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5 **Title**

6 Measurement of fine root tissue density: a comparison of three methods reveals the potential of root dry matter
7 content

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Abstract

Aims Root tissue density (RTD, the ratio of root dry mass to root volume) is a fundamental trait in comparative root ecology, being increasingly used as an indicator of plant species' resource use strategy. However, the lack of standardized method to measure this trait makes comparisons tricky. This study aims to compare three methods commonly used for determining fine RTD and to test whether root dry matter content (RDMC, the ratio between root dry mass and root fresh mass) could be used as a surrogate of fine root tissue density.

*Method*s RTD of 163 fine root samples was determined using (i) Archimedes' method, (ii) image analysis (WinRHIZO software), and (iii) using the root dry matter content as a proxy. Root samples belonged to different herbaceous species grown in different conditions.

Results RTD measured with Archimedes' method was positively correlated with RTD estimated with image analysis and with RDMC. However we demonstrated that RTD measured with Archimedes' method was better predicted by RDMC ($R^2 = 0.90$) than by RTD measured with image analysis ($R^2 = 0.56$). The performance and limitations of each method were discussed.

Conclusion RDMC is a quick, cheap and relatively easy measurable root attribute; we thus recommended its measurement as a proxy of fine root tissue density.

Keywords: Archimedes' principle, herbaceous plants, image analysis, method, root dry matter content (RDMC), root volume.

45 **Introduction**

46

47 Tissue density, defined as the amount of structural material invested by unit of volume (ratio between
48 dry mass and volume), has been traditionally regarded as a key functional trait in comparative functional
49 ecology. It is considered as an important predictor of plant strategies (Westoby 1998; Wilson et al. 1999; Craine
50 et al. 2001) since it is commonly associated with many critical aspects of plant growth and survival. Low-density
51 tissues enable a fast relative growth rate and a rapid resource acquisition as the plant can rapidly expand leaf,
52 stem or root system with a low investment on dry matter (Garnier 1992; Poorter and Bergkotte 1992; Ryser
53 1995; Ryser and Lambers 1995; Wahl and Ryser 2000; Hummel et al. 2007). However, the produced watered
54 tissue tends to have a shorter life span and is usually more vulnerable to herbivory and pathogens than the high-
55 density tissues typical of slow-growing species (Eissenstat 1991; Craine et al. 2002; Craine et al. 2005; Tjoelker
56 et al. 2005). Because of its high ecological importance, tissue density is now measured routinely in many world-
57 wide meta-analyses comparing species from contrasted growth forms and environmental conditions (Wright et
58 al. 2006; Swenson and Enquist 2008; Chave et al. 2009; Fortunel et al. 2012; Kembel and Cahill 2012). One
59 prerequisite for comparing tissue density from different studies, species and/or environmental conditions is the
60 use of standardized protocols. Methodologies employed to measure tissue density had however not received
61 enough attention (Williamson and Wiemann 2010). This is particularly evident in the case of roots, since there is
62 no standardized method to measure root tissue density; for example this trait is not included in the handbook of
63 methods for measuring functional traits (Cornelissen et al. 2003). In addition, there is even no consensus on the
64 terminology used to refer to the ratio between root dry mass and root volume. A variety of terms have been used
65 interchangeably to mean the same trait. The most common name used is root tissue density, but it has also been
66 called as root dry matter concentration (Shipley and Vu 2002), root dry matter density, root tissue mass density
67 (Wahl and Ryser 2000), root mass density (Ryser 2006), or root specific gravity (Fortunel et al. 2012).

68 The determination of root tissue density is complex mainly due to the measurement of volume of fresh
69 roots, the denominator of the ratio that defines this trait. The volume of fresh roots is particularly difficult to
70 measure since roots are usually very flexible and light, have an irregular shape and the amount of sampled roots
71 is often very low. Different methods have been used in the literature for quantifying root volume. The most
72 direct, based on Archimedes' principle, consists in measuring the weight or the volume of water displaced by
73 immersion of the roots. A literature survey conducted on 40 articles measuring root tissue density in non storage
74 roots and published between 2000 and 2012 (Appendix 1) showed that the Archimedes' method was only used

in 7% of cases. In the other 93%, root tissue density was assessed using either (i) image analysis using flatbed scanner and dedicated software (62% of cases), (ii) root dry matter content (RDMC, root dry mass per unit of root fresh mass) as a proxy for root tissue density (17% of cases), or (iii) the line-intercept method (Tennant 1975) based on manual microscopic observations (14% of cases). When image analysis softwares were used, roots were digitalized at a given resolution (400 dpi in 33% of cases) and root volume was generally calculated as the product of root length times the square of root diameter/2, assuming a cylindrical shape of roots; diameter being itself calculated by the ratio between projected area and length. When RDMC was used, it was assumed that root fresh mass is a good estimator of volume. This has been demonstrated at the leaf level in many studies (Garnier et al. 1999; Roderick et al. 1999b; Vile et al. 2005) but only once at the root level (Shipley and Vu 2002, hydroponic conditions). The only two published studies comparing root volume measured simultaneously by the Archimedes' and the image analysis methods revealed inconsistent results (Ortiz-Ribbing and Eastburn 2003; Pang et al. 2011). Methodological studies comparing the effectiveness of the three main methods commonly used for quantifying root tissue density are therefore necessary to propose a reliable protocol for accurately estimating this root attribute, which has been considered a critical trait for understanding many ecological questions.

The first objective of this study was to compare three protocols commonly used for assessing fine root volume: the Archimedes' method, the image analysis method and the root dry matter content (RDMC) method. The second objective was to test whether RDMC could be used as a proxy for root tissue density. Fine root volume and tissue density were measured on root samples from three contrasted data sets in order to cover a wide range of root tissue density values. The first data set came from species belonging to contrasted taxonomic groups and life forms grown under controlled conditions; the second one was constituted by species harvested in the field; and the third one was composed of roots collected at the community level (using soil cores harvested at different depths) along a soil resource gradient.

Material and methods

Root material: the three data sets

Pot-grown species: root material came from eighteen herbaceous species selected among the most dominant ones occurring in Mediterranean old-field successions of southern France (Appendix 2). Species were grown from seeds or ramets (according to species) in 2 L pots filled with soil and maintained in a greenhouse at the Centre d'Ecologie Fonctionnelle et Evolutive (CEFE) in Montpellier, France (43°59′N, 3°51′E). Species were harvested six to eight months later, at the peak of vegetative growth; individuals of the same species were pooled. More details are available in Birouste et al. (2012).

Field-grown species: roots from seven herbaceous species were harvested in May at the vegetative peak growth in two Mediterranean rangelands, located at the CEFE experimental garden (43°59′N, 3°51′E) and at the INRA La Fage experimental station (43°55'N, 3°05'E) (Appendix 2). Several individuals were carefully dug up with a pick to a soil depth of 15 cm and pooled together. Atypically large or small individuals were avoided.

Field-community roots: root samples were collected in the field in three contrasted plant communities from a Mediterranean rangeland located at the INRA La Fage experimental station (43°55'N, 3°05'E) (Appendix 2). Plant communities differed in species composition and abundance as well as in rooting depth. As examples, the perennial grass *Bromus erectus* was dominant in deeper soil communities (≈ 90cm depth) and represented 60 to 80% of the aboveground community biomass while the perennial grass *Festuca christiani-bernardii* was the dominant species in shallower soil (≈ 20cm depth) representing 25 to 42% of the biomass of the plant community. In July 2008 (end of the growing season), two randomly distributed soil cores (5 cm diameter) per plant community were collected to maximum rooting depth. Cores were divided into 10 cm sections obtaining a total of 30 community root samples, composed of a mixture of roots from the different species occurring in the vicinity of cores. More details are available in Pérez-Ramos et al. (2012) and Bernard-Verdier et al. (2012).

Root processing

Roots were carefully washed with water to remove adhered soil. Using a digital caliper, the finest roots (< 2 mm) were sorted and excised excluding main tap and adventious roots. For each species or core, representative subsamples of fine roots ranging from 0.02 to 0.90 g fresh mass were selected (Appendix 2; Fig.2). The subsample size was determined so that it could be: i) comparable among the three data sets and including a

continuous variation of biomass and volume within each of them; ii) placed in the sample holder (4 cm diameter) used to determine root volume with Archimedes' method; and iii) spread on one A4 sheet without exceeding the recommended scanning density (Himmelbauer et al. 2004). A total of 163 subsamples were studied, the number of subsamples per species ranged from 3 to 5 for pot-grown plants and from 8 to 10 for field-grown plants (Appendix 2). For field community roots, the fine root biomass contained in each core (5 cm diameter x 10 cm length) was especially low in deep cores and did not allow us to collect more than one subsample per core. As a consequence, the amount of fine roots in subsamples accounted for a highly variable proportion of total root biomass sampled. For each subsample, fine roots fully rehydrated were gently dried between two filter papers to remove surface water until no more water tracks remained on papers; they were then immediately weighed with a hydrostatic balance to obtain root fresh mass both in air (RFM) and in ethanol (RFM_{eth}). Pure ethanol was used to avoid root flotation; further details are provided below. Root subsample was stained by immersion in methylene blue (5 g L^{-1}) for 5 min to increase contrast during scanning, then rinsed with distilled water and carefully spread out on a transparent acetate sheet in order to avoid root overlap. The root density per area scanned ranged from 0.1 to 2.8 cm cm^{-2}. Roots were then scanned as greyscale images at a resolution of 400 dpi (pixel size = 0.063 mm) using a scanner (EPSON Expression 1680) equipped with a transmitted light source to avoid shadows (Roumet et al. 2006; Birouste et al. 2012). All roots were then recovered from the acetate sheet, oven-dried at 60 °C for 48h and reweighed to obtain the root dry mass (RDM).

Measurements of root volume (V) and root tissue density (RTD)

Each root sample was analyzed following the three methods described below.

Method based on Archimedes' principle (Arch)

This method is the most direct of the three methods since it is based on physical principle to measure sample volume. For each subsample, root saturated volume was measured using the Sartorius density determination kit (Sartorius YDK01LP, Gottingen, Germany; precision 10^{-4} g), which is based on Archimedes' principle (Buoyancy method). The weighing pan from the balance was replaced by the kit density pan stand, on which a density pan, constituted by two sample holders was hang. One sample holder (the upper one) was used to measure sample fresh mass in air (RFM); the second (lower sample holder) was immersed in a beaker filled with absolute ethanol and used to measured sample fresh mass in ethanol (RFM_{eth}), i.e. the mass as reduced by the Buoyancy force. We first tare the balance, placed the sample on the upper holder and weigh (RFM), tare the

balance again with the sample on the upper holder, then place the sample in the lower sample holder and recorded the absolute readout of the buoyancy force G = RFM – RFM_{eth} which is displayed with a negative sign. According to the Archimedes' principle, a sample (here roots) completely immersed in fluid (here ethanol) is exposed to the force of buoyancy (G), equals to the mass of ethanol displaced (M_{eth}) by roots. The volume of the displaced ethanol (V_{eth}) equals the volume of roots (V_{Arch}).

$$V_{Arch} = V_{eth} = \frac{M_{eth}}{\rho_{eth}} = \frac{G}{\rho_{eth}} \quad \text{[Equation 1]}$$

where ρ_{eth} is the density of ethanol at the temperature recorded during the measurement. M_{eth} was not directly measured but obtained as proposed in Sartorius AG (2001):

$$M_{eth} = G = RFM - RFM_{eth} \quad \text{[Equation 2]}$$

Combining equation 1 and 2, V_{Arch} and root tissue density (RTD_{Arch}) were calculated as:

$$V_{Arch} = \frac{RFM - RFM_{eth}}{\rho_{eth}} \quad \text{[Equation 3]}$$

$$RTD_{Arch} = \frac{RDM}{V_{Arch}} \quad \text{[Equation 4]}$$

Method based on root image analysis (IA)

The WinRHIZO software (WR, version 2003b, Regent Instrument, Quebec, Canada) was used to determine root length and volume in 10 diameter classes (from 0 to 2 mm, with a class width of 0.2 mm). The software is based on a skeletonization method which transforms the greyscale images into binary (i.e. black and white) and skeleton images. We selected the automatic thresholding option (recommended by Bouma et al. 2000) in order to optimize the threshold which separated grey levels in two distinct groups, root and background. For each pixel of the skeleton, the punctual diameter was measured as the smallest distance between two opposite boundary pixels in all directions at this point. The root volume was computed with the punctual diameter at the pixel position and added to the proper diameter class to obtain the root volume per diameter class (V_{IA}; Régent Instruments Inc. 2003). Total root volume (V_{IA}) and root tissue density (RTD_{IA}) were calculated as:

$$V_{IA} = \sum_{i=1}^{j} V_{IAi} \quad \text{[Equation 5]}$$

where *j* represented the number of diameter classes. The number and width of the diameter classes did not affect the V_{IA} (data not shown) because V_{IAi} was measured at each pixel independently of diameter classes.

$$RTD_{IA} = \frac{RDM}{V_{IA}} \quad \text{[Equation 6]}$$

Method based on root dry matter content (RDMC)

The root dry matter content (RDMC) is defined as the ratio between root dry mass (RDM) and root fresh mass (RFM). In this method, it was assumed that root volume could be indirectly estimated by RFM after full rehydration (i.e., V = RFM) and that root dry matter content could be used as a proxy for root tissue density (RTD):

$$RDMC = \frac{RDM}{RFM} \approx \frac{RDM}{V} = RTD \quad \text{[Equation 7]}$$

Root volume and fresh mass (RFM) are linked by root density (ρ)

$$\rho = \frac{RFM}{V} \quad \text{[Equation 8]}$$

Root volume (V) and fresh mass (RFM) would be equivalent only if root density $\rho \approx 1$. Root density (ρ) considers the fresh masses and volumes of the three phases contained in roots: solid (i.e., tissues), liquid and air (Roderick et al. 1999a). It differs from root *tissue* density (RTD) in that this latter only considers dry mass (the tissue phase). For leaves, an average leaf density of 1 had been reported for many species (i.e., Sims et al. 1998; Garnier et al. 1999; Vile et al. 2005), and the leaf dry matter content is thus commonly used as a proxy of leaf tissue density. By contrast, this relationship has been rarely studied for roots.

Statistical analyses

All analyses were performed on single root replicates. Differences in root volume and root tissue density between methods were tested for each data set using a one-way analysis of variance (ANOVA) with "method" as main factor. A post hoc test (Student-Newman-Keuls comparisons) was further applied. Major axis (MA) analyses were performed for pair-wise comparisons between the three methods since MA is particularly well adapted for testing if two methods of measurement agree, and in particular for testing whether methods scale

isometrically (Warton et al. 2006). Differences between methods were evaluated using the root mean square deviation (RMSD):

$$RMSD = \sqrt{\frac{\sum (X_1 - X_2)^2}{n}} \quad \text{[Equation 9]}$$

where X_1 and X_1 were the volumes (or RTD) measured with method 1 and 2 respectively and n the number of samples. Analyses were carried out using R 2.13.0 (R Development Core Team 2011).

Results

Root volume

Significant differences were detected in root volume when the three methods were compared (Fig. 1). Root volume measured by Archimedes' method (V_{Arch}) did not differ significantly from the root fresh mass (RFM), used as a proxy of root volume, overall and for the three datasets. Volume measured using image analysis (V_{IA}) was on average 70% higher than V_{Arch} and RFM (Fig. 1a). The effect of image analysis method varied between data sets; it was larger for pot-grown species (Fig. 1b) as compared to field-grown species (Fig. 1c) while it was not significant for field-community roots (Fig. 1d). Scatter plots with all root samples showed that V_{IA} was significantly and positively correlated with V_{Arch} (Table 1; Fig. 2a). The slopes however differed among data sets being steeper in more complex environments (0.60 for pot-grown species, 0.73 for field-grown species and 0.96 for field-community roots; Table 1). A positive relationship was also found between V_{IA} and RFM (Table 1; Fig. 2b). V_{IA} was always higher than V_{Arch} and RFM with the exception of two samples from the field-community root. The RMSD between V_{IA} and $V_{Arch,}$ and between V_{IA} and RFM averaged over 0.040 and 0.046 respectively and tended to decrease with increasing V_{Arch}. RFM was closely correlated to V_{Arch} either for the whole data set or separately for any of the three data sets (Table 1; Fig. 2c). The correlation coefficients obtained were very high regardless of the data set considered ($R^2 > 0.95$). The RMSD between RFM and V_{Arch} averaged over 0.002.

Consequences on root tissue density

Root tissue density (RTD) differed significantly between data sets ($F = 73.2$; $P < 0.001$) and methods ($F = 74.16$; $P < 0.001$). RTD measured using the Archimedes' method (RTD_{Arch}) showed a 4.5-fold variation among samples ranging from 0.153 to 0.682 g cm^{-3} (Fig. 3a). As expected, roots from pot-grown species had a lower tissue density than field-community roots and field-grown species (0.221 ± 0.005, 0.366 ± 0.017 and 0.312 ± 0.014 g cm^{-3} respectively). Root tissue density determined with the image analysis method (RTD_{IA}) presented a 10.8-fold variation, a much wider value than the range of variation observed for RTD_{Arch} (Fig. 3a) and for RDMC (Fig. 3b). Overall, RTD_{IA} was significantly correlated to RTD_{Arch} and RDMC (Table 1; Figs. 3a,b). This pattern was confirmed within each data sets except for pot-grown species (Table 1; Figs. 3a,b). RTD_{IA} was always lower than RTD_{Arch} and RDMC with the exception of the two same samples mentioned before (volume comparison). RMSD averaged 0.021 between RTD_{IA} and RTD_{Arch} and 0.022 between RTD_{IA} and RDMC. The root dry matter

240 content (RDMC) was highly correlated to root tissue density measured by Archimedes' method (RTD_{Arch}) (Table
241 1; Fig. 3c). The three data sets showed significant correlations between both variables (Table 1; Fig. 3b). RMSD
242 between RDMC and RTD_{Arch} is much lower than RMSD found between RDMC and RTD_{IA} since it averaged
243 over 0.002 and ranged from 0.001 to 0.002 among datasets. RDMC tended to be slightly higher than RTD_{Arch} in
244 pot-grown species while the opposite was observed for field-community roots).

Discussion

This study demonstrates that the three methods used to determine fine root tissue density were positively correlated with each other. The strongest correlation was found between Archimedes' method, the most direct and physical method, and the ratio between root dry mass to root fresh mass (i.e. root dry matter content), the most indirect method where root fresh mass was used as a proxy of root volume.

Estimating fine root tissue density from image analysis software

The fine root volume obtained from image analysis method was approximately 70% greater than that determined by the Archimedes' method and RDMC; leading to an average 43% decrease of RTD_{IA} as compared to RTD_{Arch} and RDMC. The only two published studies we know that compared methods reported opposite results (Ortiz-Ribbing and Eastburn 2003; Pang et al. 2011). In Ortiz-Ribbing and Eastburn (2003), root volumes of soybeans grown either in greenhouse or in the field, were respectively 2 to 3.6 times lower when measured with image analysis method (using WinRHIZO as software) as compared with those measured by Archimedes' method. In contrast, Pang et al. (2011) did not detect any significant differences between the two methods for *Cynodon sp.* grown in greenhouse. It is uncertain why these contrasted results occurred and the lack of precise information on how roots were scanned and how volume was calculated complicate data interpretation. Results obtained by image analysis are extremely sensitive to the scanning procedure (resolution and light sourced used) and to the image analysis protocol, i.e. root staining, sample density, software, thresholding and filtering of images (Bouma et al. 2000; Costa et al. 2001; Zobel et al. 2003; Himmelbauer et al. 2004; Pierret et al. 2013). In our study, although we used the protocol recommended by Bouma et al. (2000) and the volume by diameter classes suggested by Ryser (2006), the volume calculated by image analysis method was consistently higher than V_{Arch}. This might be a consequence of the resolution used. In the literature survey that we conducted (see Introduction section), 50% of the studies using image analysis method for volume estimation did not mention the resolution used. When resolution was specified 60% of these studies used a resolution of 400 dpi as we did. However, this commonly used resolution could be inadequate for quantifying the volume of very small diameter roots. Zobel (2013) recently demonstrated that commercial scanners did not have enough resolution to accurately measure fine root diameters (< 0.09 mm). According to Richner et al. (2000), the diameter of the thinnest roots should be at least three times the pixel size (i.e. 0.19 mm diameter for a 400 dpi resolution) to ensure an accurate measurement of root diameter. This was not completely followed in our study, where the proportion of very fine

roots was relatively frequent, especially in pot-grown species (60% of root length < 0.2 mm). At a resolution of 400 dpi, roots with diameter lower than one pixel (0.063 mm) were estimated using at least one pixel, leading to an overestimation of diameter and thus volume. We cannot rescanned our root samples at a highest resolution, however using another set of 16 very fine root samples (diameter ranging from 0.13 to 0.42 mm) we scanned each root sample at 400 and 1200 dpi. Our results (data not shown) demonstrated that volumes estimated at 400 dpi were 61 % higher than volumes measured at 1200 dpi, suggesting that the scanning resolution used in this study was certainly insufficient to measure accurately root volume and thereby RTD_{IA}. The resolution of 400 dpi was recommended in the years 2000 when scanners and computers performance were limited as compared with those available presently. We thus recommend using a higher resolution even if the time required for scanning and analyzing images is also higher. A new update standard protocol needs to be established to measure accurately root volume and tissue density using image analysis method.

Another potential source of error could be the automatic threshold used. A sensitive analysis reported that measurements of root length could change up to a factor of 8 according to selected values of the threshold (Bouma et al. 2000; Tajima and Kato 2011), with probable dramatic consequences on total volume and RTD estimation. This was recently confirmed by Pierret et al. (2013) who compared the performance of two image analysis packages measuring length and diameter of roots scanned at 400 dpi. Correlation between average root diameter produced by these two packages was weaker than those obtained for length due the sensitivity of diameter to thresholding.

Estimating fine root tissue density from measurement of root dry matter content

Our results showed that fine root fresh mass (RFM) did not differed significantly from root volume (V_{Arch}) measured using the Archimedes' method. Fresh mass and volume are equivalent only if the density (ρ = RFM / V) of the root is equal to 1. This was corroborated in our study, where root fresh mass scaled 1:1 with root volume V_{Arch}, and density (RFM/V) average was close to 1 (0.993 ± 0,009 g cm^{-3}, with values ranging from 0.77 to 1.56 g cm^{-3}). These estimations of fine root density are consistent with those previously reported for roots (Ryser et al. 2011) and leaves (Sims et al. 1998; Garnier et al. 1999; Vile et al. 2005). As suggested by Roderick et al. (1999a), variation of leaf density could reflect different relative proportions of the three phases that composed leaves: air, water and solid. High water content (with $\rho \approx 1$) leads to density near unity (Roderick et al. 1999b) and prevent formation of large internal air spaces. At low water content, density varied depending on the allocation of dry matter and the fractional air space. For roots, these hypotheses need to be confirmed by

304 anatomical studies. Despite small variation of root density, our study demonstrates for the first time that fine root
305 fresh mass scaled 1:1 with fine root volume and validates the use of fine root fresh mass as a surrogate of fine
306 root volume in herbaceous species. As a consequence, we also validated the use of root dry matter content
307 (RDMC) as a surrogate of fine root tissue density (RTD). This result confirmed those found by Shipley and Vu
308 (2002) on young roots of 17 species grown in hydroponic conditions. Here, we demonstrated for the first time
309 that the tight relationship between RDMC and RTD measured by Archimedes' method holds for a broad range of
310 plant species of different ages and growing under very contrasted conditions (*in situ* or in pots). RDMC slightly
311 overestimated RTD_{Arch} in pot-grown species as a consequence of the underestimation of root volume, likely due
312 to a higher proportion of air spaces. At the opposite side, RDMC is slightly lower than RTD_{Arch} in field-
313 community roots likely as a consequence of a greater presence of dense materials within the roots, which led to
314 thicker cell walls (usually associated with older root systems or field constraints).

315

316 Comparison among methods used for estimating root tissue density

317 In this study there is no way to know which technique is the more accurate and each method presents advantages
318 and disadvantages. The Archimedes' method is the most direct method considering the three dimensions of
319 roots. However, it is time-consuming and requires specific equipment (hydrostatic balance, pycnometer, digital
320 micrometer). Another disadvantage concerns the difficulty to achieve full immersion of roots in the liquid. Since
321 root density is very similar to that of distilled water, roots need to be immersed in a liquid of lower density such
322 as ethanol (density ≈ 0.8 g cm^{-3}) and this might affect root volume. Air bubbles clamping within the root sample
323 might cause additional errors. Image analysis is the most widely used method for root studies; it is an essential
324 and powerful tool to determine simultaneously many root attributes (e.g. length, area, volume, mean diameter,
325 diameter class length distributions and topology). Measurements are however strongly sensitive to the scanning
326 resolution and transformation threshold (Bouma et al. 2000; Costa et al. 2001; Himmelbauer et al. 2004; Pierret
327 et al. 2013; Zobel 2013). The measurement of RDMC is the most indirect method since it assumes a tight
328 relationship between root volume and fresh mass, which had never been demonstrated for roots at the
329 interspecific level before this study. It is easy, quick and cheap to measure; RDMC determination only requires
330 two rapid measurements with a precision balance (fresh mass and dry mass after 48h at 60°C). Fresh mass
331 determination, however, is not a very accurate measure since it depends on the degree of water saturation of root
332 tissues, the process of root drying for removing surface water and the dehydration rate in air during the

weighing. Because most of the roots are not protected against desiccation and lose water rather quickly, it is recommended to standardize the blotting procedure and to weigh roots as quickly as possible after the blotting up. Compared with the image analysis method it provides only one trait, the RDMC. Despite these inevitable disadvantages, our study demonstrates that the use of RDMC provides reliable results to estimate fine RTD, as recently demonstrated for plant residues (Iqbal et al. 2012). The choice of using a particular methodology strongly depends on the objectives of the study and materials under investigation; for studies interested in variation of fine root tissue density among species or environmental conditions, we suggest the use of RDMC to estimate root tissue density. For studies interested in variation of more morphological traits, image analysis method remained essential. These results obtained for fine roots of herbaceous species need however to be confirmed on bigger samples, using coarse roots and woody species as well as with a higher scanning resolution. Despite RDMC is by far the easiest method, it is rarely used as a proxy of root tissue density. This is in contrast with its leaf analogue, the leaf dry matter content (LDMC, the ratio of leaf dry mass to fresh mass), which is increasingly used as an indicator of plant species' resource use strategy (Wilson et al. 1999; Garnier et al. 2001; Díaz et al. 2004), leaf decomposability (Garnier et al. 2001; Fortunel et al. 2009; Kazakou et al. 2009) or soil fertility (Hodgson et al. 2011). Results from this study support the high predictive potential of RDMC for estimating fine RTD, and offer promising perspectives for root comparative ecology since RDMC enables the estimation of a key root trait from an easily measurable root attribute.

From an ecological point of view, a lot of studies have supported clear evidences that root tissue density (RTD) affects several processes of root functioning such as respiration (Makita et al. 2012; Picon-Cochard et al. 2012), growth rate (Walh and Ryser 2000; Hummel et al. 2007) and longevity (Useche and Shipley 2010). Such evidence is scarce for RDMC. Recent studies have however revealed the interest of using RDMC as an indicator of differential functional strategies. As examples, RDMC has been identified as a consistent response trait to nitrogen limitation (Pérez-Ramos et al. 2012) or soil drought (Poorter and Markesteijn 2007). Here, we demonstrated that RDMC is a good, reliable and cheap proxy of fine root tissue density. The next step is to test its importance for the prediction of ecological patterns. We strongly recommended its measurement in comparative root ecology studies, in order to strengthen the role of RDMC as a predictor of root functions and ecosystem properties and to heighten the use of this key root trait in ecological studies.

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497

498 **Table 1** Major axis regressions between fine root volume and root tissue density measured using three different
499 methods for the different data sets.
500

Data sets	Equation	R^2	*P*	RMSD
V_{Arch} vs V_{IA}				
All	$V_{Arch} = 0.70\ V_{IA} - 0.04$	0.77	***	0.0399
Pot-grown species	$V_{Arch} = 0.60\ V_{IA} - 0.03$	0.77	***	0.0362
Field-grown species	$V_{Arch} = 0.73\ V_{IA} - 0.05$	0.73	***	0.0611
Field-community roots	$V_{Arch} = 0.96\ V_{IA} - 0.04$	0.80	***	0.0060
V_{IA} vs RFM				
All	$V_{IA} = 0.75$ RFM - 0.06	0.64	***	0.0461
Pot-grown species	$V_{IA} = 0.52$ RFM - 0.02	0.72	***	0.0428
Field-grown species	$V_{IA} = 0.81$ RFM - 0.09	0.56	***	0.0706
Field-community roots	$V_{IA} = 1.12$ RFM - 0.05	0.84	***	0.0045
V_{Arch} vs RFM				
All	$V_{Arch} = 0.92$ RFM + 0.02	0.96	***	0.0016
Pot-grown species	$V_{Arch} = 1.12$ RFM - 0.04	0.99	***	0.0004
Field-grown species	$V_{Arch} = 0.89$ RFM + 0.03	0.95	***	0.0032
Field-community roots	$V_{Arch} = 0.87$ RFM + 0.004	0.97	***	0.0012
RTD_{Arch} vs RTD_{IA}				
All	$RTD_{Arch} = 1.03\ RTD_{IA} + 0.12$	0.56	***	0.0211
Pot-grown species	$RTD_{Arch} = 4.44\ RTD_{IA} - 0.24$	0.03	ns	0.0155
Field-grown species	$RTD_{Arch} = 0.93\ RTD_{IA} + 0.16$	0.53	***	0.0307
Field-community roots	$RTD_{Arch} = 0.85\ RTD_{IA} + 0.13$	0.15	*	0.0153
RTD_{IA} vs RDMC				
All	$RTD_{IA} = 0.74$ * RDMC - 0.17	0.41	***	0.0219
Pot-grown species	$RTD_{IA} = 5.44$ * RDMC - 0.33	0.03	ns	0.0207
Field-grown species	$RTD_{IA} = 0.65$ * RDMC - 0.21	0.39	***	0.0295
Field-community roots	$RTD_{IA} = 0.35$ * RDMC - 0.20	0.12	ns	0.0093
RTD_{Arch} vs RDMC				
All	$RTD_{Arch} = 1.3$ * RDMC - 0.08	0.90	***	0.0015
Pot-grown species	$RTD_{Arch} = 0.94$ * RDMC - 0.06	0.97	***	0.0005
Field-grown species	$RTD_{Arch} = 1.3$ * RDMC - 0.08	0.90	***	0.0022
Field-community roots	$RTD_{Arch} = 1.5$ * RDMC - 0.10	0.79	***	0.0024

Equations, $R^{2,}$ the significance levels and the root-mean-square deviation (RMSD) are given. Abbreviations: root volume measured using Archimedes' method ($V_{Arch,}$) or the image analysis software ($V_{IA,}$); root fresh mass (RFM); root dry matter content (RDMC); root tissue density determined using Archimedes' method (RTD_{Arch}) or the image analysis software (RTD_{IA}). Number of root samples: n=163 for all data sets, n=72 for pot-grown species, n=61 field-grown species and n=30 field-community roots. *P < 0.05; ***P < 0.001; ns, non-significant

Fig. 1 Mean and standard error of root volume for all data sets (a), pot grown species (b), field grown species (c), field-community roots (d) determined using three methods: Archimedes' method (open bars), image analysis method (grey bars), and fresh mass used here as a proxy of root volume (black bars). Root volume was measured on root samples (n=163) belonging to three data sets: pot-grown species (n = 72), field-grown species (n = 61) and field-community roots (n=30). F-value and significance level are indicated inside the plot. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Different letters indicate significant differences among methods

Fig. 2 Relationships between the fine root volume measured by (a) Archimedes' method (V_{Arch}) and the image analysis method (V_{IA}); (b) the fine root fresh mass (RFM) and image analysis method (V_{IA}) and (c) Archimedes' method (V_{Arch}) and the fine root fresh mass (RFM). Triangles represent pot-grown species, open circles field-grown species and closed circles field-community samples. R^2 of the major-axis regressions are given using the whole data as well as for each of the three data sets analyzed in this study. A log-scale is used to represent V_{Arch}, V_{IA} and RFM. For comparative purposes, the 1:1 ratio has been represented by a dotted line. ***$P < 0.001$

Fig. 3 Relationships between the fine root tissue density measured by (a) Archimedes' method ($RTD_{Arch)}$ and the image analysis method (RTD_{IA}),(b) the fine root dry matter content (RDMC) and image analysis method (RTD_{IA}) and (c) Archimedes' method (RTD_{Arch}) and the fine root dry matter content (RDMC). Triangles represent pot-grown species; open circles field-grown species and closed circles field-community root samples. R^2 of the major-axis regressions are given using the whole data as well as for each of the three data sets analyzed in this study. A log-scale is used to represent RTD_{Arch}, RTD_{IA} and RDMC. For comparative purposes, the 1:1 ratio has been represented by a dotted line. The level of significance is indicates as follows: •$P < 0.1$; *$P < 0.05$; ***$P < 0.001$; ns, non-significant

Fig1

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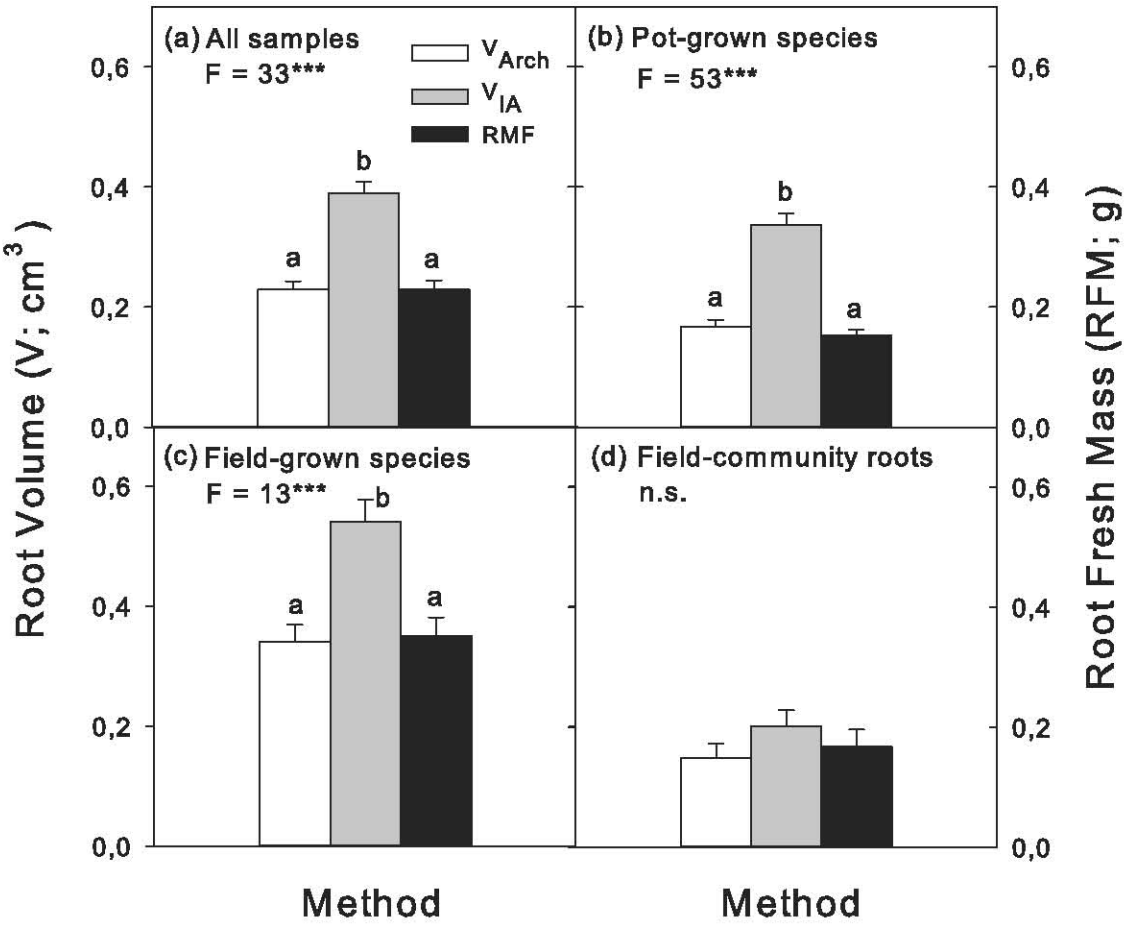

Fig2

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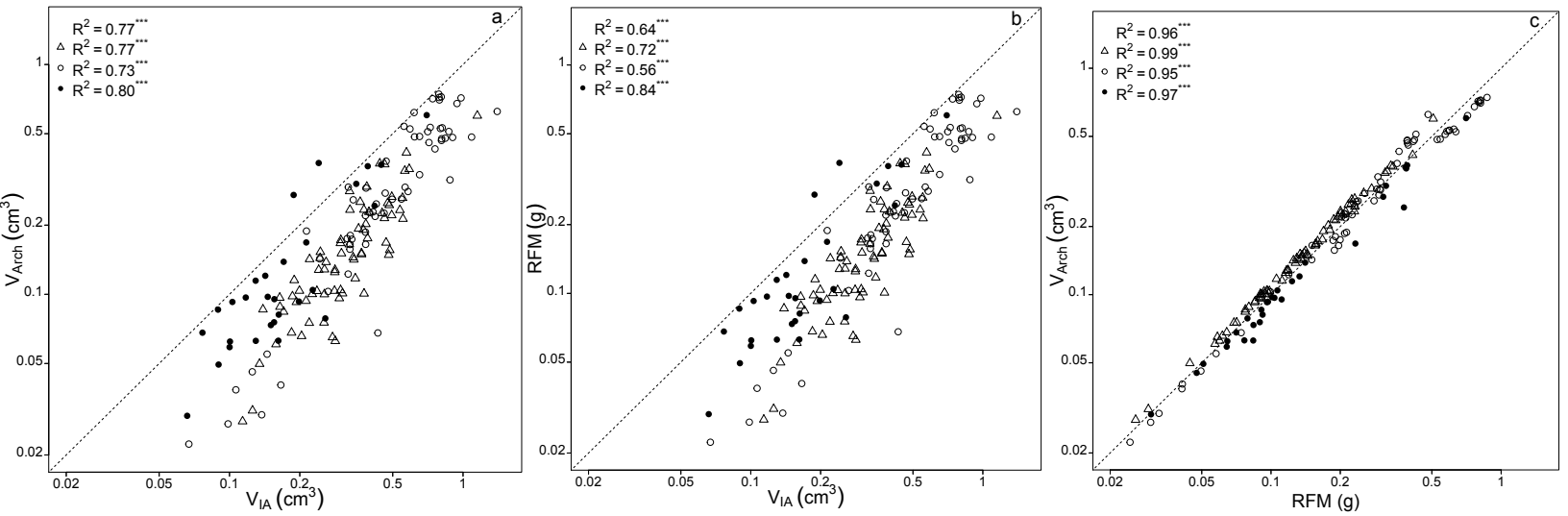

Fig3

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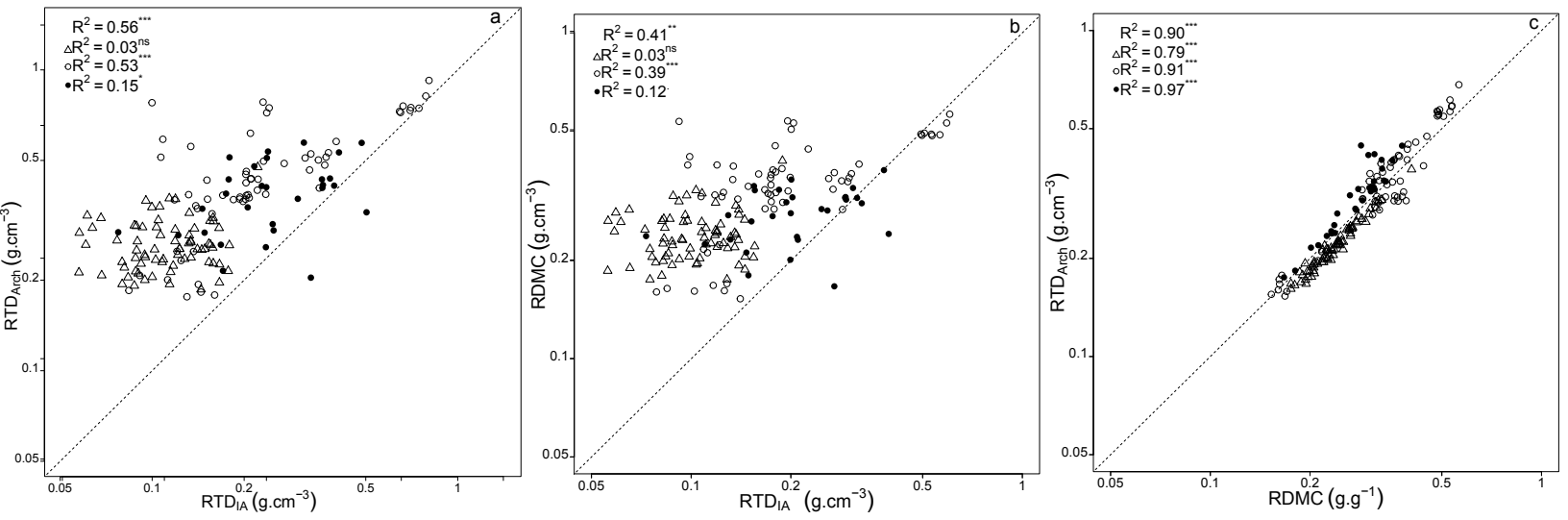

Appendix 1: List of the 40 references consulted to review methods used to assess root tissue density. Most references were found using Web of Science (Thomson Reuters) with the following combinations of words 'root tissue density', 'root dry matter content', 'root dry matter concentration' or 'root dry mass density'. The literature survey concerned only papers published between 2000 and 2012.

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Appendix 2: List of plant species or communities used to take measurements of root volume and tissue density.

Plant species or community	Botanical family	Soil Depth (cm)	n	RDM (g)	RTD_{Arch} (g cm^{-3})
Data set 1: Roots from pot-grown species					
Arenaria serpyllifolia L.	Caryophyllaceae	20	4	0.007 – 0.024	0.247 – 0.269
Bromus erectus Huds.	Poaceae	20	4	0.048 – 0.076	0.169 – 0.221
Bromus madritensis L.	Poaceae	20	4	0.018 – 0.021	0.211 – 0.284
Brachypodium phoenicoides Roem. & Schult.	Poaceae	20	4	0.039 – 0.048	0.167 – 0.195
Crepis foetida L.	Asteraceae	20	4	0.014 – 0.027	0.165 – 0.193
Clinopodium nepeta L.	Lamiaceae	20	4	0.048 – 0.128	0.213 – 0.375
Daucus carota L.	Apiaceae	20	4	0.011 – 0.034	0.200 – 0.248
Dactylis glomerata L.	Poaceae	20	3	0.018 – 0.024	0.213 – 0.246
Geranium rotundifolium L.	Geraniaceae	20	5	0.029 – 0.039	0.215 – 0.245
Inula conyza D.C.	Asteraceae	20	3	0.029 – 0.042	0.167 – 0.226
Medicago minima L.	Fabaceae	20	4	0.021 – 0.050	0.225 – 0.264
Bituminaria bituminosa L.	Fabaceae	20	4	0.033 – 0.045	0.172 – 0.234
Picris hieracioides L.	Asteraceae	20	4	0.022 – 0.032	0.182 – 0.213
Rubia peregrina L.	Rubiaceae	20	4	0.051 – 0.080	0.161 – 0.219
Trifolium angustifolium L.	Fabaceae	20	4	0.022 – 0.051	0.286 – 0.302
Teucrium chamaedrys L.	Lamiaceae	20	4	0.045 – 0.054	0.195 – 0.205
Tordylium maximum L.	Apiaceae	20	4	0.023 – 0.031	0.183 – 0.213
Veronica persica Poir.	Scrophulariaceae	20	4	0.015 – 0.025	0.201 – 0.250
Data set 2: Roots from field-grown species					
*Bromus erectus Huds.**	Poaceae	20	10	0.035 – 0.067	0.304 – 0.391
*Bromus madritensis L.**	Poaceae	20	10	0.036 – 0.060	0.153 – 0.210
Carex humilis Leyss.	Cyperaceae	20	8	0.330 – 0.420	0.547 – 0.682
Carex flacca Schreb.	Cyperaceae	20	9	0.132 – 0.188	0.282 – 0.385
Bromus erectus Huds.	Poaceae	20	8	0.047 – 0.098	0.272 – 0.345
Potentilla neumanniana Rchb.	Rosaceae	20	8	0.184 – 0.303	0.368 – 0.448
Festuca christiani bernardinii (Kerguélen)	Poaceae	20	8	0.012 – 0.040	0.402 – 0.587
Data set 3: Roots from field-community					
Community from deep soil	several	90	18	0.010 – 0.110	0.220 – 0.418
Community from intermediate soil	several	40	7	0.025 – 0.267	0.175 – 0.444
Community from shallow soil	several	20	5	0.019 – 0.078	0.216 – 0.415

Roots from field-grown species were harvested in Mediterranean rangelands at La Fage INRA experimental station, excepting the two species marked with asterisk that were harvested in Montpellier. The minimal and maximal sizes of the sample are given by the root dry mass (RDM). The minimal and maximal values of root tissue density of species or community (RTD_{Arch}) are also given.