The carboxylate composition of rhizosheath and root exudates from twelve species of grassland and crop legumes with special reference to the occurrence of citramalate.

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ABSTRACT

Aims

Low-molecular-weight organic anions (carboxylates) influence rhizosphere processes and may enhance plant phosphorus acquisition. We examined the root exudate profile of a range of pasture and grain legumes and focused on the little-investigated carboxylate, citramalate.

Methods

Twelve species of pasture legumes and herbs, including four *Lotus* spp. and two crop legumes, *Cicer arietinum* and *Lupinus albus*, were grown in a glasshouse for six weeks. The composition and amounts of carboxylates were measured in rhizosheath soil as well as in root exudates from roots washed free of their rhizosheath.

Results

Citrate and malate were found in the rhizosheath of all species. However, citramalate was present in the rhizosheath of only *Lotus* species (10–82% of total exuded carboxylates). *Cicer arietinum* had the largest amount of carboxylates in its rhizosheath and fastest rate of carboxylate exudation into the trap solution.

Conclusions

Citrate and malate were found in the rhizosheath of all species in this study, but citramalate was only found in the root exudates and rhizosheath of *Lotus* spp. Further investigation into the role of citramalate in *Lotus* spp. is merited.

Keywords: citrate, exudation, *Lotus*, organic acids, phosphorus, rhizosheath

INTRODUCTION

Plant nutrient acquisition is becoming an important topic in crop and pasture research as we respond to increasing fertiliser costs, environmental issues associated with nutrient runoff and the realisation that we are dealing with a finite resource (Bennett et al. 2001; Cathcart 1980; Johnston et al. 2014). Investigating pasture species that can yield well with less inputs and yet maintain the dietary requirements for animal production is critical to improving the efficiency

of pastures in southern Australia and elsewhere (Simpson et al. 2011, 2015). Many legume species have been introduced to southern Australian pasture systems, and initial breeding efforts largely concentrated on increasing yields under sufficient fertiliser regimes. However, rising fertiliser costs have prompted some farmers to investigate ways of reducing their inputs. Generally, in a mixed pasture–cropping system, the pasture fertilisation programme will be the first to be reduced, due to the higher financial returns of crops compared to pastures. Introduced pasture legumes such as subterranean clover (*Trifolium subterraneum*) and white clover (*T. repens*) have high nutritional requirements, and pasture productivity could be seriously affected by reducing fertiliser inputs (Burkitt et al. 2007). Species such as serradella (*Ornithopus*) and biserulla (*Biserulla pelecinus*) are included in a new breeding effort to develop cultivars that will exploit environments less suited to the more traditional species (Nichols et al. 2007). *Lotus corniculatus* is another species that has undergone extensive breeding and selection. It was introduced to southern Australia for its forage value and high condensed tannin content, which reduces bloat in sheep and cattle (John and Lancashire 1981). Other characteristics, such as tolerance to drought and infertile soils (Seaney and Henson 1970), coupled with its successful introduction in South America, also motivated its development.

Until recently, there have been few studies of the root systems of species used in southern Australian pastures. Recent studies have investigated root morphology traits of pasture species that explain clear differences in their phosphorus (P) acquisition capacities (Haling et al. 2016; Hill et al. 2010; Kidd et al. 2015; Ryan et al. 2016; Yang et al. 2015). Physiological mechanisms associated with P acquisition, namely exudation of low-molecular weight organic anions (carboxylates), have also been investigated. For instance, Kidd et al. (2015) examined 16 current and novel pasture legumes and found that the most abundant carboxylates from roots were citrate and malonate. However, while it seems that pasture legumes commonly exude carboxylates, their role in nutrient acquisition is unclear. Carboxylate exudation from roots of the white lupin (*Lupinus albus*) (Dinkelaker et al. 1989; Gardner et al. 1982; Hocking et al. 1997), for which there is convincing evidence of P solubilisation by citrate, has been examined extensively. The mobilised P is available for uptake by lupin roots and in some instances by neighbouring plants (Gardner and Boundy 1983; Horst and Waschkies 1987). The amounts of rhizosheath carboxylates in pasture legumes rarely reach the amounts recorded for *L. albus* (Kidd et al. 2015), and thus they are less likely to acquire significant amounts of P in this manner. However, there are many other

interactions that involve root exudates in the rhizosphere. These include, but are not limited to, chemotaxis (essential for legume nodulation) (Currier and Strobel 1976), as well as stimulation or inhibition of fungal and microbial communities, including root pathogens (Barber and Lynch 1977; Martin et al. 2016; Přikryl and Vančura 1980; Rovira 1959; Rudrappa et al. 2008; Weisskopf et al. 2006). These interactions with root exudates may be of equal importance to nutrient acquisition as the preservation of the root itself is vital for efficient nutrient uptake.

While much research has been undertaken on commonly reported carboxylates such as citrate, malonate, malate and oxalate, relatively little is known about a range of other carboxylates. In this experiment we focused on a little-studied carboxylate, citramalate, because it was found to be a relatively important component of exudates from *Lotus* spp. Citramalate or its conjugate, citramalic acid, is commonly associated with the accumulation of citrate in early fruit development of citrus (Bogin and Wallace 1966) and a general increase in the acidity of the fruits. It is also found in relation to anthocyanin accumulation in the skin of red apples (Fernández-Trujillo et al. 2001; Noro et al. 1988). However, it has rarely been studied in the context of root exudation (Khorassani et al. 2011; Tawaraya et al. 2014). It has been found to solubilise sparingly available P through ligand exchange, but was reported to be less effective than citrate (Khorassani et al. 2011).

The core focus of this experiment was to determine whether citramalate exudation is common to the *Lotus* genus and whether it occurs in other grassland and crop legumes.

MATERIALS AND METHODS

Experimental setup

This experiment examined 15 cultivars/lines from 12 species (Table 1). Four species were from the *Lotus* genus: commercial cultivar and breeders line of *L. corniculatus* (San Gabriel (SG) and LC07AUYF (LC)), a naturalised weed species (*L. subbiflorus*) and two Australian native species (*L. cruentus* and *L. australis*). Also included was the annual pasture legume, *Ornithopus sativus*, an *O. sativus* x *O. compressus* cross, two perennial pasture legumes (*Trifolium repens* and *Lotononis bainesii*), one pasture herb (*Cichorium intybus*) and one native Australian herb (*Ptilotus polystachyus*) known to have a remarkable ability to grow under low-P conditions (Ryan et al. 2009). Two crop pulse legumes (*Cicer arietinum* and *Lupinus albus*) were included as reference species for malonate and citrate, respectively.

The experiment was conducted under glasshouse conditions at The University of Western Australia (31°98' S, 115°81' E) from 12 March to 23 April 2015. Glasshouse temperatures were maintained between 13 °C and 30 °C (average of 20 °C) and average relative humidity was 72%. All pots from the four replicates were randomly relocated within the area occupied by the experiment weekly to minimise the influence of temperature and light gradients in the glasshouse.

Plants were grown in washed river sand (RS), which is low in most essential nutrients and is used to allow the collection of carboxylates due to its low adsorption capacity and microbial community (Ryan et al. 2012). Nutrient composition was: 2 mg kg⁻¹ ammonium-N, <1 mg kg⁻¹ nitrate-N (Searle 1984), 2 mg kg⁻¹ bicarbonate-extractable P, 20 mg kg⁻¹ bicarbonate-extractable potassium (Colwell 1963) and 7.4 mg kg⁻¹ sulfur (Blair et al. 1991). The pH in CaCl₂ was 7.8 (Rayment and Higginson 1992). *Cicer arietinum* was also grown in a red sandy loam sourced from Cunderdin (C) Western Australia (31°63' S, 117°24' E) as in previous experiments this species has performed poorly when grown in river sand (Kidd et al. 2015).

The river sand was pasteurised at 60 °C for 1 h and oven-dried at 40 °C for five days before weighing 1.2 kg into open rectangular pots (90 × 90 × 180 mm). A light gauze was placed at the bottom of the pots to retain the sand. The sand was watered to field capacity by adding 200 ml of deionised water and was then allowed to settle for two days before planting. The pots were then maintained for the duration of the experiment at 60% field capacity. Forty millilitres of nutrient solution were added to each pot and contained the following (mg): KH₂PO₄ 43.5; KNO₃ 161.6; Ca (NO₃)₂ 188.8; MgSO₄ 78.7; NH₄SO₄ 211.2; Fe EDTA 14.6; H₃BO₃ 0.57; MnCl₂ 0.36; ZnSO₄ 4.4; CuSO₄ 1.0; and Na₂MoO₄ 0.020. Following this initial application, nutrients other than P and calcium nitrate were applied in an aqueous solution at the start of week 4 (mg): KNO₃ 80.8; MgSO₄ 39.3; NH₄SO₄ 105.6; Fe EDTA 7.34; H₃BO₃ 0.3; MnCl₂ 0.18; ZnSO₄ 2.2 CuSO₄ 0.5; Na₂MoO₄ 0.010.

All seeds were surface sterilised with 50% (v/v) ethanol and germinated on damp Whatman No.5 (90 mm) filter paper. Once a radicle had appeared, five seeds were planted in each pot at 1 cm depth. These were thinned to four plants after emergence. The surface of the sand in each pot was covered with a thin layer of white alkathene beads to reduce evaporation. Four

plant-free pots were also maintained. After emergence, the legumes were inoculated using a Rhizobium strain appropriate for the legume species (Table 1).

Plant analyses

Plants were harvested after six weeks of growth. The method of rhizosheath carboxylate collection closely resembled that of Kidd et al. (2015). In brief, the four plants in each pot were removed and the bulk sand carefully excavated. The sand remaining attached to the roots was classified as the rhizosheath. The plant roots and rhizosheath were then placed into a 500 ml beaker and rinsed with a measured amount (50–100 ml) of 0.2 mM CaCl₂ solution using a 50 ml disposable syringe. A 1 ml Waters HPLC vial containing 25 µl of concentrated orthophosphoric acid was filled with the exudate solution filtered through a 0.22 µm Acrodisc syringe filter. Samples were stored at –20 °C. The remaining solution and rhizosheath soil were poured through a Whatman No.1 filter paper. Once the solution had passed through, the soil was oven-dried at 70 °C to determine rhizosheath dry weight.

Roots were gently rinsed in deionised water to remove the rhizosheath before being quickly placed into 100 ml of 0.2 mM CaCl₂ for 15 min. Plants were then removed and a 1 ml sample collected in an HPLC vial as outlined above. The roots were again rinsed briefly in deionised water before being quickly placed into 100 ml of fresh 0.2 mM CaCl₂ solution for a further 15 min. A 1 ml sample was collected as described above. Roots were then assessed for evidence of nodulation and root disease. Significant root disease was evident only on *C. arietinum* in river sand and *L. albus*, which were the only species that did not successfully nodulate. Root samples were then stored in sealed plastic bags at 4 °C until root length could be assessed using WinRhizo (Version 4.1, Regent Instruments Inc., Quebec Canada). Ten-gram soil samples were also collected from the plant-free control pots for carboxylate extraction and analysis.

Carboxylate analyses

The methodology for reverse phase liquid chromatography (RPLC) of rhizosheath and plant-exuded carboxylates was adapted from Cawthray (2003). Details for the analysis of oxalate are described in Uloth et al. (2015). The carboxylates measured and their limits of detection (µM) were: citrate 5.0, malate 7.0, malonate 8.0, oxalate 5.0, fumarate 0.06, lactate 13.0, acetate 24.0, maleate 0.05, shikimate 0.015, succinate 15.0, *cis*-aconitate 0.1, *trans*-aconitate 0.1, and citramalate 4.0. Total carboxylates were the sum of citrate, citramalate, malonate,

malate and oxalate; these were the major carboxylates present. Those listed as ‘other’ are the sum of fumarate, lactate, acetate, maleate, shikimate, succinate, *cis*-aconitate and *trans*-aconitate.

For planted pots, the amount of rhizosheath carboxylates was expressed relative to root dry mass (DM), root length and rhizosheath dry weight. The rate of carboxylate exudation was expressed per unit root length. Two species, *T. repens* and *L. australis*, grew slowly and, as such, plants were relatively small for sampling purposes. Carboxylates were only collected from the rhizosheath of these species.

Citramalate validation with reverse phase liquid chromatography (RPLC)

Initial analysis of exudates utilised a Prevail™ Organic Acid column 250 × 4.6 mm, 5 µm particle size (Grace Davison, Columbia, U.S.A) employing a mobile phase of 25 mM KH₂PO₄ at pH 2.50 and flow rate of 1 ml min⁻¹. This methodology resulted in a significant peak at a similar retention time to the succinate standard (with similar spectral properties as per the photodiode array detector) for samples from *Lotus* spp. (Fig. 1a). Quantification of this peak as succinate gave very high concentrations of a carboxylate that is not commonly recorded in plant root exudates and warranted further investigation. Based on the external calibration curve for a succinate standard, a concentration for the peak in samples was calculated; then 40% of this concentration was added by means of a 10 µl succinate standard addition to 1.0 ml of sample. Spiking the sample with succinate standard increased the peak proportional to amount added when re-analysed with the Prevail™ Organic Acid column (Fig. 1b).

Lotus corniculatus (SG) samples were subsequently reanalysed by RPLC using a different column and mobile phase being an Alltima™ C18 250 × 4.6 mm, 5 µm particle size (Grace Davison) with mobile phase of 93% 25 mM KH₂PO₄ at pH 2.50 and 7% methanol at 1 ml min⁻¹. This approach showed a peak that eluted just after the retention time for the succinate standard, still with matching spectral properties to that of succinate. Sample spiking with succinate as detailed above using the Alltima™ column gave rise to a new peak in the chromatogram that matched retention time of the succinate standard (Fig. 2).

Identification of citramalic acid by gas chromatography/mass spectrometry (GC/MS)

To identify our unknown peak, samples from *L. corniculatus* (LC and SG), *Cicer arietinum* (RS), *O. sativus*, *L. albus*, *O. pinnatus* and *P. polystachyus* were analysed by GC/MS. To 100

µl of root exudate sample, 60 µl of extraction solvent (90% methanol with three internal standards—100 µM labelled valine, 10 µM labelled sorbitol, 10 µM ribitol) were added and the resulting liquid transferred into a glass insert and evaporated to dryness in a vacuum evaporator for 3 h at room temperature. Glass inserts were transferred to a 2 ml GC vial and capped with a magnetic crimp top. A mixture of seven alkanes (100 µg ml⁻¹, C12–C36) was prepared for retention index building in AMDIS (Automated Mass Spectral Deconvolution and Identification System) software (amdis.net).

Samples were derivatised using a CTC autosampler; after 20 µl of 20 mg ml⁻¹ methoxylamine in pyridine was added, samples were incubated at 37 °C for 2 h at 750 rpm in an agitator. Following this, 20 µl N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was added and samples incubated at 37 °C for 30 min at 750 rpm in the agitator. After 1 h of incubation at room temperature, 1 µl was injected onto the GC/MS in splitless mode.

The samples were run on a 7890A GC coupled to a 5975C MSD (Agilent); the column used was a VF-5ms, 30 m × 250 µm × 0.25 µm with a 10 m guard column (Agilent J&W). The carrier gas was helium and the column flow was 1 ml min⁻¹. The temperature gradient of the oven was 70 °C for 1 min, then 7 °C per minute to 325 °C. The inlet, thermal auxiliary, MS source and MS quadrupole temperatures were set to 250 °C, 280 °C, 230 °C and 150 °C, respectively. The scan range was m/z 50–600.

The mass spectrum of the unknown peak was deconvoluted in AMDIS and sent to the NIST14 database. The compound was putatively identified by mass spectral comparison and retention index matching. A pure standard of D-(-)-citramalic acid (Fluka, Honeywell Research Chemicals) was then injected onto the GC/MS to confirm the identity of the unknown peak.

Once the GC/MS work was completed, a citramalate standard was acquired and run on the RPLC setups detailed above on both the Prevail™ and Alltima™ columns. The retention time of the citramalate standard matched that seen for the ‘unknown’ peak on both columns as well as the spectral image of the peak, thus absolutely confirming the presence of citramalate in *Lotus* samples.

Data analyses

All data were tested for variance and normality, and transformations undertaken if required. Where normality could not be attained, PerMANOVA was applied to the data. Rhizosheath carboxylate data were analysed in Past (PAST, PAlaeontological STatistics, ver. 1.89) using a one-way ANOVA to assess the effect of cultivar/line. Carboxylates below the detection limit in the RPLC method, as detailed in Cawthray (2003), were given a value of zero. Trap solution carboxylate data were analysed with a two-way ANOVA to assess the effect of cultivar/line and time (0–15 or 15–30 min following removal of the rhizosheath). The means of four replicates for each cultivar/line were graphed with standard errors of the mean using SigmaPlot version 12 (Systat Software Inc.).

RESULTS

Rhizosheath carboxylates

In rhizosheath soil, all *Lotus* species had citramalate as a significant proportion of total carboxylates (10 to 82%). No other species we studied had citramalate detected in their root exudate profile (Fig. 3). Citramalate was the dominant carboxylate for *L. corniculatus*, being 82% and 73% of the carboxylates in LC and SG, respectively (Fig. 3). The proportion of citramalate in the rhizosheath was 74% for *L. australis*, 42% for *L. subbiflorus* and 10% for *L. cruentus*. *Lotus corniculatus* and *L. subbiflorus* generally showed more carboxylates in their rhizosheath than did *L. cruentus* and *L. australis* (Fig. 4). *Lotus australis* grew poorly in this study, so while we have confidence in the composition of its rhizosheath carboxylates, any further calculations regarding the amount produced based on root morphological characters such as root dry mass or length are less reliable.

The rhizosheath of all *Lotus* species also contained citrate, with *L. subbiflorus* exhibiting an almost 50:50 ratio of citrate:citramalate (Fig. 3). Malonate was not present in the rhizosheath of *L. corniculatus*, but was dominant in that of *L. cruentus*, and small quantities were detected in the rhizosheath of *L. australis* and *L. subbiflorus*. Citrate and malonate formed the major component of the rhizosheath carboxylates in *Ornithopus* species and *L. bainesii*. Oxalate was only present in the rhizosheath of the two species with the lowest quantity of carboxylates, namely, *C. intybus* and *P. polystachyus*. Lactate and acetate were found in the rhizosheath of most species and they tended to increase in percentage composition for species that grew poorly (individual data not shown, but they are included in Figure 3 under ‘other’).

C. arietinum (C) grown in the red loam, *L. subbiflorus*, the *Ornithopus* species and *L. corniculatus* LC07AUYF had the highest rhizosheath carboxylate content when expressed per unit root DM (all $>100 \mu\text{mol g}^{-1}$ root DM) (Fig. 4a). The control species, *L. albus* and *C. arietinum* (RS), produced the least rhizosheath carboxylates per unit root DM, along with *C. intybus* and *P. polystachyus*. However, expressing rhizosheath carboxylates per unit of root length considerably increased the difference between *C. arietinum* (C) ($\sim 70 \text{ nmol cm}^{-1}$) and the other cultivar/lines ($<20 \text{ nmol cm}^{-1}$) (Fig. 4b). *Lotus corniculatus* (LC) had more rhizosheath carboxylates than the cultivar (SG) with 13 and 6.7 nmol cm^{-1} , respectively. There was little statistical difference among most of the other species. However, the carboxylates collected from *C. intybus* and *P. polystachyus* remained very low. The relative amounts of rhizosheath carboxylates were similar when expressed per unit root length and per unit weight of rhizosheath (Fig. 4c) because root length and rhizosheath dry weight were positively correlated ($R^2=0.53$ data not shown).

The amount of carboxylates collected from the rhizosheath of the control species *L. albus* and *C. arietinum* (RS) may have been adversely affected by the unexpected presence of root disease. The composition of *L. albus* carboxylates included a strong component of citrate, and *C. arietinum* (RS) included malonate, as expected. However, the rhizosheath carboxylates of these species was more dominated by lactate, acetate and fumarate (data shown as ‘other’ in Figure 3). We, therefore, have less confidence in these results as a basis for comparison. However, *C. arietinum* was also grown in a red loam, in which it produced large quantities of malonate, citrate and malate.

Carboxylates exuded into trap solutions

The exudation of carboxylates into trap solutions was measured following removal of the roots from their growth medium and gentle washing-off of the rhizosheath. Most carboxylates extracted from the rhizosheath were also exuded into the trap solutions, with two notable exceptions: lactate, which was largely absent in trap solutions (data not shown) and oxalate, which was not detected in trap solutions despite being a major carboxylate in the rhizosheath of *C. intybus* and *P. polystachyus*. There were also very large differences in the percentage composition of carboxylates between the rhizosheath and the first and second trap solution sampling periods, that is, after 0–15 min and 15–30 min following rhizosheath removal (Fig. 3). In particular, citrate was more prominent in the carboxylate profiles of most

species in the trap solutions, particularly in the second sampling period. Although, in contrast, citrate was not present in the final sampling period for both *L. corniculatus* cultivars. For the *Lotus* species, only *L. corniculatus* (LC and SG), which had relatively high proportions of citramalate in rhizosheaths, exuded citramalate into the trap solutions. Malate increased in proportion in the trap solutions sampled after 15 min, relative to the rhizosheath for *C. arietinum* (C). Carboxylates such as acetate and fumarate were more prevalent in the trap solutions of *L. bainesii* and *C. intybus* than in the rhizosheath (they are represented in ‘other’ in Figure 3).

The rate of carboxylate exudation into the trap solutions is shown in Figure 5. There was a significant effect of both cultivar/line and time due to a large reduction in exudation rate between the first sampling period and the second sampling period. The fastest exudation rate was, by far, for *C. arietinum* (C) (Fig. 5) and, in the first sampling period, it exuded an amount approaching what was found in the rhizosheath initially (Fig. 6). Only *C. arietinum* (C), *L. corniculatus* and *L. cruentus* exuded carboxylates at a rate that could have accumulated to rhizosheath levels within 1 h: the results for other species were highly variable, with some exuding at a slow rate that would have required up to 12 h to accumulate to rhizosheath levels (Fig. 6).

DISCUSSION

Citramalate

Citramalate has rarely been reported as present in rhizosheaths or root exudates, with mention in only a single publication for each of *Beta vulgaris* (Khorassani et al. 2011) and *Glycine max* (Tawaraya et al. 2014). Citramalate has been reported in extracts from roots of *L. corniculatus* (Navascués et al. 2012), but not as a component of its root exudates. We showed that citramalate was present in the rhizosheath carboxylates for the three *Lotus* species examined in this study, although its proportional representation was comparatively low in one of the species. In contrast, citramalate was not detected in any of the other pasture or grain legumes we studied.

It is possible that citramalate is rarely reported because its presence is restricted to a few genera or species. However, another reason may be that it is mistaken for succinate due to

the issues associated with its separation during RPLC with Prevail™ organic acid columns. For example, Basu et al. (1994) found succinate in *Triticum aestivum* seedlings under aluminium stress; however, there were two peaks on their chromatogram with retention times very similar to succinate that were unidentified. In addition, many studies tend to focus on the identification of well-known carboxylates exuded from plant roots, such as citrate, probably because the role and significance of these compounds is well understood (Dinkelaker et al. 1989; Gardner et al. 1982; Lipton et al. 1987).

Citramalate was not included by Dakora and Phillips (2002) in a list of organic acids identified in plant root exudates, and was not identified in a review of rhizosphere organic acids by Jones (1998). In view of more recent studies (Khorassani et al. 2011; Kidd et al. 2015; Tawaraya et al. 2014) and the present work, its potential role as a carboxylate in root exudates and the rhizosphere should now be recognised.

Nutrient acquisition in Lotus

Lotus corniculatus has a low requirement for soil P (Davis 1991) and it is that the exudation of citramalate by roots may contribute to this due to its ability to solubilise P (Khorassani et al. 2011). The amount of citramalate present in the rhizosheaths of the *Lotus* species varied, but it was consistently less than the 5 $\mu\text{mol g}^{-1}$ dry weight administered by Khorassani et al. (2011) to elicit an increase in the solubility of P in solution. Moreover, other studies on the amount of a carboxylate sufficient to facilitate P acquisition determined that values below 10 $\mu\text{mol g}^{-1}$ soil for citrate and oxalate will have little impact on P mobilisation in the soil (Gerke et al. 2000). However, in our study the rate of exudation in *L. corniculatus* (LC) approached the 22 $\mu\text{g m}^{-1} \text{h}^{-1}$ in the *Beta vulgaris* plants investigated by Khorassani et al (2011) that were shown to solubilise P. It is worth noting that there may be additive effects from citrate or malate (Oburger et al. 2009). In the present study we collected exudates from, and expressed the carboxylate levels relative to characteristics of the entire root system. However, carboxylate exudation is often concentrated at the root tips (Hoffland et al. 1992; Rovira 1969; van Egeraat 1975) with the amounts and exudation rates much higher in this localised zone.

Exudation rates

The exudation rate of *C. arietinum* (C) in the first 15 min sampling period was much faster than that of any other species ($>187 \text{ nmol cm}^{-1} \text{ h}^{-1}$). The next fastest exudation rate was for *L. corniculatus*, which barely exceeded $13 \text{ nmol cm}^{-1} \text{ h}^{-1}$. Rates of exudation reported in Wang et al. (2015) for *Brassica napus* at a whole plant level were less than $50 \text{ nmol plant}^{-1} \text{ h}^{-1}$. Thus, the rates achieved in our experiment are comparatively high. In terms of rhizosheath carboxylate amounts, *C. arietinum* (C) showed much higher values than the other cultivar/lines when carboxylates were expressed per unit of root length or per unit of rhizosheath dry weight. This difference decreased when rhizosheath carboxylates were expressed per unit of root dry weight due to the impact of the very thick roots of *C. arietinum* on calculations (Kidd et al. 2015). We suggest that rhizosheath carboxylates are best expressed per unit of root length because it better reflects differences in root exploration capacity and subsequent interaction between the plant root and rhizosheath than dry mass of the root itself which is highly influenced by root diameter.

The exudation rate for *C. arietinum* (C) would allow accumulation of the carboxylate amounts present in the rhizosheath in $<1 \text{ h}$. This rapid exudation could perhaps be a response to fast microbial degradation, as found for citrate and malonate (Martin et al. 2016), or losses through soil sorption (Suriyagoda et al. 2016). However, these factors would have had relatively little impact in the river sand used in our experiment, as soil sorption and microbial degradation are minimal (Ryan et al. 2012). The results for other species were similar, including those for *L. corniculatus*, which accumulated the carboxylate amounts present in the rhizosheath in $\sim 2 \text{ h}$. In contrast, *O. compressus* needed $\sim 8 \text{ h}$ and *L. subbiflorus* $\sim 12 \text{ h}$; in both cases, this reflects a relatively slow exudation rate. Such slow rates could perhaps indicate that mechanisms are employed to reduce losses of carboxylates from the rhizosheath (Weisskopf, 2006; Tomasi, 2008) or the microbial characteristics in the washed river sand allowed the accumulation of carboxylates beyond that which would occur in other, more biologically active soil types (Uren, 2007)

Conclusions

Citramalate was found in root exudates and rhizosheaths of the *Lotus* species examined in this study, but was not present in exudates from any of the other legumes. The contribution of citramalate to the carboxylate composition of *Lotus* root exudates was significant, but did vary substantially (10-82% of total carboxylates) among the *Lotus* spp. that were examined.

The role of citramalate in exudates from *Lotus* species, especially those where it constitutes a very high proportion of their exuded carboxylates, requires further examination.

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Table 1. List of the species, their origin and the strain of rhizobia used in the experiment.

Note that *Ptilotus polystachyus* and *Cichorium intybus* are not legumes and hence no rhizobia were applied.

Species and Authority	Cultivar / Line	Common name	Origin	Rhizobia strain
<i>Lotus corniculatus</i> L. (LC)	LC07AUYF	Birdsfoot trefoil	Europe / Asia / N. Africa	SU343
<i>Lotus corniculatus</i> L. (SG)	San Gabriel	Birdsfoot trefoil	Europe / Asia / N. Africa	SU343
<i>Lotus subbiflorus</i> Lag.	Waroona	Hairy birdsfoot trefoil	Europe / N. Africa	SU343
<i>Lotus cruentus</i> Court.	NF003	Red birdsfoot trefoil	Australia	RRI2252
<i>Lotus australis</i> Andrews.	PD01LA	Austral trefoil	Australia	RRI2252
<i>Ornithopus sativus</i> Brot.	Margurita	French serradella	Mediterranean	WSM471
<i>Ornithopus sativus</i> x <i>Ornithopus compressus</i>	Grasslands Spectra		Mediterranean	WSM471
<i>Ornithopus pinnatus</i> (Mills) Druce	Jebala	Slender serradella	Mediterranean	WSM471
<i>Cicer arietinum</i> L. (RS)	Neelam	Chick pea	Mediterranean	CC1192
<i>Cicer arietinum</i> L. (C)	Neelam	Chick pea	Mediterranean	CC1192
<i>Lupinus albus</i> L.	Kiev Mutant	White lupin	Mediterranean	WSM471
<i>Trifolium repens</i> L.	Haifa	White clover	Europe	TA1
<i>Lotononis bainesii</i> Baker.	Medina bulk	Lotononis	South Africa	WSM2598
<i>Ptilotus polystachyus</i> (Gaudich) F.Muell	MGP001	Mulla mulla	Australia	Not applicable
<i>Cichorium intybus</i> L.	Puna	Chicory	Europe	Not applicable

Figure 1. Chromatogram of the root exudates of *Lotus corniculatus* (SG) on a Prevail™ Organic Acid column with 100% 25 mM KH_2PO_4 pH 2.50 mobile phase. For the right-hand peak at ~11 min retention time: **(a)** the retention time and peak spectral properties reflect that of succinate, and **(b)** this peak increased proportional when spiking with a succinate standard

Figure 2. Chromatogram of the root exudates of *Lotus corniculatus* (SG) on an Alltima™ column with 93% 25 mM KH₂PO₄ pH 2.50 with 7% (v/v) MeOH mobile phase. For the right-hand peak with a retention time of ~7.5 min: **(a)** the elution time appears to significantly differ from the succinate standard (now to the left of the peak), and **(b)** a new peak is formed due to spiking with a 40% succinate standard (also to the left of the peak).

Figure 3. The composition of carboxylates for a range of crop and pasture legumes in: **(a)** the rhizosheath, **(b)** a trap solution after 0–15 min following removal of the rhizosheath, and **(c)** a trap solution after 15–30 min following removal of the rhizosheath (mean, n=4). Note *Lotus australis* and *Trifolium repens* were only sampled from the rhizosheath.

Figure 4 Total carboxylates in the rhizosphere (citrate, citramalate, malate, malonate and oxalate) expressed per unit: **a)** root dry mass, **b)** root length, and **c)** rhizosphere dry weight (mean $\pm$ s.e; n=4). No LSDs are presented as data were $\log_{10}$ transformed prior to analysis. One-way ANOVA showed a significant effect of species for: **a)** $P < 0.001$, **b)** $P < 0.001$ and **c)** $P < 0.0001$.

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623 **Figure 5.** Carboxylate exudation rate per unit root length. Exudates were collected in a trap
 624 solution after 0–15 and 15–30 min following removal of the rhizosheath (mean $\pm$ s.e; n=4).

625 No LSDs are presented as data were $\log_{10}$ transformed prior to analysis. Two-way ANOVA
 626 showed a significant effect of species ($P < 0.001$) and time ($P < 0.001$), but no interaction (P
 627 > 0.05).

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Figure 6. Carboxylates in the rhizosheath (citramalate, citrate, malate, malonate and oxalate) expressed per unit root length (thick hatched bars) and the time taken to reach these levels of accumulation as determined by the exudation rate in the trap solution after 0–15 min following removal of the rhizosheath (mean $\pm$ s.e; n=4).