DOI: 10.1007/s10535-017-0713-z BIOLOGIA PLANTARUM 61 (3): 587-594, 2017

Response of *Arabidopsis thaliana* root growth to phosphorus and its relation to media chemical composition

M.L. STRIEDER[1,2]*, K.G. PINTO[1,2], C. BERTOLDI[1], A.de B. SCHNEIDER[1,3], and C.A. DELATORRE[1]**

Department of Crop Science, Federal University of Rio Grande do Sul, 91501-970, Porto Alegre, RS, Brazil[1]
Institute of Developmental Genetics, Heinrich Heine University, D-40225 Düsseldorf, Germany[2]
Department of Bioinformatics and Genomics, University of North Carolina, 9201 Charlotte, NC, USA[3]

Abstract

The interaction between phosphorus (P) and other media components alters root development and masks the plant response and thus limits the ability to correctly identify *P-deficiency response (pdr)* mutants. This study aims to assess changes in root development caused by different composition of growth media normally used in *Arabidopsis* research and to study their effects on *pdr*-mutant screening. Primary root growth of four genotypes was analyzed in media differing in P concentrations: half-strength Murashige and Skoog (½ MS) and Somerville and Ogren (SO). The effects of nitrogen source and Fe on root growth were investigated in each medium separately and in a mixture. We found that the primary root length of all genotypes grown on ½ MS was reduced in comparison with plants grown on SO medium. The mutant *pdr9* was the most sensitive in ½ MS, This mutant was also hypersensitive to Fe that intensified its sensitivity to ammonium. Ammonium increased the root inhibition caused by Fe also in wild-type plants. In conclusion, on the basis of our study we recommend to use SO medium, which ensures an efficient selection to screen for *pdr* mutants through root growth. Moreover, nitrogen sources in the media other than nitrate should be taken carefully.

Additional key words: ammonium sensitivity, iron hypersensitivity, nutrient interactions, phosphate deficiency, quiescent center identity.

Introduction

Abiotic stresses are the major constraint on crop development and productivity worldwide. Among abiotic stresses, nitrogen (N) and phosphorus (P) deficiency together with drought are the three main limiting factors to crop yield. The total amount of P in the soil is usually higher than that required for adequate plant growth. However, only a small fraction of P is available to plants, due to low mobility of P in the soil and high adsorption to organic matter, clay, and iron and aluminum oxyhydroxides. Moreover, P precipitates with calcium and magnesium in the soil, reducing its availability even further. During the course of evolution, plants have evolved different mechanisms to deal with P deficiency. One common adjustment is a shallower, more branched root system (Lambers *et al.* 2006, Péret *et al.* 2014). Besides the recent advances in P signaling, little is known about the mechanism of root architecture changes upon P deficiency. The crosstalk or "interference" caused by other factors has become evident and increases the response complexity. Several factors in the medium may affect the root development under P deficiency, such as pH changes (Svistoonoff *et al.* 2007), toxic content of some micronutrients, as Fe (Svistoonoff *et al.* 2007, Ward *et al.* 2008), arsenic (Abercrombie *et al.* 2008), and high content of sucrose (Hammond and White 2011). More subtle characteristics, like contaminants in the media solidifying agents, may also affect the interpretation of the P response (Jain *et al.* 2009). Interaction among macronutrients is also relevant, *e.g.*, modification in root growth response to interaction among cytokinin, N, and P was observed in the *phosphate deficiency response1 (pdr1)* mutant (Cerutti and Delatorre 2013). Therefore, the chemical composition of the media may interfere with the ability to identify and

Submitted 23 November 2015, *last revision* 18 October 2016, *accepted* 21 October 2016.
Abbreviations: MS - Murashige and Skoog; *pdr1* - *phosphate deficiency response1*; PHR1 - phosphate starvation response 1; QC - quiescent center; SO - Somerville and Ogren; WT - wild type.
Acknowledgments: This work was supported by the Brazilian National Council for Scientific and Technological Development (CNPq) Grants No. 473050/2006-4 and 470173/2010-6. MLS received a pre-doctoral fellowship (Proc. 140725/2006-7 and 201660/2007-5) and CAD received PQ-1D fellowship (Proc. 305122/2014-2) from CNPq.
* Present address: Embrapa Wheat, 99001-970 Passo Fundo, RS, Brazil
** Corresponding author; e-mail: cadtorre@ufrgs.br

characterize P mutants (Ward *et al.* 2008). Mutants are required for elucidation of the pathways that integrate the availability of nutrients and the biochemical, physiological, and morphological responses.

Arabidopsis thaliana, the main model plant for biological studies, and its mutants have been the object of choice for dissection of P response due to their simple root anatomy. Other important traits are their short life span, self-pollination, and availability of mutants. *Arabidopsis* also does not interact with mycorrhizas and shows highly conserved mechanisms (genetic, biochemical, and molecular). Several *Arabidopsis* mutants have been identified in the last two decades and have provided helpful resources for understanding P signaling and response (Chen *et al.* 2000, Stefanovic *et al.* 2007, González-Mendoza *et al.* 2013).

This study was based on the hypothesis that media components affect the root response to phosphate (Pi) starvation and may mislead the identification of P mutants. To test the hypothesis we evaluated if the two most common media used in P research, Somerville and Ogren (1982; SO) medium and half-strength Murashige and Skoog (1962; $^{1}/_{2}$MS), would similarly affect the root growth of *Arabidopsis* in response to P limitation. Due to significant differences found between the two media, the effects of N source and Fe content on root growth were also evaluated. In order to test the hypothesis properly, the *Arabidopsis thaliana* accession Columbia (wild type) and three *phosphate-deficiency response* (*pdr*) mutants were included. Each of the three mutants belongs to a different "*phosphate-deficiency response*" phenotypic group based on root architecture as previously reported (Chen *et al.* 2000). The original screening for selection of these mutants used the SO media in which the inorganic P was substituted by organic P (nucleic acids). The media also contained vitamins, 0.5 % sucrose and 0.8 % *Phytagar* (Chen *et al.* 2000). The three mutants *pdr9*, *pdr23*, and *pdr37* displayed much shorter and branched roots than wild-type in the screening but were able, at different magnitudes, to recover root growth after transference to media containing high Pi content (Delatorre 2009, Costa *et al.* 2011). The recessive single mutants were hypothesized to contain one of the following defects: *1*) failure to grow on DNA or RNA substrates is proposed to require inactivation of redundant, Pi-starvation-inducible nucleic-acid-degrading enzymes and is likely to be caused by mutations in regulatory rather than structural plant *pho* genes; *2*) inability to secrete enzymes cleaving P from organic compounds, such as acid phosphatases and ribonucleases; *3*) inability to uptake low levels of Pi; *4*) loss-of-function of a regulatory component of Pi response; or *5*) low root growth and development (Chen *et al*. 2000).

Materials and methods

Seeds of *Arabidopsis thaliana* acc. Columbia (wild-type) and the phosphate-deficiency response mutants *pdr9, pdr23*, and *pdr37* were surface sterilized with 0.4 % sodium hypochlorite and 0.1 % (v/v) *Tween 20* for 5 min, and washed with sterile distilled and deionized water five times. The seeds were cold stratified at 4 ºC for 48 h. They were sown on disposable polystyrene square plates (*Nunc^{TM}*, *Thermo Scientific*, Waltham, MA, USA) containing solid media and sealed using gas permeable tape (*3M^{TM}*). These plates were placed vertically in a growth chamber set at a temperature of 22 ± 2 ºC, an irradiance of 100 µmol m^{-2} s^{-1}, and a 16-h photoperiod. F_3 seeds from the *AtACP5::uidA* X *pdr9* cross, *CycB1::uidA* X *pdr9* cross and *QC25::uidA* X *pdr9* cross (homozygous for both characters) were used to evaluate acid phosphatase gene expression, cell division, and root quiescent center maintenance, respectively.

Two different media were used: Somerville and Ogren (1982; SO) and Murashige and Skoog (1962). The medium SO is produced by the *Lehle Seeds* company (Round Rock, TX, USA), from which the M_2 seeds used in the selection of *pdr* mutants were obtained. The MS medium was obtained from *Caisson Laboratories* (Smithfield, UT, USA) and used in half strength (½ MS), since it is the most common formulation used in *Arabidopsis* research. The final media composition is shown in Table 1 Suppl. The MS was obtained in two commercial forms, with and without P. For both media, 0.5 % (m/v) sucrose and vitamins (0.1 mg cm^{-3} myo-inositol, 0.5 µg cm^{-3} nicotinic acid, 0.5 µg cm^{-3} pyridoxine, and 0.1 µg cm^{-3} thiamine) were added. Sucrose amount in solid media was kept low because higher amounts would affect P signaling; when liquid media were used, sucrose was omitted.

In order to remove contaminants from the solidifying agent, *Micropropagation Agar type I* (*Caisson Laboratories*) was washed four times with distilled and deionized water and anions were removed by using the ion exchange resin *Dowex 50* (*Lenntech*, Delft, The Netherlands) at 4 °C overnight. To equalize the effects of any remaining contaminants, the same batch of agar was used in all experiments. The amount of phosphorus in the agar measured by inductively coupled plasma mass spectrometry (ICP-MS) was lower than 20 µg g^{-1}. Both media were prepared using distilled and deionized water and 0.8 % agar. The pH was adjusted to 5.5, and 2.5 mM 2-(N_{22}-morpholino) ethane sulfonic acid was added as buffer. Phosphorus was added as KH_2PO_4 (to the final concentration of 0, 25, 50, 100, 250, 500, and 1 000 µM as described). When media were labeled as -P, phosphorus was removed from the media formulation and potassium was corrected with KCl.

To evaluate ammonium effect on root growth, we added either nitrate as KNO_3 or ammonium as $(NH_4)_2SO_4$ (25, 50, 100, 250, 500, 1 000, 2 500, 5 000 and 10 000 µM) to SO medium.

Three experiments were conducted to evaluate iron effect. Firstly, the interaction between iron and phosphorus was analyzed. Four concentrations of Fe-EDTA (0, 10, 50, and 100 µM) were used and two concentrations of phosphorus (-P and 2.5 mM). The second experiment evaluated the triple interaction P-Fe-N source. In this case, in addition to above mentioned concentrations of Fe and P, two sources of N were added as variables (9 mM KNO_3 or NH_4NO_3). On the third experiment, the effect of four N concentrations (0, 2, 4.5 and 6 mM) delivered as $(NH_4)_2SO_4$ was evaluated together with two Fe concentrations (0 and 50 µM) in the absence of phosphorus. For these experiments other medium components were based on SO.

Primary root length was measured eight days after germination using the software *ImageJ* (http://rsb.info.nih.gov/ij/). The number of lateral roots was counted. The number of biological replicates was 30. To evaluate cell division the number of cells in the transition from G2 to M in the root apical meristem was counted by visualizing the expression of *AtCYCB1::GUS*. Expression of *AtACP5* and *QC25* (identity for quiescent center, QC) was evaluated by GUS analysis performed following the procedures described by Vitha *et al.* (1995) and for each treatment at least 10 plants were analyzed. Representative roots were chosen and photographed with an optical microscope. For cell length measurements, the root mature zone of *Arabidopsis thaliana* wild type (WT) and the *pdr9* mutant was analyzed at day 7 using the software *Image J*. The length of 2 500 cells from 15 plants per treatment was measured.

All the experiments were conducted in a completed randomized block design. The data was tested for normality and submitted to analysis of variance by F-test ($P \leq 0.05$) and comparison of means by Fisher´s least significance test, Duncan test, or Student´s *t*-test.

Results and discussion

The comparison between the compositions of the two media mostly used in *Arabidopsis* studies, ½ MS and SO media showed significant differences. Among the micronutrients, the major differences are in the amounts of Mo, Mn, Zn, Cu, and Co, which are 2.5, 4, 27, 100, and 250 times higher, respectively, in ½ MS than in the SO medium (Table 1 Suppl.). Expressive differences between the media were also evident in macronutrient composition. The P concentration in ½ MS corresponds to ¼ of that in SO medium, however, N is three times higher in ½ MS. In addition, the source of N differs. SO media provided N only as potassium nitrate, and ½ MS

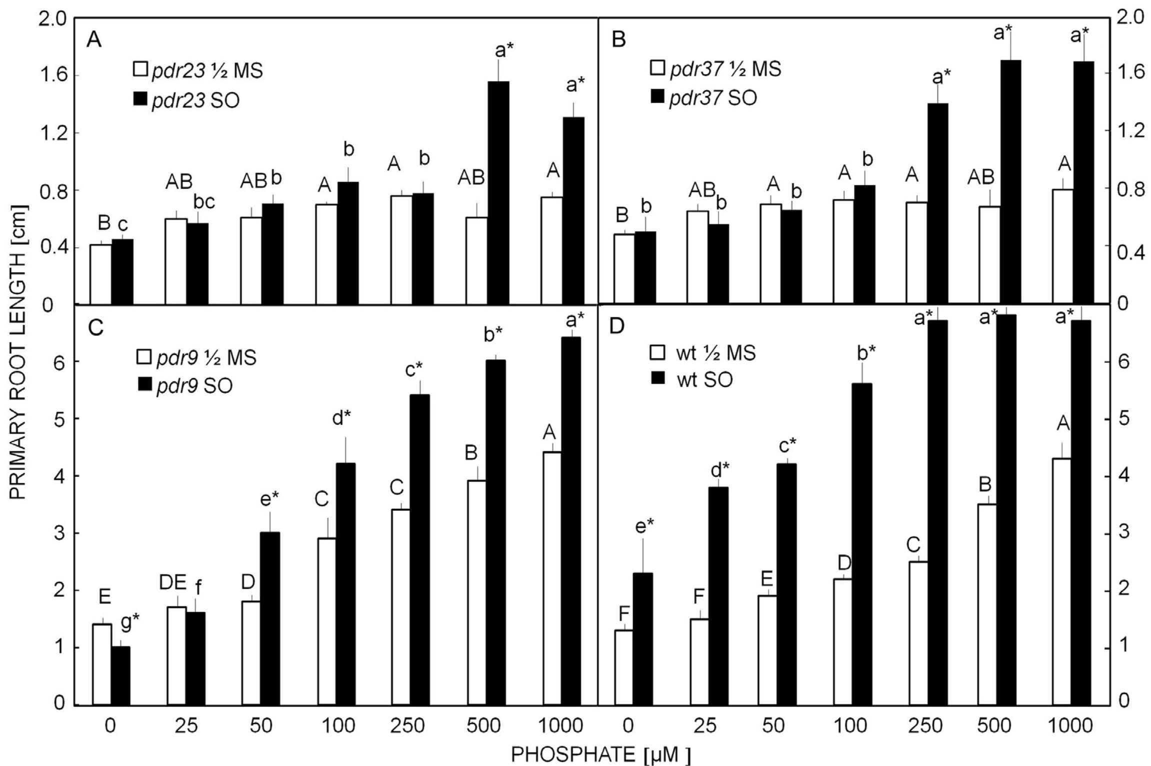

Fig. 1. Primary root length of *Arabidopsis thaliana pdr23* (*A*), *pdr37* (*B*), *pdr9* (*C*) mutants and wild-type (WT; *D*) in response to phosphate availability in SO and ½ MS media. Means ± SDs, $n = 30$. The same letters indicate no significant difference between phosphate concentrations according to LSD test ($P \leq 0.05$). Uppercase letters compares inside ½ MS media and lowercase letters inside SO media. * indicates significant difference in each phosphate concentration determined by *t*-test ($P \leq 0.05$).

also contained 1/3 of N as ammonium nitrate (9.12 mM).

The effect of the media on root growth was evaluated in four different *Arabidopsis* genotypes: the wild-type (WT) and the three genotypes carrying single mutations reduced root growth in the presence of organic sources of P (Chen *et al.* 2000). Interaction between media and genotype was significant under P deficiency conditions. Wild-type plants always showed the longest roots in both media, followed by *pdr9*, *pdr37,* and *pdr23* plants. However, longer primary roots were observed in SO media for WT (210 %), *pdr23* (160 %) and *pdr37* (160 %) plants (Table 1). Only *pdr9* plants exhibited longer roots in ½ MS (124 % in comparison to SO). In SO media the differences between the primary root length of WT and the mutants were impressive. For example, the length of *pdr9* roots was only 28 % of WT whereas it was 75 % in ½ MS. The effect of P deficiency was over-estimated on ½ MS media, reducing the differences between wild-type and the mutants.

Table 1. Primary root length [cm] of *Arabidopsis thaliana* wild type (WT) and three *pdr* mutants after 8 d on SO and ½ MS media under phosphorus starvation (-P). Means of 30 plants. Uppercase letters denote statistical difference among genotypes in ½ MS media and lowercase letters in SO media; in all cases significant differences ($P \leq 0.05$) between media were found by Fisher´s least significance test.

Genotype	½ MS	SO
WT	1.3A	2.7a
pdr9	1.0A	0.8b
pdr23	0.3C	0.5c
pdr37	0.4C	0.7b

The media composition effect on primary root growth was further evaluated by analyzing root growth under different P availabilities. Under high P concentration (1 mM), WT plants exhibited the longest roots in both media, followed by *pdr9*, *pdr37*, and *pdr23* plants, roots of which were substantially shorter (Fig. 1). These results corroborate to those obtained previously (Chen *et al.* 2000, Delatorre 2009). The WT primary root length was always smaller in ½ MS than SO medium at all P concentrations. For *pdr9* plants, ½ MS medium caused longer primary roots only under -P and at 25 µM P no difference was observed between the media. For all other P doses, primary roots were longer in SO media (Fig. 1). Moreover, using SO medium was possible to differentiate root growth between low and -P conditions. In this medium, P increments caused increase in root length of WT plants up to 250 µM P, after that no difference was found. On the other hand, response to P increments was found up to the highest dose used (1 000 µM) in ½ MS medium, suggesting that P still limited root growth even at the highest P dose (Fig. 1*D*). The maximum root length obtained in SO media was 57 % higher than that obtained in ½ MS media. The characteristic reduction in *pdr9* root growth under P limitation was only observed in SO medium (Fig. 1). Significant differences were observed at all P concentrations between WT and *pdr9*; the primary root length was always smaller for *pdr9* in SO media. However, in ½ MS media, *pdr9* did not differ from WT at 0, 25, 50 and 1 000 µM P, and its primary roots were even longer than WT at 100, 250, and 500 µM P.

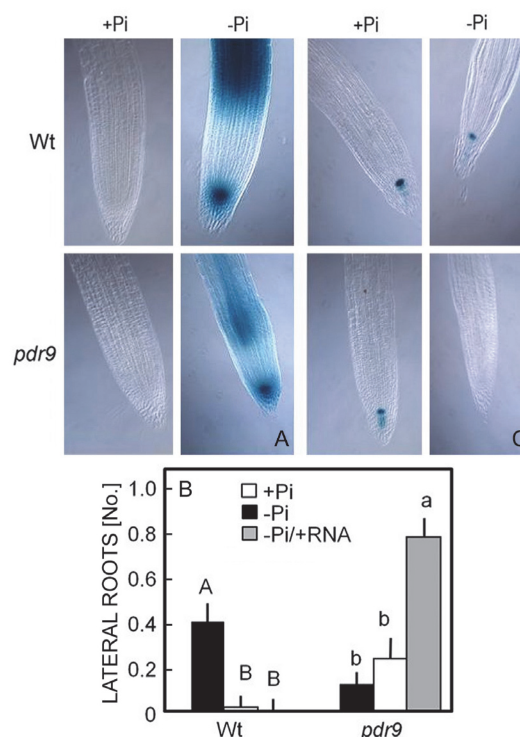

Fig. 2. Root growth in WT and *pdr9* mutant in response to P availability. *A* - Effect of P availability on *AtACP5::iudA* expression in roots of 7-d-old plants determined by GUS analysis. *B* - Number of lateral roots per plant after 7 d of growth in +Pi, -Pi and 1 mM organic P as RNA (-Pi/+RNA). Means ± SDs, $n = 90$; the same letters indicate no significant difference according to Duncan test ($P \leq 0.05$) inside each genotype. *C* - Quiescent center identity (labeled by *QC25::iudA*).

The other two mutants, *pdr23* and *pdr37*, displayed stronger root growth defect than *pdr9* (Fig. 1). The maximum primary root length in those mutants was about 25 % of WT, but even so, a difference was observed between SO and ½ MS media. The ratio between the primary root lengths at -P and 1 000 µM P was about 1.7 in ½ MS media and near three in SO media for both mutants whereas no significant difference was observed for WT. Basically, for both mutants only the growth under total absence of P was significantly different from high P in ½ MS media. Although, in SO media, it was possible to establish three categories for *pdr23*: one with very low P availability, a second with intermediate levels, and a third with the two highest P concentrations (Fig. 1*A*). Primary root length of *pdr37* plants did not differ between zero and 100 µM P in SO media, at 250 µM P it doubled its size and no further increase was

detected with P concentration up to 1 000 μM (Fig. 1*B*). These two mutants displayed very short roots in the following experiments (data not shown) hence we concentrated further analysis on the wild-type and *pdr9* plants.

The reduction in root length observed in *pdr9* at Pi deficiency could be caused by changes in cell division and cell elongation. The *pdr9* short root seems to result from changes in the quiescent center (QC) maintenance associated with reduction in cell elongation. No difference was found in the number of cells at G2/M transition, identified by the expression of *CycB1::uidA* (Table 2). However, the QC identity, labeled by *QC25::uidA,* was lost in *pdr9* roots (Fig. 2*C*) and a slight but significant reduction in cell length was found at Pi deficiency (Table 2). It would be interesting to verify if *pdr9* does interact with *altered phosphate starvation response 1* (*APSR1*). This gene was identified as required for meristem maintenance, affecting mainly cell elongation and differentiation in response to Pi (González-Mendoza *et al.* 2013). Expressions of genes induced by Pi deficiency are reduced in this mutant (Delatorre 2009), as observed for the acid phosphatase gene by *AtACP5::uidA* (Fig. 2*A*). Interestingly, the number of lateral roots in *pdr9* was lower in Pi deficiency but higher in +Pi if compared to WT plants (Fig. 2*B*).

Among the macronutrients, the difference in the two media (SO and ½ MS) for N was the most substantial. The ½ MS medium has three times more N and about 9 mM is available as ammonium (Table 1). Therefore, the effect of N added to SO media as either nitrate or ammonium was evaluated to verify if the addition of ammonium could cause the negative effect on primary root length observed in ½ MS. In WT, N dose increment up to 500 μM caused longer roots independently of the N source, suggesting that this concentration might be considered sufficient. However, the maximum root length was about 40 % higher in nitrate than in ammonium-containing media. Root length of WT was always significantly smaller under ammonium in comparison to nitrate (Fig. 3*A*). In comparison to the lowest N concentration, the addition of 0.5 mM nitrate increased the root length of WT plants 3-fold, whereas 0.5 mM ammonium increased it about 2-fold.

The effect of N source was more pronounced in *pdr9* plants. Relatively to the lowest N concentration, the

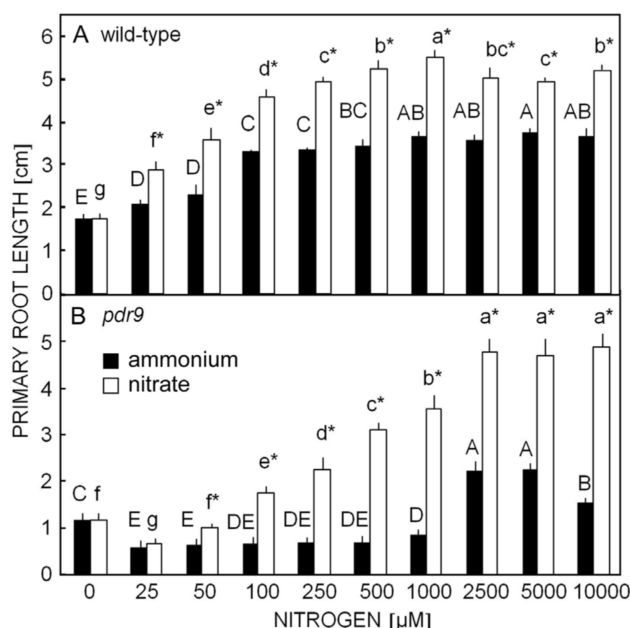

Fig. 3. Primary root length of wild-type (*A*) and *pdr9* mutant (*B*) in response to nitrogen availability in the SO media. Two different sources of N were used: potassium nitrate and ammonium sulfate. Plants were grown for 8 d. Means ± SDs, *n* = 30; the same letters indicate no significant difference according to LSD test ($P \leq 0.05$), uppercase letters compare ammonium concentrations, and lowercase letters compare nitrate concentrations, * indicates significant difference between N sources at the same N concentration (*t*-test, $P \leq 0.05$).

Table 2. Cell length of the root mature zone and number of cells in the transition from G2 to M in the root apical meristem of *Arabidopsis thaliana* wilt-type (WT) and the mutant *pdr9* after 7 d on SO media with different phosphate availability (-P and 1 mM P). Means ± SDs; *n* = 2 500 cells from 15 plants per a treatment. Uppercase letters compare phosphate concentrations and lowercase letters compare genotypes by *t*-test ($P \leq 0.05$).

Genotype	Cell length [μm]		Cell division	
	-Pi	+Pi	-Pi	+Pi
WT	67.3±1.0aB	126.5±1.3bA	6.1±0.7B	10.6±0.7A
pdr9	64.8±0.8bB	132.9±1.6aA	5.7±0.6B	10.6±1.5A

addition of 0.5 mM of nitrate increased *pdr9* root length more than 8-fold, and 0.5 mM ammonium only about 4-fold (Fig. 3*B*). The primary root growth of *pdr9* did not differ from 25 up to 1 000 µM ammonium, after that the root length increased four times and became stable from 2 500 to 10 000 µM (Fig. 3*B*). In contrast, the root growth of this mutant reacted to nitrate increments up to 2.5 mM.

Fe is a micronutrient associated with reduction of primary root length especially under the absence of P (Ward *et al.* 2008). The effect of Fe concentrations under different N sources and P availability was evaluated. Under sufficient amount of P, no addition of Fe reduced root length in nitrate fed plants for both genotypes (Fig. 4*A*). This effect was not seen in -P (Fig. 4*B*). The Fe concentration present in SO and $^1/_2$MS was the same, 50 µM. This Fe concentration at 9 mM nitrate caused maximum primary root length in both genotypes at high P, but was toxic at -P, mainly to *pdr9* (Fig. 4), corroborating the results obtained by Ward *et al.* (2008). Nitrate is an oxidant that could reduce the availability of electrons required for Fe reduction for membrane transport, and also can cause higher Fe requirement for its own reduction. Based on that, the effect of Fe doses and different N sources was examined. When NH_4NO_3 was used instead of only nitrate, the reduction of root growth due to no Fe addition on +P medium was not observed (Fig. 4*A*, 5*A*). In contrast, -P caused even stronger reduction in root growth in the presence of NH_4NO_3 at all Fe doses (Fig. 5*B*). Higher Fe availability was more harmful for *pdr9* (Fig. 4*A*,*B*). In the absence of Fe, N and P no difference was found between primary root growth of WT and *pdr9* (Fig. 5*C*). Increasing the ammonium concentration reduced primary root length for both genotypes even in the absence of Fe (Fig. 5*C*).

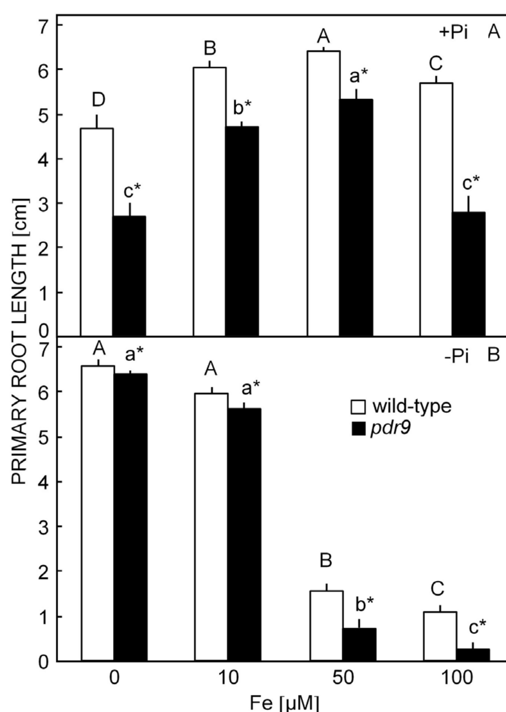

Fig. 4. Primary root length of wild-type and *pdr9* mutant in response to iron availability in in SO media with 9 mM nitrate and 2.5 mM P (+P; *A*) or without P (-P; *B*). Plants were grown for 8 d. Means ± SDs, *n* = 30. The same letters indicate no significant difference between Fe concentrations according to LSD test ($P \leq 0.05$), uppercase letters compare wild-type and lowercase letters compare *pdr9* mutant; * indicates significant difference between genotypes at the same Fe concentration according to *t*-test ($P \leq 0.05$).

Root architectural changes are common response to nutrient limitation and are quite remarkable for P starvation (Chevalier *et al.* 2003, Lambers *et al.* 2006, Péret *et al.* 2014). However, it has become clear that plant response is modulated not only by the limiting nutrient but also by its interaction with other nutrients in the media or in the soil (Hammond and White 2008, Ward *et al.* 2008, Jain *et al.* 2009, Costa *et al.* 2011, Cerutti and Delatorre 2013, Ogawa *et al.* 2014,). The majority of the studies involving phosphate starvation responses in *Arabidopsis* have been conducted using either ½ MS or SO media. Considering the low content of required micronutrients, it is possible that some of those are near toxicity in ½ MS medium when P is not present. It is known that high P concentration may reduce the uptake of some micronutrients (Kisko *et al.* 2015, Lambers *et al.* 2015). Comparison of root growth in both media demonstrated that P deficiency response was affected by the media composition in the four genotypes evaluated, demonstrating the importance of choosing the adequate conditions for mutant selection.

For example, if the ½ MS media had been used by Chen *et al.* (2000) in their genetic screen, the mutant *pdr9* would not have been selected, because its phenotype would be harder to distinguish from WT. Furthermore, for *pdr23* and *pdr37* would have been considered constitutively short roots if selected on ½ MS, and discarded in the recovery phase of the screen. Therefore the ½ MS media are not suitable for P response studies. The shorter roots observed in ½ MS suggest that one or more medium components, or their interactions limit the root growth potential.

In SO and ½ MS media composition, significant differences exist in total amount and source of N. Ammonium is not present in SO, whereas ½ MS contains about 9 mM. Despite the smaller uptake and assimilation costs of ammonium, deleterious effects of ammonium nutrition on growth have been described in several plant species (Britto and Kronzucker 2002). It has been suggested that it might be related to physiological pH, disequilibrium in the acid/base balance (Babourina *et al.* 2007), energetic imbalance from active extrusion of ammonium, the carboxylate level (Feng *et al.* 1998), or changes in hormone balance (Garnica *et al.* 2010).

N source effect was seen in both genotypes, but it was more pronounced in *pdr9* indicating the mutant as more sensitive either to ammonium or to the changes caused by ammonium interaction with other nutrients. Fe has been associated to primary root length reduction, particularly

under P limitation (Ward *et al.* 2008). Reduction of root length when Fe was not added occurred only in nitrate fed plants at +P suggesting that nitrate, but not ammonium, reduced Fe bioavailability. Pi bound the small amounts of Fe present as contamination, causing Fe-limitation. A second hypothesis is that in the absence of P plant Fe-requirements were reduced due to limited shoot growth. Recently, the transcription factor phosphate starvation response 1 (PHR1) was considered an integrator of nutrition signals not only for regulation of transporters but also for homeostasis of Pi, Fe, S, and Zn (Briat *et al.* 2015). Increment in Fe accumulation in plant tissue as well as in the abundance of transcripts from Fe excess responsive genes have been reported upon Pi limitation (Hirsch *et al.* 2006, Ward *et al.* 2008, Zheng *et al.* 2009). At -P conditions, the use of NH_4NO_3 reduced root growth even more (Fig. 5). The negative effect of ammonium on root growth for both genotypes at all N doses suggests that adjustments to Fe concentration in the media should be done based on the amount and the source of N used to assure optimal plant growth.

The similarity between WT and *pdr9* root growth in

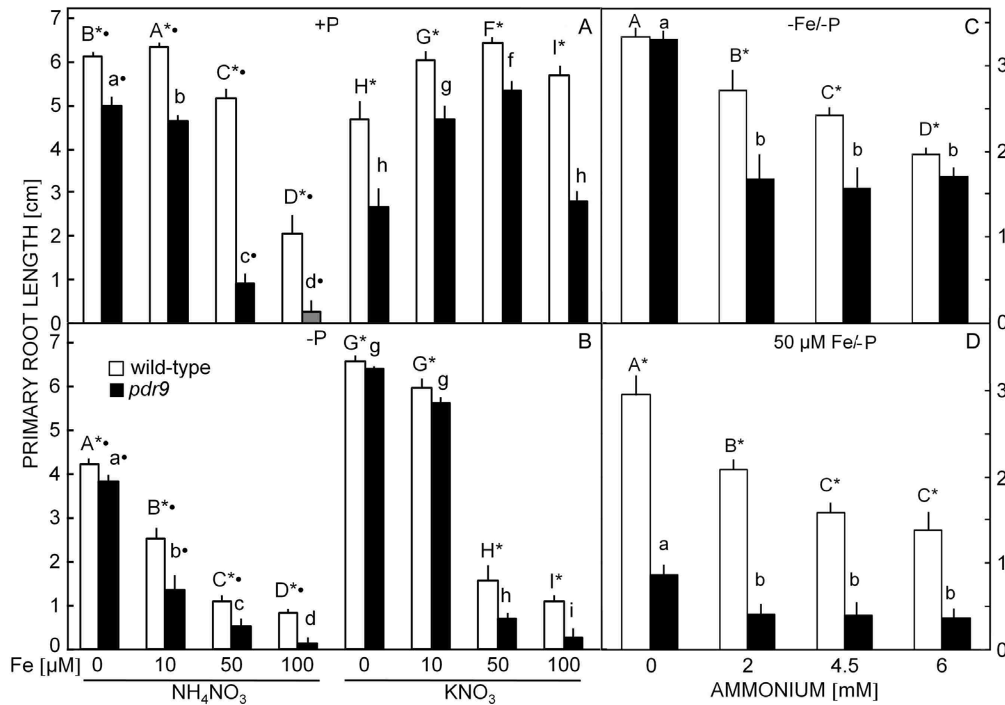

Fig. 5. Effect of the iron concentration and nitrogen source and concentration on the primary root length of wild-type and *pdr9* mutant plants (*A*,*B*). Plants were grown either at 2.5 mM P (+P; *A*) or without P (-P, *B*). Effect of different concentrations of ammonium on root growth under P and Fe deficiency (*C*) or P deficiency but 50 µM Fe (*D*). Plants were grown for 8 d. Means ± SDs, *n* = 30. The same letters in *A* and *B* indicate no significant difference between iron concentrations according to LSD test ($P \leq 0.05$) inside each genotype and N source, uppercase letters compare wild-type and lowercase letters compares *pdr9*. * indicates significant difference by *t*-test ($P \leq 0.05$) between genotypes and • difference between nitrogen sources at the same Fe concentration. The same letters in *C* and *D* indicate no significant difference by LSD test ($P \leq 0.05$) inside each genotype, * indicates significant difference by *t*-test ($P \leq 0.05$) between genotypes.

the absence of Fe, ammonium, and P (in contrast to the significant difference upon Fe addition) suggests that this mutant is hypersensitive to Fe (Fig. 5*C,D*). The *pdr9* hypersensitivity to Fe may cause the loss of QC maintenance in -P (Fig. 2*C*) It has been shown that loss of QC maintenance in *Arabidopsis* caused by high Fe:Pi ratio is under genetic control by *Low Phosphate Root 1* (*LPR1*) and *PDR2* (*pdr2* mutant was identified in the same screen as *pdr9*). LPR1 and PDR2 specifically alter Fe redox cycling and ROS production based on Fe:Pi ratios regulating callose deposition which interferes with symplastic communication and limits meristem activity (Müller *et al.* 2015). However, the negative effect of ammonium on root growth was not only due to increase in Fe bioavailability, since reduction on primary root length upon increase of ammonium dose was also observed on -Fe media for both genotypes.

All genotypes were affected by the media composition. The primary root length was significantly reduced on ½ MS. The roots of *pdr9* were the most sensitive to changes in nutritional composition. Mutants *pdr23* and *pdr37* displayed much shorter primary roots regardless of P condition in ½ MS media. If the selection of those *P-deficiency response* mutants had been done on ½ MS instead of SO, those mutants would not have been identified. Therefore, our results suggest the use of SO media instead of MS when screening for P mutants through root growth.

References

Abercrombie, J., Halfhill, M., Ranjan, P., Rao, M., Saxton, A., Yuan, J., Stewart, C.N.: Transcriptional responses of *Arabidopsis thaliana* plants to $As^{(v)}$ stress. - BMC Plant Biol. **8**: 87, 2008.

Babourina, O., Voltchanskii, K., McGann, B., Newman, I., Rengel, Z.: Nitrate supply affects ammonium transport in canola roots. - J. exp. Bot. **58**: 651-658, 2007.

Briat, J.-F., Rouached, H., Tissot, N., Gaymard, F., Dubos, C.: Integration of P, S, Fe and Zn nutrition signals in *Arabidopsis thaliana* : potential involvement of phosphate starvation response 1 (phr1). - Front.Plant Sci. **6**: 290, 2015.

Britto, D.T., Kronzucker, H.J.: NH_4^+ toxicity in higher plants: a critical review. - J. Plant Physiol. **159**: 567-584, 2002.

Cerutti T., Delatorre C.A.: Nitrogen and phosphorus interaction and cytokinin responses of the primary root of *Arabidopsis thaliana* and the *pdr1* mutant. - Plant Sci. **198**: 91-97, 2013.

Chen, D.L., Delatorre, C.A., Bakker, A., Abel, S.: Conditional identification of phosphate-starvation-response mutants in *Arabidopsis thaliana*. - Planta **211**: 13-22, 2000.

Chevalier, F., Pata, M., Nacry, P., Doumas, P., Rossignol, M.: Effects of phosphate availability on the root system architecture: large-scale analysis of the natural variation between *Arabidopsis* accessions. - Plant Cell Environ. **26**: 1839-1850, 2003.

Costa, C.T., Strieder, M.L., Abel, S., Delatorre, C.A.: Phosphorus and nitrogen interaction: loss of QC identity in response to P or N limitation is antecipated in *pdr23* mutant. - Braz. J. Plant Physiol. **23**: 219-229, 2011.

Delatorre, C.A.: Phosphate Deficiency Response: Searching for the Signaling Pathway. - Lambert Academic Publishing, Köln, Germany 2009.

Feng, J., Volk, R.J., Jackson, W.A.: Source and magnitude of ammonium generation in maize roots. - Plant Physiol. **118**: 835-841, 1998.

Garnica, M., Houdusse, F., Zamarreño, A.M., Garcia-Mina, J.M.: The signal effect of nitrate supply enhances active forms of cytokinins and indole acetic acid content and reduces abscisic acid in wheat plants grown with ammonium. - J. Plant Physiol. **167**: 1264-1272, 2010.

González-Mendoza, V., Zurita-Silva, A., Sánchez-Calderón, L., Sánchez-Sandoval, M.E., Oropeza-Aburto, A., Gutiérrez, A.D., Alatorre-Cobos, F., Herrera-Estrella, L.: *Apsr1*, a novel gene required for meristem maintenance, is negatively regulated by low phosphate availability. - Plant Sci. **205-206**: 2-12, 2013.

Hammond, J.P., White, P.J.: Sucrose transport in the phloem: integrating root responses to phosphorus starvation. - J. exp. Bot. **59**: 93-109, 2008.

Hammond, J.P., White, P.J.: Sugar signaling in root responses to low phosphorus availability. - Plant Physiol. **156**: 1033-1040, 2011.

Hirsch, J., Marin, E., Floriani, M., Chiarenza, S., Richaud, P., Nussaume, L., Thibaud, M.C.: Phosphate deficiency promotes modification of iron distribution in *Arabidopsis* plants. - Biochimie **88**: 1767-1771, 2006.

Jain, A., Poling, M.D., Smith, A.P., Nagarajan, V.K., Lahner, B., Meaghe,r R.B., Raghothama, K.G.: Variations in the composition of gelling agents affect morphophysiological and molecular responses to deficiencies of phosphate and other nutrients. - Plant Physiol. **150**: 1033-1049, 2009.

Kisko, M., Bouain, N., Rouached, A., Choudhary, S.P., Rouached, H.: Molecular mechanisms of phosphate and zinc signalling crosstalk in plants: phosphate and zinc loading into root xylem in *Arabidopsis*. - Environ. exp. Bot. **114**: 57-64, 2015.

Lambers, H., Hayes, P.E., Laliberté, E., Oliveira, R.S., Turner, B.L.: Leaf manganese accumulation and phosphorus-acquisition efficiency. - Trends Plant Sci. **20**: 83-90, 2015.

Lambers, H., Shane, M.W., Cramer, M.D., Pearse, S.J., Veneklaas, E.J.: Root structure and functioning for efficient acquisition of phosphorus: matching morphological and physiological traits. - Ann. Bot. **98**: 693-713, 2006.

Müller, J., Toev, T., Heisters, M., Teller, J., Moore, K.L., Hause, G., Dinesh, D.C., Bürstenbinder, K., Abel, S.: Iron-dependent callose deposition adjusts root meristem maintenance to phosphate availability. - Dev. Cell **33**: 216-230, 2015.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-479, 1962.

Ogawa, S., Valencia, M., Ishitani, M., Selvaraj, M.: Root system architecture variation in response to different NH_4^+ concentrations and its association with nitrogen-deficient tolerance traits in rice. - Acta Physiol Plant **36**: 2361-2372, 2014.

Péret, B., Desnos, T., Jost, R., Kanno, S., Berkowitz, O., Nussaume, L.: Root architecture responses: in search of phosphate. - Plant Physiol. **166**: 1713-1723, 2014.

Somerville, C.R., Ogren, W.: Isolation of photorespiration mutants in *Arabidopsis thaliana*. -In: Edelman, M. (ed.): Methods in Chloroplast Biology. Pp. 129-138. Elsevier Biomedical Press, Amsterdam 1982.

Stefanovic, A. Ribot, C., Rouached, H., Wang, Y., Chong, J., Belbahri, L., Delessert, S., Poirie, Y: Members of the *pho1* gene family show limited functional redundancy in phosphate transfer to the shoot, and are regulated by phosphate deficiency *via* distinct pathways. - Plant J. **50**: 982-994, 2007.

Svistoonoff, S., Creff, A., Reymond, M., Sigoillot-Claude, C., Ricaud, L., Blanchet, A., Nussaume, L., Desnos, T.: Root tip contact with low-phosphate media reprograms plant root architecture. - Nat. Genet. **39**: 792-796, 2007.

Vitha, S., Benes, K., Phillips, J.P., Gartland, K.M.A.: Histochemical GUS analysis. - In: Gartland, K.M.A., Davey, M.R. (ed.): *Agrobacterium* Protocols. Pp. 185-193. Humana Press, Totowa 1995.

Ward, J.T., Lahne,r B., Yakubova, E., Salt, D.E., Raghothama, K.G.: The effect of iron on the primary root elongation of *Arabidopsis* during phosphate deficiency. - Plant Physiol. **147**: 1181-1191, 2008.

Zheng, L., Huang, F., Narsai, F., Wu, J., Giraud, E., He, F., Cheng, L., Wang, F., Wu, P., Whelan, J., Shou, H.: Physiological and transcriptome analysis of iron and phosphorus interaction in rice seedlings. - Plant Physiol. **151**: 262-274, 2009.