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# Root-released organic acids and phosphorus uptake of two barley cultivars in laboratory and field experiments

Tara S. Gahoonia [a,*], Farouq Asmar [b], Henriette Giese [b], Gunnar Gissel-Nielsen [b], Niels Erik Nielsen [a]

[a] *The Royal Veterinary and Agricultural University, Department of Agricultural Sciences, Plant Nutrition and Soil Fertility Laboratory, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Copenhagen, Denmark*

[b] *Risø National Laboratory, Department of Plant Biology and Biogeochemistry, PO Box 49, DK-4000 Roskilde, Denmark*

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**Abstract**

A major portion of phosphorus (P) applied as fertilizers is bound in soils as P compounds of variable adsorption strength, reducing the effectiveness of P fertilization. Plant genotypes equipped with mechanisms for utilizing the adsorbed P more efficiently can, therefore, enhance the effectiveness of P fertilization. Such genotypes will also enrich plant gene pools for further analysis and upgrading of P efficiency by selection and breeding. We studied the variation and the mechanisms of P uptake of two winter barley (*Hordeum vulgare* L.) cultivars Marinka and Sonate (parents of existing 200 haploid progeny lines), by laboratory and field experiments. After cultivation in nutrient solution for 21 days, Marinka produced more roots than Sonate, but similar amounts of dry shoots of lower P content (Marinka $3.4 \pm 0.4$ mg $g^{-1}$, Sonate $4.9 \pm 0.6$ mg $g^{-1}$). The total P uptake per plant did not differ between the cultivars. Marinka retained more P in roots as indicated by the higher concentration of P in the roots (Marinka $3.9 \pm 0.3$ mg $g^{-1}$ and Sonate $3.0 \pm 0.4$ mg $g^{-1}$). In sterile nutrient solution culture, the cultivars differed mainly in release of organic acids from the roots, with Marinka releasing three times more citric acid and nearly two times more acetic acid than Sonate. The cultivars had similar root hair lengths and they did not differ ($P > 0.05$) in depletion of available soil P fraction (extracted with 0.5 M $NaHCO_3$) in the rhizosphere. Marinka absorbed nearly twice as much P from the strongly adsorbed soil P fraction (extracted with 0.1M NaOH). Also under field conditions, Marinka absorbed more P and produced more shoot dry matter. The higher P uptake by Marinka than Sonate can be attributed to its ability to acquire P from strongly adsorbed soil P by releasing more organic acids, especially citric acid, from its roots. 

*Keywords:* Barley genotypes; Organic acids; Phosphorus efficiency; Rhizosphere; Root exudation; Root hairs

## 1. Introduction

Concerns about depletion of high-grade phosphate rock, the necessary fertilizer raw material

* Corresponding author. Tel.: +45-35283497, fax: +45-35283460.

*E-mail address:* tsg@kvl.dk (T.S. Gahoonia)

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(Cathcart, 1980), cost of P fertilization in developing countries and potential environmental problems associated with P inputs in developed countries, have stimulated the search for means of saving and utilizing P more efficiently. In addition to the agronomic measures (Mengel, 1997), the need of selecting and breeding P-efficient crop varieties is receiving increased attention (Lynch, 1998). Soil phosphorus problems are particularly severe in developed countries, where rapid growth in human population is expected. For securing food to the increasing population, the need for P fertilization is also expected to increase (Brynes and Bumb, 1998). However, when P fertilizers are applied to replenish soil fertility, about 70–90% of the P fertilizers is adsorbed and retained in soil in various P compounds. The mechanisms of P adsorption in soils are not fully resolved (Sample et al., 1980), but for addressing the bioavailability of soil P, it can be divided into easily desorbable plant available P and strongly adsorbed P fractions by using sequential extraction procedure (Hedley et al., 1982). Although, in the long-term, the adsorbed soil P may not be considered lost, but as a phosphorus capital (Sanchez and Leakey, 1997), it is not immediately available for supporting plant growth. This reduces the effectiveness of P fertilization, inducing the lack of economic incentives to apply costly P fertilizers (Brynes and Bumb, 1998).

Plant species and their cultivars possess diverse root morphological (Gahoonia et al., 1997) and physiological (Neumann et al., 1999) processes for adapting to low P supply, but the relative importance of the P mobilizing processes may differ with plant species and cultivars. For example, white lupine (Braum and Helmke, 1995) and Pigeon pea (Ae et al., 1990) possesses specific ability to mobilize and use adsorbed soil P, not available to other plants. Plant roots release a variety of organic acids in the rhizosphere (Jones and Darrah, 1995). The release increases during P stress (Lipton et al., 1987; Hoffland et al., 1992). Bolan et al. (1990) reported that organic acids increase soil P availability by decreasing adsorption of P and by increasing dissolution of relatively insoluble P compounds. Other reports (Dinkelaker et al., 1995) suggest that root-released citric acid increase the availability of mineral-bound P by solubilizing Ca, Fe, and Al phosphates.

The role of chemical organic acids (Gerke, 1992; Hue, 1991) and root-released organic acids by different plant species in mobilization of soil P is documented (Jones and Darrah, 1994). However, the information is sparse (Römer and Schenk, 1998), whether the release of organic acids from roots of barley cultivars differs and how it affects rhizosphere P mobilization and uptake by plants. Such information is important for elucidating the plant genetic factors, which may increase the release and uptake of P from insoluble soil P fractions, improving the effectiveness of P fertilization. In this paper, we report the variation in root-released organic acids and their relationship to phosphorus uptake of two barley cultivars Marinka and Sonate in laboratory and field studies.

## 2. Materials and methods

### 2.1. Soil

The soil used for the field and rhizosphere studies was a sandy clay loam, which had received no P since 1966. The soil contained 15% clay, 18% silt, 65% sand, total C = 11.5 mg $g^{-1,}$ total N = 1.3 mg $g^{-1}$. Inorganic P extractable with 0.5 M $NaHCO_3$ ($NaHCO_3$-$P_i$) was 0.45 mmole P $kg^{-1}$ soil and inorganic P extractable with 0.1 M NaOH (NaOH-$P_i$) was 2.45 mmole P $kg^{-1}$ soil. The soil solution P concentration was 3 μM. Soil pH (0.01 M $CaCl_2$) was 5.6 and cation exchange capacity = 8.4 $cmol_c$ $kg^{-1}$ soil at pH 7.

### 2.2. Cultivars

The two winter barley cultivars (*Hordeum vulgare* L. cv. Marinka and Sonate) were chosen for the study, because 200 double haploid progeny lines of crosses Marinka × Sonate and basic maps including Restriction Fragment Length Polymorphism (RFLP) markers are available. Therefore, if differences in P uptake between the two cultivars are characterized, the progeny lines can be useful for genetic analysis of P efficiency in barley.

### 2.3. Nutrient solution experiments

#### 2.3.1. Root length determination

Because of concerns of persistent holes in the small low-P plots (also used for other purposes) sampling of soil cores for root studies was not done. The ability of the cultivars to produce roots was determined in a nutrient solution experiment. Plants were grown in a basic nutrient solution (Table 1) in 5 l plastic pots in a glass-house as described by Gahoonia and Nielsen (1997). The electrical conductivity of the solution was maintained at 0.63 mS $cm^{-1}$ by adding a maintenance solution (Table 1). Roots were harvested after 21 days and total root system length was measured using a scanner (ScanJet IIcx) and Dt-Scan software (Delta-T Devices, Cambridge, England). All shoots and roots were washed thoroughly with distilled water for determining P concentration.

#### 2.3.2. Determination of root-released organic acids

To assess the variation in root-released organic acids, 40 seeds of each cultivar were sown individually in sterile sand in Eppendorf vials and supplied with sterile nutrient solution as described by Asmar and Gissel-Nielsen (1996). After 7 days, the Eppendorf vials with the seedlings were placed in the holes (10 mm diameter) made on the lids of 35 ml bottles, containing 30 ml of the sterile maintenance nutrient solution (Table 1), where the P concentration was reduced to 0.005 mM. The bottles were wrapped in aluminum foils and transferred to a growth chamber with a night/day regime of 8/16 h, 15/20°C, relative humidity 65–75%, and light intensity 460 μE $s^{-1}$ $m^{-2}$. After 10 days, the roots of the plants were first rinsed 2–3 times with a sterile 225 μM $CaCl_2$ solution (pH 4.3) for 30 min in a laminar-flow-hood. The bottle lids with the plants were transferred into 10-ml sterile bottles. The roots protruding from the Eppendorf vials were dipped into 7 ml of a sterile 450 μM $CaCl_2$ solution (pH 7). The $CaCl_2$ solution maintains the structural integrity of root membranes during the growth under P stress. After 12, 24 and 48 h, 12 plants of each cultivar were harvested, and the solutions were filtered through millipore filters, then freeze-dried and extracted with 20 and 98% ethanol for 30 min at 80°C (Jones and Darrah, 1995). The solutions were analyzed for organic acids with HPLC using Aminex HPX-87H column. Filtrate samples (100 μl) were injected into the HPLC using a glass syringe and eluted isocratically with 4 mM $H_2SO_4$ at a constant flow rate 0.6 ml/min for 20 min at 20°C. Peaks for the organic acids were detected at a wavelength of 210 μm and the organic acids were identified by comparison to the retention times obtained for pure organic acids injected as standards. From the peak areas, the quantity of organic acids in the samples was calculated and expressed as mg organic acid, $g^{-1}$ root dry weight, $day^{-1}$.

### 2.4. Rhizosphere experiment

To study P acquisition from different soil P fractions in the rhizosphere, soil samples of known distances from roots were obtained by thin-slicing the rhizosphere soil as described by Gahoonia and Nielsen (1991). The cultivars were first pre-grown for 12 days in vermiculite filled in PVC tubes (length 10 cm, diameter 4.4 cm) closed at the bottom by nylon cloth, which was impervious to roots. Two ceramic fiber wicks were placed along the inner sides of the tubes to supply nutrient solution of a defined composition. The tubes, along with the plants having uniformly developed root mats were transferred to soil columns filled into PVC tubes (length 3 cm, diameter 5.6 cm). A nylon screen of mesh size 53-μm (open space, 22%) separated each column into 3-cm test soil below and 1-cm soil layer above the screen. The soil columns (bulk density 1.3 g $cm^{-3}$) were maintained at defined moisture ($\theta = 0.21$) by placing them over small, cup-shaped sand baths, each fitted with a wick dipping into a distilled water reservoir. For some columns, six 1-mm holes were made in the nylon screen to measure root hairs on the roots grown in the soil as described in the next section. After transplantation, new root mats developed over the nylon mesh, representing a root surface area of 24.6 $cm^{-2}$. No roots, but only root hairs penetrated into the soil. The supply of the maintenance solution (Table 1) to the plant roots in the vermiculite was continued via the two

Table 1
The composition and concentration of basic and maintenance solutions

| Nutrients | $NO_3$ (mM) | $NH_4$ (mM) | P (mM) | S (mM) | K (mM) | Ca (mM) | Mg (mM) | Cl (μM) | Na (μM) | Fe (μM) | Mn (μM) | Zn (μM) | Cu (μM) | B (μM) | Mo (μM) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Basic | 5.0 | 0.0 | 0.005 | 0.44 | 0.58 | 1.92 | 0.78 | 0.10 | 0.001 | 50 | 7.0 | 0.7 | 0.7 | 2.0 | 0.7 |
| Maintenance | 13 | 3.2 | 0.54 | 0.61 | 7.16 | 1.40 | 0.35 | 0.40 | 0.24 | 15 | 12 | 6.0 | 0.9 | 4.6 | 0.2 |

wicks at 20-cm water tension. The solution was adjusted to match the average nutrient ratio in dry matter of cereals. Based on previous studies (Gahoonia and Nielsen, 1991, 1992), the solution creates moderate P deficiency and avoids deficiencies of other nutrients. The equal water uptake by plants from the external nutrient solution maintained equal supply of nutrients to both the cultivars. Water uptake also occurred via the soil column ($10 \pm 2\%$ of the total water uptake). The percentage of total N as ammonium in the supply solution was adjusted to maintain a constant rhizosphere soil pH (Gahoonia and Nielsen, 1992). The experiments were conducted under controlled conditions (light intensity 280 $\mu E\ s^{-1}m^{-2}$, light/dark period 16/8 h, temperature 18/15°C, relative humidity 75%). After 14 days, the soil columns were separated from the root mats, quickly frozen in liquid nitrogen and sliced with a freezing microtome to obtain rhizosphere soil samples at distances 0.2, 0.4, 0.6, 0.8, 1.0, 1.5. 2.0, 2.5, 3.5 and 4.5 mm from the mesh surface.

*2.4.1. Root hair determination in soil*

To measure root hairs of the cultivars, they were grown as described above in the rhizosphere experiment, but six holes (ca. 1 mm) were made at different places in the nylon screen so that about six roots penetrated and grew into the soil columns. After 7 days, the soil columns were immersed in water in dark at 5°C overnight. The roots were then removed using a kitchen sieve and subjected to an ultrasound treatment in an Ultrasound bath (Branson 5200, 120W, 47K Hz) for 5–10 min to remove the remaining soil particles without damaging the root hairs. Root hairs were measured using Quantimet $500_+$ Image Processing and Analysis System (Leica) at $10\times$ magnification as described in Gahoonia and Nielsen (1997).

*2.4.2. Phosphorus analysis of rhizosphere soil*

Soil P was separated into easily desorbable plant available (NaHCO3-$P_i$) and strongly adsorbed P (NaOH-$P_i$) fractions (Hedley et al., 1982). For determining NaHCO3-$P_i$, 5 ml of 0.5 M $NaHCO_3$ (pH 8.5) was added to 0.5 g of air-dry soil in a centrifuge tube. The tubes were shaken by rotation for 2 h and centrifuged (3000 rpm). Inorganic P in the supernatant was determined immediately by the method of Murphy and Riley (1962). For determining NaOH-$P_i$, the residual soil in the centrifuge tube was suspended in 25 ml of 0.1 M NaOH and shaken for 17 h and centrifuged. Inorganic P in the supernatant was again determined immediately. Unplanted soil samples were analyzed as controls.

*2.5. Field experiment*

*2.5.1. Experimental design*

A randomized complete block design with two replicates was used. The plot size was $1.5 \times 10$ m. Fertilizer applications were 60 kg N and 60 kg K $ha^{-1}$, applied one week before sowing.

*2.5.2. Field sampling of plant material*

Aerial parts from 1 $m^2$ per plot were harvested six times during the growing period, i.e. 24, 36, 52, 66, 84 and 94 days after germination.

*2.5.3. Plant analysis*

Shoots were dried at 80°C to constant weight, ground and 0.3 g was digested in $H_2SO_4$–$H_2O_2$–$HNO_3$ mixture. Phosphorus was determined as described by Murphy and Riley (1962). Phosphorus uptake in field was calculated from shoot dry matter and P concentrations.

Statistical analysis was performed with Statistical Analysis System Institute (1989) and Microsoft Excel software as found appropriate.

## 3. Results

In the nutrient solution experiment, Marinka produced nearly two times more root length than Sonate (Table 2), but did not produce more shoot dry matter. The concentration of P in shoot of Marinka was lower than Sonate. The concentration of P in the roots of Marinka was higher indicating that it retained more P in their roots. The root hair lengths of cultivars were same (Table 2).

Of the organic acids released from roots of Marinka and Sonate, the significant differences

between the cultivars were found only in release of citric acid and acetic acid (Fig. 1). Marinka released three times more citric acid and nearly twice as much acetic acid from the roots as compared to Sonate. Both cultivars released large quantities of fumaric acid (Fig. 1), but the difference between the cultivars was not significant ($P > 0.05$).

Table 2
Root parameters and concentration of phosphorus (P) in shoot dry matter of two barley cultivars in nutrient solution experiment

| Parameter | Marinka | Sonate |
|---|---|---|
| Root length (m $g^{-1}$) | $109 \pm 10$ | $61 \pm 7$ |
| Root hair length (mm) | $0.67 \pm 0.17$ | $0.79 \pm 0.20$ |
| Root dry weight (g $plant^{-1}$) | $0.52 \pm 0.06$ | $0.38 \pm 0.07$ |
| P in roots (mg $g^{-1}$) | $3.9 \pm 0.3$ | $3.0 \pm 0.4$ |
| Shoot dry matter (g plant) | $0.78 \pm 0.10$ | $0.70 \pm 0.04$ |
| P in shoot dry matter (mg $g^{-1}$) | $3.4 \pm 0.4$ | $4.9 \pm 0.6$ |

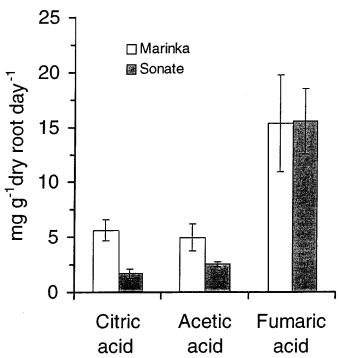


Fig. 1. Root-released organic acids of two winter barley cultivars, Marinka and Sonate, in sterile nutrient solution culture.

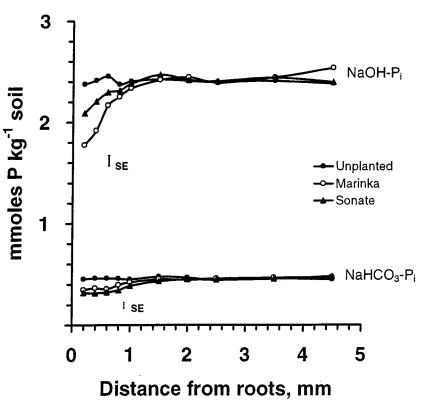


Fig. 2. Depletion of available P ($NaHCO_3$-$P_i$) and strongly adsorbed P (NaOH-$P_i$) in the rhizosphere soil of two winter barley cultivars in 14 days.

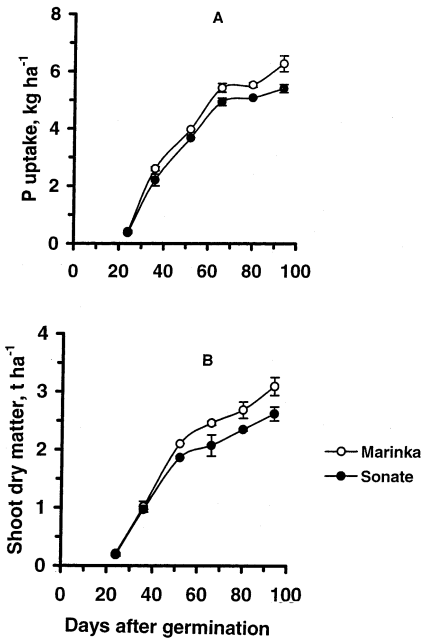


Fig. 3. Time-course of phosphorus uptake (A) and the corresponding shoot dry matter production (B) of two winter barley cultivars, Marinka and Sonate, in a low-P field.

The rhizosphere depletion profiles of easily desorbable plant available P ($NaHCO_3$-$P_i$) did not differ, showing that the two cultivars did not differ in absorbing P from this P fraction (Fig. 2). However, Marinka decreased the concentration of strongly adsorbed soil P (NaOH-$P_i$) by 0.7 mmole P $kg^{-1}$ (from 2.4 to 1.7 mmoles P $kg^{-1}$) in the rhizosphere soil. The decrease was only 0.3 mmole P $kg^{-1}$ soil with Sonate. Marinka could mobilize and deplete twice as much P from NaOH-$P_i$ than Sonate (Fig. 2), showing that Marinka possesses greater ability to release and absorb P from the strongly adsorbed P in soil.

Under field conditions, over the growth period of up to 94 days after germination, Marinka absorbed more P than Sonate (Fig. 3A) and also produced more shoot dry matter (Fig. 3B).

## 4. Discussion

Marinka was superior to Sonate in depletion of P from strongly adsorbed soil P fraction in the rhizosphere (Fig. 2) and the P uptake in the field (Fig. 3A), but not in nutrient solution experiment (Table 2). Variation in P uptake may result from variation in root development and/or due to the ability of the roots to release organic acids into the rhizosphere. In nutrient solution experiment, Marinka produced nearly twice as much roots as Sonate (Table 2). But despite the greater root length, Marinka neither produced more shoot dry matter, nor it has a higher plant P concentration (Table 2). Marinka retained more P in roots. Hence, total P uptake did not differ between the cultivars in the nutrient solution experiment, where all P was readily available in solution without diffusion limitation. The superior P uptake of Marinka was associated with its ability to extract P from NaOH-$P_i$ in soil (Fig. 2), as revealed by rhizosphere studies.

The release of organic acids differed between the two cultivars (Fig. 1), which corresponded to the higher depletion of P from the NaOH-$P_i$ (Fig. 2). Citric acid is known to be effective in desorbing strongly bound soil P by ligand exchange of phosphate against citrate (Gerke, 1992), whereas acetic acid has only a negligible effect (Earl et al., 1979). The concentration of soil solution P in the vicinity of roots may reach very low and close to the $C_{min}$ i.e. minimum effective concentration below which nutrient uptake stops (Nielsen, 1976). When P supply is lowered, enhanced root exudation of organic acids is observed (Lipton et al., 1987). The increased exudation of organic acids in the vicinity of roots may release P from the strongly adsorbed P fractions for maintaining soil solution P concentration above the $C_{min}$ value (Nielsen and Schjørring, 1983).

The longer root hairs of other barley cultivars were related to the higher depletion of $NaHCO_3$-$P_i$ from rhizosphere soil (Gahoonia and Nielsen, 1997). In contrast, root hair lengths of Marinka and Sonate were same (Table 2) and the rhizosphere P depletion profiles of $NaHCO_3$-$P_i$ were not different (Fig. 2). The rhizosphere technique (Gahoonia and Nielsen, 1991) applied to study depletion of soil P fractions implies that rhizosphere pH remains unchanged (Gahoonia and Nielsen, 1992). Therefore, the differential ability of the cultivars to deplete P from NaOH-$P_i$ fractions (Fig. 2) can not be attributed to the root-induced pH change. It may be mentioned that both $NaHCO_3$ and NaOH, used for fractionation of soil P, are alkaline, which favor P desorption. However, the results of this study show that the higher release of citric acid from roots of Marinka favors desorption and uptake of P from strongly adsorbed soil P fraction.

Rhizosphere phosphatase activity may increase mobilization and depletion of soil organic P (Asmar et al., 1995). Marinka is reported to have higher activity of root-released extracellular phosphomonoesterase and phosphodiersterase than Sonate in both sterile and non-sterile conditions (Asmar and Gissel-Nielsen, 1996). Therefore, the possibility of higher mobilization of P from soil organic P by Marinka can not be ruled out, which might have contributed to the higher P uptake of Marinka in the field (Fig. 3A). Soils may differ in available, insoluble and organic P fractions, which can be more effectively utilized by selecting cultivars with longer root hairs (Gahoonia et al., 1999), releasing of higher quantity of organic acids (Fig. 1) and inducing higher phosphatase activity (Asmar et al., 1995) in the rhizosphere, respectively.

## 5. Conclusions

The results showed that the barley cultivar Marinka releasing more citric acid could extract and absorb more P from strongly adsorbed soil P fraction. In tropical regions, where P fertilization is necessary, a large amount of P is fixed as strongly adsorbed P in soils. In developed countries, where it is desirable to reduce fertilizer P inputs, the proportion of P fixed in non-available P fractions may increase. The barley cultivars releasing higher amounts of organic acids, such as citric acid, in the rhizosphere, can have added advantage of bypassing P fixation process in soils for utilizing soil and fertilized P more efficiently.

This can increase the effectiveness of P fertilization, hence may add to the incentives of using P fertilizers, where they are necessary. In addition to the possibility of maintaining yield in low P soils and the identified P efficient genotypes will also increase plant gene pools for upgrading the P efficiency of barley by breeding.

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