High Resolution Synchrotron Imaging of Wheat Root Hairs Growing in Soil and Image Based Modelling of Phosphate Uptake

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Summary

- Root hairs are known to be highly important for uptake of sparingly soluble nutrients, particularly in nutrient deficient soils. Development of increasingly sophisticated mathematical models has allowed uptake characteristics to be quantified. However, modelling has been constrained by a lack of methods for imaging live root hairs growing in real soils.

- We developed a plant growth protocol and used Synchrotron Radiation X-ray Tomographic Microscopy (SRXTM) to uncover the 3D interactions of root hairs in real soil. We developed a model of phosphate uptake by root hairs based directly on the geometry of hairs and associated soil pores as revealed by imaging.

- Previous modelling studies found that root hairs dominate phosphate uptake. By contrast, our study suggests that hairs and roots contribute equally. We show that uptake by hairs is more localised than by roots and strongly dependent on root hair and aggregate orientation.

- The ability to image hair-soil interactions enables a step change in modelling approaches, allowing a more realistic treatment of processes at the scale of individual root hairs in soil pores.

Introduction

Crop productivity is significantly constrained by poor bioavailability of sparingly-soluble nutrients, especially phosphate (P) (Barber 1984; Tinker & Nye 2000; Vaccari 2009). The efficiency of P acquisition, in which root hairs play a critical role, is important in addressing global food-security issues that arise from increasing world population and climate change. Advances in genetics provide the capability to breed plants with improved root-hair phenotypes, with manipulation of length and spatial patterning providing potential routes to enhanced P uptake (Gahoonia & Nielsen 2004; Datta et al. 2011). Discovery of optimal traits for P uptake is particularly important because rock P resources are predicted to be exhausted within 50-100 years, meaning alternatives to traditional fertilization are imminently required. Despite the importance of root hairs to P uptake, the absence of published studies imaging undisturbed hairs growing *in situ* within soil has hindered the validation of mathematical models describing their function in the soil.

The proliferation of root hairs along major root axes has been implicated in a range of processes including enhanced nutrient and water uptake, root-microbial signal exchange, allelochemical release and plant anchoring (Bates & Lynch 1996; Ehrhardt et al. 1996; Datta et al. 2011). Despite their ubiquity throughout the plant kingdom, the role of root hairs in plant-soil processes remains poorly understood due to the difficulties inherent to studying their behavior under field conditions. This poor understanding has limited the targeted selection of root hair traits, particularly for enhanced nutrient acquisition in the field (Gahoonia & Nielsen 1997; Gahoonia & Nielsen 1998; Gahoonia et al. 1999; Gahoonia & Nielsen 2004; Datta et al. 2011).

Previous morphological, image-based and genetic studies of root hairs have been conducted either in artificial hydroponic/agarose gel systems or by destructively

79 washing roots from the soil (Ma et al. 2001) or cryo-scanning using an electron
80 microscope (Watt et al. 2005). Clearly, hydroponic/agarose growth conditions are
81 radically different from those existing in soil (*e.g.* in terms of mechanical resistance)
82 and it is therefore unclear how data from these studies translates into real soil
83 environments. Though experiments with mutants of various species in real soils
84 have linked root hair density and length to P uptake, the hair-scale mechanisms of
85 uptake are indistinguishable using these approaches (Bates & Lynch 2001). Washing
86 of soil-grown roots is limited as a technique due to the small scale and fragile nature
87 of root hairs (~3-6 μm, see review in Leitner et al. (2010)), making it difficult to
88 estimate the degree of loss or damage during the washing process. Resultingly,
89 observation of root hairs growing in situ in real soil requires the use of non-invasive
90 methods such as X-ray computed tomography (CT), using either a laboratory
91 microfocus scanning system (μCT), or synchrotron radiation-based systems (SRCT).
92 μCT scanners produce X-rays for imaging by bombarding a metal target with
93 thermionically emitted electrons, producing a polychromatic, incoherent beam of
94 photons in a cone or fan arrangement. Though satisfactory for many imaging
95 applications, μCT systems have their limitations, particularly in terms of available X-
96 ray flux. Whilst a variety of commercial microfocus scanners now operate at
97 sufficient resolution to image structures at the root hair scale (≈1 μm), scan durations
98 at such resolutions, with good contrast-to-noise ratios, are inevitably in the order of
99 hours. During such lengthy scans, movement due to plant growth and/or soil
100 changes (*e.g.* local water movement) almost inevitably compromise image quality,
101 making it problematic to use microfocus scanners for root hair imaging. By contrast,
102 SRCT uses X-rays generated by electromagnetic deflection of an extremely high-
103 energy (relativistic) electron beam. A photon beam produced by a contemporary

104 synchrotron source is typically highly monochromatic, coherent and has a far greater
105 photon flux than that of a microfocus sources. These characteristics allow acquisition
106 of radiographs at high resolutions, with adsorption and/or phase contrast, using
107 greatly reduced exposure times. The consequent reduction in scan times down to the
108 order of ≈ 5 minutes allows small, unstable structures to be imaged without gross
109 intra-scan movement. Furthermore, phase contrast effects can be exploited to enhance
110 edges between materials of similar adsorption contrast. For these reasons, SRCT was
111 used in this work to image the root hairs of wheat plants grown in soil at a resolution
112 of ~1 μm.
113 Modelling of P uptake by root hairs has been hampered by a lack of reliable data
114 about the geometric configuration of hairs with respect to soil particles, with the result
115 that most models contain unsubstantiated assumptions, reducing their reliability and
116 accuracy. Previous models (Barber 1984; Tinker & Nye 2000; Ma et al. 2001; Leitner
117 et al. 2010) all present cases in which soil is treated as a continuum around the root
118 hairs, sharing the underlying assumption that root hairs are much larger than soil
119 aggregates. As can be seen from SRXTM imaging of root hairs and soil particles in
120 this study (Figs 1 and 2), this assumption is false. Zygalakis et al. (2011) (and Leitner
121 et al. (2010) for hydroponic growth) do not make this assumption, instead using
122 multi-scale homogenization theory to derive model equations for the situation where
123 root-hair spacing is comparable to soil aggregate size. These models take the
124 influence of soil pore-space on P diffusion into account, including not only soil
125 aggregate influences, but also geometric hindrance arising from the root hairs (Leitner
126 et al. 2010; Zygalakis et al. 2011). Whilst multi-scale homogenization provides a very
127 useful tool for understanding the impact of small periodic structures on the large-scale
128 behavior it fails to provide sufficient information regarding factors influencing root-

129 hair P uptake at the soil-pore scale. Optimisation of models at the whole-plant scale
130 will require validation of pore-scale uptake behaviour, including whether hairs or
131 roots dominate P uptake, or whether root and hair contributions are comparable
132 (Leitner et al. 2010). This study presents a complete work-stream and methodology
133 for imaging living root hairs using SRXTM and translating resulting image data for
134 assessment of plant P uptake using image based modeling.
135
136 **Materials and Methods (see SI for full description)**
137 ***Plant Growth Microcosm***
138 Winter wheat seeds *Triticum aestivum* (cv. Santiago; KWS, Thriplow Cambridge UK)
139 were pre-germinated on wetted tissue paper for 48 hours. Seven 1 ml syringe barrels
140 were clustered, inserted into a larger tube of 3 cm diameter, and filled with soil,
141 including a ≈5 mm layer covering the barrel openings. The soil used was a
142 Dunnington Heath series Chromi-Abruptic Luvisol from the University of
143 Nottingham field site at Sutton Bonington (52° 49' 52", -1° 15' 13"). A single pre-
144 germinated wheat seed was then planted in each assembly, such that individual roots
145 could grow into the syringe barrels (Methods S1 and Fig. S1). Plants were grown for
146 10 days in a controlled growth environment (Fitotron SGR, Weiss-Gallenkamp,
147 Loughborough, UK). Day conditions (16 h) were 23^{o}C and 60% humidity and night
148 conditions (8 h) were 18^{o}C and 55% humidity, with both temperature and humidity
149 ramped between states over a 30 minute period. Plants were initially top-watered with
150 5 ml of water, following which 3 ml was added every 48 h to maintain water at close
151 to field capacity.
152 ***Imaging***

153 Following growth, CT imaging was carried out at the TOMCAT Beamline on the
154 X02DA port of the Swiss Light Source at the Paul Scherrer Institute, Villigen,
155 Switzerland. Plant and soil material protruding above the barrel openings was excised
156 using a scalpel, and individual syringe barrels were separated from the cluster
157 immediately prior to scanning at 20 KeV, with 1501 equiangular projections acquired
158 through 180^{o}. The projections were post-processed to generate light and dark
159 corrected sinograms, which were in turn converted to a stack of 2D, 8bit tiff files for
160 each scan. Features were extracted from data using a combination of automated tools
161 for soil classification, and manual segmentation for root hairs using a graphical tablet
162 and AVIZO Fire 7 software. The resulting segmented regions were used to generate
163 volume finite element (FE) meshes for COMSOL Multiphysics using ScanIP.

164 ***Modelling***

165 In COMSOL the uptake of P by root surfaces and desorption from the particle
166 surfaces was modeled, based on previous work (Schnepf et al. 2011). Though the
167 idealised geometry used previously (Schnepf et al. 2011) was simpler than the
168 situation in this study, their mechanistic approach represents the closest model in the
169 literature to that required for image based modelling, i.e., it considers P uptake and
170 spatially distributed binding reactions on soil particle surfaces, rather than soil bulk
171 binding reactions.

172 The sub-region used to generate the finite element (FE) model for simulations was
173 defined according to the following procedure. The constraints of available
174 computational power required that the model consist of $< 9\times10^{6}$ elements. Choosing
175 meshing parameters which preserved soil surface morphology, the maximum
176 allowable sub-region volume was found to be $\approx 1\times10^{-18}$ m^3. For reliable comparison
177 with future data, a segment geometry was selected as a repeatable geometry, with

centerline aligned to that of the root. A number of constrains determined the positioning of the segment for the simulation. The image region was required to (a) be unaffected by visible blurring artifacts, (b) exclude lateral roots, (c) exclude edge effects at the soil/tube boundary. In the regions satisfying these criteria, soil structure was relatively homogenous as a result of preparation method. Since hairs were also evenly distributed over the root surface, the specific segment position (determined randomly) is qualitatively representative of the soil and hair characteristics of the entire dataset. Following definition, a multi-region, finite element volume mesh was generated from the segment RAW data using the +FE-Free algorithm in ScanIP (Simpleware Ltd., Exeter, UK) for input to COMSOL (Version 4.2a, COMSOL Inc., Burlington, MA, USA).

The following equations and boundary conditions were applied to the FE model.

Diffusion of P in soil solution:

Eqn. 1
$$\frac{\partial C_l}{\partial t} = D\nabla^2 C_l,$$

where C_l is the concentration of P in the soil pore water measured in mol m^{-3} , and D is the P diffusion constant in water in $m^2 s^{-1}$.

Mass conservation on soil surface is given by the following first order reaction:

Eqn. 2
$$\frac{dC_a}{dt} = -k_d(C_a - \frac{k_a}{k_d}C_l)$$

where C_a is the amount of P bound per unit surface area of soil aggregate in mol m^{-2}, k_a is the rate of sorption in m s^{-1}, and k_d is the rate of desorption in s^{-1}.

The root hair (and root) P uptake boundary condition is described by Michaelis Menten kinetics:

Eqn. 3
$$D\underline{\hat{n}}.\nabla C_l = \frac{F_m(C_l - C_{\min})}{K_m + (C_l - C_{\min})}$$

where $C_{\min}$ is the soil solution P concentration when the P uptake by plant stops, F_m is the maximum rate of P uptake in mol $m^2 s^{-1}$, K_m is the concentration when uptake is half of maximum possible in mol m^{-3}, and $\underline{\hat{n}}$ is a vector normal to the root surface, pointing into the root domain, i.e., all surfaces in three dimensions have two unit

normals pointing in opposing directions; the only difference being the sign. We assume that the root and root hair P uptake properties are the same all over the surface. This might not necessarily be true since P transporters can potentially be heterogeneously distributed, but at this time we are unable to quantify such characteristics, and thus do not take heterogeneity into account. However, due to the spatially explicit nature of our model it would be very easy to include if and when experimental data becomes available.

The boundary condition at the soil particle surface boundary reflects the binding reactions at that location. Hence it is intrinsically coupled to Eqn. 2, i.e.,

Eqn. 4
$$D\underline{\hat{n}}.\nabla C_l = k_d(C_a - \frac{k_a}{k_d}C_l),$$

where $\underline{\hat{n}}$ is a vector normal to the soil aggregate surfaces.

We take a zero flux boundary condition on the external surfaces of the domain of integration:

Eqn. 5
$$D\underline{\hat{n}}.\nabla C_l = 0.$$

Initially the soil and associated P surface density and concentration are assumed to be in equilibrium, i.e. $\frac{k_a}{k_d} = \frac{C_{a0}}{C_{l0}}$, where C_{a0} and C_{l0} are the initial concentrations on the soil surface and in the fluid respectively. Though the quantity C_{l0} must be derived experimentally, only the volume concentration, C_{av}, in the soil can be directly measured. We thus convert it into a surface concentration using $C_{a0} = C_{av}\rho V / S$, where V is the soil volume in m^3, S is the soil surface area in m^2 and ρ is the bulk soil density in kg m^{-3}.

Soil Chemical Characterisation

Adsorption and desorption constants (k_a, k_d) for the simulations were parameterised using standard soil tests (Murphy & Riley 1986; Giesler & Lundström 1993) which determined the fractions of bound and soluble P (Methods S1 and Fig S2). The methods used to define these parameters are fully described in the on-line Supplementary Information. The bulk total binding rates were measured and the bound mass was distributed evenly to all imaged soil aggregate surfaces as the initial condition for the numerical simulation. Parameters for root and hair P uptake were

obtained from the literature (Barber 1984; Tinker & Nye 2000). A list of all parameter values used for simulation are given in Table S1.

Postprocessing of Simulation Results

The pore-scale simulation results were post-processed in Paraview and VGStudio MAX, and surface integration was conducted in COMSOL Multiphysics.

Results

Representative examples of unprocessed image data are shown in Figs 1 and 2 (and supplementary Methods S1 Fig S3 and Movie S1). These reveal the intricate and varied nature of root hair and soil particle interactions. In some instances, root hairs traverse large macro-pores, while others infiltrate intra-aggregate micro-cracks. The crucial question arising is what the geometric interplay between root hairs and soil aggregates implies about root and root hair P uptake from the soil; especially given that significant amounts of P are bound to the soil aggregate surfaces (Tinker and Nye 2000). We used three-dimensional, image-derived root, root hair and soil aggregate geometries to compute P uptake by the root and root hairs in a qualitatively typical sub-region representing 550 μm of root length over an angular range of ~75^{o} (Figs. 1 and 2) whilst taking into account dynamic binding on the soil particle surfaces and diffusion in the soil pore space. The finite element simulation results (Fig. 3) were then analysed to determine P transport characteristics in the soil pore space.

Initial quantities of bound P on the soil were parameterised using the agronomic indexing system adopted by the UK government to regulate use of P fertilizers (Defra 2010). Index P3 implies that the total amount of P in the soil is 39 mg P l^{-1}, which should be sufficient to produce crops, rendering additional fertilization inadvisable. Indices P2 and P1 reflect soils with lower total levels of P, 23 mg P l^{-1} and 12 mg P l^{-1}, respectively. Simulations were run for all three different agronomic soil P indices,

assuming that the soil P binding properties were the same in each case. Initial concentration was also assumed to be homogenous over all soil surfaces. For all three P index scenarios, cumulative total P uptake from both root and all root hairs was comparable, with uptake by both decreasing to very low values after 5 hours (Fig. 4). However, the uptake contribution by the root was around 15% higher than for root hairs in all cases.

Though the predominant paradigm in the literature (Barber 1984; Tinker & Nye 2000; Ma et al. 2001; Leitner et al. 2010) that root hairs dominate P uptake, this result confirms that P uptake by root hairs and the root are intimately coupled. This calculation is with respect to the single root scale, but the behavior at the root system scale might differ, since not all root surfaces are likely to be covered by hairs. Hence on the root system scale, the importance of the hairs might be reduced, especially in the latter stages of development when the root system is more mature and thus more extensive. The area of the root surface in the simulation was 1.96 x 10^{-7} m^2 and the total area of root hairs was 1.66 x 10^{-7} m^2, such that the difference in P uptake is attributable to the difference in the total surface area rather than to the soil volume into which the hairs penetrate. This is a significant finding as it contradicts previous studies which concluded that root hairs dominate P uptake (Barber 1984; Tinker & Nye 2000; Ma et al. 2001; Leitner et al. 2010).

Plotting diffusion streamlines following paths of maximum concentration gradient from the soil particles to root and root hair surfaces allowed the flux of P to be visualised (Fig. 3B). Mapping depletion zones on soil particle surfaces to the corresponding root and root hair uptake surfaces allowed the degree of depletion gradient localization to be quantified. Sets of seed points were defined on the soil surface with inter-seed separation length $\delta_0 = 0.25\mu m$, and the paths of maximum

285 concentration gradient from seed point to uptake surface were computed. This
286 allowed P regions on the soil surfaces to be mapped to specific sites where the
287 released P was absorbed by the plant. Neglecting any jumps between different soil
288 aggregates the average distance between streamline termination points on the soil
289 surface is δ_L. The ratio of distances between streamlines on the soil aggregate and
290 the root or root hair surfaces can now be estimated using $\mathbb{R} = \delta_L / \delta_0$. Similarly the
291 average length of the streamlines can be easily obtained by summing the arc lengths
292 along the streamline trajectories. The mean ratios for the streamline sets, together
293 with the error in the mean for both root and root hair surfaces is shown in Fig. 4. The
294 sensitivity of the calculations to δ_0 was measured by halving δ_0 and repeating the
295 calculations for a subset of the sampled points. This produced no noticeable
296 difference in the results for average streamline length or distance ratio. The data show
297 that root hairs draw P from a particle surface region whose area is roughly comparable
298 to between two and three times that of the uptake area on the root hair surface. By
299 contrast, the root extracts P from a much larger soil surface area of approximately five
300 to ten times that of the root surface. This occurs because the root surface creates large
301 global diffusion gradients in soil pores by comparison to the smaller gradients very
302 close to soil particle surfaces created by root hairs. Root hairs have a high length to
303 diameter ratio and are generally spatially closer to the surfaces of soil particles, such
304 that associated P fluxes are more localized and influenced to a greater extent by hair
305 diameter and curvature. A comparison of mean diffusion path lengths for the root
306 surface and root hairs confirmed that dependent on time, soil surface to root paths
307 were approximately 5-9% longer than those from soil surfaces to the root hairs.
308 Additional insight arises from the dynamic nature of the error bounds on the mean
309 measure of the soil particle to root and root hair area ratio. Variation is initially

greater (though it decreases with time), since during the early stage diffusion interacts dynamically with the binding of P to the soil particle surfaces, i.e., the uptake-driven diffusion profile in the soil pore domain has begun to influence the soil surfaces, but the surface binding reactions have not yet reached equilibrium. It is also evident that after the soil particle surface-binding reactions reach equilibrium, there is a level of adjustment between the surface concentrations (via particle to particle interaction) at around 2-3 hours, after which the entire soil pore and soil surface domain reaches equilibrium (Fig. 4).

Discussion

This study has demonstrated that *in situ* imaging of living roots in soil is possible using SRXTM, and that consequent resolution of individual root hairs with respect to soil aggregates can be achieved. Through use of suitable protocols and imaging methodology, the workflow from living plant to modelling results has been established for nutrient uptake for the first time. Assumptions must still be made regarding initial distribution of P, but a step change in modelling has been brought about by removing the assumption of idealized geometry.

Clearly, all scientific methodology is party to limitations, and this is true for our work presented here. In particular, to achieve the spatial resolution necessary for imaging root hairs, it was necessary to develop a growth assay that was small (≈5 mm in diameter). Due to the physical constraints involved in imaging with X-rays, this requirement for small sample sizes is unlikely to change radically in the near future. Additionally, even a very powerful computer enabled us to simulate only a small section of the entire volume imaged. We envisage that this particular constraint will disappear in the very near future due to rapid improvement in software and hardware, such that within 1-2 years, the possibility of running image based simulations on the

entire imaged domain can be envisaged. Finally, there are specific ways in which validation of the model could be achieved. One particularly promising approach for validation of our P modelling results is the use of X-ray fluorescence tomography, which should in theory allow mapping of the distribution of P in the soil. Though promising, the technique is still in its infancy, but our growth assay is highly suitable for investigation as soon as the technique becomes fully accessible.

Thus, the image data acquired, combined with modeling in this study, have been utilized to substantially improve upon existing P uptake models, showing that P uptake by root hairs is a rapid process, occurring on the same timescale as P uptake by the root. Quantification of the zones from which root hairs extract P from the soil shows that the majority is extracted from soil surfaces immediately adjacent to root hairs, with very little acquired from particle surfaces oriented away from them. These results have major implications for our understanding of how root hairs should be modelled at the plant and crop scale, and indicate that previous modelling studies should be re-visited using an imaging-based, multi-scale homogenization approach. Understanding of P uptake grounded in hair-scale mechanisms can now be used to optimize plant models at larger scales, where resolution of single hairs is impossible. This research also opens up possibilities for assessing how different soil surface binding reactions and geometries might influence different rhizosphere processes. Furthermore, the methodology developed in this study will now enable investigations of rhizosphere processes, involving many replicates of different plant and soil treatments. It also raises the possibility of imaging live mycorrhizal hyphae in the soil together with host roots. Mycorrhizal hyphae are of similar size to root hairs (≈10 μm in diameter), but the structure is much more complex (Smith & Read 2002). We believe our assay could be extended and modified to address questions of

mycorrhizal-plant interaction, thus enabling the validation of plant mycorrhizal models (Schnepf et al. 2005; Schnepf & Roose 2006; Schnepf et al. 2008a; Schnepf, et al. 2008b; Schnepf et al. 2011; Thonar et al. 2011). Another area where our technology could be applied is the assessment of mucilage and root exudates in the rhizosphere; thought to be highly important in P uptake (Hinsinger et al. 2005). Whilst X-ray CT is not necessarily suitable for imaging mucilage and exudates due to their near-identical density to that of water, other methods such as neutron tomography are well suited to such investigations. The plant growth assay outlined here could be applied to this technique, which like X-ray CT has been hampered by the small sample sizes necessary to achieve the spatial resolution needed for rhizosphere studies and model development (Esser et al. 2010).

Supplementary Information is linked to the online version of the paper.

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Supporting Information Information

Methods S1: Document describing the experimental and modelling methods in detail.

Figure S1. Plant growth assay. (A) Bundle of 7 syringe barrels with tabs removed. (B) Addition of foil collar. (C) Bundles are filled with Luvisol, with a 5 mm depth of compost added above the barrel tops. (D) Bundles are housed in larger 50 ml Falcon tubes. (E) Individual syringe barrels are retrieved for imaging following dismantling.

Figure S2. Curves fitted to (C_{sor}) data for determination of parameters A and κ. Data were determined for two concentration via a soil assay.

Figure S3. Representative sub-regions of raw data. (A-D) Interactions of root hairs with pores and soil particles seen in x,y plane of data at different z positions.

Table S1: Values of parameters used in simulation

Movie S1: Movie showing the imaging and modelling workstream.

460
461 **Figure Legends**
462
463 **Figure 1**
464
465 Region selection and classification for rhizosphere simulation from synchrotron data.
466 (A) A segment is defined, with centreline aligned with the centre axis of the root, (B)
467 All other voxels are removed, leaving only the defined segment , (C) root hairs, soil,
468 fluid and root surface regions are individually defined using different discrete grey-
469 levels, (D) a volume mesh is generated, with root hairs, soil, root surface and water
470 defined separately.
471
472 **Figure 2**
473
474 Digitally rendered 3D volume with the soil phases partially cut away to reveal ≈3mm
475 section of a seminal root of *Triticum aestivum,* including lateral roots and root hairs.
476
477 **Figure 3**
478 (A) Estimated surface concentrations of phosphate (C_a) on the soil particle surfaces
479 after 10 hours of uptake by root and root hairs, (B) streamlines showing phosphate
480 transport paths from soil surfaces to root hairs (magenta) and root (blue).
481
482 **Figure 4**
483
484 Consolidated simulation results (over 10 hours). Left axis indicates cumulative P
485 uptake, plotted separately for root surface and root hairs, with $C_{a,0}$ values
486 corresponding to phosphate indexes P1, P2 and P3. Right axis shows ratios of
487 polyline distance between groups of streamline start points (on soil surfaces), and
488 groups of corresponding end points (on root and root hair surfaces), where streamlines
489 follow maximum C_l concentration gradients (shown in Figure 3B). Plotted points
490 are mean values for all sets, calculated separately for root and root hairs (standard
491 error in means indicated by error bars).
492