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Short communication

Flavonoids of white lupin roots participate in phosphorus mobilization from soil

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ABSTRACT

The impact of flavonoids released by phosphorus-deficient white lupin roots on inorganic P and soil microorganisms is largely unknown. We report that flavonoids isolated from white lupin roots mobilized inorganic phosphorus and decreased soil microbial respiration, citrate mineralization, and soil phosphohydrolase activities, but did not reduce the soil ATP content. The results suggest that white lupin's release of flavonoids into the rhizosphere plays a significant role in its efficient P-acquisition strategy by solubilizing Fe-bound P and by limiting the microbial mineralization of citrate.

Availability of soil P is controlled by various soil properties (e.g pH, Al-Fe-(hydro)oxydes, clay mineralogy, organic matter content) and microbial activity (Brady, 1990). White lupin (*Lupinus albus* L.) can increase P availability by forming cluster roots and by releasing high amounts of citrate (Shen et al., 2003; Shane and Lambers, 2005). The citrate chelates divalent cations, releasing inorganic P (P_i) from insoluble (hydro)oxides (Ryan et al., 2001) and is acquired by roots as an inorganic anion. Under conditions when Pi is limiting, white lupin roots also release large amounts of flavonoids (Neumann et al., 2000), identified by Weisskopf et al. (2006a) as mainly genistein- and hydroxygenistein-derivated. As flavonoids, and especially genistein, are secreted in higher amounts from cluster than from non-cluster roots and from P-deficient than from P-sufficient plants (Weisskopf et al., 2006a), it can be hypothesized that flavonoids might help the plant to cope with P deficiency either directly by solubilizing P_i or indirectly by inhibiting the mineralization of citrate by rhizosphere microorganisms (Neumann and Römheld, 2007). We tested these hypotheses by determining the effects of purified flavonoids from white lupin cluster roots on the P_i availability, on microbial biomass and respiration, citrate mineralization and soil hydrolase activities.

To achieve these aims, white lupin plants were grown hydroponically for five weeks in the absence of Pi and roots from different stages were sampled as described by Massonneau et al. (2001). Roots of P-deficient plants were divided into clusterized and non-clusterized roots; the first were separated into apices of the clusterized roots (Apex cr), juvenile, premature, mature and senescent clusters, see also Table 1. From the non-clusterized roots only the apices were collected (Apex ncr). Flavonoids were extracted from the different root stages by gently shaking the different root parts in 80% methanol for 1 h (for the detailed extraction procedure, see Weisskopf et al., 2006a). The HPLC-ESI-MS analysis showed that flavonoids, and especially isoflavonoids, were by far the most abundant phenolic compounds in white lupin roots. A comparison between root contents and root exudates revealed that the same flavonoids were present in and excreted from white lupin cluster roots; furthermore, genistein was the major compound both in root tissues (about 2 mg g^{-1} root FW) and in exudates (0.4 mg g^{-1} root FW h^{-1}) (Weisskopf et al., 2006a).

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Table 1
P and ^{59}Fe mobilization by citrate or flavonoids, and Fe(III) reduction by flavonoids extracted from different types of root collected from P-deficient white lupin plants

Treatment	Mobilization (μmol mg^{-1}h^{-1})		$Fe(OH)_3$ reduction (μmol Fe mg^{-1}h^{-1})
	P	^{59}Fe	
Citrate	9.76±2.50 c	7.96±0.62 d	*Not Determined*
Apexncr	1.02±0.09 a	1.34±0.10 a	0.07±0.05 a b
Apexcr	3.67±0.79 b	1.55±0.41 ab	0.03±0.01 a
Juvenile	3.27±0.68 b	2.06±0.29 b	0.12±0.02 b
Premature	4.54±0.90 b	2.14±0.18 b	0.18±0.01 b
Mature	4.82±1.04 b	2.04±0.35 b	0.15±0.05 b
Senescent	2.94±0.71 ab	0.76±0.72 ab	0.19±0.11 b

It is also reported a scheme of a typical clusterized root.
Flavonoids extracted from: apices of non-clusterized (Apex ncr) and of clusterized roots (Apex cr), cluster roots at different developmental stages (juvenile, premature, mature and senescent). Data are means ± standard deviation ($n = 3$). Different letters indicate significant differences ($P < 0.05$) within each column. Data of P or ^{59}Fe mobilization were calculated as differences between the mobilization level of each treatment (150 μg of flavonoid or citrate) and its control (P: 0.675 ± 0.05; ^{59}Fe: 0.91 ± 0.04 μmol h^{-1}) and then expressed per mg of flavonoids or citrate.

For mobilization experiments, vivianite ($Fe_3(PO_4)_2 \cdot 8H_2O$) suspension was prepared as described by Rosado et al. (2002); for the Fe-mobilization experiments, Fe was labeled with ^{59}Fe (specific activity 133 kBq μmol^{-1} Fe). Mobilization of P_i and ^{59}Fe was determined at pH 6.0 as described by Cesco et al. (2000), putting vivianite inside a dialysis tube in the absence (control) or presence of 150 μg of flavonoid or citrate. Since flavonoids were dissolved in dimethyl sulfoxide (DMSO), all the treatments, control and citrate included, were performed in presence of DMSO at a final concentration of 0.1%. Episodically, samples were taken from the solution outside the dialysis tube and phosphate was measured according to Forbusch (1983) and ^{59}Fe was quantified by liquid scintillation counting. The flavonoids' ability to reduce amorphous Fe-hydroxide was measured spectrophotometrically using the bathophenanthrolinedisulfonate (BPDS) reagent (Chaney et al., 1972). Fifty micrograms of flavonoids were applied on Fe-hydroxide ($Fe(OH)_3$, 6.7 μmol) prepared using the method described by Cesco et al. (2000).

The effects of flavonoids on soil microbial biomass and respiration, citrate mineralization and hydrolase activities were tested using an Eutric Cambisol (World Reference Base for Soil Resources, 1998), agricultural soil with a classical crop rotation since 1974. Soil main characteristics were 81% sand, 6% silt, 13% clay, 0.7% total organic C, 0.07% total N, $pH_{(H_2O)}$ 6.0, CEC, 12.2 cmol kg^{-1}, dithionite–citrate–bicarbonate extractable Fe and Al 4.21 and 0.79 g kg^{-1}, respectively. The soil was sieved (<2 mm) and incubated at 40% WHC for 7 d. After preincubation, 10 g of soil (dry weight basis) were moistened with 1 ml of root flavonoid solution (containing 0.02% DMSO), to reach a final flavonoid concentration of 100 ng g^{-1} soil. Control samples were amended with equivalent amounts of solution containing only 0.02% DMSO. Six replicates were prepared for the addition of flavonoids from each root stage. Three out of these six replicates were amended with citric acid at the rate of 100 mg C kg^{-1} soil, three served as controls. Soils were incubated at 25 °C in the dark in air tight jars provided with valves for gas sampling. After 4 d evolution of CO_2 was measured by gas chromatography (Blackmer and Bremner, 1977) and ATP content was determined according to Ciardi and Nannipieri (1990). Acid and alkaline phosphomonoesterase activities were assayed according to Tabatabai and Bremner (1969) and phosphodiesterase activity as reported by Browman and Tabatabai (1978). Urease activity was assayed as described by Nannipieri et al. (1974), the protease activity was determined according to Ladd and Butler (1972), and the β-glucosidase activity was assayed according to Tabatabai (1982). The statistical significance of differences among the means was assessed by one-way analysis of variance with a confidence interval of 95%.

Table 2
Soil basal respiration, citrate mineralization and ATP content

Treatment	Soil respiration (mg CO_2–C kg^{-1})		ATP content (μg kg^{-1})	
	−Citrate	+Citrate	−Citrate	+Citrate
Control	41.7 ± 2 b	96.9 ± 4.7 c	743 ± 146 a	724 ± 69 a
Apex ncr	26.7 ± 1.4 a	57.4 ± 0.9 b	812 ± 71 a	815 ± 96 a
Apex cr	27.3 ± 1.5 a	43.6 ± 2.0 a	774 ± 75 a	876 ± 59 a
Juvenile	26.7 ± 0.4 a	35.6 ± 0.6 a	804 ± 36 a	801 ± 92 a
Premature	28.5 ± 0.6 a	62.3 ± 2.3 b	757 ± 87 a	713 ± 46 a
Mature	26.9 ± 0.5 a	53.9 ± 0.9 b	710 ± 40 a	758 ± 85 a
Senescent	45.5 ± 1.03 c	103.7 ± 1.13 d	810 ± 137 a	637 ± 79 a

Flavonoids extracted from: apices of non-clusterized (Apex ncr) and of clusterized roots (Apex cr), cluster roots at different developmental stages (juvenile, premature, mature and senescent). Data are means ± standard deviation ($n = 3$). Different letters indicate significant differences ($P < 0.05$) within each column.

Results show that flavonoid additions to the ^{59}Fe–vivianite suspension enhanced the release of both ^{59}Fe and P_i in the external solution (Table 1), with citrate having the highest mobilization rates, probably through ligand exchange (Gerke et al., 1994). The ^{59}Fe/P ratio values were generally <1, suggesting that part of the Fe mobilized remained in solution and part precipitated as Fe-oxide. This might be due to an incongruent dissolution of the vivianite (Roldán et al., 2002). Flavonoids reduced Fe (III) when supplied as $Fe(OH)_3$ (Table 1). This result supports the hypothesis

Table 3
Enzyme activities of the soil with or without flavonoids, amended or not with citrate

Treatment	Enzyme activities											
	Acid phosphatase (mg *p*-NP kg^{-1} h^{-1})		Alkaline phosphatase (mg *p*-NP kg^{-1} h^{-1})		Phosphodiesterase (mg *p*-NP kg^{-1} h^{-1})		β-Glucosidase (mg *p*-NP kg^{-1} h^{-1})		Urease (mg NH_4^+–N kg^{-1} h^{-1})		Protease (mg NH_4^+–N kg^{-1} h^{-1})	
	−Citrate	+Citrate	−Citrate	+Citrate	−Citrate	+Citrate	−Citrate	+Citrate	−Citrate	+Citrate	−Citrate	+Citrate
Control	12,280 b	12,934 c	1281 b	1296 c	832 b	**896 c**	496 a	482 ab	8.5 ab	6.5 a	29.7 ab	21.8 a
Apex ncr	8845 a	**10,852 b**	1119 a	1218 bc	674 a	**818 b**	476 a	552 b	8.7 ab	10.8 b	26.2 ab	27.9 b
Apex cr	9075 a	10,088 ab	1004 a	979 a	678 a	732 a	481 a	532 ab	8.6 ab	10.2 b	20.0 ab	24.8 b
Juvenile	8013 a	9434 a	1019 a	1014 ab	641 a	**741 ab**	457 a	466 a	9.8 b	11.4 b	25.2 b	29.8 b
Premature	8407 a	8808 a	1076 a	1020 ab	691 a	724 a	516 a	456 a	10.1 b	10.9 b	21.6 b	26.2 b
Mature	8569 a	9502 a	1085 a	1088 ab	664 a	718 a	486 a	470 a	9.4 ab	11.4 b	23.8 ab	29.3 b
Senescent	12,486 b	**13,635 c**	1559 c	**1359 c**	854 b	835 bc	528 a	492 ab	9.5 ab	7.6 ab	26.6 ab	27.4 ab

Flavonoids extracted from: apices of non-clusterized (Apex ncr) and of clusterized roots (Apex cr), cluster roots at different developmental stages (juvenile, premature, mature and senescent). Data are means ($n = 3$). Different letters indicate significant differences ($P < 0.05$) within each column; bolded values indicate significant effect ($P < 0.05$) of citrate addition within each enzyme activity.

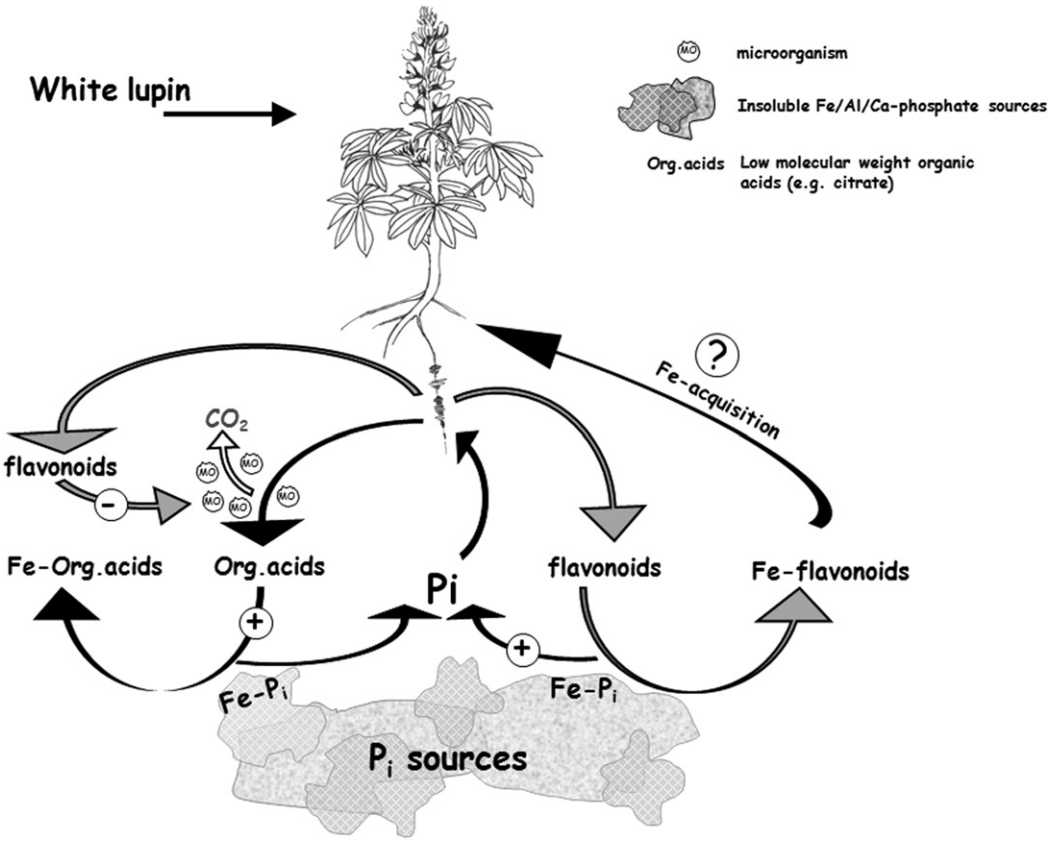

Fig. 1. Proposed role of cluster-root flavonoids in white lupin's strategy for the Pi acquisition from Fe–P sources.

that flavonoids released into the rhizosphere can lead to the reduction of the Fe^{3+} co-precipitated with phosphate (Neumann et al., 2000). Overall, these results confirm that plant-secreted flavonoids can solubilize soil P like synthetic phenolics do (Hu et al., 2005), supporting the findings that limited Fe availability also enhances cluster-root development (Hagström et al., 2001) and root accumulation of phenolics (Liang and Li, 2003), and may partly explain why secretion of phenolics enhances the reutilization of the root apoplastic Fe by Fe-deficient red clover plants (Jin et al., 2007).

Flavonoids, with the exception of those extracted from senescent cluster roots, decreased the cumulative soil basal respiration and the citrate mineralization, whereas soil ATP content was not significantly affected by treatments with flavonoids or citrate (Table 2). These results suggest that flavonoids could affect microbial metabolism without biocidal effects. However, because soil ATP content estimates the whole soil microbial biomass, our results do not contradict the adverse effects of flavonoids detected on specific microbial groups (Hong et al., 2006; Rivera-Vargas et al., 1993; Weisskopf et al., 2006b).

Among the measured soil hydrolases, with the exception of those extracted from senescent cluster roots, flavonoids significantly decreased the phosphatase activities regardless of citrate addition, did not affect protease and β-glucosidase activities, and significantly increased urease activity (Table 3). It is well known that flavonoids can inhibit tyrosine kinase, topoisomerase, and α-glucosidase activities, and affect the cellular turnover of phosphorylated compounds of vertebrates (Wang et al., 2004; Weber et al., 1997). To our knowledge, no data of inhibition of soil phosphatase activity by natural flavonoids have been reported so far. Such selective inhibition of phosphohydrolases may help the plant to compete for P with rhizosphere microorganisms. However, because the used methods do not allow to discriminate between different enzyme locations (Burns, 1982), caution is needed in the interpretation of these results. In general, no clear influence of root type (Apex cr or ncr versus other root samples) and cluster-root stage on flavonoid activities could be evidenced, except for senescent cluster roots, where effects were usually lower than for other roots. This lack of difference in most parameters studied might be due to the fact that the same amount of flavonoid extracts was used in this work for all root types, whereas cluster-root stages actually vary in their amounts of exuded flavonoids, being the highest in the youngest ones (juvenile and premature) (Weisskopf et al., 2006a).

In conclusion, our results show that flavonoids released from the roots of P-deficient white lupins are involved in P-acquisition both directly by mobilizing insoluble Fe-bound P and indirectly by reducing the microbial citrate mineralization and the activity of enzymes involved in microbial P acquisition (Fig. 1).

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