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SHORT COMMUNICATION

Influence of different trap solutions on the determination of root exudates in *Lupinus albus* L.

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Abstract White lupin is very often used as a model plant for root exudation studies due to its capability to release huge amounts of organic acids and flavonoids. The complex nature of these organic compounds makes not only their analytical determination difficult but also their extraction from soil samples. For these reasons simplified approaches, as hydroponic-based systems are widely used to study the root exudation. Therefore, the composition of a trap solution is crucial to limit artefacts causing over/underestimation of exudation rates and/or a biased molecular composition of the collected compounds. The present study was aimed at assessing the influence of different trap solutions and collection times on the quali- and quantitative root exudation pattern of white lupin (*Lupinus albus* L.) grown under phosphorus (P) and iron (Fe) deficiency. Our results suggest that, in works aimed at studying root exudation processes, water is the most effective trap solution to collect the exudates like organic acids and flavonoids, especially in short time (e.g. 2 h). For longer times, low concentrations of Ca could be helpful to limit osmotic stress and possible passive leakage and/or diffusion. The use of bacteriostatic compounds as NaN_3 and Micropur bias the results, due to interferences either with the metabolism or inhibition of the exudation processes, especially in the case of flavonoids such as quercetin. Also, the use of a pH buffer solution like 2-(N-morpholino)ethanesulfonic acid (MES) should be avoided for its undesired interferences with the release.

Keywords Root exudates · Trap solutions · *Lupinus albus* L. · Organic acids · Flavonoids

Introduction

It is well known that roots are able to influence significantly the chemical, physical and biological characteristics of the surrounding soil (rhizosphere) also through the release of organic compounds (Uren 2000; Colombo et al. 2014). These latter, named root exudates, can be (a) passively released by roots (diffusates) thanks to the concentration gradient between root cells and rhizosphere, or (b) actively excreted/secreted in response to nutrient deficiency, metal toxicity and/or interactions with microorganisms (Jones et al. 2004; Bais et al. 2006). Anyway, independently on the release mechanism involved, root exudates comprise a complex mixture of low (organic acids, amino acids, sugars, phenolic acids, flavonoids, phytosiderophores, etc.) and high molecular weight (polysaccharides, enzymes, etc.) organic compounds (Lynch 1970; Pinton et al. 2007; Cesco et al. 2012; Mimmo et al. 2014) with very different biological and chemical properties. These exudates represent a root-to-rhizosphere carbon efflux that can reach values up to 10 % of the net assimilated carbon by plants (Jones et al. 2004). For instance, only recently some authors showed that maize roots released up to 166 kg C ha^{-1} as rhizodeposited-C belowground, 50 % of which was recovered in the upper 10 cm (Pausch et al. 2013). The qualitative and quantitative exudation pattern is however affected by several environmental factors, including pH (Meharg and Killham 1990), soil type (Van Veen et al. 1985), oxygen status

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(Wiedenroth and Poskuta 1981), light intensity, soil temperature (Graham et al. 1982), nutrient availability (Kraffczyk et al. 1984), the presence of microorganisms (Meharg and Killham 1991) and plant species (Lambers 1987). It is interesting to note that, as a consequence of this variability in the type, amount and chemical properties of root exudate release, the rhizosphere of a specific plant species can vary consistently along the root both in time and in space (Terzano et al. 2014).

White lupin is a plant species well-known for its capability to exude huge amounts of organic acids and flavonoids under nutritional disorder (Weisskopf et al. 2006a; Tomasi et al. 2008; Mimmo et al. 2011); this process is mainly localized in clusters of closely spaced lateral rootlets (named cluster or proteoid roots), characterized for their limited growth and dense cover with root hairs (Gardner et al. 1983; Dinkelaker et al. 1989). For these characteristics, white lupin is able to survive and to complete the life cycle also in the presence of barely available nutrient sources (Lambers et al. 2006). This plant species has been very often used as a model plant for studies aiming at a deeper and more detailed comprehension of the root exudation process (Florez-Sarasa et al. 2014). However, for the other plant species as crops, this process is still poorly defined since root exudates are barely quali-quantified, both at the rhizosphere level and at the field scale. Furthermore, the complex nature of these organic compounds makes not only their analytical determination difficult but also their extraction from soil samples, due to their capability to interact with soil particles. For these reasons, in studies dealing with the root exudation process, simplified approaches, like the culture-based systems, have been widely used (Neumann and Römheld 1999; Cesco et al. 2002; Gottardi et al. 2012). In these studies, plants were grown in either pure nutrient solution or in semi-hydroponic conditions with a solid support (e.g. quartz sand, perlite, etc.); therefore, exudates were collected by immerging the root system in a trap solution (water, $CaCl_2$, $CaSO_4$, etc.) for a time period ranging from 2 min to 25 days (Vranova et al. 2013). This methodological approach is very simple and can be managed and adapted easily. It has also the advantage of allowing frequent measurements, as those necessary for the estimation of kinetic parameters of an enzymatic process like exudation mechanism mediated by active transport (Tomasi et al. 2009). In addition, with hydroponic systems, it is possible to limit root injuries (very easy and frequent in the case of root removal from soil, even when plants are grown in rhizoboxes) that can be the cause of an overestimation of the root exudation process. Also, the risk of microbial contamination/degradation due to the presence of soil particles can be prevented with hydroponic approaches. Considering these methodological aspects (related to the quali-quantitative analysis of root exudation process), it is interesting to note that plant roots grown in solution cultures can be morphologically and physiologically different from those of soil-grown plants and can exhibit a stimulated root exudation (Boeuf-Tremblay et al. 1995; Groleau-Renaud et al. 1998). Moreover, the sterile conditions in the collection of root exudates, often desired in order to preserve the presence of these molecules in the trap solution, might bias their quantification. For instance, non-sterile conditions might influence the exudation pattern in plants exposed to stress like nutritional disorders. Therefore, considering all these aspects, it appears evident that the composition of the trap solution is crucial to limit artefacts causing over/underestimation of exudation rates and/or a biased molecular composition of the collected exudates. Nonetheless, this aspect is very important to understand rhizosphere processes related to root exudation dependent nutrient acquisition by crops, yet to date not clearly defined.

For these reasons, the present study was aimed at assessing the influence exerted by the different composition of the trap solution and collection times on the quali- and quantitative exudation pattern of white lupin (*Lupinus albus* L.) grown under control condition and phosphorus (P) and iron (Fe) deficiency. Here, we aimed at comparing distilled water, MES-KOH usually used in nutrient solution as buffer (Tomasi et al. 2009), Micropur used as a bacteriostatic agent in hydroponics (Tomasi et al. 2012) and sodium azide (NaN_3) which to date has only been used as a bacteriostatic agent added in soil or soil solutions (Oburger et al. 2014). To our knowledge, to date, there is no comparative study regarding the effect of the trap solution on the determination of root exudates which could bias the results.

Materials and methods

Plant growth

White lupin seeds (*Lupinus albus* L. cv. Amiga; Südwestdeutsche Saatzucht, Rastatt, Germany), were soaked for 24 h in an aerated 0.5 mM $CaSO_4$ solution and germinated for 4–5 days in the dark at 22 °C between two layers of filter paper moistened with 0.5 mM $CaSO_4$. Homogeneous seedlings were selected and transferred in black pots (in order to limit photochemical reduction phenomena, Zancan et al. 2006) containing an aerated nutrient solution with the following composition: mM, 0.25 KH_2PO_4, 5 $Ca(NO_3)_2$ 4 H_2O, 1.25 $MgSO_4$, 1.75 K_2SO_4, 0.25 KCl; μM: 20 Fe(III)-EDTA, 25 H_3BO_4, 1.25 $MnSO_4$ 7H_2O, 1.5 $ZnSO_4$ 7H_2O, 0.5 $CuSO_4$ 5H_2O, 0.025 $(NH_4)_6Mo_7O_{24}$ 4H_2O. The nutrient solution in the pots was renewed every 3 days. After one week of plant growth in a full nutrient solution, the plants were transferred either to a full nutrient solution (control), to an Fe-free (−Fe) or to a P-free (−P) nutrient solution. The plants were grown in a growth chamber under controlled conditions for additional

4 weeks (day 14 h 24 °C 70 % RH, night 10 h 19 °C 70 % RH). Experiments were carried out in triplicate.

Collection of root exudates

At harvest, the plants were removed from the nutrient solutions and roots were washed several times with distilled water in order to remove any traces of nutrient solution. The plants were then transferred in smaller pots containing 100 ml of trap solution. The following trap solutions were used in the experiment: H_2O MQ, Micropur 1 mg L^{-1} (Tomasi et al. 2012), NaN_3 1 mM, MES-KOH (2-(N-morpholino)ethanesulfonic acid) 10 mM pH 6 (Tomasi et al. 2009). Root exudates were collected for 2 and 24 h, respectively, continuously aerating the trap solutions and covering the pots with aluminium foil to maintain the roots in the dark. After 2 h/24 h, the plants were removed, the root systems weighed and stored at −80 °C until further analysis. The trap solutions were filtered at 0.45 μm (Spartan RC, Whatman), frozen at −20 °C, lyophilized and resuspended in either distilled water (for organic acid determination) or methanol (for flavonoid determination).

Root extractions

Samples of white lupin roots were incubated in 10 ml of 100 % (*v/v*) methanol for 1 h at room temperature under gentle shaking to extract the internal content of organic acids and flavonoids. The roots were then removed and the remaining solution filtered at 0.45 μm (Spartan RC, Whatman). The methanol solutions were afterwards placed overnight under a hood to evaporate the solvent. After that, the extracts were resuspended in distilled water for the organic acid determination and in methanol for the determination of phenolic compounds.

Organic acid and flavonoid analysis

Organic acids were separated by high-performance liquid chromatography (HPLC) using a cation exchange column (Rezex ROA, Phenomenex; 300×7.8 mm), with an isocratic elution with 10 mM H_2SO_4 as carrier solution at a flow rate of 0.6 ml min^{-1}. The organic acids were detected at 210 nm using a Waters Photodiode array detector (PDA 2998, Waters Spa, Italy). Standard acids were prepared as individual stock solutions using Sigma free acids and then combined to give diluted reference standards. The organic acids were identified by comparing retention times of unknowns to pure organic acids and by standard additions (Sandnes et al. 2005).

Flavonoids were separated by HPLC with a XBridge Shield-C18 column (Waters), using an isocratic elution with 60 % solvent A (acetonitrile 100 %) and 40 % solvent B (phosphoric acid 0.1 %) as mobile phases at a flow rate of 1 ml min^{-1} and detected at 254 nm (PDA 2998 Waters Spa, Italy). Standard solutions were prepared as individual stock solutions using Sigma HPLC-grade reference compound for genistein and Extrasynthese HPLC-grade for quercetin and then combined to give diluted reference standards. The flavonoids were identified by comparing retention times of unknowns to pure compounds and by standard additions. Method modified from Inderit et al. (2008). We employed an isocratic elution to eliminate variability among peak areas that occurs when employing an increasing gradient elution.

Statistical analysis

The results are presented as means of at least three replicates± standard deviation (SD). Statistical analysis was performed using Statgraphics (Statpoint Technologies, Inc., Warrenton, VA, USA). Data were analysed by analysis of variance (ANOVA), and means were compared using SNK's test at $P<0.05$ to determine the significance of differences found.

Results

Release of organic acids

Citric and malic acid could be identified in the root exudates of white lupin. Oxalic acid was identified in some samples but was however below the limit of quantification. Figure 1a and b show the release of citrate measured in root exudates of white lupin collected after 2 or 24 h of contact with the trap solutions, respectively. This release was significantly affected by collection time ($P<0.001$), treatment ($P<0.001$, Fe and P deficiency vs control) and type of trap solution ($P<0.05$) resulted significant. Overall, the release of citrate is significantly higher in solutions collected from –Fe plants, both after 2 and 24 h. Lowest concentrations of citrate were found in samples collected from control plants. The composition of trap solutions affected the release of citrate. In fact, after 2 h, the concentration of citrate in –Fe samples was at least two times higher in water compared to samples collected in Micropur, NaN_3 and MES. Overall, Micropur reduced citrate release, especially in exudates collected in 24 h (Fig. 1b).

Concerning malate (Table 1), after 2 h, malic acid could not be detected in every analysed samples and was not influenced significantly by the nutrient deficiency treatments. In control plants, the highest concentrations of this organic acid were determined in solutions of NaN_3 followed by water, Micropur and MES. A similar pattern was observed after 24 h where malate concentration in NaN_3 solutions was higher than in the other trap solutions; in this case, no significant difference between nutritional treatments was observed. Root exudates

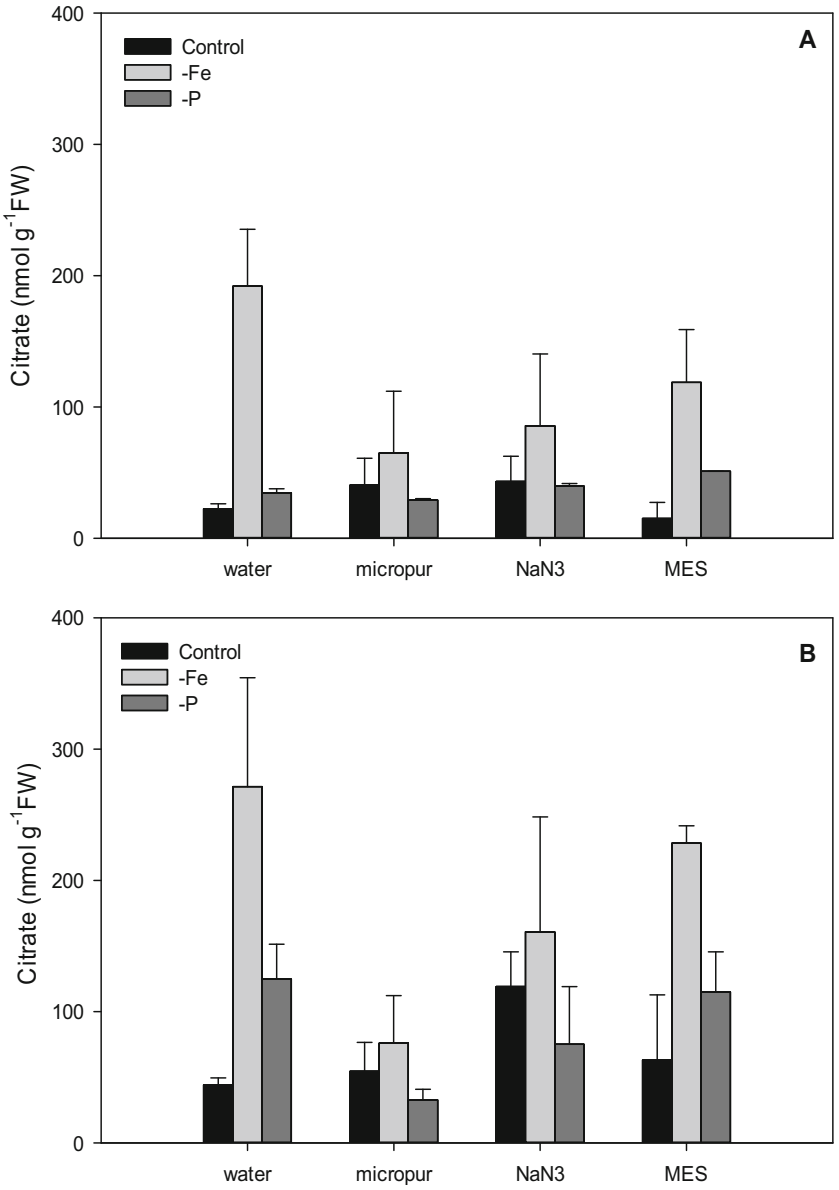

Fig. 1 Citric acid detected on root exudates collected from lupin roots in 2 (**a**) and 24 h (**b**), (mean ±SD, n=3); Three-way ANOVA results: collection time (P<0.001), treatment (P<0.001) and type of trap solution (P<0.05); all the interactions resulted not significant

released by control plants in water contained three times lower malate than those analysed in roots immersed in NaN_3 containing trap solution; this behaviour was less pronounced when plants were exposed to a nutritional disorder (−Fe and –P). Exudates collected in the Micropur solutions showed instead the lowest contents of malate (at least five times lower) in all treatments (Table 1).

Release of flavonoids

Two types of flavonoids could be determined in the root exudates of white lupin, identified as genistein and quercetin by comparison with standard references and standard additions. Other chromatographic peaks were detected, but the concentration was too low for an unambiguous identification. Table 1 shows the effect of the four different trap solutions and three plant nutritional treatments on the concentration of genistein and quercetin in root exudates collected after 2 and 24 h, respectively.

In the case of 2 h root exudate collection, the genistein concentration of control plants measured in water was the highest, while the level of this flavonoid in exudates collected in the presence of NaN_3 resulted the lowest (four times lower than water). Among the three nutritional states, the highest concentrations of genistein were detected in samples of Fe-deficient plants, with the release having the following order: NaN_3 > MES > water ≥ Micropur. Overall, the lowest levels of genistein were found in exudates collected from P-deficient plants, irrespectively from the composition of the trap solution.

Table 1 Malate, genistein and quercetin release given in nmol g^{-1} FW (mean±SD, n=3) determined in root exudates collected from lupin roots in 2 and 24 h, respectively

	2 h			24 h		
	Control	-Fe	-P	Control	-Fe	-P
Malate						
Water	5.72±0.20	< LOD	< LOD	8.27±3.80	20.10±2.43	21.03±2.34
Micropur	1.51±1.24	4.44±1.86	< LOD	5.49±2.62	< LOD	1.67±0.87
NaN_3	13.24±3.95	1.58±1.58	5.69±0.13	26.77±2.98	31.19±3.23	24.07±3.56
MES	2.88±1.80	< LOD	< LOD	6.68±1.84	< LOD	< LOD
Genistein						
Water	29.12±0.41	21.38±4.80	9.77±4.19	17.13±5.43	24.44±3.15	26.45±0.55
Micropur	15.36±5.29	20.08±0.61	6.30±0.78	20.25±6.11	29.55±10.52	7.87±1.79
NaN_3	7.49±0.44	35.51±2.76	8.81±4.15	18.88±3.22	69.82±11.98	39.07±6.79
MES	19.89±5.65	30.42±2.48	10.88±3.60	11.83±1.57	< LOD	43.76±8.73
Quercetin						
Water	12.49±0.30	21.38±4.80	< LOD	9.34±2.23	24.44±3.15	14.09±2.14
Micropur	10.96±1.54	20.08±0.61	5.31±0.82	13.47±3.31	29.55±10.52	53.84±17.68
NaN_3	9.71±2.38	53.51±3.45	< LOD	31.50±14.33	69.82±11.98	58.61±9.30
MES	23.38±11.17	30.42±2.48	< LOD	15.10±0.32	< LOD	< LOD

Three-way ANOVA results for malate: collection time (P<0.001), treatment not significant (ns), type of trap solution (P<0.05), type of trap solution × treatment (ns), type of trap solution × collection time (P<0.01), treatment × collection time (P<0.05), treatment × collection time × type of trap solution (ns); Three-way ANOVA results for genistein: collection time (P<0.01), treatment (P<0.001), type of trap solution (P<0.01), type of trap solution × treatment (P<0.001), type of trap solution × collection time (ns), treatment × collection time (P<0.05), treatment × collection time × type of trap solution (ns); Three-way ANOVA results for quercetin: collection time (P<0.05), treatment (P<0.05), type of trap solution (ns), type of trap solution × treatment (ns), type of trap solution × collection time (ns), treatment × collection time (ns), treatment × collection time × type of trap solution (ns)

LOD limit of detection

After 24 h, the release of genistein was differently affected by trap solutions: exudates of control plants showed the lowest genistein concentration compared to those collected from -Fe and –P plants. Considering the composition of trap solution, the lowest level of this flavonoid was recorded in MES-trap solutions, while in the other three conditions, the concentrations of genistein was essentially the same. With respect to the nutritional plant states, the highest concentrations of genistein were found in samples of Fe-deficient plants, with a peak when NaN_3-containing trap solution was used (2.5 times higher than water and micropur). Differently, samples of exudates collected from P-deficient plants exhibited concentrations of genistein in MES and NaN_3 trap solutions higher than the other two conditions (water and Micropur), being the lowest value when Micropur trap solution is considered.

Quercetin was the second flavonoid detected in the exudates. This flavonoid was mainly detected in exudates of controls and Fe-deficient plants after 2 h of collection (Table 1). All the treatments lead to highest concentrations in trap solutions containing MES (23.38 and 30.42 nmol g^{-1} FW in control and –Fe plants, respectively), while in the other trap solutions, the concentrations of quercetin were halved and not significantly different among them. In P-deficient plants, quercetin was detected only in trap solutions containing Micropur (Table 1).

After 24 h, the maximum release of quercetin was determined in samples collected in –Fe plants, in particular when Micropur and NaN_3 were present in the trap solutions; in water the concentration of quercetin was about halved (24.48 nmol g^{-1} FW). The highest concentration of this flavonoid was detected in NaN_3 solution of control plants, while the minimum was found when water was used for the collection of the exudates. The same behaviour was observed in samples collected from roots of P-deficient plants (Table 1).

Root exudates vs root content ratio

Organic acids (citrate and malate) and flavonoids (genistein and quercetin) were also determined in root tissues at the end of the experiment, and these values have been used to calculate the ratios between the release (R) and the root content (C) of these compounds, after 2 and 24 h, in the different

nutritional plant states and as a function of the diverse trap solutions (Online resource 1).

Regarding citrate, all ratios were lower than 1 with the only exception of the samples of –P plants collected in Micropur for 24 h. At this collection time, R/C were higher in samples collected in water and Micropur trap solutions of –Fe and –P plants as compared to those of control plants; differently, no significant differences were found among the nutritional states using NaN_3 and MES-trap solutions. Overall, a significant increase in the values of R/C, comparing same treatments and solutions, was observed after 24 h. In particular, the R/C obtained with of Micropur and NaN_3 trap solutions was up to 20 times higher (Micropur –P), while a decrease was detected only in –P water and MES samples.

As for citrate, the values of R/C were lower than 1 (Online resource 1); however, not all R/C values could be calculated since many concentrations were < LOD. However, higher R/C values were found in NaN_3 solutions for all treatments after 2 h. Considering controls, R/C increased after 24 h in all trap solutions considered (Online resource 1).

As seen with the other compounds detected in the trap solutions, also the R/C values for genistein were lower than 1. After 2 h, the highest R/C were determined in solutions obtained from Fe- and P-deficient plants in water. Control samples showed the lowest values. Comparing the R/C values at 2 and 24 h, respectively, a huge increase (sixfold) for genistein was detected in solutions containing NaN_3 of Fe- and P-deficient plants. Other ratios have not shown significant differences.

The second flavonoid detected, quercetin, showed R/C values higher than 1 (with the exception of Micropur solution in control samples) after 2 h (Online resource 1). Ratios were fluctuating over treatments and different solutions; however, the highest R/C values were detected in NaN_3 solutions in Fe- and P-deficient plants. After 24 h, the ratios were all below 1 in controls, and all decreased in Fe-deficient plants; P-deficient plants showed instead stable R/C values, with the exception of MES solution, which increased.

Discussion

In the present study, for a better comprehension of root exudation, we collected root exudates released by white lupin (*Lupinus albus* L.) roots of intact plants grown under three different nutritional states (control, Fe- and P-deficiency) for 2 and 24 h. The root exudates were collected using four different trap solutions (distilled water or NaN_3-, MES- and Micropur-containing solutions) by analysing the exudation pattern (in terms of citrate, malate, genistein and quercetin).

Distilled water used as a trap solution guaranteed generally higher values of exudates, as compared to the other three trap solutions. Considering the higher R/C values in plants exposed to distilled water with respect to those treated with the other three trap solutions, it appears evident that the improved performance of the distilled water is probably ascribable, at least in part, to an osmotic stress imposed to roots by the distilled water. For longer collection periods than 2 h, an addition of an electrolyte as Ca might overcome excessive osmotic stress stabilizing the membranes (Schapire et al. 2009). On the other side, considering the aim of these collections, it is necessary to point out that the addition of ions to the trap solution might interfere with the subsequent quantitative analysis of the exudates. In fact, for the generally low concentrations of exudates in the trap solution, vacuum evaporation or lyophilization of the liquid samples are commonly required. This concentration step can on one side favor the detection of the exudates but on the other, it might easily lead to very high salt concentrations interfering in turn within the analytical method and/or cause irreversible precipitation of certain exudate compounds (Neumann and Römheld 2007). As a consequence, the exudate pattern by roots can be altered giving an indication very different of what really occurs in the rhizosphere. The use of distilled water as a trap solution should thus be favored, especially for short collection times to avoid artefacts, both during the collection and analysis phases.

Considering the time of exudate collection, the results here presented show that with distilled water the longer collection time (from 2 to 24 h) of the root contact with the trap solution affected only weakly the exudate detection, particularly when this value is compared with those obtained with the other trap solutions. These slightly lower concentrations observed in distilled water could be ascribed to a higher microbial degradation, reducing especially the concentration of organic acids (Jones 1998). To limit this phenomenon, the addition to the trap solution of compounds like Micropur or NaN_3 as bacteriostatic agents could represent a useful strategy. However, the results here presented clearly show that the addition of Micropur to the trap solution impaired the release of malate, citrate and genistein. There are other evidence in literature indicating a negative effect of the presence of Micropur in the trap solutions (Neumann and Römheld 2007), although these effects were detected using high concentrations of the bacteriostatic agent (2.5–10 mg L^{-1}). In any case, considering that in the present work, Micropur was used at 1 mg L^{-1}, this evidence seems to discourage the use of this compound in the trap solution for exudate collection. In contrast to Micropur, when NaN_3 was added to the trap solution, a higher detection of root exudates than those in the other trap solutions was observed. This phenomenon was particularly evident when the collection was prolonged up to 24 h and in white lupin plants exposed to nutrient deficiencies. Bacteriostatic agents as sodium azide is very often used to prevent degradation once the exudates are collected (Oburger et al. 2014; Gent et al. 2005), but its effect on the root exudation of intact roots has not been investigated yet. In this respect, it is interesting to

point out that a weakening of the membrane integrity of plants can occur as a consequence of P-deficiency (Shen et al. 2011). In the conditions of our study, the combination of NaN_3 and P-deficiency might thus further affect the stability of the membranes, allowing a passive diffusion and/or a leakage of exudates in the trap solution. Moreover, the difference in root contents of organic acids and flavonoids after the exposure to NaN_3 seems to indicate an action of this bacteriostatic also at the level of plant metabolism related to the synthesis of these metabolites. For these reasons, it is reasonable that the higher concentrations detected in root exudates detected in NaN_3 trap solutions are most likely due to these secondary and undesirable effects rather than to the bacteriostatic action of this compound in favor to the preservation of the exudates.

Besides water, the addition of MES to the trap solution has been widely used for the collection of root exudates (El-Baz et al. 2004; Tomasi et al. 2009, 2012) since it has the advantage to maintain the pH stable. In this work, MES was on one side able to guarantee efficiently the pH buffering during the collection but on the other gave contrasting results regarding the exudate concentrations detected. A similar effect of MES was also described by El-Baz et al. (2004). Thus, considering the difficulties in interpreting and comparing data obtained with MES as the trap solution with those present in literature, the use of this compound for the purposes similar to those here described should therefore not be recommended, at least when organic acids and flavonoids are considered.

The release of huge concentrations of organic acids and flavonoids under P- and Fe-deficiency both in hydroponic (Tomasi et al. 2008) and soil conditions (Mimmo et al. 2011) has been well-described in white lupin. This exudation process is spatially and temporally localized for some compounds like citrate (Neumann and Römheld 1999; Neumann et al. 1999; Tomasi et al. 2009), while only partial information is available for other compounds such as flavonoids (Weisskopf et al. 2006b). Comparing the two nutritional disorders, data here presented showed that Fe-deficiency induced in white lupin roots mainly released citrate, as also described by Hagström et al. (2001). The citrate release was higher prolonging the collection time to 24 h, indicating a continuous release of this compound during the day. An exception to this behaviour among the four trap solutions here considered has been recorded with the solution containing Micropur, where the concentration of citrate released was essentially the same in the two time intervals considered. With respect to malate, its release in Fe-deficiency was mainly detected in water after 2 h while it was not detectable in the Micropur- and MES-containing trap solutions. The huge release of malate measured after 24 h in the presence of NaN_3 could indicate alterations of the integrity and permeability of the membranes leading to an uncontrolled leakage of compounds including malate. In Fe-deficiency, also the release of both flavonoids genistein and quercetin was very pronounced. Considering the redox and complexing properties of these compounds (Cesco et al. 2010, 2012), this effect could be part of the strategy adopted by this plant species to cope with the micronutritional deficiency.

With respect to P-deficiency, the results here presented show that the release of citrate by plants exposed to this nutritional disorder is generally higher than that measured in control plants but never reaching the extents measured in Fe-deficient roots. Also in the case of malate, a similar pattern of exudation was observed; however, the extent of malate release was always lower than that measured for citrate. Concerning the release of flavonoids in white lupin plants exposed to this nutritional stress, the low level of genistein detected in the trap solution after 2 h (with an extent even lower than that measured in P-sufficient control plants) is followed by a huge release after 24 h. If this effect could be related to a time-dependent exudation pattern for genistein in P-deficient white lupin, the evidence here presented needs further experimental evidence.

Conclusions

In conclusion, the results here presented suggest that in works aimed at studying root exudation processes, distilled water is the most suitable trap solution to collect the exudates like organic acids and flavonoids. Even though there might be a slight degradation of these compounds in water, sterile or semi-sterile conditions could be of great benefit in limiting this process. Moreover, the addition of low concentrations of $CaSO_4$ or $CaCl_2$ could help in contrasting osmotic stress; with this respect, the ion concentration adopted is crucial in restricting the risk of salinity (high salt concentration), a limiting factor during further processing of the samples (e.g. lyophilisation and then analysis). The use of MES and NaN_3 should be avoided for their undesired interferences with the root exudate release process as well as Micropur for its capacity to inhibit the process. This study highlighted that one of the most crucial aspects in the root exudate determination is the method adopted for their collection. Caution should therefore be taken in the interpretation of data from exudation studies and comparisons using different collection times and procedures.

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