented significant higher P acquisition compared to Tukan plants, regardless of the P treatment (P<0.001; Fig. 2c, S2). P fertilization increased P acquisition in both cultivars, being the highest increase observed in Crac plants (by 5.4 and 4.3 times in Crac and Tukan, respectively; Fig. 2c). Unfertilized Crac plants accumulated 70% more P in their tissues and produced more roots and shoots - by 47%

and 42%, respectively - compared to Tukan. Accordingly, P acquisition was highly correlated with biomass production under this condition (R^2=0.82, *P*<0.001; Fig. S3a)

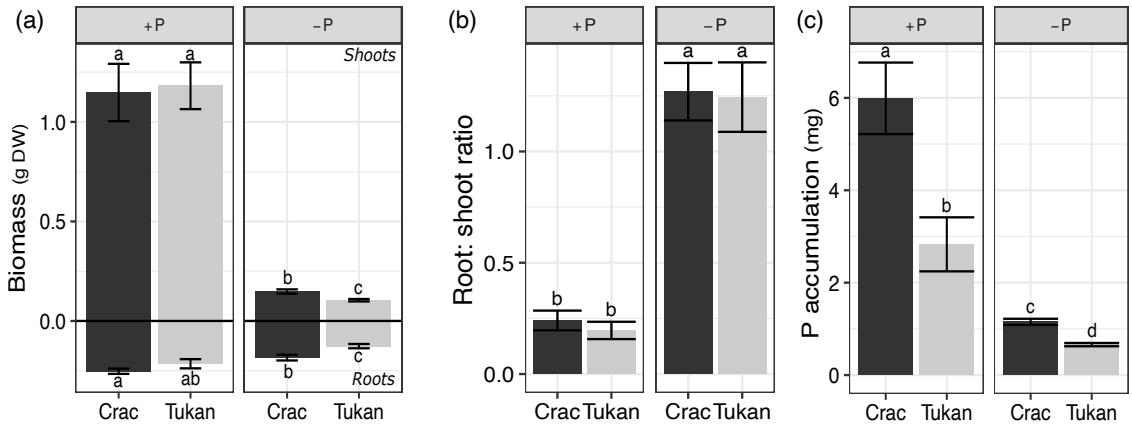

Figure 2. Biomass production (a), root: shoot ratio (b), and phosphate accumulation (c) of wheat cultivars Crac (dark bars) and Tukan (light bars) growing in an Andisol either amended or not with an equivalent of 44 kg P ha^{-1} of Pi fertilizer. Plants were harvested at Zadoks growth stage 23 (Zadoks et al., 1974). Data represent the means of five independent replicates (±SE). Letters indicate the significance of the differences between means as determined by LSD test (*P*<0.05).

4.3.2 Assessment of the root system morphology

Overall, Crac plants presented enhanced root morphology compared to Tukan. They showed a larger, wider, thinner, and more branched root system regardless the P treatment (*P*<0.05; Fig. 3a-d; S2). Accordingly, Crac root system presented 64% more length, 46% more surface area, and 56% more forks than Tukan ones when plants were fertilized. These differences were increased under unfertilized conditions. Here, 102% more length, 70% more surface area, and 79% more forks were observed in Crac. Remarkably, these parameters were highly correlated to P acquisition under both P conditions (Fig. S3b). Pi fertilization significantly increased root growth of both cultivars (*P*<0.05 for all variables, except average diameter). Interestingly, fertilized Tukan plants presented similar root

morphological parameters as non-fertilized Crac ones, suggesting higher root plasticity under different soil P conditions in Crac. In order to assess the root system efficiency in acquiring P, root P efficiency (P uptake per root area) was also calculated. Crac plants presented higher root efficiency (P<0.05; Fig. 3e), especially when extra P was applied. Thus, Crac root systems accumulated 50% more P per root area than Tukan ones under +P condition.

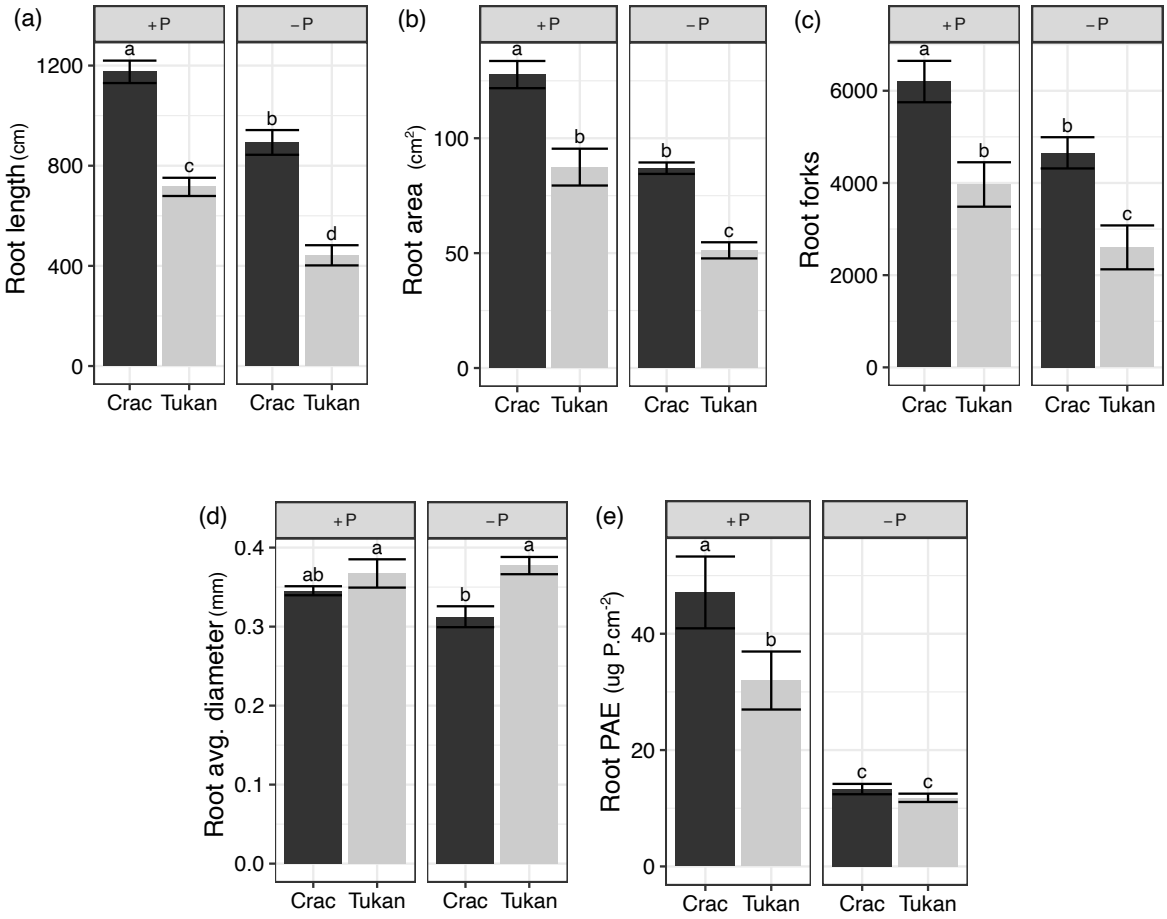

Figure 3. Root system morphology of wheat cultivars Crac (dark bars) and Tukan (light bars) growing in an Andisol either amended or not with an equivalent of 44 kg P ha^{-1} of Pi fertilizer. Plants were harvested at Zadoks growth stage 23 (Zadoks et al., 1974). Graphs showing total length (a), root surface area (b), number of forks (c), root average diameter (d), and root P acquisition efficiency (e). Data represent the means of five independent replicates (±SE). For statistics see legend in Fig. 2.

4.3.3 Arbuscular mycorrhizal symbiosis in Crac and Tukan

Both cultivars presented similar root mycorrhizal colonization (around 45%) when extra P was applied (Fig. 4a). P-deficiency increased mycorrhizal colonization in Crac plants in 20%, while Tukan plants showed no response to this stress condition (Fig. 4a). Pi fertilization decreased mycorrhizal hyphal length density (HLD) in both genotypes (Fig. 4b). Even though Crac plants presented overall slightly more HLD, no significant differences were observed within P treatments.

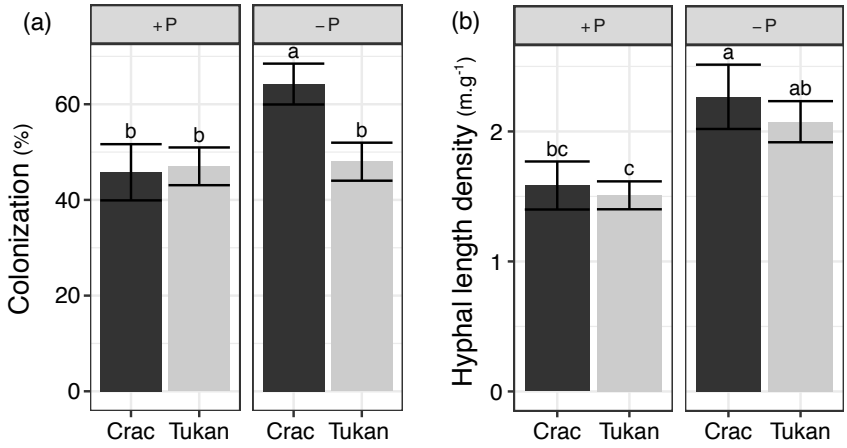

Figure 4. Mycorrhizal root colonization (a) and hyphal length density (b) of wheat cultivars Crac (dark bars) and Tukan (light bars) grown in an Andisol either amended or not with an equivalent of 44 kg P ha^{-1} of Pi fertilizer. Plants were harvested at Zadoks growth stage 23 (Zadoks et al., 1974). Data represent the means of five independent replicates (±SE). For statistics see legend in Fig. 2.

Analysis of 100 clones of the gene library covered about 85% of total AM species diversity in our experimental system, as shown by rarefaction analysis (Fig. S4). A fraction of the sequences representing each pattern was sequenced, blasted against MaarjAM (MaarjAM database; Öpik et al. 2010). AM fungal identity was further confirmed by phylogenetic analysis with type strains as shown in Fig. S5. Phylogenetic analysis clustered the sequences into 7 different OTUs closely related to *Archaeospora* (Arch), *Paraglomus*

(Par), *Gigaspora* (Gig1 and Gig2), *Claroideoglomus* (Clar1 and Clar2), and *Rhizophagus* (Rhiz) genus. Among them, Rhiz, Par, and Gig1 were found in the majority of the samples (Fig. 5a,b). Interestingly, 80% of Tukan plants were colonized by Arch, while in Crac only 40% of the samples showed the presence of this ecotype (Fig. 5a). In general, no differences in the AM community composition were observed within the treatments (Fig. 5; Table 3). Although unfertilized Crac possessed relatively less species colonizing their roots, no significant differences were observed in the richness indexes evaluated in this study (Table 3).

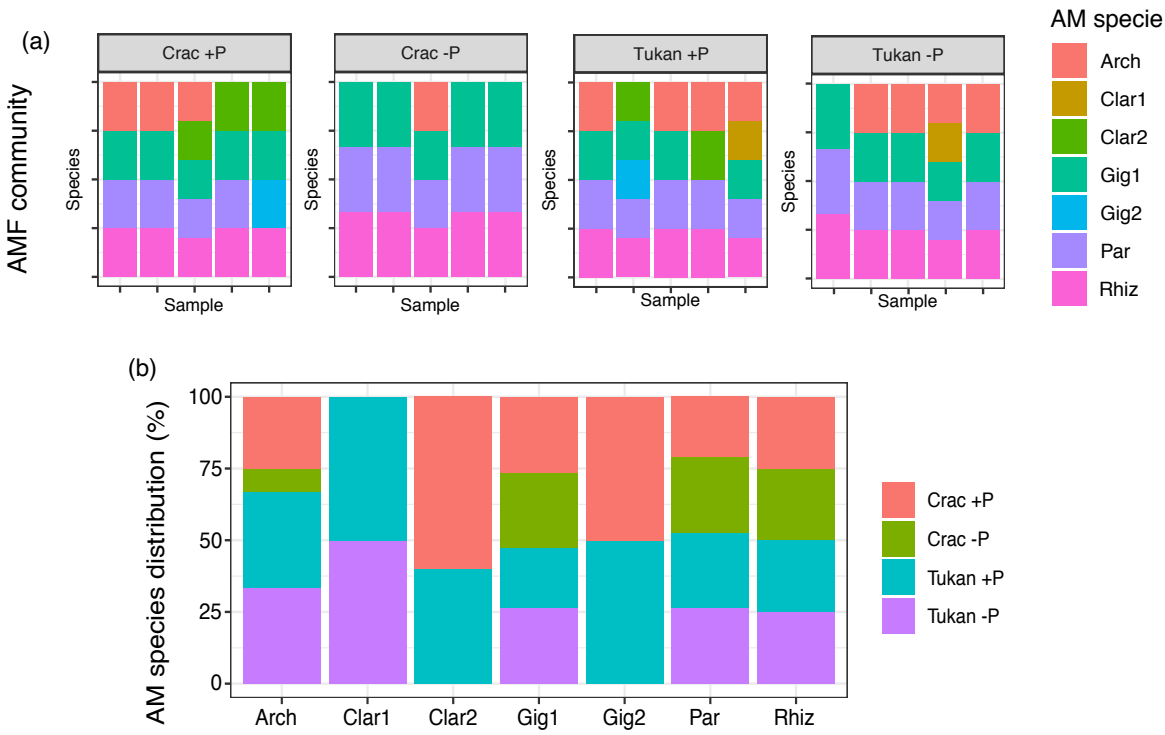

Figure 5. Arbuscular mycorrhizal community analysis of AMF effectively colonizing the roots of wheat cultivars Crac (dark bars) and Tukan (light bars) grown in an Andisol either amended or not with an equivalent of 44 kg P ha^{-1} of Pi fertilizer. Plants were harvested at Zadoks growth stage 23 (Zadoks et al., 1974). Graphs representing overall community composition (a) and AMF species distribution within different treatments (b).

4.3.4 Analysis of the root exudation

Rhizospheric acid phosphatase (P-ase) activity was higher in Tukan than in Crac plants (P<0.05), even though no significant differences were observed within the P treatments (Fig. 6a). Notwithstanding, P-ase activity tended to show a negative correlation with P-acquisition, especially under the deficient condition (Fig. S3d). To further assess the differences between the genotypes, the P-ase exudation of the different root zones (top and bottom roots) was assessed (Fig. 6b). P limitation significantly increased P-ase exudation on top roots of both cultivars, being Tukan levels higher than Crac regardless the P conditions (Fig. 6b). On the other hand, P limitation induced Crac P-ase exudation on bottom roots by 6.4 times, while Tukan plants showed no response. Thus, Crac plants presented 200% more exudation in this root zone under Pi limitation when compared to Tukan (Fig. 6b).

Table 3. Different diversity indexes used to analyze the AMF community effectively colonizing wheat roots.

Cultivar	Treatment	Richness	Extrapolated richness	H	Simpson´s	Beta diversity
Tukan	+P	4.2	11.2	1.42	0.75	0.67
	-P	4.0	10.2	1.16	0.69	0.25
Crac	+P	4.2	11.2	1.42	0.75	0.19
	-P	3.2	6.8	1.37	0.74	0.25

Results of organic acid exudation into the rhizosphere revealed a significant amount of oxalate on all samples. Crac plants showed 47% more of this LMWOAA in the non-fertilized soil compared to Tukan ones (P<0.05; Fig. 6c). Under this condition, oxalate concentration showed a partially significant correlation with P acquisition (R^2=0.33, P<0.1; Fig S3e). Interestingly, P addition increased oxalate rhizospheric concentration in

Tukan plants by 100% (P<0.01), while no differences were observed in Crac plants between P conditions. On the other hand, an interestingly exudation pattern between different root zones was found on P-fertilized Crac plants. This cultivar maintained a high exudation rate on top roots, while reducing it on bottom ones (Fig. 6d). In this context, oxalate exudation on top roots was highly and significantly correlated with P accumulation under fertilized condition (R^2=0.63, P<0.05; Fig. S3f).

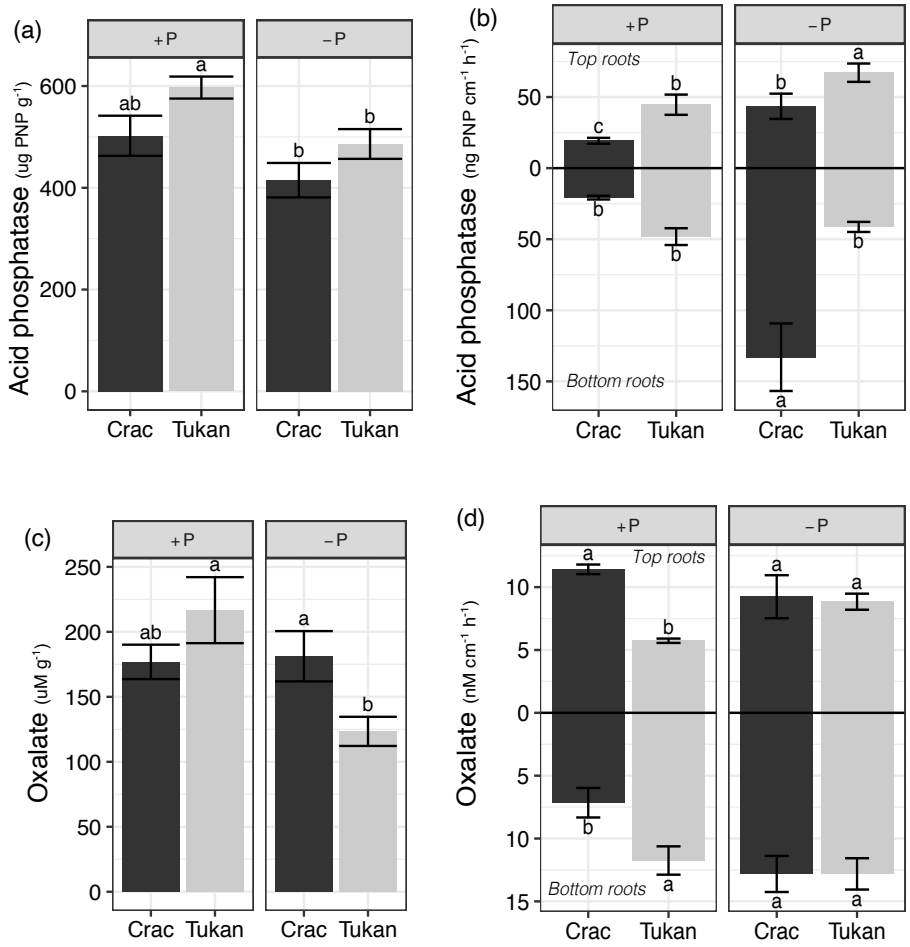

Figure 6. Acid phosphatase activity (a) and oxalate concentration (c) in the rhizosphere soil and their exudation rate on two root zones – top and bottom (b and d, for P-ase and oxalate exudation respectively) of wheat cultivars Crac (dark bars) and Tukan (light bars) grown in a Andisol either amended or not with an equivalent of 44 kg P ha^{-1} of Pi fertilizer. Plants were harvested at Zadoks growth stage 23 (Zadoks et al., 1974). Data represent the means of five independent replicates (±SE). For statistics see legend in Fig. 2.

4.3.5 Soil P availability

Pi fertilization of an equivalent of 44 kg P ha^{-1} (common fertilization rate in Chile) increased Olsen-P levels in almost 10 mg kg^{-1} (Table 4), thus increasing the recommended soil P levels for wheat cropping from almost low to high (Acevedo et al., 2011). In the absence of Pi fertilizer, both cultivars slightly increased Olsen-P levels in the rhizosphere soil, although no significant differences were found between cultivars and the control soil under that condition. On the other hand, Crac plants depleted available P in 5.8 mg kg^{-1} when extra P was applied, while Tukan plants showed no significant differences compared to amended pots without plants (Table 4). The rhizosphere soil pH was significantly increased during the experiment; however, no significant differences were observed between cultivars (Table 4).

Table 4. Available phosphate (Olsen-P) and pH of the rhizosphere soil of Crac, Tukan and pots without plants after 9 weeks in an Andisol either amended or not with an equivalent of 44 kg P ha^{-1} of Pi fertilizer.

Treatment	cv.	Olsen-P ($mg.kg^{-1}$)	pH
-P	Crac	15.4 ± 1.1^{b}	5.3 ± 0.05^{ab}
	Tukan	13.4 ± 0.5^{b}	5.2 ± 0.04^{b}
	Control	12.2 ± 0.4^{b}	4.9 ± 0.03^{c}
+P	Crac	15.9 ± 1.3^{b}	5.5 ± 0.02^{a}
	Tukan	20.8 ± 2.1^{a}	5.4 ± 0.04^{a}
	Control	21.7 ± 1.1^{a}	5.0 ± 0.04^{c}

Data represent the means of five independent replicates (±SE). Letters indicate the significance of the differences between means as determined by LSD test ($P<0.05$).

4.4 Discussion

Pi fertilizer quality and production is going to reduce in the near future due to the high pressure over this non-renewable resource (Cordell et al., 2009; Mogollón et al., 2018). In

this scenario, the use and development of P acquisition efficient genotypes would help to increase the lifespan of this resource until new solutions are discovered. Here, we analyzed the P acquisition associated to root traits of two commercial wheat cultivars differing in their response to Pi fertilizers (Seguel et al., 2017) in order to identify those traits with higher influence.

Crac plants acquired more P under optimal and limiting P conditions; however, no differences in biomass production were observed under P fertilization. This fact could be explained since plants with higher P accumulation in their tissues tend to store this nutrient in the vacuole, not contributing to the metabolism and growth (Veneklaas et al., 2012). Nevertheless, it might constitute an important P reservoir to sustain growth at later periods of P limitation as it concentration on plant tissues normally decrease along with development due to a reduction on absorption processes and an increase on internal remobilization – in which up-to 50% of the P from old leaves is transported to sink tissues (Aerts, 1996; Greenwood et al., 2001). In this sense, remobilized P can be an important nutrient source for growth, especially in soils with high P-fixing capacity where P supplied by the fertilizer is sorbed in the soil matrix along time (Veneklaas et al., 2012).

In addition to helping the plant to set in the soil, the root system is responsible for nutrient and water acquisition from the soil matrix through a complex interplay with the biogeochemical properties of the former (Ahmadi et al., 2014). Roots need a high degree of plasticity in order to maintain these functions under a wide range of stress scenarios. Unlike other nutrients that are not highly fixed in the soil matrix and can be reached by the root system via mass flow and diffusion, P is highly immobile (Syers et al., 2008). Therefore, only a small fraction of P demand (1-5%) is usually delivered by mass flow

(Lambers et al., 2006). To complete plant requirements, roots have to grow beyond the depletion zone to find distant P patches and facilitate it diffusion to the root surface (Lambers et al., 2015). Here, we found an improved root growth in Crac plants– regardless of P treatment and experimental condition – which showed the highest correlation with P accumulation (Fig. 3, S2, S3b). In agreement with our results, Nobile et al. (2019) and Wen et al., (2019) studying P-acquisition root mechanisms of several plants species also found that root morphological adaptations were the main drivers of P accumulation in wheat cropping. In addition, root morphology was found be to highly correlated with P acquisition in other plant species, as in annual pastures and *Brassica oleracea* (Haling et al., 2016; Hammond et al., 2009). Finally, experiments at the field level found that root anatomical and morphological traits can considerably enhance cereal crop yield stability under limited water and nutrient resources (Lynch et al., 2014; York et al., 2013), highlighting the importance of considering root traits in breeding programs focused on increase P acquisition efficiency.

Crac plants presented higher root P acquisition efficiency ($P<0.05$), especially when extra P was applied. In agreement with our results, Sandaña and Pinochet (2014) also reported an increase in P acquisition per root length of P-fertilized wheat plants compared to unfertilized ones. In this sense, this increase of efficiency could be explained by that fertilizers are usually applied in localized zones, therefore when roots intercept this area P acquisition highly increases - or at least faster than roots growth. Even though no significant differences were observed in the absence of the Pi fertilizer, Crac accumulated more P in their tissues due to its higher root growth. The absence of differences between Crac and Tukan in this condition could be explained by the intrinsic low Pi availability in

this soil, which restricts uptake efficiency, and the high difference in root growth and morphology between Tukan and Crac - in where Crac showed 70% more root surface area – reducing the ratio between P acquisition and root area in the former. Taken together, the higher efficiency in Crac could be attributed to its higher *PHT1* expression**,** as shown in chapter V.

Wheat is a highly mycotrophic plant, especially under P deficient conditions, in which colonization levels ranges from 20 to 90% in young plants, depending on plant genotype/AMF treatment (Covacevich et al., 2007; Graham and Abbott, 2000). The root colonization levels found in our greenhouse experiments agreed with an observation made in a field trial with the same soil and cultivars, in which 90 days-old Crac and Tukan plants (at the end of tillering) presented high colonization levels (up-to 60%), being P-deprived Crac plants more colonized than Tukan ones (Seguel et al., 2017). Here, mycorrhizal colonization showed a partial correlation (R^2=0.34; P<0.1; Fig. S3c) with P acquisition under P-limited conditions. It is well accepted that P nutrition is one of the main regulators of AM symbiosis, being its establishment and efficiency generally reduced with increasing soil Pi availability (Richardson et al., 2011; Smith et al., 2011). P deficiency induces the exudation of strigolactones by plant roots, which stimulate metabolic activity and hyphal branching in AM spores, and therefore increase the probability of encounter between AM fungi and plant roots to initiate the symbiosis (Bouwmeester et al., 2007; López-Ráez et al., 2017). In this context, the higher colonization of Crac plants in the absence of Pi fertilization could be attributed to its higher strigolactone production, as shown in Chapter V.

AM fungi are found in almost every ecosystem and can provide benefits to plants through multiple mechanisms, including pathogen resistance, and nutrient and water acquisition (Smith and Read, 2010). Despite that, AM fungal diversity only involves ~250 morphologically and 350 to 1000 molecularly defined AM species (Kivlin et al., 2011; Öpik et al., 2013). In a global survey, Öpik et al. (2006) showed that AM richness in plant roots of different ecosystems is overall low, with only an average of 18 fungal taxa colonizing each plant species in tropical forests and only 5 AM species in agricultural fields. Here, we found 7 different AM species colonizing the roots of the wheat cultivars, belonging to 5 different genera, with an average richness of 4 species (Fig. 5, Table 3). So far, few studies have assessed mycorrhizal community colonizing wheat roots, being richness varying from 1 to 8.7 AM species (Dai et al., 2014; Liu et al., 2012b; Mao et al., 2014). In these studies, *Claroideoglomus*, *Rhizophagus*, *Funneliformis* and *Glomus* were the most common genera reported to be colonizing wheat roots. Accordingly, the OTUs found in this study were within the genera mentioned above, and also within the ones morphologically described by Aguilera et al. (2014, 2017) in a previous AM survey under acidic soils. Here, a preferential association between Tukan plants and *Archaeospora* species was observed (Fig. 5). The effects of mycorrhizal symbiosis with *Archaeospora* species on plant growth and tolerance to stress have been poorly studied, with responses varying depending on the host plant (Bennett and Bever, 2007; Helgason et al., 2002). Further studies should be carried out in order to analyze whether there is any preferential association between Tukan and *Archeospora* species, and if this association can confer improved wheat growth under high P-fixing conditions to further consider the incorporation of this trait in future breeding programs.

Since P is present on fixed sources and/or unavailable forms, plants having higher root growth do not necessarily must show increased P acquisition. In this case, root biochemical responses play a major role on accessing P from sparingly available pools in soil (Campos et al., 2018; Fenesi et al., 2019; Tarafdar and Claassen, 2003). It is well-known that plants often release more phosphatases and LMWOAA from their roots when subjected to P deprivation (De Andrade et al., 2011; Vance et al., 2003). Here, rhizospheric activity of both phosphatase and oxalate accumulation were in general lower in the unfertilized soil – especially in Tukan plants. However, this effect can be mainly due to the high stress level and reduced root growth of this cultivar under Pi limitation, as top roots exudation was shown to be increased under this same condition (Fig. 6b, d). Overall, even though Tukan showed higher exudation (and therefore relatively more available P through mineralization/solubilization), it also presented reduced root growth and P acquisition efficiency, therefore not taken advantage of the higher P availability. This fact was further confirmed in the analysis of available soil P, where no depletion of available P was observed by this cultivar under optimal P conditions (Table 4). This suggests a neutral balance between P solubilization/mineralization and acquisition.

Oxalate concentration in this study ranged from 100-250 μM g^{-1} soil, which is 100 times less than the concentration proved to effectively solubilize Pi by artificial spiking into soil (Ryan et al., 2014). Interestingly, in a recent study, Xu et al. (2019) showed that even low differences in carboxylates exudation of wheat plants were able to affect microbial activity and the magnitude of soil priming effect, and consequently nutrient dynamics, including P availability. Accordingly, in our experiment oxalate concentration

in the P-limited soil was partially correlated with plant growth (not shown) and P acquisition (Fig. S3e).

It is generally accepted that root exudates are mainly concentrated in the root apical zone, decreasing its exudation rate with root age (Hodge et al., 2009; Personeni et al., 2007). Nevertheless, there are few studies assessing root exudation over different root zones, and most of them are focused on exudation of Proteaceae plants (Hajiboland et al., 2005; Neumann and Römheld, 1999; Nuruzzaman et al., 2006). Here, we found an interestingly pattern for oxalate exudation in fertilized Crac plants, where this cultivar showed a higher exudation rate in top roots (near the fertilizer placement) compared to exudation near the root tips (Fig. 6d). Accordingly, Hajiboland et al. (2005) observed an increase in malate exudation with increasing distance from root tips in a Zn-efficient rice cultivar in absence of stress conditions. Here, a high correlation between oxalate exudation on top roots and P accumulation was observed under fertilized condition (Fig. S3f), however, further studies are necessary to assess whether this trait remains functional at the crop level to agronomically exploit this mechanism.

Wheat growth and yield are highly correlated with P availability in soils, especially on P-limited soils (Deng et al., 2018; Sandaña and Pinochet, 2014; Seguel et al., 2017). In this context, Andisols possess high P-fixing capacity, therefore, reducing P availability to plants (Borie et al., 2019). However, the P-sorption and -desorption rate in these soils are highly influenced by pH (Vistoso et al., 2012). Here, we observed an increase of both rhizospheric pH and Olsen-P levels in the unfertilized plants during the experiment. These increases would be mainly due to the nutrient solution used in this experiment, which possesses nitrate as the main nitrogen source, thus, affecting pH and P-sorption and -

desorption rate (Gahoonia et al., 1992). In agreement, Paredes et al. (2011) also observed an increase of pH and Olsen-P in rye grass and tall fescue plants under nitrate fertilization. Nevertheless, no significant differences in pH and Olsen-P between the cultivars under unfertilized condition were observed here. Conversely, Crac presented a higher depletion of soil P levels when fertilizer was applied, suggesting a higher fertilization recovery. Remarkably, usually only about 10-30% of the P fertilizer is taken up by roots in the first year of its application (Syers et al., 2008). Therefore, this higher recovery could configure an important step to increase P fertilization efficiency in high-input system.

Finally, it is important to mention that the relative contribution of each mechanism mentioned above on P-acquisition depends on both plant species and environmental conditions (Lynch and Ho, 2005; Raven et al., 2018). Compared to other crop species, wheat seems to rely more on root morphological traits (Nobile et al., 2019; Wen et al., 2019). On the other hand, Deng et al. (2018) showed that wheat cultivars with similar growth and yield potentials could rely on different root strategies. Indeed, in their experiment, modifications on root morphology and efficiency were the most important mechanisms for one cultivar, while the other cultivar depended on its higher P-ase activity. Therefore, further studies should be carried to assess if the different P acquisition mechanisms shows similar behavior under a wide range of environmental conditions, and the outcomes in plant performance under P-limiting conditions if different advantageous traits could be combined (*e.g.*, higher exudation of Tukan plants with higher root growth and efficiency of Crac plants).

4.5 Conclusions

In this work, it was demonstrated that root morphological traits are the most important ones in order to increase P acquisition in high P-fixing soils, in both presence and absence of Pi fertilizers. In this sense, unfertilized Crac plants showed higher growth, which was highly correlated with root morphological traits and higher P acquisition. Even though fertilized Crac plants did not present higher plant biomass production at this stage, its higher P acquisition could be an important source to sustain growth over later periods of P-limitation. Moreover, mycorrhizal symbiosis and root exudates could be also considered as important mechanisms to enhance P acquisition under limiting conditions, becoming important targets for future breeding programs orientated to generate cultivars adapted to low P input systems. On the other hand, the higher depletion of the Pi fertilizer by Crac plants, especially through their higher root growth and top roots exudation, should be considered in breeding programs in order to increase Pi fertilization recovery in high P-fixing soils.

Supplementary data

Fig. S2. Principal component analysis (PCA) summarizing the main results of the rhizobox experiment.

Fig. S3. Regression analysis between the main variables analyzed in this study.

Fig. S4. Rarefaction curve of AM species colonizing roots.

Fig. S5. Phylogenetic tree of AM species found in the pot experiment.

Acknowledgments

This work was supported by funding from FONDECYT 11160385 and 1170264 grant from Comisión Nacional de Investigación Científica y Tecnológica (CONICYT-Chile), CONICYT DOCTORADO NACIONAL 2016 (21161474), CONICYT + PAI/ CONCURSO NACIONAL TESIS DE DOCTORADO EN EL SECTOR PRODUCTIVO 2017 (T7817120011), CONICYT/FONDAP/15130015, and AGL2015-64990-C2-1R (MINECO-Spain). We also acknowledge Dr. Gustavo Curaqueo for the analysis on the root scanning system, Campex Baer® for their agronomical support and BIOREN-UFRO for providing their facilities.

CHAPTER V

"Phosphate acquisition efficiency in wheat is related to root: shoot ratio, strigolactone levels, and PHO2 regulation"

Article published in *Journal of Experimental Botany* (2019) 70: 5631–5642

doi: 10.1093/jxb/erz349

Phosphate acquisition efficiency in wheat is related to root: shoot ratio, strigolactone levels, and pho2 regulation

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ABSTRACT

Inorganic phosphorus (Pi) fertilizers are expected to become scarce in the near future; so, breeding for improved P-acquisition related root traits would decrease the need for fertilizer application. This work aimed to decipher the physiological and molecular mechanisms underlying the differences between two commercial wheat cultivars (Crac and Tukan) with contrasting P-acquisition efficiencies (PAE). For that, four independent experiments with different growth conditions were conducted. When grown under non-limiting Pi conditions both cultivars performed the same. Crac was less affected by Pi-starvation, presenting higher biomass production, root growth, root: shoot ratio, and root efficiency for Pi-uptake under this condition. In this sense, Crac higher PAE correlated with its higher expression of Pi-transporters *TaPht1;2* and *TaPht1;10*. We also analysed the *PHO2* signalling and homeostasis pathway, of which Crac presented a faster and higher induction upon Pi-starvation. Based on our findings and in the related literature, we suggest a model for long-term modulation of this pathway. In addition, we analysed internal strigolactones levels and found that Crac presented overall higher concentration, increasing the evidence of direct relationship between SLs and P-responses. Finally, we suggest that the higher PAE of Crac is related to a fine-tune regulation of the P starvation responses, especially the P homeostasis pathway through *PHO2* modulation.

5.1 Introduction

Phosphorus (P) is the second most limiting nutrient in plants besides nitrogen, being involved in numerous cellular processes as protein activation, energy transfer, signalling and regulation of carbon metabolism (Lan et al., 2017; Xu et al., 2018). However, unlike nitrogen, which can be fixed by microorganisms, the amount of P available for agriculture is finite (Bovill et al., 2013). Moreover, when compared to other essential macronutrients, P is one of the less-abundant elements in the lithosphere (0.1% of the total), highlighting the need of supplying P fertilizers to sustain modern agricultural production (Campos et al., 2018). As a consequence, P fertilizers consumption has increased worldwide in the past decades. P fertilizers are made from non-renewable resources as rock phosphates, which are expected to become scarce in the near future as few mining sites are discovered and demand is expected to further increase by 50-100% in the next 30 years (Cordell et al., 2009; Ulrich and Frossard, 2014). P is present in plants either as organic phosphate esters or as free inorganic orthophosphate form (Pi). Remarkably, Pi has high-affinity to both soil mineral particles and organic matter; therefore, its availability in agroecosystems is generally below plant´s demand, even in fertilized sites, where up to *ca*. 90% of the applied P fertilizer is not taken up by the roots in the first year (López-Arredondo et al., 2014; Syers et al., 2008). Therefore, although a huge amount of P fertilizers are used, plants are normally subjected to stress due to the deficiency of this essential nutrient. Nevertheless, when P fertilization exceeds soil holding capacity, environmental problems associated with eutrophication due P-leaching are likely to occur (Bennett et al., 2001). In addition, these fertilizers can contain heavy metals, such as cadmium that may accumulate in arable soils as a result of the addition of rock phosphate (van de Wiel et al., 2016).

Plants have developed an array of complex regulatory mechanisms to adapt themselves to low Pi availability in the soil, known as P starvation responses (PSRs), aiming to optimize its external and internal use (Ham et al., 2018; Puga et al., 2017). These responses include changes at genetic, biochemical, physiological, morphological and rhizospheric levels (Puga et al., 2017). PSRs include alterations in shoot and root morphology, growth and development, exudation of low molecular weight organic acids anions and Pi-releasing enzymes, modifications in lipids and carbohydrate metabolism, association with soil microorganisms, as well as the regulation of expression and activity of high-affinity Pi transporters (PHT) (Campos et al., 2018; Lambers et al., 2015). Nevertheless, in order to respond accurately, plants need first to sense the P-status both locally and systemically in order to orchestrate the appropriate responses (Lan et al., 2017; Scheible and Rojas-Triana, 2015). PSRs are themselves complex, with a large set of genes (>1000) being regulated. However, new genomic findings have contributed to shed light over some mechanisms of P sensing, signalling and homeostasis, especially in the model plants *Arabidopsis thaliana* and rice (*Oryza sativa*) (Lan et al., 2017; Liu et al., 2012a). It is well established that the transcriptional activator *PHOSPHATE STARVATION RESPONSE 1* (*PHR1*) in Arabidopsis and its orthologous *OsPHR2* in rice play a key role regulating the expression of numerous Pi-starvation-induced (PSI) genes (Rubio et al., 2001; Zhou et al., 2008). Among them, special attention has been paid to the microRNA miR399, which expression is highly induced by Pi deprivation (Pant et al., 2008). This regulator has been shown as a key systemic cue between plant tissues by modulating the activity of *PHO2*, which encodes an ubiquitin-conjugating E2 enzyme (UBC24) implicated in protein degradation (Lin et al., 2008). Down-regulation of *PHO2* prevents the

degradation of the Pi transporter *PHO1*, involved in Pi xylem loading, and some transporters of the *PHT1* family, associated to P acquisition and translocation within the plant (Huang et al., 2013; Liu et al., 2012a). Another key PSI gene family involved in P signalling and homeostasis is *At4*/*IPS1* in Arabidopsis and rice, respectively. These genes affect the miR399-*PHO2* interaction by sequestering free miR399 through a target mimicry mechanism, preventing its binding to *PHO2* transcripts and, thus, its degradation (Fig. 1) (Franco-Zorrilla et al., 2007). Therefore, P acquisition and distribution within the plant is regulated mainly by the interaction of the triad *IPS1*-miR399-*PHO2*, which serves to fine-tune Pi-starvation responses (Fig. 1).

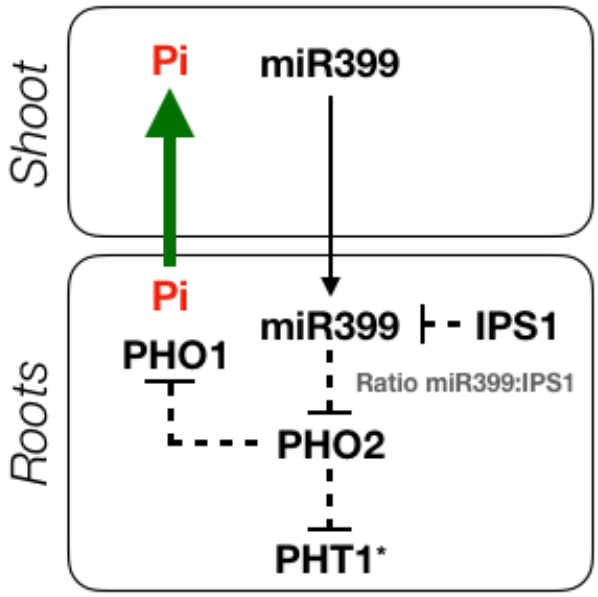

Figure 1. Schematic summary of the P signalling and homeostasis pathway in plants. Upon Pi deficiency, expression of the miRNA miR399 is induced in the shoot. miR399 moves downwards, inactivating PHO2 in the roots. Regulation of PHO2 prevents the degradation of the Pi transporters PHO1 and PHT1, which increase Pi uptake and translocation. Pi deficiency also induces the expression of IPS1, which binds miR399, modulating this response. Based on Puga et al. (2017).

Wheat (*Triticum aestivum* L.) is one of the most important food crops in the world, with global grain production of 7.5×10^{14} g in 2016, making it the 3rd most harvested crop

worldwide, after sugarcane (*Saccharum* spp.), and maize (*Zea mays* L.) (FAO, http://faostat3.fao.org/home/E Accessed: June 01, 2018). However, wheat production is highly dependent on P fertilizers, leading to a higher consumption per area when compared to other major crops (Heffer, 2013). Therefore, improving P fertilization efficiency in wheat cropping is a major goal in order to achieve a more sustainable agricultural production. The last can be achieved by improving P fertilizers availability in soil, as by avoiding Pi sorption to soil particles, and/or by the development of P use/acquisition efficient plants (Campos et al., 2018). While the first option increase operational costs and require modern technology, many times not accessible for producers, breeding for P acquisition efficient root systems would provide benefits to both high and low-input systems (Rose and Wissuwa, 2012; Wissuwa et al., 2001). In recent years, some molecular mechanisms underlying P signalling and homeostasis in wheat has been revealed. Wang and co-workers characterized *TaPHR1*, and showed that over-expressing lines had improved root growth, enhanced Pi uptake, and higher yield (Wang et al., 2013). In another breakthrough, the *IPS1*-miR399-*PHO2* system was shown to be functional in wheat (Ouyang et al., 2016). In that study, *TaPHO2* expression was found to be related to root and shoot growth, shoot Pi accumulation, and activity of some *PHT1* transporters (Ouyang et al., 2016). In this context, the Pi transporter gene family *PHT1* from wheat has been recently identified, consisting of 16 phylogenetically distinct transporters (Grün et al., 2018).

On the other hand, it is well known that phytohormones such as auxin, cytokinin, abscisic acid, ethylene, and specially strigolactones (SLs) play synergistic roles in the regulation of P homeostasis when plants are subjected to P stress, through modulation of

the P signalling and homeostasis-associated pathways and ultimately root functioning (Chien et al., 2018; Waters et al., 2017). SLs are the latest class of phytohormones described, and have been shown to function as regulators of plant development/architecture and as signalling molecules in the rhizosphere to recruit arbuscular mycorrhizal fungi under Pi limitation (López-Ráez et al., 2017; Waters et al., 2017). Indeed, their biosynthesis is highly promoted under this stress condition (López-Ráez et al., 2008; Yoneyama et al., 2007, 2012). Recently, it has been shown that exogenous application of the synthetic SL analogue GR24 induced root hair elongation, anthocyanin accumulation, production of acid phosphatases, and reduced plant weight (Ito et al., 2015), which are characteristic PSRs, suggesting a potential overlap between these two signalling and homeostasis pathways in plants. Although the molecular mechanisms that regulate P signalling and homeostasis, and their associated plant morphological changes and Pi uptake capacity are being established at the laboratory level, only a few studies have verified these findings in commercial cultivars so far. In the present work, we aimed to characterize at the physiological and molecular level two commercial wheat cultivars - Crac and Tukan - with different P acquisition efficiencies, and to relate these phenotypes with their ability to regulate P signalling and homeostasis under Pi limited conditions. The results provide new insights into the regulation of P signalling and homeostasis in plants and suggest new potential targets for future breeding strategies in plant production.

5.2 Material and methods

5.2.1 Plant material and growth conditions.

In a previous study, a screening of wheat cultivars commonly used in Chile revealed high variations in P acquisition and grain yield when grown in a high Pi-fixing Andisol (Seguel et al., 2017). From that study, we selected two cultivars - Crac and Tukan (formerly known as TCRB14 and STKI14, respectively) - showing contrasting P acquisition efficiency (PAE) under Pi deficient conditions. Seeds of the wheat cultivars were surface sterilized in 4% sodium hypochlorite, rinsed thoroughly with sterile distilled water and germinated for 72 h on moistened filter paper at 25°C in darkness. Precise phenotyping for optimal root system characteristics is difficult and time-consuming as root traits are hidden under the soil, making difficult their extraction for observation (Zhu et al., 2011). Therefore, different 'artificial' growing methods are used under lab conditions to facilitate its access, such as growing plants in liquid culture (hydroponics) or in transparent surfaces (rhizoboxes). These methods, although they do not fully represent the root growth in soil, give valuable clues to understand general features, and the physiological and genetic background behind them (Hargreaves et al., 2009). In order to access the effects of Pi-deficiency on plant development and P acquisition of these cultivars, seedlings of each genotype were grown hydroponically (Fig. S6a) for 2 week with a standard nutrient solution (Taylor and Foy, 1985) containing 200 μM of Pi in 1 L containers and then half of the plants were submitted to Pi-starvation (10 μM of Pi in nutrient solution) for 3 weeks. In parallel, seedlings of each genotype were transferred to 0.5 L plastic pots with a mixture of autoclaved substrate of sand and vermiculite (1:1) and were watered manually with standard nutrient solution low in Pi (10 μM) for 33 days (Fig. S6b). In addition to the experiments previously mentioned, another set of plants were grown for 8 weeks in rhizoboxes (30 cm height, 20 cm width, and 0.7 cm depth) filled with an acidic high P-fixing soil without P-fertilization (Fig. S7a).

Plants were grown under greenhouse conditions with temperatures ranging from 16-23ºC during the day and 10-18ºC at night and were harvested at Zadoks growth stage 23 (Zadoks et al., 1974).

For gene expression analysis and SL quantification, six seedlings of each cultivar were grown hydroponically in 3 L containers, containing a modified Long Ashton nutrient solution with 150 μM of Pi (Hewitt, 1966) in a greenhouse for a total of 5 weeks (Fig S6c). Nutrient solution was replaced twice in a week. After 4 weeks, half of the plants were transferred to a modified nutrient solution without P and were let grown for another week. For the time course Pi starvation experiment, plants were grown for 4 weeks under normal P conditions, and then, half of the plants subjected to 2, 4, and 7 days Pi deprivation. Six independent plants were grown per treatment and time point. Shoots, roots, and root exudates were collected, weighted, frozen with liquid nitrogen and kept at -80ºC until use.

5.2.2 Root morphological measurements

For the phenotyping experiments, root systems were cleaned after harvest, arranged to minimize overlaps (Yao et al., 2009), placed in an A3-sized Perspex tray filled with water, and scanned in both grey-scale and colour in a Epson Expression 11000XL calibrated for Image Analysis. The images were then subjected to software analysis (WinRhizo; Regent Instruments, Quebec), and root length, specific area and average diameter were assessed.

5.2.3 P acquisition

After root morphology determination, plants were separated into roots and shoots and both parts weighted and dried at 65ºC in a forced-air oven for 72 h. After drying, the roots and

shoots samples were weighted, crushed, ground, ashed in a furnace at 550ºC, and digested using an H_2O:HCl:HNO_3 mixture (8:1:1, v:v:v). Then, Pi content was determined using the vanadate-molybdate colorimetric method (Murphy and Riley, 1962).

5.2.4 RNA extraction and gene expression analysis by qPCR

Total RNA from roots was extracted using TRIsure™ (Bioline, Toronto, Canada) according to the manufacturer's instructions. The RNA was treated with RQ1 DNase (Promega, Madison, WI, USA) and purified through a silica column using the RNA Clean & Concentrator™5 (Zymo Research, California, USA). Before storage at -80ºC, RNA was quantified using a Nanodrop 2000C spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and its integrity checked by gel electrophoresis. The first-strand cDNA was synthesized with 1 µg of purified total RNA using the PrimeScript™ RT Master Mix (Clontech, Fremont, CA, USA) according to the manufacturer's instructions. The expression of marker genes for different P signalling and homeostasis pathways (Table S1) was analysed by real-time quantitative polymerase chain reaction (qPCR). All reactions were performed using TB Green™ Premix Ex Taq™ (Kusatsu, Shiga, Japan) on an iCycler iQ5 system (Bio-Rad), using 5 µL of single-stranded cDNA (diluted 1:50) and specific primers for each gene, except for *TaPht1;10* and tae-miR399b, where a dilution 1:5 was used. In the case of the gene *TaPht1:2,* it is present in chromosomes A and B, showing high sequence similarities among them; therefore, it was not possible to design specific primers able to differentiate the expression of the two alleles (Grün et al., 2018). The amplification protocol included an initial denaturation at 95ºC for 3 min followed by 35 cycles of 95ºC for 30 s, 58ºC for 30 s, and 72ºC for 30 s. The specificity of the different amplicons was checked by a melting curve analysis (from 65 to 100ºC) at the end of the

amplification protocol. Five independent biological replicates were analysed per treatment and time point, and each PCR reaction was done in duplicate. Relative quantification of specific mRNA levels was performed using the comparative $2^{-\Delta Ct}$ method. Expression values were normalized using the housekeeping gene *TahnRNPQ* (Grün et al., 2018).

5.2.5 Strigolactone quantification

Germination bioassays were performed using extracts from frozen roots from plants grown for molecular analysis. For SL extraction, root extracts were processed as described by López-Ráez et al. (2008). SLs are germination stimulants of root parasitic plants of the family Orobancheaceae (Bouwmeester et al., 2007; López-Ráez et al., 2017). Therefore, germination assays with seeds of these parasitic weeds is an indirect way to estimate SLs levels. Germination bioassays with pre-conditioned seeds of the parasitic plants *Phelipanche ramosa* were performed as described by López-Ráez et al. (2008). The synthetic SL analogue 2-*epi*-GR24 and demineralized water were included as positive and negative controls, respectively. Extract dilutions of 1:10 and 1:20 were tested for seed germination. After 7 d, the germinated and non-germinated seeds were counted using a binocular loupe. In addition to the germination bioassays, SLs were quantified by UHPLC-MS/MS using GR24 as internal standard as described by Rial et al. (2019). Briefly, 0.1 g of ground root material was extracted with 1 mL of ethyl acetate in an ultrasonic bath for 10 min, centrifuged for 10 min at 5000 rpm, concentrated in a rotary evaporator and stored at -80°C. Before the chromatographic analysis, extracts were dissolved with MeOH (1:1, v:v) and GR24 added to each sample to a final concentration of 10 $\mu g\ L^{-1}$. Chromatographic analyses were carried in a Bruker EVOQ Triple Quadrupole Mass Spectrometer with an electrospray ionisation (ESI) source in positive mode. The mobile phase consisted of

solvent A (water, 0.1% formic acid) and solvent B (MeOH, 0.1% formic acid) and the flow rate was set to 0.3 $mL \cdot min^{-1}$. The optimised linear gradient system was as follow: 0-0.5 min, 50% B; 0.5-5 min, to 100% B; 5-7 min, 100% B; 7-7.5 min, to 50% B; 7.5-10.5 min, 50% B. The injection volume was 5 μL. The instrument parameters were set as described by Rial et al. (2019)

5.2.6 Statistical analysis

Means for plant growth, root morphology measurements, P acquisition, gene expression analysis by qPCR and SL production were obtained from the results of five replicates. Data were assessed for normality, transformed when necessary, and significant differences between means were analysed by independent Student's t test or ANOVA followed by Tukey LSD when suited. Correlations among the different variables were performed using the r Pearson coefficient. All statistical analyses were carried out with R software.

5.3 Results

5.3.1 P acquisition and root system morphology in Crac and Tukan

In acidic soils, including Andisols in Chile, Pi-bioavailability is rather low, many times due to high levels of iron and aluminium, which greatly affects plant productivity. In a previous study, the most efficient cultivar - Crac - yielded almost three times more grains than the less efficient cultivar - Tukan - at low Pi fertilization levels (Seguel et al., 2017). In the present work, we aim to decipher the physiological and molecular mechanisms behind such phenotypes. For that, plants of these two wheat cultivars were grown in different substrates and under different P conditions, and their P acquisition capacity

compared. Similar results were obtained with the different growing conditions. When grown in hydroponics (Fig. S6a) with sufficient Pi, both cultivars accumulated and allocated P in a similar manner to shoot and roots. Pi-starvation reduced Pi uptake in both cultivars. However, despite the loss of Pi accumulation in both organs, the loss was significantly lower in Crac than in Tukan (Tables 1 and S2). Indeed, Pi-starved Crac plants accumulated 25% and 17% more Pi in the shoots and in the roots, respectively, than the less efficient cultivar Tukan. Differences in Pi uptake between the two cultivars were even higher when using pots with inert substrate, where Crac accumulated 40% and 60% more Pi in shoots and roots, respectively, than Tukan (Table 1). The same pattern was also observed in soil-grown plants in rhizoboxes, although in a lesser extent (Fig. S7b). Interestingly, despite the different Pi uptake capacity of the two cultivars under Pi limitation, no significant differences were observed in their root Pi concentration (Table 1), suggesting that the increased P accumulation in Crac was associated to a larger root system.

5.3.2 Shoot and root growth under Pi deficiency

One of the main symptoms of Pi deficiency in plants is the enhancement of the root: shoot ratio; either by a reduction in shoot growth, an increase in root production, or both (Chien et al., 2018; Ericsson, 1995). To ascertain whether the differences observed in PAE between Crac and Tukan are associated to plant growth, their shoot and root morphology were analysed. As expected, no significant differences in growth were observed under normal Pi conditions when grown hydroponically (Fig. 2a). Under Pi-starvation, plants showed a clear reduction in shoot growth ($P<0.01$), with a concomitant increase of root

Table 1. Phosphate (Pi) uptake, concentration and root efficiency in accumulating Pi in shoots, roots and the whole plant in Crac and Tukan. Plants were grown in Pi-sufficient conditions (+P; 200 µM, hydroponically) and under Pi-starvation (-P; 10 µM, hydroponically and in pots) and were harvested at Zadoks growth stage 23 (Zadoks et al., 1974).

		Hydroponic (+P)			Hydroponic (-P)			Pot (-P)		
Tissue	**cv**	**P** uptake (µg)	**P** conc. (mg. g^{-1})	**Root Efficiency** (mg P. m^{-2})	**P** uptake (µg)	**P** conc. (mg. g^{-1})	**Root Efficiency** (mg P. m^{-2})	**P** uptake (µg)	**P** conc. (mg. g^{-1})	**Root Efficiency** (mg P. m^{-2})
Shoot	Crac	1060±158	1.57±0.23	61.57±1.60	600±32*	1.18±0.09*	17.73±0.55	678±43**	1.06±0.04**	22.41±1.61*
	Tukan	1130±43	1.41±0.04	74.17±5.05	480±22	0.92±0.05	17.04±1.99	406±28	0.89±0.02	18.48±0.77
Roots	Crac	180±16	1.01±0.08	10.51±1.02	120±02*	0.44±0.03	03.65±0.11	120±18**	0.40±0.06	3.61±0.51*
	Tukan	180±24	1.12±0.14	11.94±1.31	100±06	0.51±0.03	03.76±0.23	46±5	0.30±0.02	2.28±0.16
Plant	Crac	1240±157	1.45±0.13	72.07±6.56	720±29**	0.92±0.01**	21.38±0.45	797±44***	0.88±0.04**	26.46±2.05*
	Tukan	1310±47	1.36±0.02	86.10±5.17	580±15	0.81±0.02	20.80±1.81	421±41	0.73±0.01	20.76±0.71

Data represent the means of five independent replicates (±SE). Asterisks indicate the significance of differences between the cultivars in the same condition, as determined by Student' s t test analysis: * P<0.05, ** P<0.01, *** P<0.001.

production compared to plants growing under normal Pi conditions (Fig. 2a and Table S2). The effect was more severe in the less efficient cultivar Tukan, with a 37% reduction in shoot biomass, while the reduction was only a 23% in Crac. The opposite effect was observed in the roots, where under Pi-starvation an increase in biomass of 30% was found in Tukan, while the increase was up to 59% in Crac (Fig. 2a; Table S2). Taken together, hydroponically grown plants displayed an average increase of the root: shoot ratio of 128% and 87% for Crac and Tukan, respectively (Fig. 2b; Table S2). The same trend was observed in plants grown in pots with substrate, where Crac showed more shoot (34%) and root (42%) biomass than Tukan under Pi-starvation, giving rise to a 20% higher root: shoot ratio in Crac (Fig. 2b). A similar pattern was also observed in plants grown in soil (Figs. S7d,e). Therefore, a positive correlation between Pi accumulation and root: shoot ratio ($R=0.87$, $R=0.95$, and $R=0.85$, $P<0.01$, for hydroponic, pot, and rhizobox experiments, respectively) under Pi-starvation was observed in all growing conditions.

Additionally, parameters associated to root system morphology, such as total root length and root area were evaluated (Fig. 3). In hydroponics, Pi deprivation increased root length in both cultivars by 113% and 80% in Crac and Tukan, respectively (Fig. 3a and Table S2). Root surface area also increased under Pi-starvation, by 98% and 73%, respectively (Fig. 3b and Table S2). In the pot experiment, root length (about 65%) and surface area (about 50%) were also greater in the most efficient cultivar - Crac - than in Tukan (Fig. 3). The same behaviour was observed in plants growing in rhizoboxes, with Crac showing wider root systems (Figs. S7f,g). Differences in the diameter of the roots were also observed, being roots of the cultivar Crac significantly thinner (2.27 mm) than those of Tukan (2.41 mm) ($P<0.01$). These differences on average diameter were also

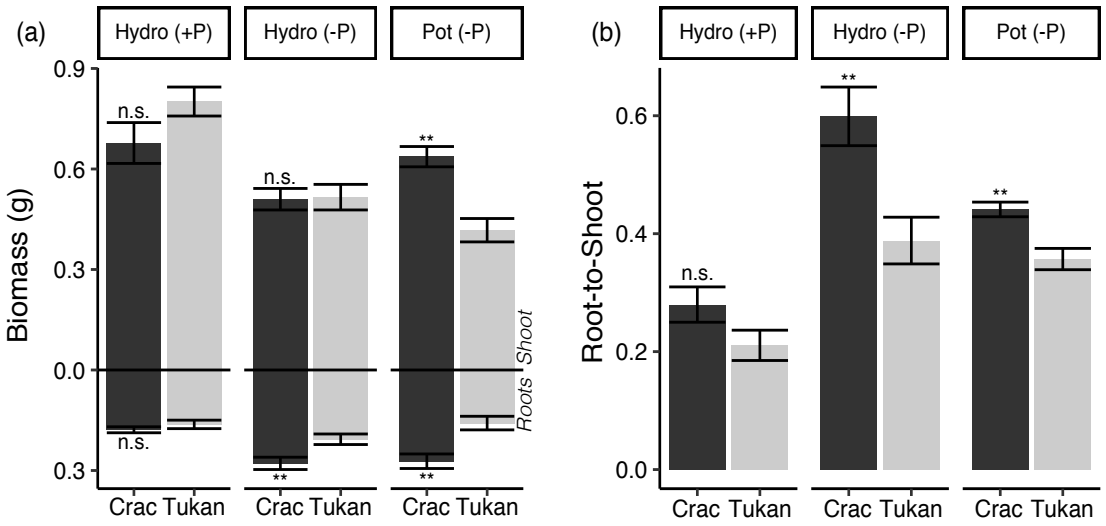

Figure 2. Growth rate of wheat cultivars Crac (dark bars) and Tukan (light bars) in sufficient Pi conditions (+P; 200 µM, hydroponically) and under Pi starvation (–P; 10 µM, hydroponically and in pots) harvested at Zadoks growth stage 23 (Zadoks et al., 1974). Graphics represent shoot and root biomass (a) and root: shoot ratio (b). Data represent the means of five independent replicates (±SE). n.s, non-significant differences. Asterisks indicate the significance of the differences between the cultivars in the same condition as determined by Student's t-test: $^{**}P<0.01$.

detected in plants growing in rhizoboxes with soil (Fig. S7h). In order to assess the root system efficiency in acquiring Pi, root efficiency (Pi uptake per root area) was calculated. Although little differences were observed among cultivars in the hydroponic experiment, the losses of efficiency from Pi-sufficient to Pi-deficient conditions in Tukan were significantly higher for all the experimental variables compared to those observed in Crac (Table S2). Nevertheless, Crac plants growing in pots and rhizoboxes significantly acquired more Pi per root area compared to the other cultivar (Table 1 and Fig. S7i). Together, these results suggest a more developed and efficient root system for this genotype under P deficiency.

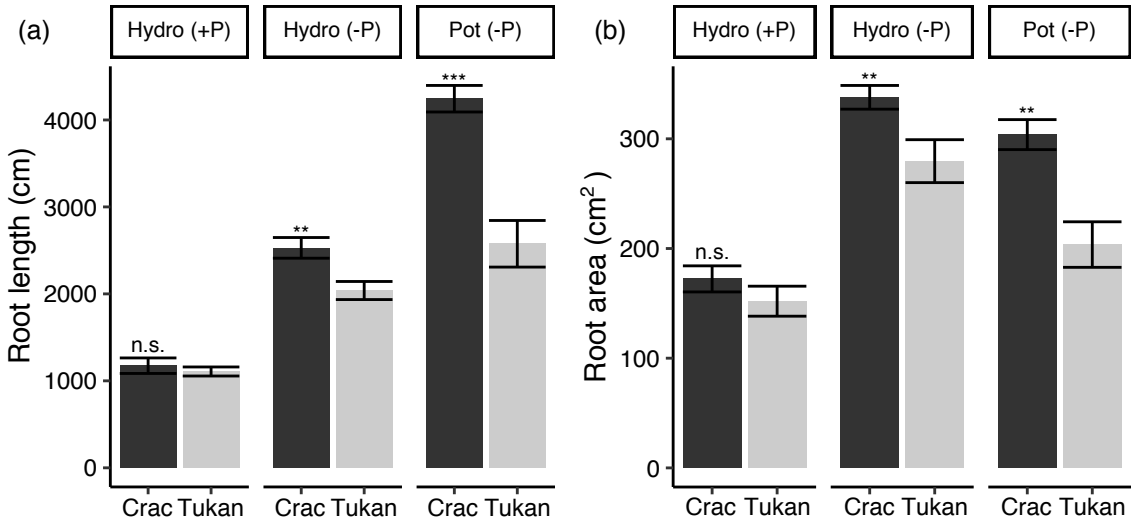

Figure 3. Root system morphology of wheat cultivars Crac (dark bars) and Tukan (light bars) grown in sufficient Pi condition (+P; 200 µM, hydroponically) and under Pi starvation (–P; 10 µM, hydroponically and in pots) harvested at Zadoks growth stage 23 (Zadoks et al., 1974). Graphics show total root length (a) and root surface area (b). Data represent the means of five independent replicates (±SE). n.s, non-significant differences. Asterisks indicate the significance of differences between the cultivars in the same condition as determined by Student's t-test: $^{**}P<0.01$, $^{***}P<0.001$.

5.3.3 Gene expression of Pi transporters

We investigated whether the higher root efficiency observed for Crac was related to a higher induction of Pi transporters. For that, a new experiment under normal and Pi deficient conditions was carried out in hydroponics, and gene expression levels of different Pi transporters were analysed by qPCR. Wheat has 16 phylogenetically distinct Pi transporters, being seven of them induced by Pi-deprivation (Grün et al., 2018). We analysed the expression profile of two of them, *TaPht1;2a/b* and *TaPht1;10*. The first one is the most expressed, and it was described as a fast responsive Pi marker. The other shows low expression levels, and its expression is increased along the time (Grün et al., 2018). The remaining Pi-inducible *TaPHT1s* show similar expression patterns either to *TaPht1*;*2a/b* or *TaPht1;10*, but with lower expression levels (Grün et al., 2018; Teng et al.,

2017). For that reason, they were not analysed in this work. Expression analysis revealed major differences among the two cultivars and their responses to Pi deprivation, with Crac showing higher basal levels of both transporters. The expression of *TaPht1;2a/b* was about 140 times higher in Crac than in Tukan, and Pi-starvation induced its expression levels 6 and 13 times in Crac and Tukan, respectively (Fig. 4a). Therefore, the levels of *TaPht1;2a/b* upon Pi stress were about 50-fold higher in Crac. A similar pattern was observed for *TaPht1;10*, although its expression levels were much lower than that of *TaPht1;2a/b* (more than 70 times in Crac). In this case, basal transcripts levels of *TaPht1;10* in Crac were 3 times higher than in Tukan, and the induction by Pi deficiency was 10 and 2 times in Crac and Tukan, respectively, giving rise to a 15-fold more expression in the former under stress conditions (Fig. 4b).

5.3.4 Crac and Tukan show different regulation of P signalling and homeostasis

All adaptive responses that plants have evolved to cope with Pi deficiency are regulated through P signalling and homeostasis mechanisms, which begins with the integration of the information of the extracellular Pi concentration and its levels in the different organs (Puga et al., 2017). Here, the *IPS1*-mediated signalling cascade, including *PHR1-IPS1*-miR399-*PHO2*, plays a pivotal role in P homeostasis regulation by coordinating the activities of Pi uptake and its root:shoot translocation through the transporters *PHT1s* and *PHO1* (Fig. 1) (Ham et al., 2018; Ouyang et al., 2016). To investigate whether Crac and Tukan presented differences in the P signalling and homeostasis pathway, we analysed the gene expression of *TaIPS1*, tae-miR399 [specifically from tae-miR399b family members, with confirmed expression and regulation activity in wheat roots under Pi-starvation (Ouyang et al., 2016)], *TaPHO2* and *TaPHO1* in roots of the two cultivars after 7 days of

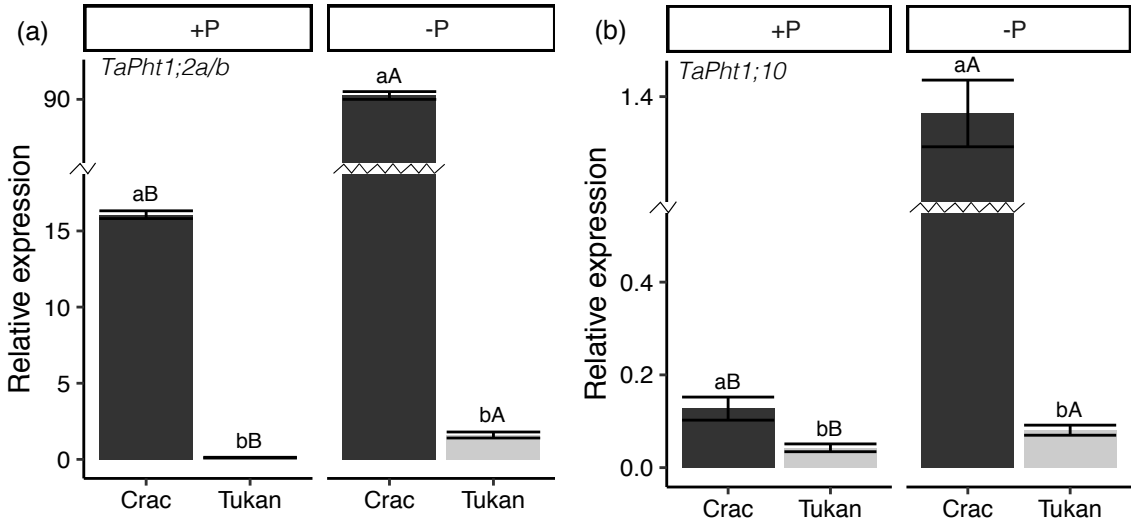

Figure 4. Gene expression analysis of two *TaPHT1s* Pi transporters in roots of Crac (dark bars) and Tukan (light bars) plants grown in nutrient solution with Pi (+P; 150 µM) and without Pi for the last week (–P) harvested at Zadoks growth stage 24 (Zadoks et al., 1974). Graphics represent expression of *TaPht1;2a/b* (a) and *TaPht1;10* (b). Expression levels were referenced to the expression of the housekeeping gene *TahnRNPQ*. Bars represent the means of five independent replicates (±SE). Lower case letters indicate differences between cultivars in the same condition, and upper case letters indicate differences within the same cultivar under normal and deficient Pi conditions as determined by Student's t-test analysis (P<0.05).

Pi deprivation and under normal conditions. No differences in the transcript levels of *TaIPS1* were found under optimal Pi conditions, but a clear induction by Pi starvation was observed in both cultivars (Fig. 5a). Interestingly, a 10-fold higher induction was observed in Crac compared to Tukan. As for *TaIPS1*, Pi-starvation promoted tae-miR399b expression in both cultivars, being this increase much higher in Crac (128-fold) than in Tukan (about 2-fold). Thus, the number of transcripts under stress conditions was more than 10 times higher in the most efficient cultivar Crac (Fig. 5b). The same behaviour was observed for *TaPHO2* under Pi limitation. Here, an increase of 6 and 2 times was observed for Crac and Tukan, respectively, resulting in almost 5-fold higher transcript levels in Crac (Fig. 5c). We further assessed the expression of the three *TaPHO2* alleles present in the

wheat genome (1A, 1B, and 1D), using specific primers (Table S1). Different patterns were observed for the three alleles regarding P responses (Fig. S8a-c). Overall, Crac *TaPHO2* alleles presented a higher induction in Pi starvation, especially *TaPHO2* 1B, with an increase of 25-fold. Tukan *TaPHO2* 1B and 1D showed a small, but significant increase in its expression under Pi deprivation (2.8- and 2-fold, respectively), while no differences were observed for *TaPHO2* 1A expression. No differences in gene expression were observed for the transporter *TaPHO1* (Fig. 5d).

In order to assess the dynamics of Pi signalling in the two cultivars, a time-course experiment was performed under the same conditions as described before, but harvesting plants after 2, 4, and 7 days of Pi deprivation. Interestingly, Crac presented an induction of *TaPHO2*, *TaIPS1* and tae-miR399b since day 2, which was steadily increased over time. However, in Tukan the increasing response of these genes by Pi starvation was only observed at day 7 (Fig. 6), indicating a faster response from Crac to Pi deprivation.

5.3.5 Strigolactone levels in Crac and Tukan

Since root morphology of Crac and Tukan was different under Pi-starvation, we assessed if SLs were involved in such changes. We first performed a germination bioassay with *P. ramosa* seeds using root extracts from the two cultivars. GR24 (10^{-8} and 10^{-9} M), used as positive control, induced high germination, while water (negative control) did not induce any germination. Under Pi-sufficient condition, Crac induced twice the germination of *P. ramosa* than Tukan (Fig. 7a), suggesting a higher basal level of SLs in that cultivar. Pi-starvation increased germination in both cultivars: 7% and 10% for Crac and Tukan, respectively (Fig. 7a). Orobanchol was detected in the root extracts of both cultivars. This

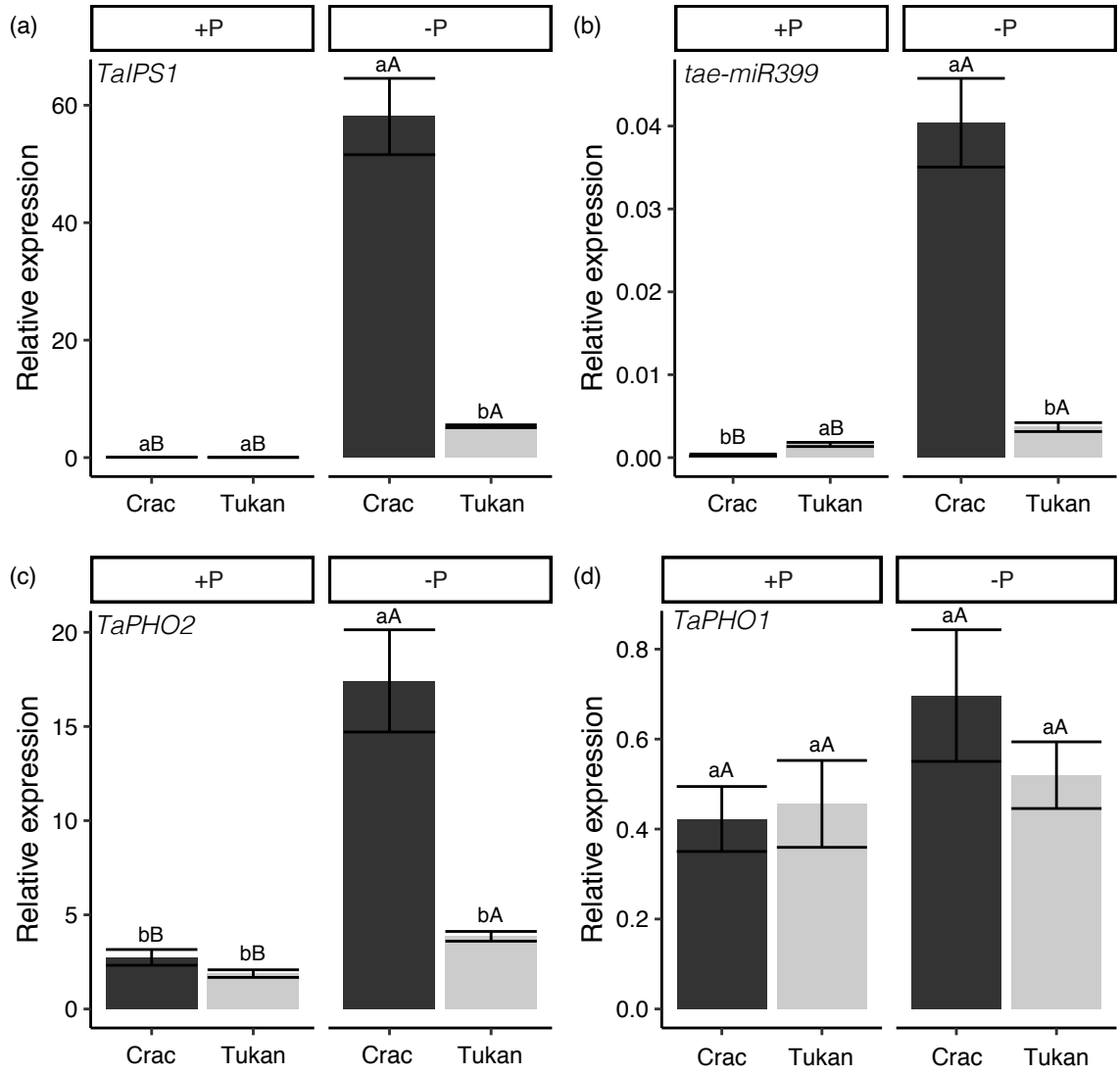

Figure 5. Expression levels of genes involved in P signalling and homeostasis in roots of Crac (dark bars) and Tukan (light bars) plants grown in nutrient solution with Pi (+Pi; 150 µM) and without Pi for the last week (-P) harvested at Zadoks growth stage 23 (Zadoks et al., 1974). Graphics representing gene expression of *TaIPS1* (a), tae-miR399 (b), *TaPHO2* (c), and *TaPHO1* (d). Expression levels were referenced to the expression of the housekeeping gene *TahnRNPQ*. Bars represent the means of five independent replicates (±SE). Small letters indicate differences between genotypes in the same condition and capital letters indicate differences within the same genotypes under normal and deficient Pi conditions, as determined by Student's t test ($P< 0.05$).

SL was reported as the main SL present in wheat exudates (Yoneyama et al., 2012). Orobanchol levels were significantly increased about 80% in Crac plants subjected to Pi-

starvation (P<0.01; Fig. 7b). This stress also increased the amount of orobanchol in Tukan, which were not detected under Pi-sufficient conditions. Here, orobanchol levels under Pi deficiency were similar to those in Crac in the absence of stress, indicating lower levels of SLs in Tukan, as previously observed in the *P. ramosa* germination bioassays (Fig. 7a). In addition to orobanchol, the SL fabacyl acetate was also detected in trace amounts in some samples. It was only detected in extracts from plants grown under Pi limitation, indicating that its biosynthesis was also promoted by Pi-starvation, and also showing higher contents in Crac (data not shown).

To explore if the elevated levels are related to a higher activity of the SL biosynthetic pathway in the Crac cultivar, the expression of two key genes involved in SL biosynthesis - *TaD27,* and *TaCCD8* - was analysed. The sequential action of these two enzymes gives rise to carlactone, the precursor of all the canonical SLs, including strigol- and orobanchol-type SLs (Al-Babili and Bouwmeester, 2015; Zhang et al., 2018). The search for the wheat *D27* gene was conducted using BLAST against its orthologue sequence from rice (LOC107276001). Two complete sequences were found for copies in the chromosomes 7A and 7D (Accession numbers: KX168420.1 and KX168421.1), showing 52 and 54% homology, respectively, with rice *D27* (Fig. S9). The same strategy was applied for *CCD8*, however no direct match was found. Therefore, the sequence encoding the putative wheat *CCD8* was searched in the wheat genome database (IWGSC database), using BLAST against its orthologue sequence from *Zea mays* (*ZmCCD8*). One sequence for each chromosome was found (3A, 3B, and 3D) (Fig. S10). The sequences obtained for *TaD27* and *TaCCD8* were checked for the presence of the functional domain DUF4033 and RPE65 respectively, and their homology with wheat close relatives

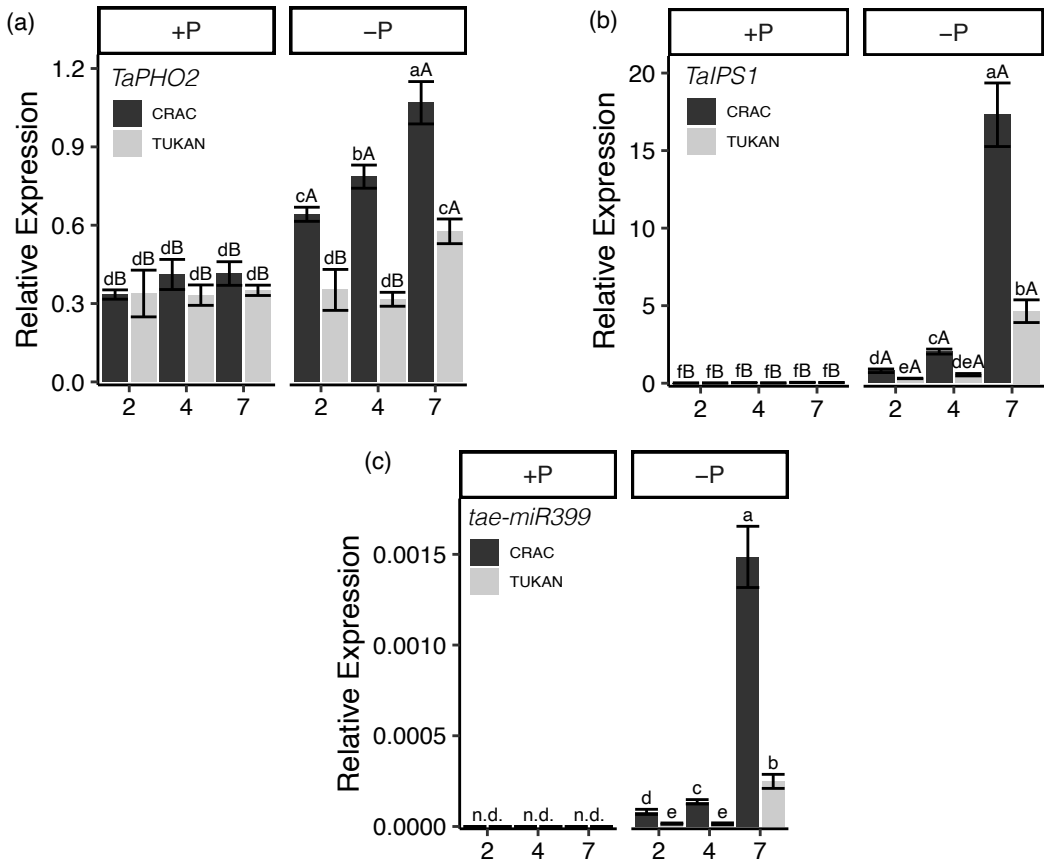

Figure 6. Expression levels of genes involved in P signalling and homeostasis in roots of Crac (dark bars) and Tukan (light bars) plants grown in nutrient solution with Pi (+P; 150 µM) and without Pi (-P) harvested after 2, 4 and 7 days of Pi deprivation. Graphics representing the gene expression of *TaPHO2* (a), *TaIPS1* (b), and, tae-miR399 (C). Expression levels were referenced to the expression of the housekeeping gene *TahnRNPQ*. Bars represent the means of five independent replicates (±SE). Small letters indicate differences between cultivars in the same condition and capital letters indicate differences within the same cultivar and day between +P and –P conditions, as determined by Student's t test (P<0.05). n.d, non-detected.

was assessed (Figs. S9a and S10b). Specific primers for *TaD27* and *TaCCD8* were designed to perform qPCR (Table S1). The basal expression of *TaD27* under Pi-sufficient conditions was almost 4-fold higher in Crac roots than in Tukan (Fig. 7c). However, no differences in basal expression levels of *TaCCD8* were found between the two cultivars

(Fig. 7d). Pi deprivation induced *TaD27* transcript levels about 40 times in Crac, while only 3 times in Tukan (Fig. 7c). A similar trend was observed for *TaCCD8*, with an increase in the expression levels of about 5-fold in Crac and only about 2-fold in Tukan (Fig. 7d). These results confirm that Crac produce higher basal levels of SLs than Tukan, and respond more efficiently to P starvation through a stronger promotion of SL biosynthesis, which would favour a greater and faster growth of the root system in response to Pi limiting conditions.

5.4 Discussion

5.4.1 Crac has an improved root system morphology and a higher P acquisition capacity

In the present work we analysed the physiological and molecular traits of two commercial wheat cultivars - Crac and Tukan - showing differential PAE under Pi-limiting conditions (Seguel et al., 2017). Interestingly, under that stress conditions grain yield parameters correlated with root biomass and P acquisition (Seguel et al., 2017). Here, when plants were grown under Pi-sufficient conditions, no differences in P acquisition and biomass production/partitioning were observed, highlighting that the differences further discussed are specifically related to contrasting responses to Pi limitation. In this sense, our results are in agreement with the previous study in plants grown in an acidic high Pi-fixing soil (Seguel et al., 2017). We show that Crac also presents a higher Pi accumulation in shoots and roots when subjected to Pi deprivation (Table 1). Alterations in shoot and root growth and/or morphology are the most spread plant adaptations to Pi-starvation, affecting the root:shoot ratio (Chien et al., 2018; Haling et al., 2016). Interestingly, under these

conditions, Crac showed smaller losses on shoot biomass production and greater increments in root growth, giving rise to higher root:shoot ratios, which correlated with total P acquisition in all experiments. In addition to greater root:shoot ratios, Crac showed larger and thinner roots than Tukan (Fig. S7 and S11). Altogether, these phenotypic differences would allow Crac to have a higher soil exploration capacity in search of Pi patches under limiting conditions.

5.4.2 Improved PAE is associated to higher expression of *PHT1* Pi transporters

Several studies correlate higher Pi accumulation and, in most cases, plant growth with higher expression of Pi transporters of the *PHT1* family (Ham et al., 2018; Liu et al., 2013; Wang et al., 2013). Recently, it has been shown that under Pi-starvation, transcript levels of the gene *TaPht1;2* were the most abundant of all the *PHT1* transporters described in wheat (Grün et al., 2018). This transporter is the orthologue of the rice *OsPht1;2*, which is also highly expressed in Pi-deprived roots, and it is characterized as a low-affinity Pi transporter, mainly involved internal in Pi translocation (Ai et al., 2009). The other Pi transporter analysed was *TaPht1;10,* which expression levels were lower (about 25-fold) than those of *TaPht1;2*. *TaPht1;10* and its orthologues in rice - *OsPht1;9* and *OsPht1.10* - are considered as high-affinity Pi transporters, and are mainly induced at long-term under Pi-starvation (Ai et al., 2009; Grün et al., 2018). As expected, both genes were highly induced by Pi deficiency in both cultivars, confirming their role in Pi uptake and distribution under nutritional stress. In agreement with this, it has been shown that transgenic *Nicotiana tabacum* plants overexpressing a *Pht1;2* gene displayed higher Pi content and better growth than the corresponding wild-type (Cao et al., 2018). These plants also showed enhanced Pi in the xylem sap, pointing out that this transporter is involved in

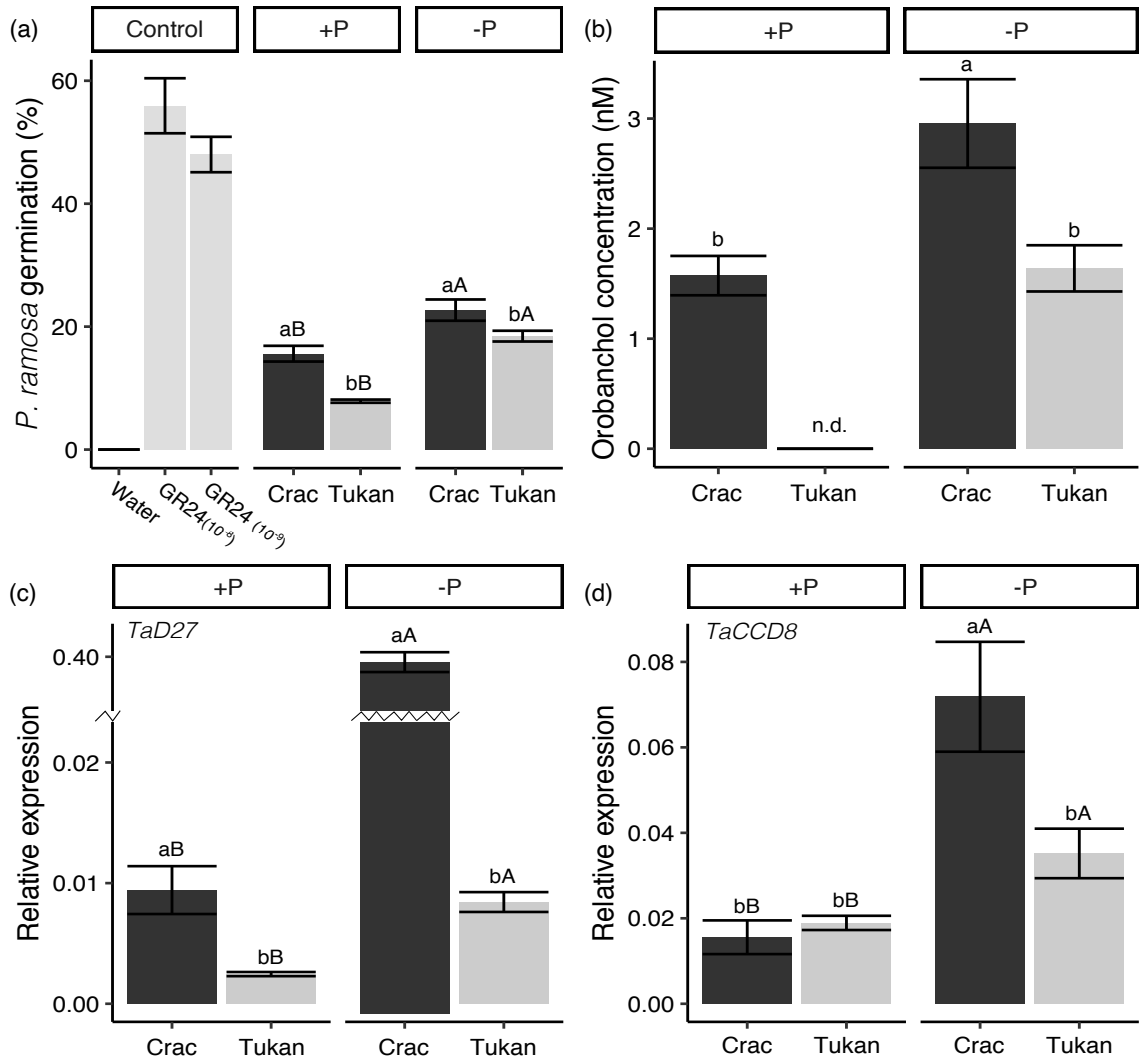

Figure 7. Analysis of SL levels in roots of Crac and Tukan plants grown in nutrient solution with Pi (+P; 150 µM) and without Pi for the last week (-P) harvested at Zadoks growth stage 23 (Zadoks et al., 1974). (a) Germination of *P. ramosa* seeds induced by extracts from Crac (dark bars) and Tukan (light bars). (b) Orobanchol levels in root extracts determined by UHPLC-MS-MS. Data represent the means of three independent replicates (±SE). Different letters indicate significant differences between means, as determined by Tukey LSD test analysis. n.d, non-detected. Expression levels of two SL biosynthesis genes: (c) *TaD27*; (d) *TaCCD8*. Bars represent the means of five independent replicates (±SE). Small letters indicate differences between genotypes in the same condition and capital letters indicate differences within the same genotypes under normal and deficient Pi conditions, as determined by Student's t test (P<0.05).

root: shoot Pi translocation. No differences in gene expression were observed for *TaPHO1*, component of the other family of Pi transporters and involved in Pi xylem loading (Franco-

Zorrilla et al., 2007). However, this was not surprising since it was previously shown that this transporter is regulated post-transcriptionally by the action of *PHO2* (Fig. 1) (Huang et al., 2013; Lin et al., 2008). Noticeably, basal levels of the two genes encoding *PHT1* transporters under sufficient Pi conditions were higher in the most efficient cultivar Crac, and the final levels under Pi-starvation were much higher than in Tukan (Fig. 4). In this sense, these differences could be related to the internal SL levels, which are directly linked to *PHT1* expression and other PSI genes in Arabidopsis (Ito et al., 2015). These results, together with the enhanced root system of Crac, indicate that this cultivar is better suited to readily response to this type of nutritional stress by an improved Pi uptake and root: shoot translocation capacity.

5.4.3 P signalling and homeostasis, and its relationship with SLs

It is generally accepted that *PHO2* activity is rapidly reduced under Pi deficiency due to high induction of miR399 in the first hours of stress, which overcomes protection by *IPS1* (Ajmera et al., 2018). However, there are only few studies evaluating the effect of long-term Pi deprivation in the regulation of this pathway. In a time-course study in rice, Ajmera et al. (2018) found that the number of *IPS1* transcripts increased slowly, but stronger than those of miR399, leading to a relative increase of *PHO2* levels after one week of stress. A similar pattern was observed in barley plants after 16 days of Pi-starvation, where cultivars showing high levels of *HvIPS2* matched with those with higher *HvPHO2* expression (Huang et al., 2011). In the present study, an induction of tae-miR399b was observed after one week of Pi deficiency. However, the total number of transcripts at this time point was much lower than those of *TaIPS1* (more than 1000 times), leading to a complete sequestration of tae-miR399 transcripts. This blockage would explain the high induction

of *TaPHO2* observed in our system (Fig. 5). Accordingly, *A. thaliana IPS1*-overexpressing lines also presented a enhanced *PHO2* accumulation due to higher *IPS1*-mediated miR399 sequestration (Franco-Zorrilla et al., 2007). Therefore, it seems that the regulation of P signalling and homeostasis by the triad *IPS1*-miR399-*PHO2* is dynamic, showing a different regulation over time. Based on the responses observed in wheat seedlings and other model plants at different developmental stages (Ajmera et al., 2018; Huang et al., 2011; Ouyang et al., 2016), we propose a model to explain the behaviour of these three regulators during early and late responses upon Pi-starvation (Fig. 8). According to this, during the first hours of stress there might be a rapid induction of miR399 levels, which mediates the cleavage of *PHO2* transcripts, probably to increase the relative amount of *PHT1* members to promote Pi uptake from the soil, with the corresponding translocation to the shoots. In case that Pi limitation continues over time, transcripts of *IPS1* would increase highly to lock miR399, and probably to exert other regulatory functions as well, with the concomitant increase in *PHO2* levels. This increase will trigger late Pi responses related to the improvement of Pi uptake and modification of root morphology, probably to search for new Pi 'hotspots', among others. In agreement with this hypothesis, higher *PHO2* levels under Pi-starvation in barley correlated with a higher root: shoot ratio (Huang et al., 2011). Conversely, wheat plants blocked at *TaPHO2* showed a lower root: shoot ratio (Ouyang et al., 2016). The initial down-regulation of *PHO2* proposed in our model was not observed in the time-course experiment, probably because this regulation may occur within hours after Pi deprivation at this developmental stage. On the other hand, the relationship *IPS1*-miR399 did not fully explain the variation on *PHO2* levels, which was

in accordance with the responses observed in rice (Ajmera et al., 2018). We suggest two possibilities to

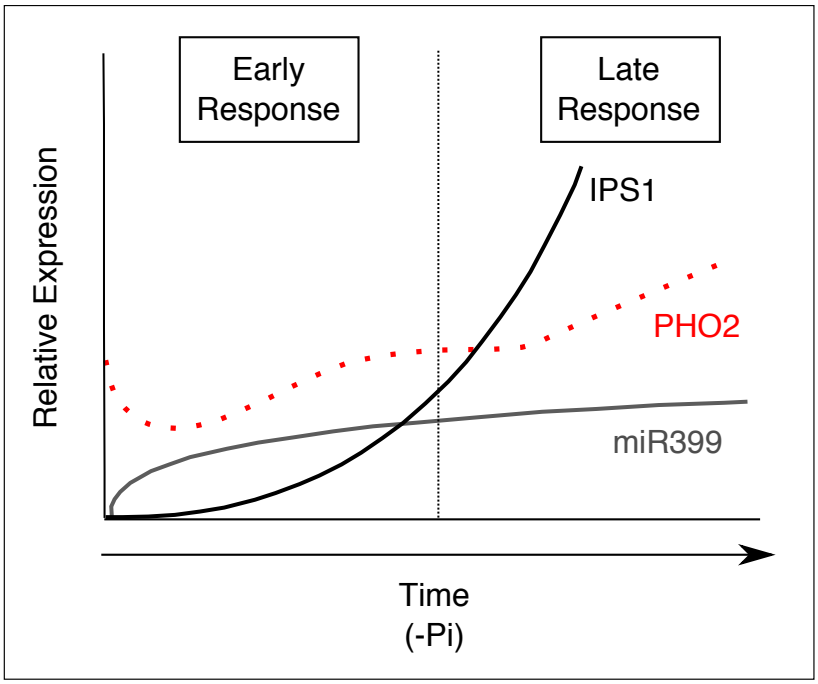

Figure 8. Proposed model for the regulation of P signalling and homeostasis by the module *IPS1*–miR399–*PHO2*. At early stages of Pi starvation, the high miR399 induction favours *PHO2* transcript degradation and triggers early phosphate starvation responses (PSRs). As Pi starvation follows, the miR399:*IPS1* ratio diminishes due to sustained high expression of *IPS1*, increasing *PHO2* degradation, and initiating late PSRs. Black and grey lines represent *IPS1* and miR399 expression, respectively, and the dotted line represent *PHO2* expression.

explain this fact: i) there must be more genes involved in this complex regulation; and/or ii) when *IPS1* transcripts reach a certain level, they are sufficient to sequester miR399. Thus, further increase of these transcripts would not affect *PHO2* degradation. Further studies are required to fully decipher how this signalling and homeostasis pathway works, especially at longer periods of Pi-starvation and at different plant developmental stages.

As for the *TaPht1;2* and *TaPht1;10* transporters, the levels of the three regulators *IPS1*-miR399b-*PHO2* under Pi limitation were much higher in the cultivar Crac with enhanced PAE than in the less efficient cultivar Tukan. However, the basal transcript levels of these genes were similar in P-sufficient conditions. Again, these results show that Crac is more efficient in the response to Pi deficiency, and that this enhanced efficiency is due to a better and faster Pi-starvation signalling and homeostasis regulation. In agreement with this, Crac showed increased basal levels of SLs than Tukan (Fig. 6). SLs, together with other phytohormones, are involved in the regulation of P homeostasis under Pi limitation by modulating P signalling-associated pathways and root growth (Chien et al., 2018; Waters et al., 2017). Therefore, it might be that the higher SL levels in Crac would act as a priming signal under Pi-starvation to boost plant responses to the stress. However, further research is needed to clarify the connexion between SLs and P signalling.

5.5 Conclusions

Understanding the physiological mechanisms of improved PAE and the genetic basis therein will allow breeders to select more P-efficient cultivars. This knowledge will help to diminish the use of P fertilizers in agriculture, thus reducing costs and alleviating the excessive consumption of this non-renewable resource. However, traits associated to PAE are complex and context dependent. In the present study, we suggest that the higher PAE of the commercial cultivar Crac might be related to the relationship SLs-P signalling and homeostasis through a fine-tune modulation of *PHO2* activity. This modulation, at long-term would relatively reduce shoot Pi loading, favouring the development of an enhanced root system and giving rise to an increased soil exploration capacity in search of Pi patches under limiting conditions and to increase P acquisition in high Pi-fixing soils.

Further research is needed to understand the relationship SLs-P signalling to develop new strategies for improved plant performance under P stress conditions.

Supplementary Data

Table S1. Primer sequences used in the qPCR analysis.

Table S2. Effects (%) of Pi-starvation on hydroponics grown plants.

Fig. S6. Example of the different experimental conditions.

Fig. S7. Main results of the effects of Pi-starvation in the rhizobox experiment

Fig. S8. Expression levels of *TaPHO2* alleles

Fig. S9. Phylogenetic analysis of *D27* sequences and amino acidic sequence

Fig. S10. Phylogenetic analysis of partial *CCD8* sequences and amino acidic sequence

Fig. S11. Example of Crac and Tukan root systems.

Acknowledgements

This work was supported by funding from FONDECYT 11160385 and 1170264 grant from Comisión Nacional de Investigación Científica y Tecnológica (CONICYT-Chile), CONICYT DOCTORADO NACIONAL 2016 (21161474), CONICYT + PAI/ CONCURSO NACIONAL TESIS DE DOCTORADO EN EL SECTOR PRODUCTIVO 2017 (T7817120011), CONICYT/FONDAP/15130015, and AGL2015-64990-C2-1R (MINECO-Spain). We also acknowledge Dr. Koichi Yoneyama and Dr. Xiaoan Xie for kindly provide the natural strigolactones used to set up their analytical quantification, Dr. Gustavo Curaqueo for the analysis on the root scanning system, Campex Baer® for their

agronomical support, BIOREN-UFRO for providing their facilities, and Dr. María J. Pozo and Dr. Francisco Macías for critical reading of the manuscript and support.

CHAPTER VI

General discussion and concluding remarks

6.1 General discussion

As mentioned in the previous chapters of this Doctoral Thesis, phosphorus (P) is an essential macronutrient for plant development, playing a crucial role in its metabolism. However, more than 50% of the soils worldwide, including many agricultural ones, are limiting in this nutrient (MacDonald et al., 2011; Syers et al., 2008). Thus, enhancement of P acquisition efficiency (PAE) of major crops – as wheat (*Triticum aestivum* L.) – represents an emerging opportunity to improve agricultural sustainability at national scale, but also worldwide. In this context, we studied the P acquisition-related root traits (*i.e*. root morphology, organic acid and phosphatase exudation, and root mycorrhizal colonization) of two wheat cultivars with contrasting PAE to generate a trait-based background which could be incorporated on breeding programs oriented to the generation of more efficient cultivars. The effects of each major trait on P acquisition are discussed below.

It is generally accepted that AM symbiosis improves plant' P accumulation, however, its effects depend on plant genotype, fungal species and environmental conditions, being the mechanisms that dictate the magnitude of the responses unclear (Feddermann et al., 2010; Munkvold et al., 2004; Smith and Smith, 2015). In this Doctoral Thesis, we assessed whether the effects of AM symbiosis differed at cultivar level (Chapter III), and if cultivars showing differential PAE possessed differences in mycorrhizal colonization and community composition under soil conditions that could confer higher P accumulation (Chapter IV).

According to our results, plants response to AM symbiosis (including P accumulation) depends on both plant genotype and AM fungal isolate. Accordingly,

symbiosis of the both wheat cultivars by *Claroideoglomus claroideum* highly increased P accumulation, while colonization by *Rhizophagus intraradices* significantly increased calcium (Ca) accumulation. In agreement with our data, lettuce plants colonized by the same *C. claroideum* isolate also showed increased P acquisition, while acquisition of other nutrients did not change or were even reduced (Santander et al., 2019). Interestingly, this higher nutrient acquisition showed no significant correlation with mycorrhizal colonization, hyphal length, and with changes on root morphology and exudation pattern. In this context, Jansa et al. (2005) and Thonar et al. (2011) also found no correlation between mycorrhizal parameters and P acquisition. The authors suggested that the differences observed in their experiments were related to differences in hyphal branching pattern and on hyphal efficiency in acquiring P. Notwithstanding, systemic effects of AM symbiosis in the expression of plant' specific nutrient transporters have also been reported, as the induction of the expression of some Pi transporters and the consequent increase in P accumulation - regardless of the colonization extent - in tomato plants (Poulsen et al., 2005). However, further studies are necessary to elucidate whether differences in hyphal traits and/or AM induction of specific transporters are the main drivers of such responses.

In a second experiment, we assessed whether the wheat cultivars (contrasting in PAE) presented differences in their mycorrhizal colonization, hyphal length density, and on community composition under P-sufficient and P-limiting conditions. There, we observed no significant differences in mycorrhizal colonization and hyphal length density between the cultivars, except for a significantly higher colonization level on Crac plants subjected to P-limitation. Indeed, mycorrhizal colonization showed a partial correlation with P acquisition under that condition. The latter can be related to higher colonization

frequency of AM species that stimulate Pi transporters expression or with higher hyphal efficiency in acquiring P (Munkvold et al., 2004; Poulsen et al., 2005). The high colonization levels observed in the efficient cultivar is probably related to its higher strigolactone (SL) production under P-deficiency. SLs are phytohormones whose production and exudation are stimulated by P starvation, modulating plant responses under this stress condition (Kumar et al., 2015; López-Ráez et al., 2008). SLs also function as *ex-planta* signaling molecules in the rhizosphere, promoting AM spore germination and initial hyphal branching, thereby enhancing the probability of contact with roots (Liao et al., 2018). In this context, plant mutants with lower SL production showed reduced AM colonization, while synthetic SL application rescued the phenotype (Gomez-Roldan et al., 2008).

As previously shown, AM symbiont identity significantly impact P accumulation (Jansa et al., 2008; Lendenmann et al., 2011). Here, we found an average of four AM species colonizing the roots of the wheat cultivars, which agrees with the average species richness found to be colonizing crop plants at field conditions (Öpik et al., 2006). However, no differences were observed in overall community composition between the cultivars and P treatments, except for a higher presence of an *Archaeospora* species in Tukan plants. In agreement with our results, Mao et al. (2014) by analyzing AM community in twenty-one wheat hybrids also found no differences in species richness among cultivars, but a preferential association between cultivars sharing common traits and some AM genus, with no broader impact on yield. Unfortunately, the effects of *Archaeospora* colonization on plant traits have been poorly studied (Bennett and Bever, 2007; Helgason et al., 2002); therefore no clear correlation with P acquisition responses can be further inferred.

Contrary to the differences found in the mycorrhizal profile of these cultivars – which were mainly observed under P-deficient conditions, the P-acquisition efficient cultivar (Crac) presented higher root length, root surface area, number of forks, and a smaller average diameter compared to the less-efficient cultivar (Tukan) regardless the P condition (deficient and sufficient). Remarkably, all of these parameters were highly correlated with P accumulation in the soil experiments. In this context, root morphological traits have been shown to be the main contributors for P acquisition in wheat plants (Nobile et al., 2019; Wen et al., 2019), as well as in other plant species (Haling et al., 2016; Hammond et al., 2009).

Great efforts have been made to unravel the genetic background of improved root traits; however, only few genes have been functionally characterized (Wissuwa et al., 2016). Here, the efficient genotype presented a faster and higher induction of the P-signaling and homeostasis pathway. In this context, the differential PHO2 activity have been shown to affect root growth and P accumulation both in barley and wheat plants (Huang et al., 2011; Ouyang et al., 2016). In addition, we also proposed a key role of SLs on this differential modulation, as the cultivars under study presented contrasting SL production and mutants with low SL production showed alterations in the regulation of this pathway, which was recovered upon exogenous SL application (Chien et al., 2018; Waters et al., 2017).

Even though root biomass and growth are generally highly correlated with P acquisition, another important aspect to be considered is the root efficiency in acquiring P (mg P cm^{-2} of root system; Mori et al. 2016). In this context, root efficiency can be improved either by increasing the number of Pi transporters and or by producing

transporters with high affinity (Ai et al., 2009; Zhou et al., 2008). Here, we observed a higher root efficiency in the efficient genotype, which was directly correlated with a higher expression of *TaPht1;2* and *TaPht1;10* under P-limitation. Again, this differential expression could be related to the higher endogenous SL levels in this cultivar, as PHT1 expression was found to be affected by SLs in the model plant *Arabidopsis* (Ito et al., 2015).

On the other hand, exudation of acid phosphatases (P-ase) and low molecular weight organic acid anions (LMWOAAs) were in general higher in the less-efficient genotype. Nevertheless, despite the potential higher P availability of this cultivar, negative correlations were found between P-ase activity and P accumulation. The latter is probably related to their reduced Pi transporter expression (*i.e.* a lower root efficiency), limiting P acquisition rate despite presenting relatively higher availability in the soil.

Due to its low mobility and high fixation to soil constituents, P usually presents a "patchy" distribution. Therefore, roots need to be able of sensing this heterogeneity to allocate their resources efficiently (Shu et al., 2007). This fact has great relevance in agricultural systems, where nutrients tend to be concentrated in first centimeters or localized in bands (Li et al., 2014). Here, we analyzed the exudation of acid P-ase and LMWOAAs in two different root zones, namely top roots and bottom roots. Interestingly, the efficient cultivar showed a higher oxalate exudation in the top roots under fertilization, which was highly correlated with P acquisition under that condition. Besides its direct effects on increasing P solubilization, low concentrations of LMWOAAs were shown to affect microbial activity, to promote priming effect, and thus, nutrient dynamics - including P mineralization (Fenesi et al., 2019). Unfortunately, root exudation at different root zones has been poorly analyzed, being most of the studies focused on Proteaceae plants, which

possess a very particular exudation behavior (Dessureault-Rompré et al., 2007; Peñaloza et al., 2002; Spohn and Kuzyakov, 2013a). The few studies performed on non-Proteaceae plants possessed different objectives and experimental designs (Neumann and Römheld, 1999; Nuruzzaman et al., 2006), making it difficult to compare results between experiments. In this context, Hajiboland et al. (2005) found an increase in malate exudation with increasing distance from root tips in a Zn-efficient rice cultivar. However, no inference to nutrient acquisition neither to growth responses was reported.

6.2 Concluding remarks and future directions

A deeper understanding of the mechanisms related to improved P acquisition and the genetic basis therein would allow breeders to select more P-efficient cultivars. This knowledge would help to diminish the use of P fertilizers in agriculture and also to optimize its application efficiency. Here, we observed a differential response to AM symbiosis between the wheat cultivars and the AM species under study, in which high PAE responses were obtained when inoculated by *C. claroideum*, regardless of the plant genotype. Thus, the first hypothesis - the growth and nutritional responses (especially on P) of wheat cultivars colonized by AM fungi will depend on species-specifics interactions between the host plant genotype and the AM ecotype - was accepted. Accordingly, the selection of "optimal" AM fungal strains (context-dependent), their establishment, and maintenance under field conditions arise as an opportunity to improve the efficiency and productivity of wheat cropping under P-limiting conditions. In this way, even though great advances have been achieved by bio-inoculant industry in the last decades, extensive inoculation of annual crops is neither technically nor economically feasible with current technology; therefore, research on how management practices – like crop rotation, tillage system, intercropping,

and others – influence AM community composition and function are necessary to fully "benefit" from the symbiosis.

The second hypothesis - higher P acquisition under P-deficient condition will be related either to an enhanced root growth, higher organic acid and phosphatase exudation, or increased root mycorrhizal colonization. - was also accepted considering that root morphological parameters were much more developed in the efficient cultivar, and that it showed a high correlation with P acquisition, both under deficient and sufficient P conditions. In addition, even though no broad differences in AM community composition and hyphal length density were observed, mycorrhizal colonization extent showed a partial correlation with P acquisition under P-limiting conditions. More detailed studies should be carried to analyze the differences in the frequency of colonization of each AM species and its effects on P accumulation, especially under deficient conditions. In addition, as previously mentioned in Chapter IV of this Doctoral Thesis, the relative contribution of each P-acquisition root trait will depend both on plant genotype and environmental conditions, therefore, it is important to assess whether the different P acquisition mechanisms shows similar behavior under a wider range of conditions and at different phenological stages.

Based on the SL production profiles of the cultivars under study and the current information available in the literature, we proposed a major role of this phytohormone in the improved Pi starvation responses observed in the efficient cultivar, especially in the P homeostasis and signaling pathway and on the expression of Pi transporters. Thus, the third hypothesis - strigolactone production will be higher in the P-efficient cultivar, affecting Pi-starvation responses and improving P acquisition - was accepted. This improved

modulation would increase relative P levels in the root system, allowing to maintain a higher root growth under P limiting conditions to search for new P "hotspots" in the soil. As before, further research is needed to assess the basis of higher SL production (*e.g.*, differences in promoters, gene sequence, duplication/loss of function, among others) and whether improved SL modulation is present in other wheat cultivars and their correlation to root morphology and efficiency in order to fully understand the SLs–P signaling relationship and to develop new strategies for improved plant performance under P stress conditions.

Even though this Doctoral Thesis focused on the improved root traits of the efficient cultivar under P limiting conditions, the higher oxalate exudation at the top roots of the efficient cultivar could comprehend an important mechanism to increase fertilizer recovery in high-input systems. In addition, the less-efficient genotype also presented interesting traits (*i.e.*, overall higher exudation rate) that could increase P efficiency by utilizing part of the organic- and residual-P present in the soils if combined with a more efficient root system. The less-efficient genotype also showed a preferential association with *Archaeospora* species. However, the outcomes of this preferential association must be addressed. Also, whether this association occurs over a wider range of conditions (*i.e.* different soil conditions, propagule density, etc.) must be taken into account to be considered as a functional trait.

Overall, after analyzing the P-acquisition related root traits at different scales (*i.e.* morphological, physiological, and molecular), this Doctoral Thesis provides new insights regarding plant Pi starvation responses using wheat cultivars with contrasting responses to P fertilization at field conditions. It also provides novel precedents to local breeders with

potential application focused on increasing sustainability in wheat cropping both in low- and high-input systems and raises new questions whether other P acquisitions traits could confer higher P acquisition in local wheat cultivars growing under P-limiting conditions rather than those found here, and the outcomes in plant performance under P-limiting conditions if different advantageous traits are combined (*e.g.*, higher exudation of Tukan plants with higher root growth and efficiency of Crac plants).

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Supplementary data

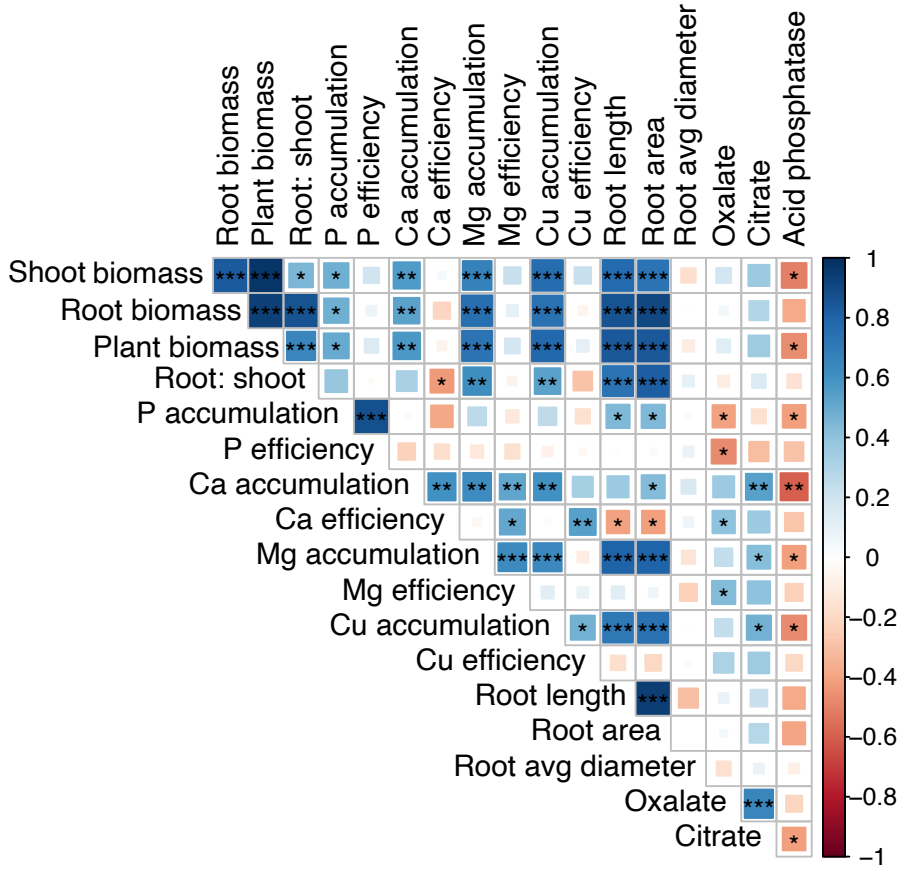


Figure S1. Pearson correlation coefficients between the main variables under study of Crac and Tukan plants inoculated with *R. intraradices* (RI) and *C. claroideum* (CC) or non-inoculated (NI). * $P< 0.05$; ** $P < 0.01$; *** $P< 0.001$.

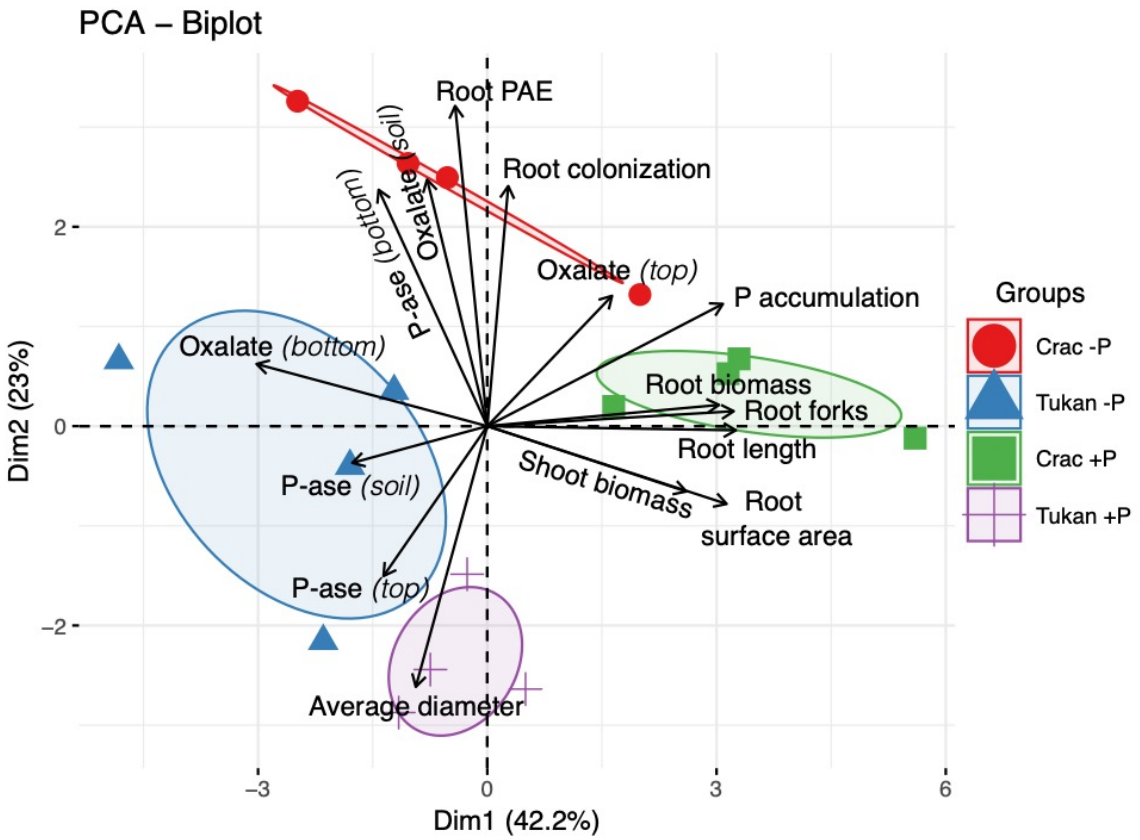


Figure S2. Principal Component Analysis reflecting the formation of groups clustering according to experimental variables assessed in the rhizobox experiment. A trade-off between root morphological traits, shoot biomass, and P acquisition with P-ase activity and oxalate on bottom roots was seen along PC1 (42.2% explained variance), and was, as well, related to main differences between wheat cultivars. A second trade-off could also be seen along PC2 (23% variance explained) where P efficiency and root mycorrhizal colonization were faced with root average diameter. In general, PCA showed a clear separation between Crac and Tukan plants, in where Crac plants showed higher P accumulation and root morphological parameters regardless the P treatment.

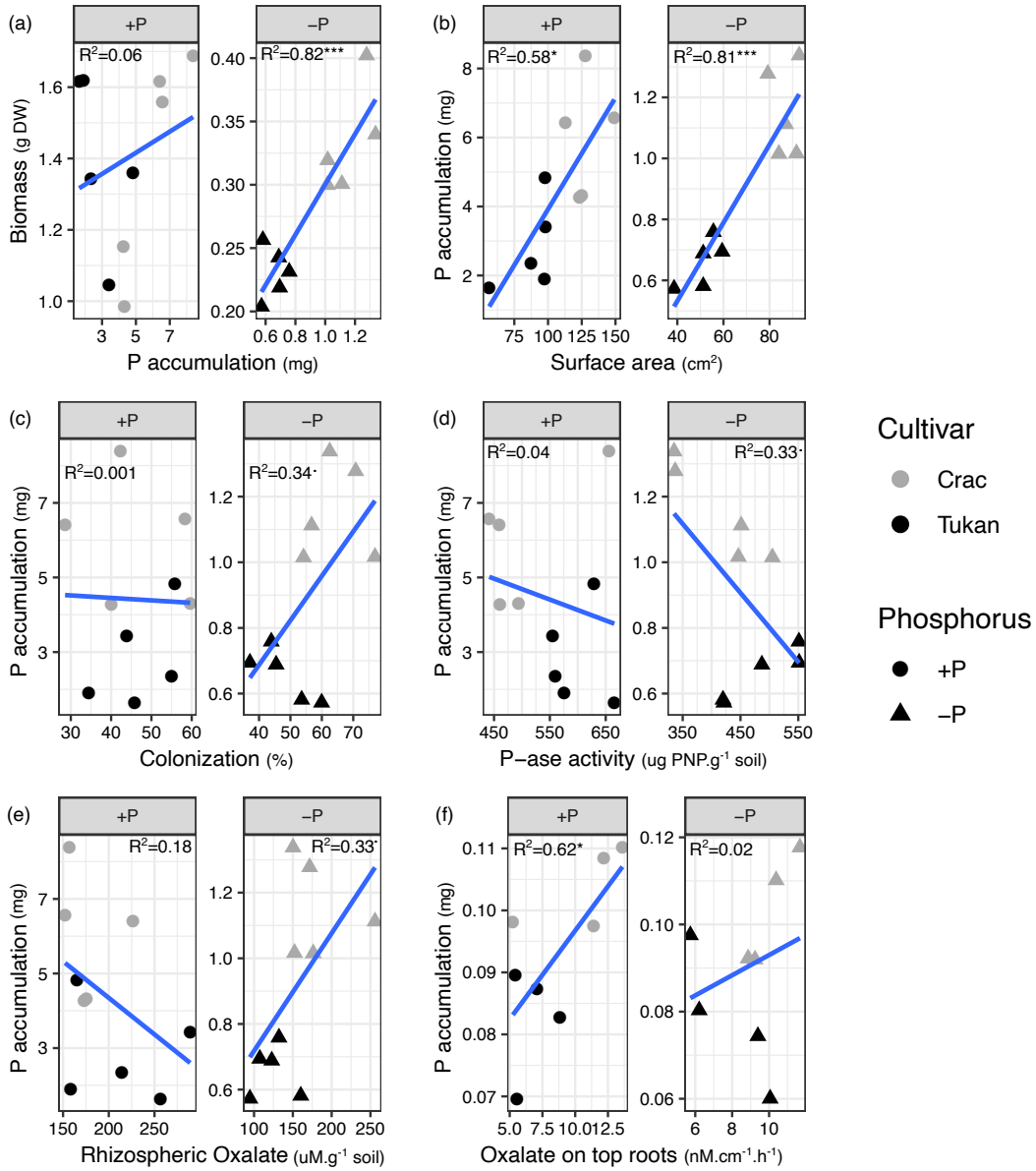


Figure S3. Regression analysis between the main variables under study. ·P<0.1, *P<0.05, ***P<0.001.

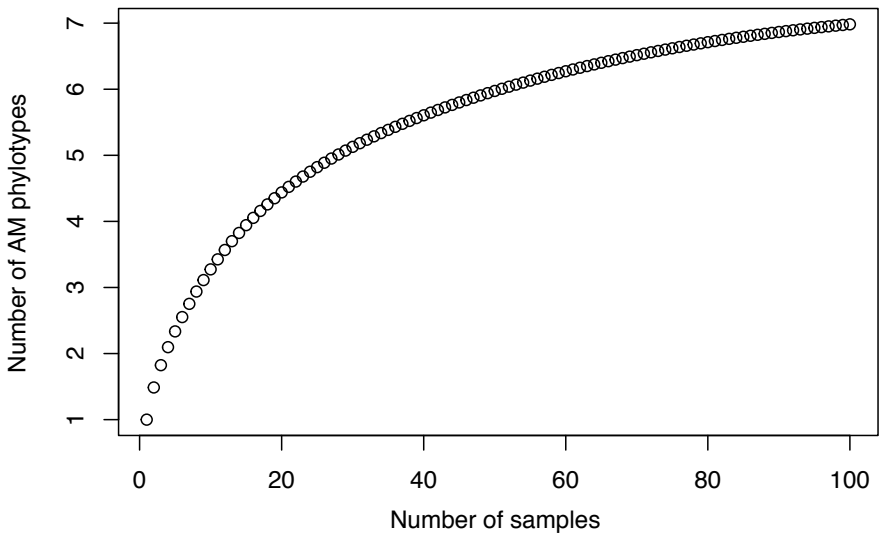


Figure S4. Sampling effort curve for AM phylotypes detected colonizing roots of two wheat cultivars grown in an Andisol either amended or not with an equivalent of 44 kg P ha^{-1} of Pi fertilizer.

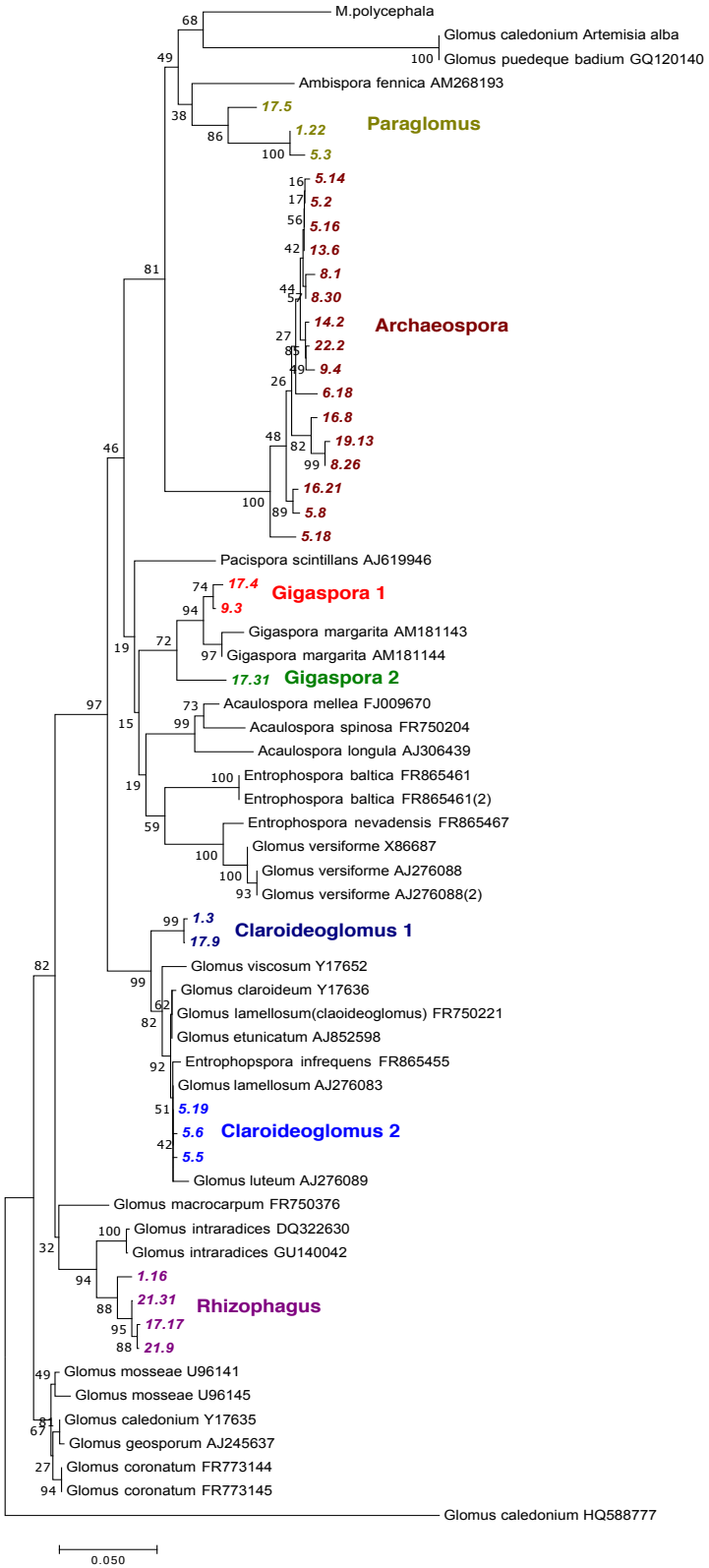


Figure S5. Phylogenetic analysis of representative sequences of each fragment pattern obtained by RFLP analysis. The OTUs found in this work are represented by different colors.

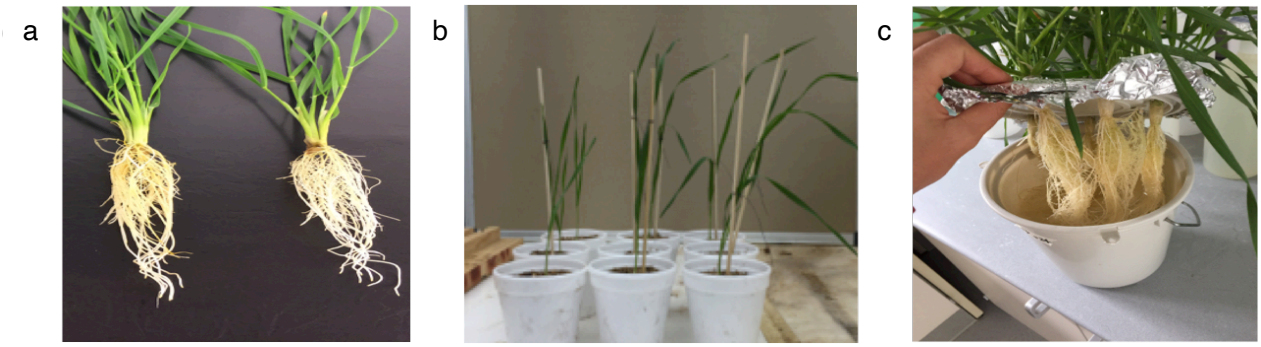


Fig. S6. Growth performance wheat plants under different experimental conditions. (a) Hydroponically and (b) pot grown plants for phenotyping experiments; and (c) hydroponically grown plants for molecular analysis.

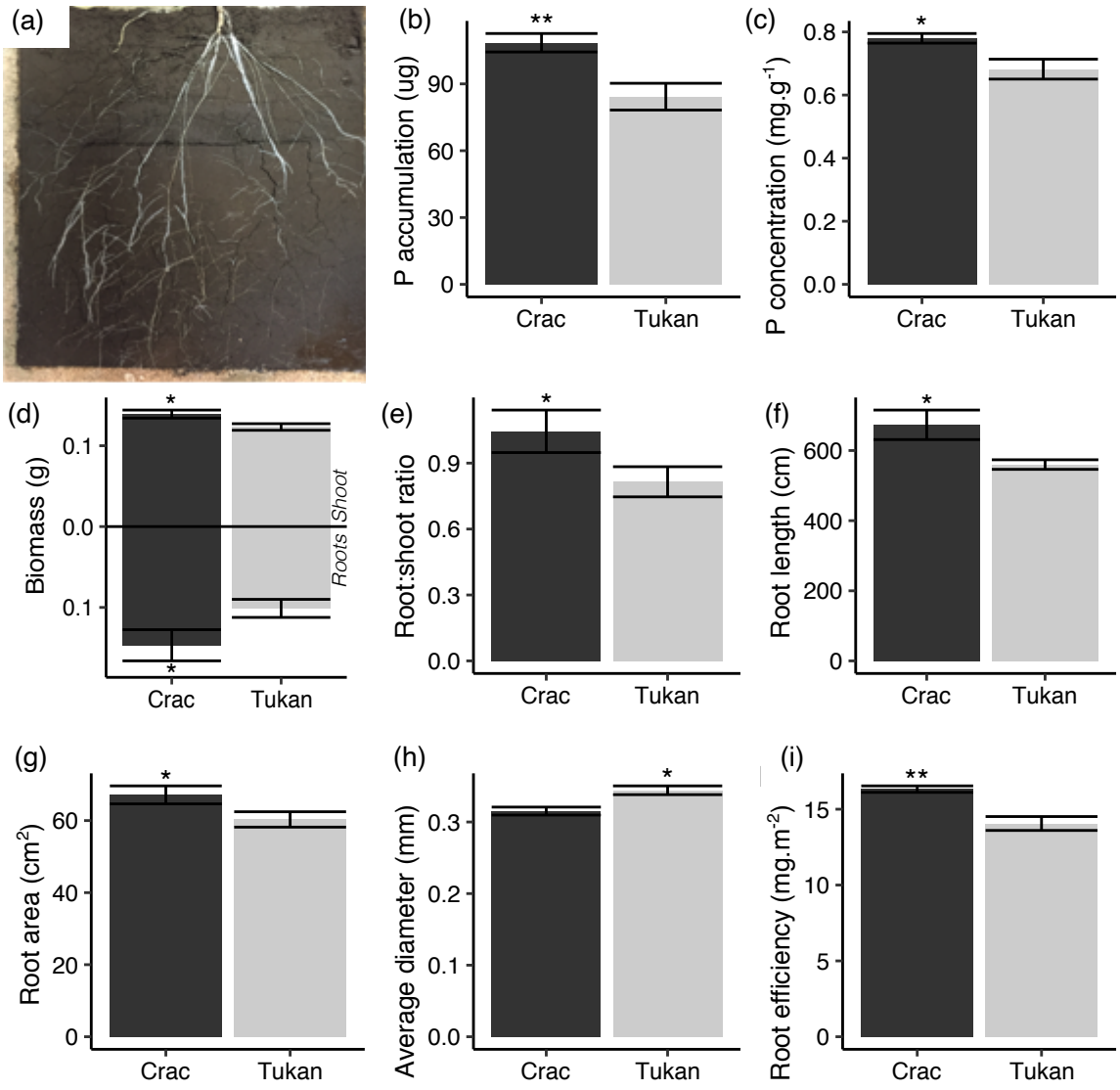


Fig. S7. Rhizobox experiment to analyze the effect of Pi starvation in Crac (dark bars) and Tukan (light bars). Plants were grown for 8 weeks in a high P-fixing acidic soil without Pi addition. (a) Visual representation of rhizobox growth system; (b) Shoot Pi accumulation; (c) Shoot Pi concentration; (d) Shoot and root biomass production; (e) Root: shoot ratio; (f) Total root length; (g) Root area; (h) Average root diameter; and (i) Root efficiency in Shoot Pi accumulation. Data represent the means of five independent replicates (±SE). Asterisks indicate the significance of differences between the genotypes, as determined by Student's t test analysis: * P<0.05, ** P<0.01, *** P<0.001.

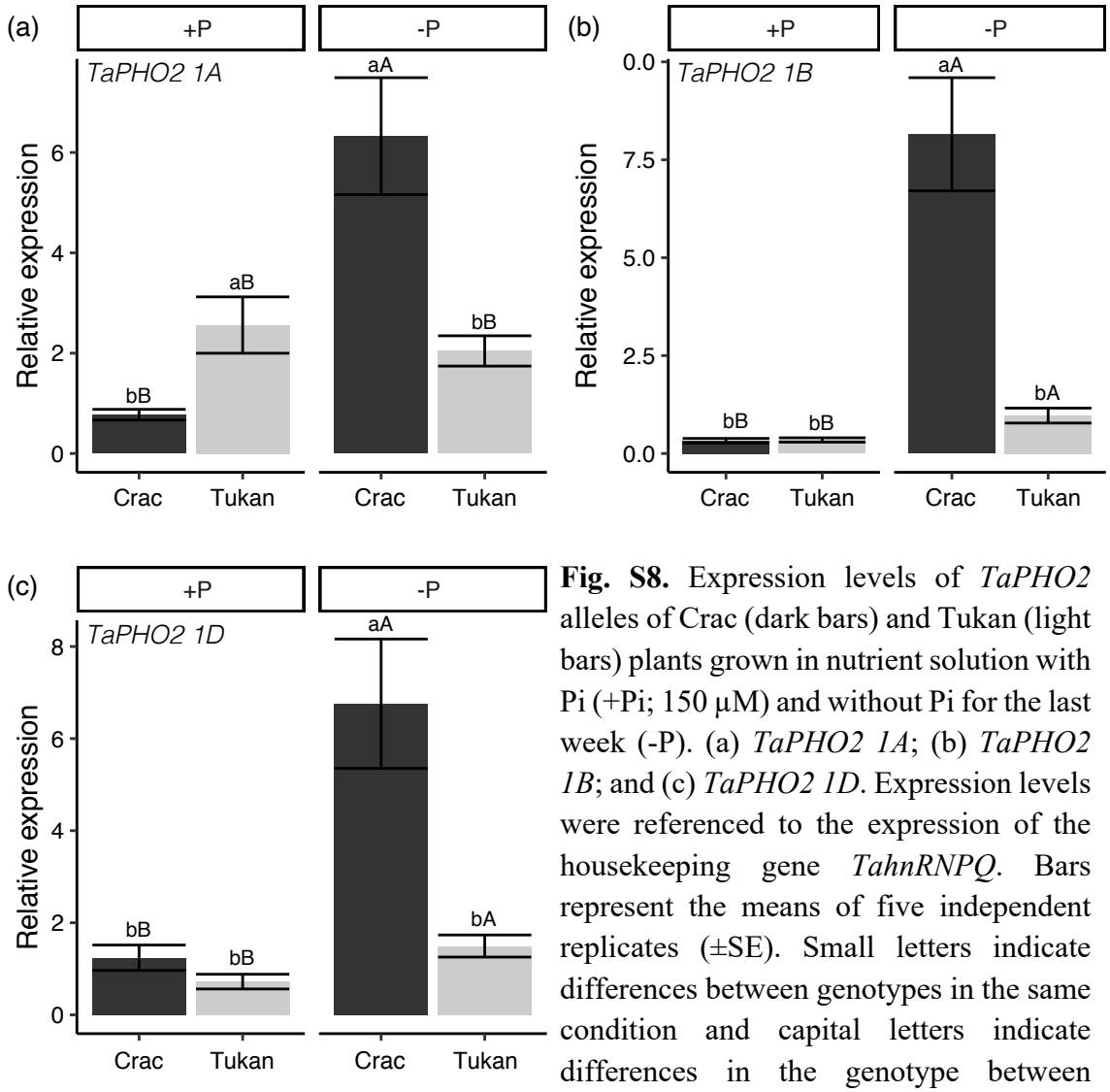
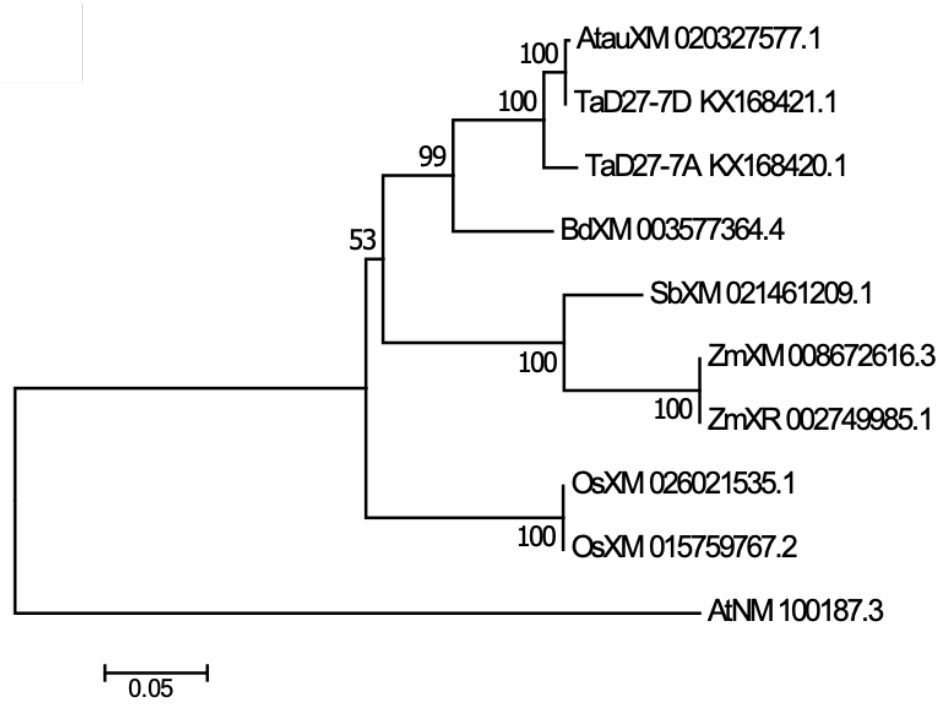


Fig. S8. Expression levels of *TaPHO2* alleles of Crac (dark bars) and Tukan (light bars) plants grown in nutrient solution with Pi (+Pi; 150 µM) and without Pi for the last week (-P). (a) *TaPHO2 1A*; (b) *TaPHO2 1B*; and (c) *TaPHO2 1D*. Expression levels were referenced to the expression of the housekeeping gene *TahnRNPQ*. Bars represent the means of five independent replicates (±SE). Small letters indicate differences between genotypes in the same condition and capital letters indicate differences in the genotype between conditions, as determined by Student's t test (P<0.05).

(a)

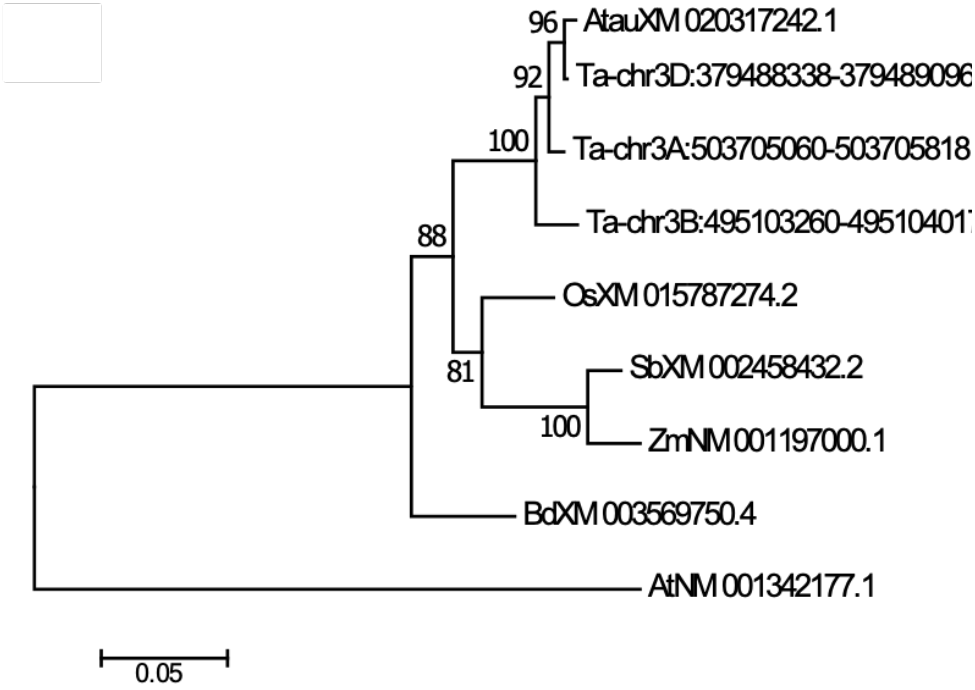


(b)

A I N **Met** E A T A L V L L P H S H S G L T A R A P P C V G G S S A **Met** K F
S Y V R R I K R S S T V R G V **Met** A R P Q E A T L A R V P A P A P T R P V
R E T A A A T T T T K T V Y H D T W F D N L A I G Y L S R K L Q E A S G I K
G K H G Y Q G L I E A A V A I S R I F R L D T Q C E V V A G A L E F
A **Met** P S Y I V T **Met** I K V **Met** **Met** P P S K F S R E Y F A A F T T I F F F
W L V G P C E V R E S E V D G T R E K N V V Y I P K C R F L E S T N C V
G **Met** C T N L C K I P S Q K F **Met** Q D S L G V S V Y **Met** S P N F E
D **Met** S C E **Met** I F G Q Q P P E D D P A L K Q P C F S T K C I A K Q D Y
G V N C **Stop**

Fig. S9. Phylogenetic analysis of *D27* sequences from *Aegilops tauschii* (Atau), *Arabidopsis thaliana* (At), *Brachypodium distachyon* (Bd), *Oryza sativa* (Os), *Sorghum bicolor* (Sb), *Zea mays* (Zm), and *Triticum aestivum* (A); and Amino acidic sequence of *TaD27* (B). In red sequence encoding for DUF4033 superfamily. The tree was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches (Fellsenstein, 2009). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

(a)



(b)

ALLQDGTYLRNGPGLWDLGDYGFRHLFDGYATLVRV
SFRDGRAVGAHRQIESEAYKAARRHGKVCYREFSEV
PKPDSFKSFVGQLASLFSGNSLTDNSNTGVVRLGDG
RVLCLTETVKGSIVVDPDTLDTVSKFEYEDKLGGLIH
SAHPIVTDTEFWTLIPDLIRPGYVVARMetDAGSNERQ
FVGRVDCRGGPAPGWVHSFPVTENYVVVPEMetPLR
YCAANLLRAEPTPLYKFQWHLESGSYMetHVMetCKAS
GKI

Fig. S10. Phylogenetic analysis of partial *CCD8* sequences from *Aegilops tauschii* (Atau), *Arabidopsis thaliana* (At), *Brachypodium distachyon* (Bd), *Oryza sativa* (Os), *Sorghum bicolor* (Sb), *Zea mays* (Zm), and *Triticum aestivum* (a); and Partial amino acidic sequence of *TaCCD8* (b). In red sequence encoding for RPE65 superfamily. The tree was constructed as described in Figure S9.

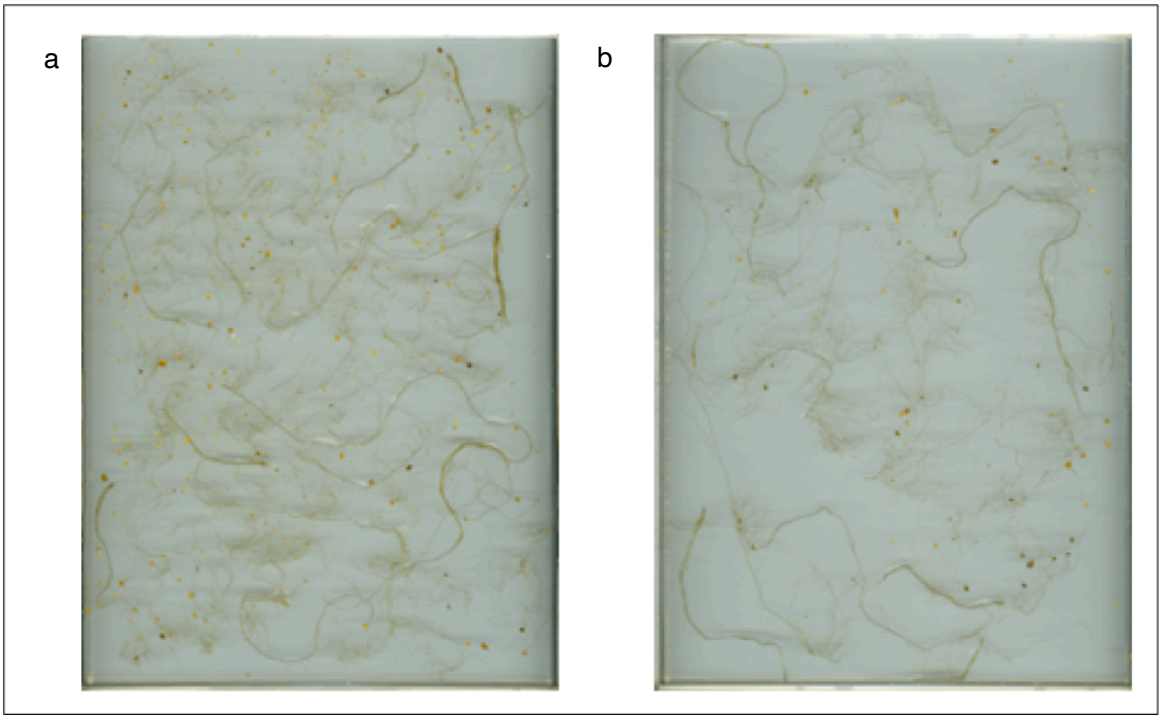


Fig. S11. Transparent tray containing the root systems of a single Crac (a) and Tukan (b) plant grown for 5 weeks in pots filled with sand/vermiculite and exposed to low Pi stress (10 µM). Root systems were cut before the analysis in order to minimize overlaps.

Table S1. Primer sequences used in the real-time qPCR analysis.

Gene	primer (5' → 3')	˚C	Reference
TaPht1;2a/b	CGACACCATTGCTCCGACTG TCAARCACACCAACMATGCACG	58	Grün et al. (2017)
TaPht1;10	CTAACTCTGACGCCCAAGAG CGGAACTGCTTATGCGTSG	58	Grün et al. (2017)
TaIPS1	CAGTACCAGCTGCATGCCTG CTAGCCAACGCCGGATCCA	58	Ouyang et al. (2016)
Tae-miR399	GGAGGCATGCATGTACTGATG GGCAATTCTCCTTTGGCACG	58	Ouyang et al. (2016)
TaPHO2	GGAGAAGAACTCCATCACGTACAACG GGCAAGTGAAGTGCTCCTTGACGA	54	Ouyang et al. (2016)
TaPHO1	GAGTGGCTACCACAAATTGAATC TATTTTTACATCCATGTCAAAGGTG	58	Ouyang et al. (2016)
TaD27	CATGTGAGGTCAGGGAATCTG TCATCTCGCAGCTCATGTCT	55	This work
TaCCD8	AAGGGCTCCATCGTCGTC CAGCGTCCAGAGGTCGGTGTC	58	This work
TaPHO2 1A	GTATAAGGATGATGGAATTGAAGTA CATTCTTAGTACTCTCATGGTGAT	55	Ouyang et al. (2016)
TaPHO2 1B	GGTTTAGCTTCAGTCCTGTCAG CAGCCTTTGAACAGCGGTC	58	Ouyang et al. (2016)
TaPHO2 1D	CTCGGCGGTGATCTCATTG AGGCGATCCCAGCTTCGC	58	Ouyang et al. (2016)
TahnrRNPQ	TCACCTTCGCCAAGCTCAGAACTA AGTTGAACTTGCCCGAAACATGCC	58	Grün et al. (2017)

Table S2. Effects of Pi starvation in Crac and Tukan grown in hydroponics. Plants were grown in Pi-sufficient conditions (200 μM) for 2 weeks, and then half of them subjected to Pi deprivation (10 μM) for 3 weeks. Values (%) of growth rate, root system architecture, and Pi acquisition were calculated as the difference between individual plants in both conditions. Asterisks indicate the significance of differences between the genotypes in the same condition, as determined by Student's t test analysis: * $P<0.05$, *** $P<0.001$.

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cv	Shoot Weight	Root Weight	Total Weight	Root: Shoot	Root Length	Root Area	Shoot P (mg)	Shoot P ($mg.g^{-1}$)	Root P (mg)	Root P ($mg.g^{-1}$)	Plant P (mg)	Plant P ($mg.g^{-1}$)
Crac	-23%	59%	-11%	128%	113%	98%	-46%	-25%	-34%	-49%	-46%	-36%
Tukan	-37%	31%	-26%	87%	80%	77%	-57%	-36%	-48%	-60%	-57%	-45%
p-value	*	*	***	*	*	*	*	n.s.	*	*	*	*