Plant Soil (2013) 363:383–398
DOI 10.1007/s11104-012-1350-0

REGULAR ARTICLE

A randomization method for efficiently and accurately processing fine roots, and separating them from debris, in the laboratory

Laura E. Koteen · Dennis D. Baldocchi

Received: 16 February 2012 / Accepted: 25 June 2012 / Published online: 9 August 2012

Abstract
Background and Aims We developed a method for processing roots from soil cores and monoliths in the laboratory to reduce the time and cost devoted to separating roots from debris and improve the accuracy of root variable estimates. The method was tested on soil cores from a California oak savanna, with roots from trees, *Quercus douglasii,* and annual grasses.
Methods In the randomized sampling method, one isolates the sample fraction consisting of roots and organic debris<= 1 cm in length, and randomizes it through immersion in water and vigorous mixing. Sub-samples from the mixture are then used to estimate the percentage of roots in this fraction, thereby enabling an estimate of total sample biomass.
Results We found that root biomass estimates, determined through the randomization method, differed from total root biomass established by meticulously picking every root from a sample with an error of 3.0 % +/− 0.6 %s.e.
Conclusions This method greatly reduces the time and resources required for root processing from soil cores and monoliths, and improves the accuracy of root variable estimates compared to standard methods. This gives researchers the ability to increase sample frequency and reduce the error associated with studying roots at the landscape and plant scales.

Keywords *Quercus douglasii* · Population sampling · Randomization analysis · Root processing methods · Root separation from debris

Responsible Editor: Angela Hodge.

L. E. Koteen (✉) · D. D. Baldocchi
Department of Environmental Science, Policy and Management, University of California, Berkeley, CA 94720-3114, USA
e-mail: lkoteen@berkeley.edu

Introduction

An understanding of root distribution and dynamics is critical to understanding ecosystem structure and function. Because roots supply plants with water and nutrients, they play an essential role in governing most important ecosystem processes, including net primary productivity, species competitive dynamics, ecosystem carbon storage, and carbon, water and energy flux rates. Roots also form mycorrhizal associations which mediate nutrient uptake and draw carbon belowground (Jones et al. 2004; Lynch and Whipps 1990). In addition, roots anchor plants in the soil, influence soil structure, and provide soil stability (De Baets et al. 2008; Reubens et al. 2007; Tisdall and Oades 1982). And, root processes link the plant canopy with soil organisms, as photosynthesis fuels root exudation, influencing soil chemistry and leading

to microbial booms and busts (Hogberg et al. 2001; Nguyen 2003; Paterson et al. 2007; Vogt et al. 1995). At landscape to regional scales, root depth and distribution often help explain hydrologic cycles in arid and semi-arid regions, and in locations that undergo seasonal water scarcity (Chaves et al. 2002; Huxman et al. 2005; Jackson et al. 1996; Nepstad et al. 1994). For example, deep roots that tap into groundwater sources can enable tree growth in locations where soil moisture reserves alone would be inadequate (Canadell et al. 1996; Lewis and Burgy 1964; Miller et al. 2010). Moreover, the rooting depth, plasticity and root distribution of community dominants will be a primary factor in determining how ecosystems respond to climate change, and how resistant community structure and carbon cycling are to changes in water availability (Baker et al. 2008; Huxman et al. 2005). As global change progresses, there is increased appreciation for the importance of root and rhizosphere processes in governing ecosystem carbon cycling, and greater urgency to understand the role of root distribution and function in imparting ecosystem resistance and resilience to change (Chapin et al. 2009; Jackson et al. 2000).

Despite their importance, roots are understudied in ecology because they are hard to characterize well and because processing roots in the laboratory from traditional soil cores and monoliths consumes considerable time, human and financial resources (Bohm 1979; Pierret et al. 2005). What's more, once roots have been extracted and processed, ecologists may have little confidence in the dataset they've compiled because roots are spatially heterogeneous in the soil and most fine roots grow and turnover on the order of weeks to months (Gill and Jackson 2000; Publicover and Vogt 1993). Therefore, to quantify root biomass and root processes well requires frequent sampling in space and time, and core sizes with larger diameters or amply-replicated monoliths are preferable (Bohm 1979; do Rosario et al. 2000). Yet, the spatial extent, size and frequency of root samples are constrained because root processing is so laborious.

The most commonly-used method to process root samples in the lab is to rinse samples of soil and mineral particles by pressing them through sieves of different mesh size and collecting the root and organic debris that is caught by the sieve. Another common method used to separate roots from soil is the use of an elutriator, which is touted as superior by some because it leaves lab workers free to attend to other tasks while the device performs soil-root separation (Smucker et al. 1982). From this stage, the roots, many of which are now fragmented, must be separated by hand from the non-root organic debris with forceps. This task is the rate-limiting step in the whole process and can take from minutes to days for each core sample, constraining core sample size and number. To reduce the per-sample workload, and because there are diminishing returns with the amount of root biomass recovered over time, one frequently sets a limit on the roots they will pick, based on root segment length. When only root fragments remain that are the length of the chosen limit (i.e. <= 0.5 cm), one stops picking, and assumes the remaining roots contribute little to the total. However, this assumption is often incorrect because the remaining short fragments often constitute a significant fraction of the core sample, leading to significant underestimation of root biomass. The choice around when to stop picking is also often subjective in practice, and can vary by core sample or lab worker.

To address drawbacks of standard root processing methods, newer techniques have been developed which employ scanners and image processing software, and these can provide generally good accuracy when core samples are not highly contaminated by debris. However, commercial scanners are expensive and yield better information about root length than biomass, and they cannot distinguish among vitality classes (Benjamin and Nielsen 2004). Moreover, and most importantly, they do not free researchers from the need to separate root material from debris, particularly for root biomass estimation.

We also evaluated another, more recently proposed method for reducing the time of root processing in the lab (Metcalfe et al. 2007), referred to as the temporal prediction method by the study's authors. This method relies on a maximum likelihood approach to estimate the total root biomass of each core sample, and assumes that the diminishing amounts of root mass recovered in each timed picking interval provides a basis for total root mass estimation. However, in practice, we found this method inaccurate because: 1. roots remain in the soil matrix and are often difficult to distinguish from soil and 2. we cannot reconcile the assumptions of this method with our observation that a large number of small

fragments can remain once larger fragments are removed. At least with our current sample set and also with roots we have processed in the past, we have found the distribution of root fragment sizes is often highly skewed towards smaller, shorter roots. (For the current study, we found that roots less than 1 cm in length represented 57 %±20 %s.d. of total core sample root biomass). Therefore, the decline in root mass lab workers recover with each successive timed interval may be due to greater obscurity of root segments, rather than reduced number (i.e. darker, finer and shorter roots may be less visible).

Attempts to circumvent the difficulties associated with processing roots from soil cores and monoliths have led to several alternative methods to estimate root variables, including the use of minirhizotrons and a number of indirect approaches based on carbon budgets or flux measurements (Clark et al. 2001; Vogt et al. 1998). The use of minirhizotrons is the most widely-used among them, and can be the preferred method depending on the objectives and constraints of the root study. For example, the use of minirhizotrons is preferable if repeated destructive sampling in small plots must be avoided, and is of particular use for root turnover estimates, and for enabling a direct view of root dynamics in situ (Majdi et al. 2005). However, minirhizotrons are frequently found to underestimate root biomass and cumulative root length when compared with estimates from sequential coring or monolith methods, although loss of root mass can also occur during sieving. Minirhizotrons are also known to influence the soil environment and pattern of root growth at the interface of the soil with the wall of the observation tube (Pierret et al. 2005; Vogt et al. 1998). Moreover, post-image processing of minirhizotron data is likewise laborious, depending on sampling interval and tube number, and discrimination between live and dead roots is difficult until roots disappear (Clark et al. 2001; Vogt et al. 1998).

Here we outline a promising approach to root processing which can greatly reduce the time required to process root samples and also improve the accuracy of root estimates, and which should be widely-applicable across root samples of all types. Moreover, the amount of time required to process a core sample of large diameter versus one of small diameter will be small, thus allowing researchers to extract larger cores, as well as a greater number of cores, which are needed to better characterize root distribution both spatially and temporally. This method still requires careful separation of root and other organic debris from soil through sieving, or through the use of a root elutriator system. However, the smaller and most unruly fraction of both root and non-root material is randomized and sub-sampled in a method that preserves all of the original material from the core sample. The sub-samples are then used to provide an estimate of the percent of this fraction that is root material. Variance in sub-sample estimates of the root component can also be used to construct confidence intervals to quantify the method error.

The objectives of this study were: 1. to test the accuracy of the randomization method on a set of mixed tree and grass root cores extracted from soils at an oak savanna site from the Sierra Nevada foot hills of Northern Central California, 2. to construct confidence intervals for core sample root estimates, and 3. to provide the reader with the tools required to estimate the appropriate sub-sample number and sampling parameters to accurately characterize fine roots in individual systems.

Materials and methods

The method we developed for processing roots from a California oak savanna is similar to the traditional method, but increases the time devoted to sieving material, while greatly decreasing the time spent picking roots. Our intent was to obtain quantitative estimates for root density as it varies by depth and with distance from the bole of the tree, or from the center of tree clusters. We sampled roots according to a radial design from five trees, with cores extracted from the north, south, east and west of the tree at distances of half the mean canopy radius, at the mean canopy radius and 1.5 × the mean canopy radius. By depth, we sampled at intervals form 0–10 cm, 10–20 cm, 20–40 cm, 40–60 cm, and from 60 cm to the depth of the bedrock. Bedrock depth varied from 40 cm to 90 cm on average. Two tree clusters were also sampled in radial intervals from a point at the approximate center of the trees within the cluster. We assumed that root growth did not vary

by cardinal direction. Therefore, there were four replicates per depth, per distance from the tree bole and per cluster center. We used a root corer with an inside diameter of 5 cm. We took an additional set of core samples in the surrounding grassland at a distance of 1.5 times the canopy radius plus 1 m. For these "grass cores", we used a soil corer with a diameter of 3.5 cm and sampled at depth intervals of 0–10 cm, 10–20 cm, and 20–50 cm. Depth intervals differed from the tree/grass root cores because the roots of California's annual grasses are concentrated near to the top of the soil profile, but more evenly distributed with depth where trees are found (Holmes and Rice 1996; Koteen et al. 2011). Once extracted, core samples were brought to the lab and stored at 5 °C until processing began. Here, we report data from 323 tree/grass root core samples, and from 72 core samples representing the grasses.

Site description

The roots for this study were obtained from Tonzi Ranch, located in the foothills of the Sierra Nevada Mountains, near Ione, California, (38.4311_N latitude, 120.966_W longitude, 177-m altitude) (Baldocchi et al. 2004). The vegetation type is an oak/grass savanna, with a tree canopy composed primarily of blue oak, *Quercus douglasii,* over an understory of non-native annual grasses that have been naturalized in this region for over a century (D'Antonio 2007; Heady 1977). This area possesses a Mediterranean type climate, with cool wet winters and hot dry summers. Annual rainfall is variable, with a 30 year average from the closest weather station of 562 mm, and an average annual temperature of 16.5 °C. The soil is of the Auburn soil series, very rocky silt loam, weathered from a bedrock of schist. Soil organic matter content varies from less than 1 % to 15 %. The oaks are winter deciduous and leaf out in late March each year. Grasses grow from seed when rain begins in autumn and senesce and die with the onset of summer drought (Ma et al. 2007). Tree roots grow rapidly with the initiation of warmer temperatures each spring. They cease growth when soil water potential falls to app. −0.3 MPa (Gershenson, A, pers. comm.). The water year that began in October of 2010 was one of high rainfall and with a growing season greater in length than is typical. We timed our sampling to coincide with expected peak annual root biomass in both trees and grasses.

Core sample processing: sieving

As with traditional methods, this method begins with separating roots from soil cores through sieving, (Fig. 1). We used sieves with two mesh sizes; a 1 mm mesh sieve on top and a 0.25 mm mesh beneath it. Because several studies indicate a clear tradeoff between sieve mesh size and root biomass recovery, we chose to pass all materials through the 0.25 mm mesh sieve (Amato and Pardo 1994; Livesley et al. 1999). We found that the 1 mm mesh caught larger and longer root pieces, and let most of the non-root organic debris pass through. The 0.25 mm mesh caught virtually all the root and other organic detritus and small rocks, but let the soil pass through. Our procedure was to wash the sample carefully until all the soil was removed, breaking up any larger aggregates by hand, without the use of chemicals to disperse soil aggregates. For these particular samples, the most practical solution was to process the material caught by each sieve separately, as most of the organic debris is caught by the finer mesh sieve and only a small amount of non-root debris is trapped on the coarser. We then rinsed the material from the 1 mm mesh into a rectangular white enamel pan, (33 × 20 cm). We chose this container because it was large enough for organic material to float freely without obscuring other roots and because the dark roots and debris were easily visible against the white of the pan. To separate out heavier mineral particles, which naturally settle, we poured the sample back and forth between the sieve and the container, and added more water as necessary.

We used forceps to pick all roots greater than 1 cm in length from the 1 mm mesh sieve, and place them into a labeled sampling tin. We also removed and discarded all sizable detritus from this fraction. Any remaining organic material from the 1 mm mesh sieve was then added to the finer mesh size sieve, and the process was repeated with this fraction. At the end of this step, virtually all rock particles were removed from the sample. The residual fraction of the sample contained only roots and debris 1 cm or less in length. In practice, we found the removal of larger and longer roots from the sample by hand took up to ½ hour, and frequently much less time.

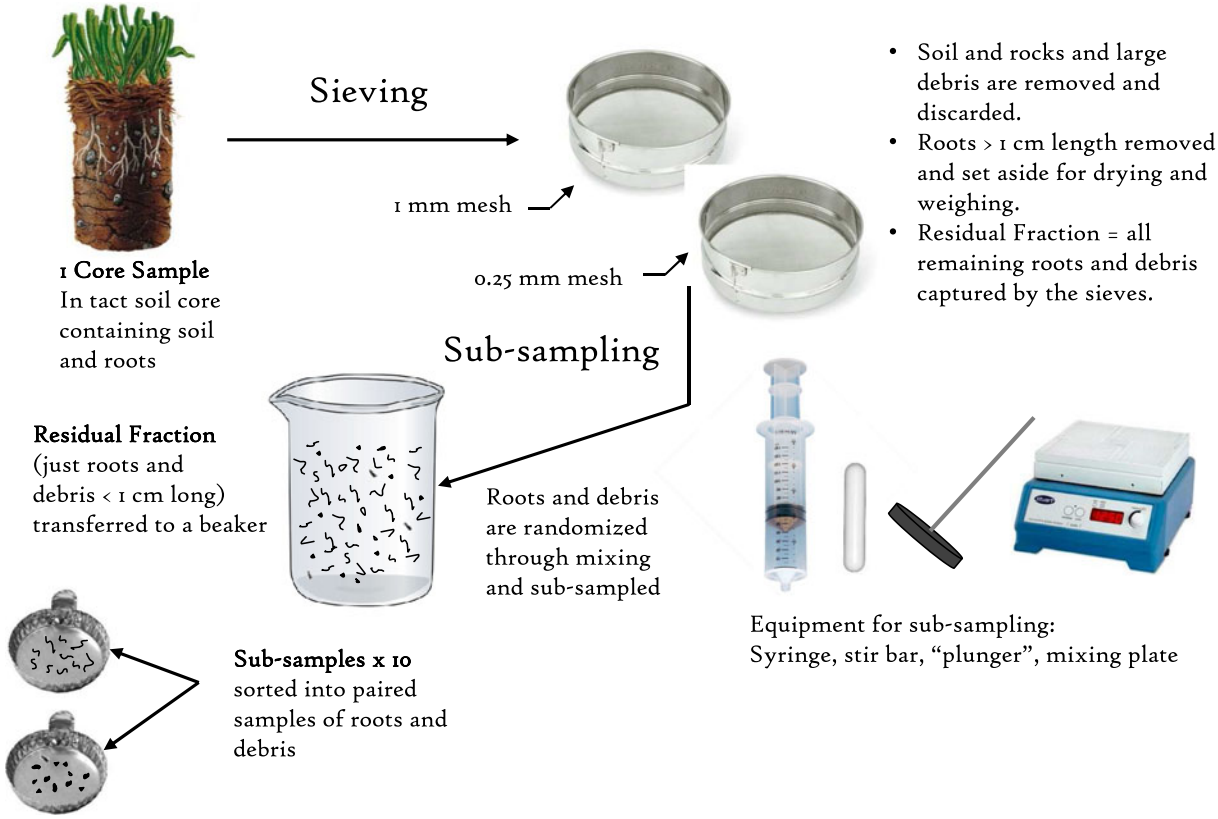

Fig. 1 Diagram of the procedure for processing roots through the randomization method. First, rocks and soil are removed through sieving. Roots greater than 1 cm in length are picked and set aside. Roots and debris that remain make up the "residual fraction" and are transferred to a beaker, randomized through mixing and plunging, and sub-sampled. Sub-samples are sorted into paired tins of root and debris, dried and weighed

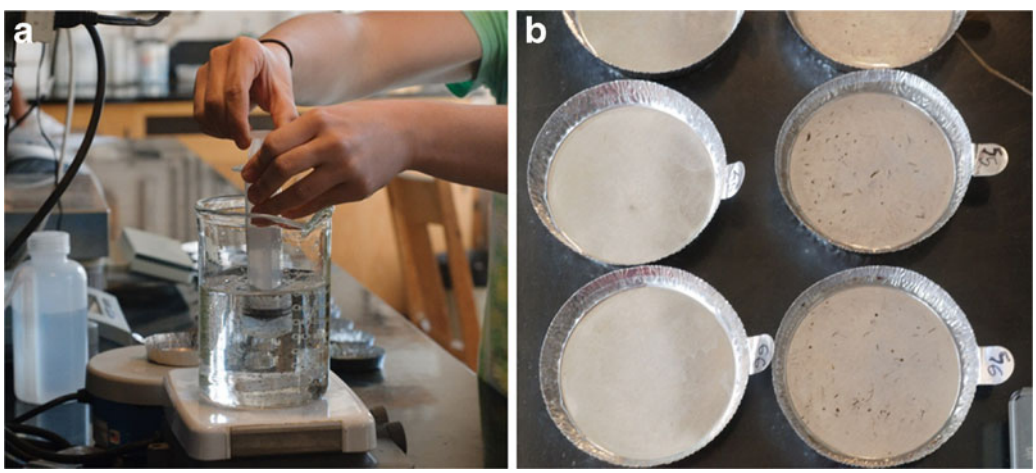

Fig. 2 Sub-sampling from beaker with syringe after randomization of residual sample (**a**), and paired sub-sample tins with roots and debris on the right to be separated (**b**)

Core sample processing: sub-sampling

We refer to the residual root and organic matter that remains after the longer root and debris are removed, as the "residual fraction", R_f, (Figs. 1 and 2). This residual fraction can represent a substantial percentage of the total root biomass per soil core, and has therefore presented the greatest challenge for researchers to process consistently and accurately. To remove every small piece of root can take from several hours to days per sample. The alternative; to stop picking roots from a sample based on a root length threshold, can vary arbitrarily from one worker to another, and from one sample to another, introducing a potentially large source of sampling error. Our solution to this sampling problem was to immerse the residual fraction of root and debris particles in water, to stir the mixture sufficiently to suspend the material randomly throughout the water column, and then to extract several sub samples from the mixture with a syringe. We then sorted each sub-sample into paired samples of root and non-root material using the naked eye, (Figs. 1 and 2). After drying at 65 °C for 48 hours, we weighed each sub-sample fraction and calculated the ratio of root to non-root organic material for each sample pair. From these sub-samples we derived a mean and standard error for the percent of the residual fraction that was roots. We then applied this percentage to the dried remains of the residual fraction that was not removed through sub-sampling, R_{-ss}, and then added back the sum of roots that were removed through sub-sampling to compute a total estimate for the short root portion of root biomass, f_{sr}, for the core sample:

$$f_{sr} = \mu_r * R_{-ss} + \sum_{i=1}^{n_{ss}} r_i, \quad \text{where} \quad \mu_r = \frac{1}{n_{ss}} \sum_{i=1}^{n_{ss}} \frac{r_i}{r_i + nr_i} \tag{1}$$

where n_{ss} is the number of sub-samples, r_i and nr_i are the dry weights of the root fraction and non-root fraction of sub-sample i respectively, and the second term on the right side of the equation 1 is the sum of the roots removed through sub-sampling. It was then possible to estimate the total root biomass of the core sample, R_t, as:

$$R_t = f_{lr} + f_{sr} \pm s_r \tag{2}$$

where f_{lr} represents the dry weight of the long roots picked manually from the sample before sub-sampling. And the standard error for the whole sample based on this estimation was the percent standard error of the sub-sample mean, μ_r, multiplied by R_{-ss}.

$$s_r = R_{-ss} * \sqrt{\frac{\left(\frac{1}{n_{ss}} \sum_{i=1}^{n_{ss}} \left(\mu_r - \frac{r_i}{r_i + nr_i}\right)^2\right)}{n_{ss}}} \tag{3}$$

For the actual mixing and sub-sampling, we placed the residual fraction in a water filled beaker on a mixing plate and added a magnetic stir bar. We mixed the water and organic material on the highest setting in order to bring all roots and debris into suspension, including those particles that floated on the surface or sank to the bottom of the beaker. Just before sampling, we slowed the mixing and submerged a plunger into the solution to disperse the particles that collected in the vortex during mixing to ensure the solution was fully randomized. The plunger was a flat circular disc with a diameter slightly smaller than the inside diameter of the beaker and attached to a rod, which we constructed in the lab. After plunging, we submerged a 40 ml syringe into the solution and extracted our sub-sample. Before use, we sawed off the end of the syringe, leaving a circular hole, 1 cm in diameter; this size was chosen to be compatible with the maximum length of roots in the residual fraction (<=1 cm). We recommend no larger than a 1 cm diameter hole because it was the largest size at which none of the solution dripped from the syringe when held vertically while we transferred the sub-sample from beaker to sampling tin. It was also large enough to draw in a representative amount of root and debris. We continued sub-sampling until finished, mixing and plunging between each sub-sample. Finally, this method assumes that the size of the sum of the sub-samples will be much smaller than the size of the total residual fraction, and if this assumption is not met, the traditional method of manual picking of roots should be used instead. A detailed video of the randomization

method for processing roots is available at this web location: https://www.youtube.com/watch?v=Xi1EW2LMkUM&feature=youtu.be

Core sample processing: test phase

This method introduces several variables that may vary across root type and core size, for which we recommend testing to achieve the optimal combination for a given set of root samples. These include 1. The size of the beaker, 2. the amount of water in the beaker, 3. the size of the sub-samples, and 4. the number of sub-samples. . That said, the sub-sampling parameters we used will likely be applicable to many sample sets. We tested three samples using two different combinations of beaker/sub-sample size variables, and found very small differences in sample estimates. Therefore, we do not believe that this method is sensitive to variable choices within reasonable bounds. However, in order to determine the most workable values for a specific sample set and to minimize the time spent processing samples, we recommend testing different variable quantities.

For guidance, the size of the cores we extracted were 5 cm in diameter and ranged in volume from app. 785 to 2400 cm^3, depending on the depth range of the soil core. We used two beaker sizes for large and small samples, 1000 ml and 2000 ml, and filled each three-quarters full. (Here, large and small refer to the size of the residual fraction, which is visually determined, not the size of the initial soil core.) We generally adhered to a standard sub-sample size of 20 ml, however, for very large or very small amounts of debris we varied by +/− 5 ml, opting to change beaker size instead. We defined our constraints as striving to minimize the time devoted to picking individual sub-samples on the one hand, and ensuring that the weight of any sub-sample root or non-root fraction not fall below the detection limit of the micro-balance in our lab, (Mettler Toledo Model AE240, readability: 0.1 mg) on the other.

In our test phase, we sub-sampled 10 soil cores, representing a range of core locations and depths. We then picked out, dried and weighed all of the root and debris in these samples, with the sub-sample roots added back in, to determine the actual ratio of root to debris and to determine the accuracy of our estimate.

Determination of sub-sample number

To determine the optimal number of sub-samples necessary for accurate estimation of each core sample, we performed a randomization analysis in Matlab to compare sub-sample sets of varying number, similar to the type of randomization analyses described in (Manly 2007), (Appendix 1). We began by sub-sampling five core samples with large residual fractions. We set the initial sub-sample number to twenty for these five core samples as an upper bound on the number of sub-samples required. There may still be a small amount of error associated with the mean of the 20 sub-samples, however, verification of the efficacy of the overall method supports the assumption that this error is very small, (Table 1). Twenty was also chosen because with a sub-sample size of 20 ml, and an initial beaker water volume of 750 ml, greater than half of the sample is mobilized for sub-sample processing. Therefore, we reasoned that in order for this method to deliver actual time savings, at least half of the time devoted to root/debris separation should be achieved. For each of these twenty sub-samples, we separated roots from non-root debris, and dried and weighed both fractions to determine the percent of roots and non-roots in each sub-sample. We used the list of the percent root values from each of the 20 sub-samples as our initial distribution, $f_{r1}\ldots\ldots f_{r20}$. Within Matlab, we then randomly sampled from this distribution100 times, without replacement, to produce sub-sample sets of length 19. We then tested for differences between the sample means of each of the simulated distributions with the mean of the initial distribution of 20 sub-samples using a two-tailed *t*-test, in turn setting α=0.05, 0.10, 0.15, 0.20, and 0.25. We then repeated this analysis with randomly-generated sub-sample sets of length 18, 17…. 5, assuming an absolute lower bound of five sub-samples. We decided the sub-sample number for which 95 % of the simulated sample means were not significantly different than that of the initial distribution at α=0.25 to be the appropriate number of sub-samples, (Fig. 3).

Outlier removal

Several factors can introduce variability into the sub-sampling process and yield individual sub-samples that inaccurately reflect the actual ratio of root to

Table 1 Statistics and Characteristics of Samples Processed using the Randomization Method during the Test Phase*

	Total Sample			Residual Fraction				
Sample Number	Total Sample (Estimate) (g/core)	Total Sample (Actual) (g/core)	Total Sample % Error	Root Mass (Estimate) (g/core)	Root Mass (Actual) (g/core)	% standard deviation of sub-samples	Mass of Residual Fraction (g/core)	% of total sample roots that is estimated
1	0.481	0.481	0.14	0.121	0.120	6.5	0.201	25.0
2	2.168	2.134	1.60	0.156	0.122	8.1	0.378	7.2
3	0.608	0.640	5.07	0.426	0.458	5.7	0.688	70.1
4	0.269	0.261	2.91	0.126	0.127	9.3	0.578	47.0
5	1.019	0.972	4.89	0.281	0.233	3.9	0.813	22.9
6	0.253	0.258	1.69	0.104	0.108	3.0	0.569	42.0
7	1.049	0.994	5.47	0.569	0.514	6.1	1.289	51.7
8	1.122	1.084	3.40	0.328	0.288	3.0	1.406	6.4
9	2.889	2.863	0.89	0.171	0.146	9.2	0.258	5.9
10	2.177	2.147	1.39	0.318	0.292	6.2	0.944	12.2
Mean			3.0 (1.8)			6.1 (2.3)		31.1 (6.4)

*Even numbered samples each had 10 sub-samples. By chance, odd numbered samples had one outlier removed from each, and therefore had 9 sub-samples. Numbers in parentheses represent one standard error.

non-root particles in the residual fraction. For example, incomplete mixing of the residual fraction, or settling of root and non-root particles after mixing can lead to sub-sample error and high levels of variance in ratio estimates. Weighing errors also sometimes occur, as root weights are small and expressed in milligrams. In recognition of these factors, we subject the data to an outlier analysis. The process consisted of comparing the standard deviation of the percent of roots (or non-roots) for all the sub-samples $r_1 \ldots r_i$, of an individual core sample, and then recalculating the standard deviation with n-1 values; omitting each sub-sample value in turn. If the standard deviation, σ_{ss}, dropped by 2 percent with the removal of any one value of r_j, then that value was eliminated. So, if

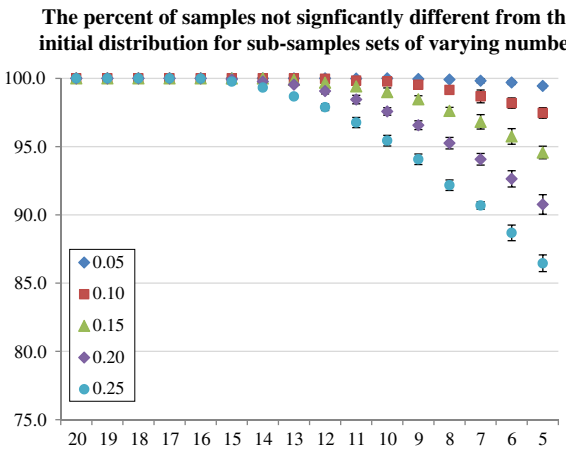

Fig. 3 Confidence intervals for the percent of randomly simulated samples for which the mean is not significantly different from the mean of the initial distribution (n=20) from a two-tailed *t*-test at the values of α given in the legend. Presented are the mean and standard error of five samples. All data were square root and arcsine transformed

$$\sigma_{ss} = \sqrt{\left(\frac{1}{n_{ss}}\sum_{i=1}^{n_{ss}}\left(\mu_r - \frac{r_i}{r_i + nr_i}\right)^2\right)}, \tag{4}$$

then we can compute a set of mean and standard deviation values for which

$$[\mu_{r_{-i}}] = \frac{\left(\left(\sum_{i=1}^{n_{ss}} \frac{r_i}{r_i+nr_i}\right) - \frac{r_j}{r_j+nr_j}\right)}{n_{ss}-1} \; for\ each\ j = 1\ to\ n_{ss},\ \text{and} \tag{5}$$

$$[\sigma_{ss_{-i}}] = \sqrt{\left(\frac{1}{n_{ss}-1}\left(\sum_{i=1}^{n_{ss}}\left(\mu_{r_{-i}} - \frac{r_i}{r_i+nr_i}\right)^2\right) - \left(\frac{r_j}{r_j+nr_j}\right)^2\right)}, \tag{6}$$

Then, for all $\sigma_{ss_{-i}}$, if $\sigma_{ss} - \sigma_{ss_{-i}} \geq 2$, then r_j and nr_j are treated as outliers. We used a simple Matlab script to perform this analysis, however, it can also be done quite easily in Excel.

Results

For the test phase of method development, we sub-sampled ten samples, and then picked them clean using forceps, carefully separating out the root and debris from the residual fraction to test the accuracy of our estimation. The estimates and actual root fractions appear in (Table 1). On average, the method predicted total core sample root biomass, including picked (f_{lr}) and sub-sampled roots (f_{sr}) with an error of 3.0 +/− 0.6 %s.e., and with a small tendency to overestimate root biomass.

We also measured the time savings achieved by employing the randomization method versus picking the sample clean for the ten core samples. On average, the picking phase of the sub-samples requires between one and two hours for a single worker. For the ten samples that we sub-sampled and picked clean by hand, it took 21 h +/− 5 h (s.d.) for complete root removal to be achieved.

Our outlier analysis revealed that for the 190 tree root samples that were processed through the randomization method, 51 % had no outliers, 42 % had one outlier removed, 6 % had two outliers removed and 1 % had three. An additional 40 % of samples, 133 of a total of 323, violated the assumptions of the method, which requires that the sum of the sub-samples be much smaller than the total size of the residual fraction. For these samples we picked out all the roots and discarded sample debris.

We looked at the variability in the percent root estimate for each of the ten sub-samples from each core sample as an indicator of the degree to which the residual fraction was adequately randomized before each sub-sample was extracted. We reasoned that if the percent root estimates were largely consistent among sub-samples that the sample was well-randomized, with each sub-sample reflecting near to the true ratio of roots to debris in the residual sample. These values and non-normalized standard error for the entire sample also appear in (Table 1).

Lastly, the results from our analysis to determine the number of sub-samples required to accurately predict the root biomass in each core sample appear in (Fig. 3). We found 10 sub-samples per core sample to be the smallest number of sub-samples that satisfied our test criteria.

Discussion

We found the randomization method to be highly accurate across the ten soil core samples we tested when attention to important root variables and equipment limitations were carefully considered. The randomization method is based on the physical dispersal of organic matter in water, and as a physical process, it should apply equally well to fine root samples of other species and various organic debris types (i.e. leaves, twigs, fungal hyphae).

In analyzing the sampling error we found that the method overestimated root mass eight out ten times; although for four of the eight samples in which overestimation occurred, the total sample error was less than 3 %. And this error was small considering uncertainties in distinguishing root from non-root particles in very small organic fragments. This tendency to overestimate root mass may result from losses in material associated with the order of the processing steps we followed. We first sub-sampled, picked, dried and weighed the samples. We then added the sub-sampled roots back to the total sample and picked these samples clean. Small amounts of material may have been lost in the sub-sampling step, leading to a smaller estimate from hand picking the entire sample. Based on the 190 core samples processed using the randomization method, our overall % standard deviation among sub-samples was 6.1 % (0.2 s.e.). However, the standard deviation among sub-samples fell over time as we refined our steps, and as lab workers grew comfortable with the procedure, ($\overline{\sigma_{ss}} = 7.2\%$ for the first third of core samples processed and $\overline{\sigma_{ss}} = 5.6\%$ for the remaining two-thirds). Overall, we found that 60 % of core samples met the criteria for sub-sampling. By

definition, the samples that did not meet these criteria were those that required less time for hand separation of root and debris.

With regard to time savings, we found sub-sampling soil cores saved approximately 20 h of work per sample. However, the samples we chose for method verification all had large residual fractions, and we picked these samples until every last root fragment was removed. Under traditional picking methods, one would cease picking when only very small fragments remained and the majority of the root biomass had been removed; a practice which would consume less time than the exercise undertaken in our lab for verification purposes. Yet, stopping short of picking the entire sample would also reduce the accuracy of the total sample estimate compared to the randomization method.

All in all, compared to direct root separation by hand, the randomization method provides an alternative with the potential to provide considerable time savings and improved accuracy, while reducing integrated labor costs. However, it requires careful effort to avoid procedural error. The attention invested in each step will determine the accuracy of root estimates. For example, because the entire sample is dried and weighed, including roots and debris, it is important to diligently separate mineral from organic particles when washing away soil. If not discarded, these particles will be incorporated into the residual fraction, inflating the value of $R_{\_ss}$ in equation (1). Another potential source of error exists in the sub-sampling step. Achieving a well-randomized mixture of roots and debris is important for accurately assessing the root to debris ratio. We found that the heavier particles of the residual fraction fell out quickly once vigorous mixing and plunging in the beaker ceased, and therefore it was important to sample quickly once the beaker movement slowed and the plunger was applied. Another possible solution is to increase the viscosity of the mixture by submersing the residual fraction in a liquid other than water, such as isopropyl alcohol (μ=2.4). Only substances that co-distill with water when evaporated in the oven should be attempted, however. We tested increasing solution viscosity by adding guar gum to the solution, a common food thickener, but found it created the additional problem of needing to subtract its weight from each sub-sample. We opted to use water for simplicity of disposal.

Sorting roots into live and dead components could also be facilitated by the randomization method, although it would require one additional step. Once the root and debris of each sub-sample are sorted, a second round of separation can be initiated in which the live and dead fractions of the root portion of the sub-samples are determined before the sample is put in the oven for drying. Ratios of live to dead material can be obtained and likewise applied to the entire residual fraction for an estimate of the total sample live to dead root ratio.

Perhaps the most significant benefit of this method is that it allows for the collection of more roots with a lower time investment and high accuracy. Both a greater number of core samples and larger diameter core samples can be collected. Moreover, larger diameter core samples can be processed with only a marginal increase in time investment over smaller diameter samples. As a result, the use of this method could change sampling protocols, causing a shift towards larger diameter cores. A shift in the scale of analysis could, in turn, yield a different set of ecological insights. Because roots are highly heterogeneous in soil, more and larger diameter cores may provide for reduced uncertainty in root biomass and productivity estimates.

Acknowledgements The authors would like to thank Naama Raz-Yaseef, Melanie Hahn and Ricardo Lopez for dedicated work in the field; Hanna Buechi and Joseph Verfaillie for work and creative input in the field and the lab, and Siyan Ma for work in the field and helpful comments on this manuscript. We thank Russell Tonzi for the use of his ranch as a study site. We also thank Elizabeth Bojorquez, Peggy Chuang, Christine Han, Janet Hsiao, Joseph Ko, Cindy Lam, MinhThao Luong, LanThao Nguyen, Nhat-Than Nguyen, Erika So and Phung Ton for their assistance in lab processing and method development. This work was funded by the US Department of Energy Grant DE-SC0005130 and by support from the Kearney Soil Science Foundation of the University of California.

Appendix 1

This is a matlab script for determining the correct sample size for root sub-sampling analysis using the randomization method.

```
%
data=xlsread('SSinput.xlsx',2);      %user must change input file name.
mn1=mean(data)*100; sd1=std(data)*100;
%
for k=1:16
        for i=1:100
                snums(1:21-k,i,k)=randsample(data,21-k,false);
        end
end
%
n(1:20,1:100,1:16)=0;
for m=1:16
        for j=1:100
                for i=1:20
                        if(snums(i,j,m)==0)
                        n(i,j,m)=1;
                        end
                end
        end
end
```

```
%
for m=1:16
        for j=1:100
                nct(j,m)= sum(n(:,j,m));
        end
end
%
snums_rt=asin(sqrt(snums));
%
for m=1:16
        for j=1:100
                stest_5(j,m)=ttest2(snums_rt(1:20-nct(j,m),j,m),asin(sqrt(data)),.05);
                stest_10(j,m)=ttest2(snums_rt(1:20-nct(j,m),j,m),asin(sqrt(data)),.1);
                stest_15(j,m)=ttest2(snums_rt(1:20-nct(j,m),j,m),asin(sqrt(data)),.15);
                stest_20(j,m)=ttest2(snums_rt(1:20-nct(j,m),j,m),asin(sqrt(data)),.2);
                stest_25(j,m)=ttest2(snums_rt(1:20-nct(j,m),j,m),asin(sqrt(data)),.25);
        end
end
%
for m=1:16
        snumsall_5(m,1)=sum(stest_5(:,m));
        snumsall_10(m,1)=sum(stest_10(:,m));
        snumsall_15(m,1)=sum(stest_15(:,m));
        snumsall_20(m,1)=sum(stest_20(:,m));
```

```
49          snumsall_25(m,1)=sum(stest_25(:,m));
50  end
51  snums_allalpha=[snumsall_5'; snumsall_10'; snumsall_15'; snumsall_20'; snumsall_25';]';
52
53
54
```

Line 6: Reading in the 20 data points. The data is in decimal percent form and represents the fraction of the sample that is roots for each of 20 sub-samples. (If an initial data size of greater than 20 samples is desired for testing, this program will need to be altered to reflect that.)

Line 7: Determine mean and standard deviation and multiply each value by 100 for ease of looking at the data.

Lines 9-13: In this loop, the program randomly samples the actual sub-sample values (with replacement) a decreasing number of times, and repeats this step 100 times for each sub-sample size,(i.e. it first randomly draws 20 sub-sample values from the sampled distribution, 100 times. Then it draw 19, then 18...5.

Lines 15-24: Here I create a 3D matrix of zeroes. This step is added because the variable **snums** has all zeroes in the locations where there are not values. (i.e. if the sample size is 12 values, the last 8 values are zero because the matrix size is 20 rows x 100 columns. In this loop, if a value in **snums** = 0, it is set = 1.

Lines 26-30: Here I create a new variable, **nct** that has 1 row and 100 columns and 16 sheets. **nct** = the sum of the rows for each of the columns in **n**. It is simply the number of zero values in each of the columns in **snums**. I use it in the next step to set the number of values that I average to get a mean value for each of the columns that represents a set of sub-samples.

Line 32: Here I arcsine transform the generated data.

Lines 34-42: Here I perform a ttest using each generated distribution with from 5 to 20 values to test for significant differences in sub-sample means against the actual sub-sample distribution of 20 values. I incresaingly adjust the significance value of the test easing the criteria for a significant difference finding. I also arcsine transform the original data for comparison.

Lines 44-50: Here I sum the number of significant differences found with each of the 100 generated sample comparisons for each set of sub-samples of varying length. Because I chose a sub-sample frequency of 100, the sum can also represent a percentage value without further normalizing. I sum the ttest results for each of the different alpha values.

Variables:

'SSinput.xlsx' is the file name where the 20 sub-sample values are stored for each sample processed in the lab.

snums is the dataset of generated distributions randomly assembled by repeatedly sampling the data 19 times...5 times.

snums_rt is the snums data arcsin transformed to satisfy criteria of normality for the ttest.

stest_5...stest_25 is the outcome (0 or 1) of repeated ttests of generated distributions against the original data.

snumsall_5...snumsall_25 represents the percent of times the null hypothesis is rejected for datasets of sample sizes of different lengths 19....5.

snums_allalpha is variable for printing the output in a format that can be easily copied and pasted into a spreadsheet.

References

Amato M, Pardo A (1994) Root length and biomass losses during sample preparation with different screen mesh sizes. Plant Soil 161:299–303

Baker IT, Prihodko L, Denning AS, Goulden M, Miller S, da Rocha HR (2008). Seasonal drought stress in the Amazon: Reconciling models and observations. J. Geophys. Res.-Biogeosci. 113.

Baldocchi DD, Xu LK, Kiang N (2004) How plant functional-type, weather, seasonal drought, and soil physical properties alter water and energy fluxes of an oak-grass savanna and an annual grassland. Agric For Meteorol 123:13–39

Benjamin JG, Nielsen DC (2004) A method to separate plant roots from soil and analyze root surface area. Plant Soil 267:225–234

Bohm W (1979) Methods of Studying Root Systems. Springer, Berlin, p 188

Canadell J, Jackson RB, Ehleringer JB, Mooney HA, Sala OE, Schulze ED (1996) Maximum rooting depth of vegetation types at the global scale. Oecologia 108:583–595

Chapin FS, McFarland J, McGuire AD, Euskirchen ES, Ruess RW, Kielland K (2009) The changing global carbon cycle: linking plant-soil carbon dynamics to global consequences. J Ecol 97:840–850

Chaves MM, Pereira JS, Maroco J, Rodrigues ML, Ricardo CPP, Osorio ML, Carvalho I, Faria T, Pinheiro C (2002) How plants cope with water stress in the field. Photosynthesis and growth. Ann Bot 89:907–916

Clark DA, Brown S, Kicklighter DW, Chambers JQ, Thomlinson JR, Ni J (2001) Measuring net primary production in forests: Concepts and field methods. Ecol Appl 11:356–370

D'Antonio CM (2007) Ecology of invasive non-native species in California grasslands. In: Stromberg M, Corbin JD, D'Antonio CM (eds) California Grasslands: Ecology and Management. UC Press, Berkeley, CA, pp 67–83

De Baets S, Poesen J, Reubens B, Wemans K, De Baerdemaeker J, Muys B (2008) Root tensile strength and root distribution of typical Mediterranean plant species and their contribution to soil shear strength. Plant Soil 305:207–226

do Rosario M, Oliveira G, van Noordwijk M, Gaze SR, Brouwer G, Bona S, Mosca G, Hairiah K (2000) Auger sampling, ingrowth cores and pinboard methods. In: Smit AGBAL, Engels C, van Noordwijk M, Pellerin S, van de Geijn SC (eds) Root Methods: A Handbook. Springer, Berlin, p 601

Gill RA, Jackson RB (2000) Global patterns of root turnover for terrestrial ecosystems. New Phytol 147:13–31

Heady HF (1977) Terrestrial vegetation of California. In: Barbour MG, Major J (eds) Valley grassland. Wiley and Sons, New York, pp 491–514

Hogberg P, Nordgren A, Buchmann N, Taylor AFS, Ekblad A, Hogberg MN, Nyberg G, Ottosson-Lofvenius M, Read DJ (2001) Large-scale forest girdling shows that current photosynthesis drives soil respiration. Nature 411:789–792

Holmes TH, Rice KJ (1996) Patterns of growth and soil-water utilization in some exotic annuals and native perennial bunchgrasses of California. Ann Bot 78:233–243

Huxman TE, Wilcox BP, Breshears DD, Scott RL, Snyder KA, Small EE, Hultine K, Pockman WT, Jackson RB (2005) Ecohydrological implications of woody plant encroachment. Ecology 86:308–319

Jackson RB, Canadell J, Ehleringer JR, Mooney HA, Sala OE, Schulze ED (1996) A global analysis of root distributions for terrestrial biomes. Oecologia 108:389–411

Jackson RB, Schenk HJ, Jobbagy EG, Canadell J, Colello GD, Dickinson RE, Field CB, Friedlingstein P, Heimann M, Hibbard K, Kicklighter DW, Kleidon A, Neilson RP, Parton WJ, Sala OE, Sykes MT (2000) Belowground consequences of vegetation change and their treatment in models. Ecol Appl 10:470–483

Jones DL, Hodge A, Kuzyakov Y (2004) Plant and mycorrhizal regulation of rhizodeposition. New Phytol 163:459–480

Koteen LE, Baldocchi DD, Harte J (2011) Invasion of non-native grasses causes a drop in soil carbon storage in California grasslands. Environ Res Lett 6:044001

Lewis DC, Burgy RH (1964) Relationship between oak tree roots and groundwater in fractured rock as determined by tritium tracing. J Geophys Res 69:2579

Livesley SJ, Stacey CL, Gregory PJ, Buresh RJ (1999) Sieve size effects on root length and biomass measurements of maize (Zea mays) and Grevillea robusta. Plant Soil 207:183–193

Lynch JM, Whipps JM (1990) Substrate flow in the rhizosphere. Plant Soil 129:1–10

Ma S, Baldocchi DD, Xu L, Hehn T (2007) Inter-annual variability in carbon dioxide exchange of an oak/grass savanna and open grassland in California. Agric For Meteorol 147:157–171

Majdi H, Pregitzer K, Moren AS, Nylund JE, Agren GI (2005) Measuring fine root turnover in forest ecosystems. Plant Soil 276:1–8

Manly BFJ (2007) Randomization, Bootstrap and Monte Carlo Methods in Biology. Chapman & Hall. Taylor & Francis Group, Boca Raton, FL, p 455

Metcalfe DB, Williams M, Aragao LEOC, da Costa ACL, de Almeida SS, Braga AP, Goncalves PHL, de Silva AJ Jr (2007) A method for extracting plant roots from soil which facilitates rapid sample processing without compromising measurement accuracy. New Phytol 174:697–703

Miller GR, Chen X, Rubin Y, Ma S, Baldocchi DD (2010). Groundwater uptake by woody vegetation in a semiarid oak savanna. Water Resour. Res. 46

Nepstad DC, Decarvalho CR, Davidson EA, Jipp PH, Lefebvre PA, Negreiros GH, Dasilva ED, Stone TA, Trumbore SE, Vieira S (1994) The role of deep roots in the hydrological and carbon cycles of amazonian forests and pastures. Nature 372:666–669

Nguyen C (2003) Rhizodeposition of organic C by plants: mechanisms and controls. Agronomie 23:375–396

Paterson E, Gebbing T, Abel C, Sim A, Telfer G (2007) Rhizodeposition shapes rhizosphere microbial community structure in organic soil. New Phytol 173:600–610

Pierret A, Moran CJ, Doussan C (2005) Conventional detection methodology is limiting our ability to understand the roles and functions of fine roots. New Phytol 166:967–980

Publicover DA, Vogt KA (1993) A comparison of methods for estimating forest fine root production with respect to sources of error. Can J For Res 23:1179–1186

Reubens B, Poesen J, Danjon F, Geudens G, Muys B (2007) The role of fine and coarse roots in shallow slope stability and soil erosion control with a focus on root system architecture: a review. Trees-Struct Funct 21: 385–402

Smucker AJM, McBurney SL, Srivastava AK (1982) Quantitative separation of roots from compacted soil profiles by the hydropneumatic elutriation system. Agron J 74:500–503

Tisdall JM, Oades JM (1982) Organic matter and water-stable aggregates in soils. J Soil Sci 33:141–163

Vogt KA, Vogt DJ, Asbjornsen H, Dahlgren RA (1995) Roots, nutrients and their relationship to spatial patterns. Plant Soil 169:113–123

Vogt KA, Vogt DJ, Bloomfield J (1998) Analysis of some direct and indirect methods for estimating root biomass and production of forests at an ecosystem level. Plant Soil 200:71–89