



Comparing two field protocols to measure individual shrubs' root density distribution

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Abstract

Purpose A large fraction of a plant's biomass is belowground, especially in shrublands that typically occur in episodically water-limited climates. Nonetheless, we have no standardized method to map the distribution of the root density (i.e., biomass per soil volumetric unit) of plant individuals (hereafter, Individual-level Root Density Distribution, IRDD). This type of information is difficult to collect, especially in woody plant communities in natural conditions

where roots of different individuals can be highly intermingled.

Methods We assess three methods to map IRDD of field shrubs: soil drilling to extract roots, plant injection with dyes, and microsatellite analysis for individual-level root identification. Using the resulting data, we fitted IRDD models obtaining comparable predictions of the root density of shrubs for each method.

Results The proportion of identified roots was higher using plan injection, but the cost per linked roots was two orders of magnitude higher using microsatellite. Model results show that microsatellite markers had a similar success as compared to plant injection for those plant individuals for which it worked well, but it failed completely for several genotypes or individuals.

Conclusions Core drilling machines and plant injection with dyes of different colors to link root fragments from the sample pool to plant individuals represent an affordable, reliable way to study the foraging behavior of woody plants which roots are highly intermingled.

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Introduction

Shrublands cover about 7–8% of Earth's land surface (Lal 2004; Maestre et al. 2021), and are expanding

because of anthropogenic desertification (Eldridge et al. 2011; Huang et al. 2020). Shrubs are an important functional type of vegetation amid largely undervalued by humankind (Tomaselli 1977; Kemper et al. 1999) and understudied by ecologists, as compared to forest and grassland systems (Wullschleger et al. 2014; Fusco et al. 2019; Schrader-Patton and Underwood 2021). Many shrubs grow in resource-poor soils, and allocate much of their biomass to their roots to acquire water and soluble nutrients (Schenk and Jackson 2002; Bardgett et al. 2014). As most plants, they absorb through their roots and compete for water, nitrogen, phosphorus, and many other soil resources that are subsequently transported to the leaves to photosynthesize (Lambers et al. 2008; Kirkham 2014). Compared to aboveground plant organs, we know very little about roots, since roots cannot be directly observed and are very difficult to measure (Jones et al. 2011; Lux and Rost 2012). Additionally, most of what we know about plant roots come from experiments conducted in controlled conditions, yet rooting behavior of plants may be very different in natural conditions (Poorter et al. 2016). Developing, testing, and standardizing methodologies to measure root traits in the field is a salient pending task for ecologists to address (Addo-Danso et al. 2016).

While many root variables can be measured (Freschet et al. 2021a, b), a map of the distribution of root density in soil (i.e., biomass per soil volumetric unit) of plant individuals (hereafter, Individual-level Root Density Distribution, IRDD) would provide the most comprehensive ecological information about plant foraging strategies (Cabal et al. 2021). IRDD maps allow researchers to study the root density distribution of neighboring plants in the soil and, by integrating such densities in three-dimensional space, the plant allocation of biomass to belowground tissues (Cabal et al. 2020). Estimating an IRDD would require samples of roots from known spatial coordinates and depths, and the assignment of roots in the sample to the surrounding individual plants.

While several techniques exist to study roots in the field, such as the use of stable isotopes (Stahl et al. 2013), anatomical or chemical phenotype markers (Roumet et al. 2006; Lei and Bauhus 2010), tomography (Zenone et al. 2008; Weigand and Kemna 2017), or rhizotron systems (Arnaud et al. 2019) to name a few, only a few may allow researchers to map IRDD of plants from root mixture samples (Cabal et al.

2021). Core sampling allows obtaining root density information, as researchers can weight the extracted roots and know the volume of the sample. As for the identification of the roots at the individual-level (not the species-level), a few studies have used microsatellite analyses to link root samples to individual plants (Brunner et al. 2001; Saari et al. 2005; Lang et al. 2010). Dying plant roots with different colors by injecting dye into their aboveground stems might be a cheaper and easier alternative to DNA analysis. This technique has been used successfully in several experiments in controlled conditions, in plants grown in pots in the greenhouse (Murakami et al. 2006; Cahill et al. 2010; Cabal et al. 2020) and in tomato plants grown in outdoor containers (Murakami et al. 2011), but has never been used in woody plants in the field.

In this study, we evaluate three field methods to map IRDD in three mediterranean shrub species growing in central Spain. Firstly, we adapted a diamond core-drilling engine designed for the construction industry as a method for root extraction. Secondly, we compared two different root identification methods to link root fragments from soil samples to aboveground plants: microsatellite analyses and plant injection with dyes. Based in model predictions, we compare the root identification methods with each other to determine which is the most advisable to map the IRDD of plants in the field.

Materials and methods

Study site description

This study was carried out in a mediterranean shrubland in ‘Las Tejoneras’ (40°06′42.69″ N, 5°16′32.46″ W, 329 m.a.s.l.), Candeleda (Ávila, Spain), a small isolated granitic mountain that rises about 50 m above the surrounding plains. Leptosols and superficial bedrock are the dominant soils. The area presents a meso-mediterranean, sub-humid ombroclimate (Rivas-Matínez and Armaiz 1984) with characteristic arid summers, an annual precipitation of 797.9 mm, and a mean temperature of 16.17 °C during the last decade (from January 2010 to December 2019 data from the AEMET meteorological station in Candeleda, 40°08′21″ N, 5°18′41″ W, 350 m.a.s.l.). The vegetation growing in this terrain is a biodiverse

mediterranean closed-canopy shrubland with over a dozen shrub species. Three dominant shrub species were selected: gum rockrose (*Cistus ladanifer* L.), rosemary (*Salvia rosmarinus* Schleid.), and hairy-fruited broom (*Cytisus striatus* [Hill] Rothm).

Field data collection

We selected seven 2×2 m plots based in the occurrence of individuals of the targeted species and in order to represent a range of plant sizes within each species from small to the largest in the region. We sampled one monospecific rosemary plot in the summer of 2018, accounting for all the 14 plants in and around the plot. Roots from this plot that could be linked to the species using DNA barcoding were identified using microsatellite markers (Segarra-Moragues and Gleiser 2009). We sampled six plots in the summer of 2019, with seven rosemary, eleven rockrose, and six broom plants of different sizes, whose roots were identified by root injection with dyes. In the latter six plots, a maximum of five focal individuals per plot were selected given that we used five different dye colors, but more individuals of the same and other species were found in and around the plot. We measured the total dry weight of photosynthetic and structural aboveground biomass of the selected focal plants from each plot.

Subsequently, we extracted soil cores of a maximum depth of 800 mm (minimum depth could be as low as 100 mm in the event of finding a large rock underground), and diameter of 104 mm, with a core drilling machine designed for construction, and adapted to the field (Fig. SM 1–3). We extracted a variable number of cores from each plot following a regular spatial pattern. We chose to sample following a regular pattern to represent all the parts of the plot and representing equally the different sides and distances from the focal plants. We obtained several soil samples from each core at different depths. We sifted soil samples using a 2 mm sieve, recovering mineral material (gravel and stones) whose volume was measured, and large root fragments whose diameter and dry weight was measured. We recovered the organic matter from the fraction of sample that passed through the sieve by flotation. Such organic matter was oven dried, we separated fine roots—all with mean diameter < 0.5 mm—from other materials, and weighed these roots in bulk.

We located the relative position of the insertion points of the stem of each focal plant to the soil

surface (plant insertion) and the centroid of each cylinder-shaped soil sample (sample centroid) in the plot in three-dimensional space. To that end, we used a combination of drone photography, laser level measurements of the slope in the plot, and information about the minimum and maximum depth of each soil sample (Fig. SM 4–5).

Individual-level identification of roots

In the first plot, we analyzed 14 leaf samples representing the plant individuals and 904 root fragments extracted from 42 soil samples from 23 soil cores, linking roots to aboveground tissues using DNA analysis. Given the large number of collected root fragments, and the high cost of DNA analyses, we only retained root fragments with a diameter > 1 mm for the DNA analysis (415 root fragments). We analyzed the selected roots individually, following a dual approach. First, root DNA was isolated, and the region of the chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene was amplified by polymerase chain reaction (PCR) and sequenced. We compared the resulting sequences with barcode records available in two public reference databases to verify that the samples belonged to rosemary plants. Then, the roots with detectable rosemary barcodes (217 root fragments), together with the 14 leaf samples, were genotyped using six different microsatellite loci in four multiplex reactions.

In the six remaining plots, we injected 24 plants with dyes of different colors before soil coring. We extracted 2,840 root fragments from 216 soil samples in 81 soil cores and linked them to the plants based on the dye colors (Fig. SM 6). Because woody plants have never before been injected with dyes in order to link root fragments to plants, we developed a hydraulic model to estimate the velocity of dye in the stem vessels ($\overline{v_{10}(f)}$). This velocity is a function of the mean plant vessel diameter, the root length that the researcher wants to stain, the soil water potential, and the osmotic potential of the dyes used (Fig. SM 7). We obtained measures of the vessel diameter of the three focal species of our study and measured the osmotic potential of the dyes used with a psychrometer. The model assessment suggested a staining approach exerting 1.5 bar over a period of 30 h in our particular system. Other researchers aiming to use the plant injection method can use our model to estimate the

pressure and time required in their own system. We identified root fragments in the lab using two methods (Fig. SM 8). First, we visually identified the color inside the roots under the bark. All roots for which color was not visible with the naked eye were introduced in a small plastic zip bag with water and kept at a constant temperature of 50 °C in a water bath. Bags were checked after five minutes, two hours, and one day, to see if any colorant dissolved in the water. When we detected a dye inside a root, visually or in dissolution in water, the root was linked to the plant stained with the same color in the plot.

Data processing

We calculated the Euclidian distance between every focal plant insertion and all soil sample centroids in the same plot, which allowed us to link data from the soil samples and plants. We sorted roots into diameter classes, differentiating fine (<2 mm) and coarse roots (≥ 2 mm), and, within fine roots, diameters of: S (1.0 to 1.99 mm), XS (0.50 to 0.99 mm), and XXS (<0.50 mm). For each soil sample, we calculated rooting volume (small mineral and organic particles and pores within) by subtracting the measured volume of gravel and stone from the total volume of the cylindrical sample, and root density as the root dry biomass (mg) per rooting volume (cm^3) of the soil sample. Root densities (RD) could thus be calculated for the various diameter classes and for each individual plant that was dyed or genetically analyzed, or for all roots collectively.

Data analysis

We fitted generalized linear mixed (GLMM) models predicting plants' individual root density in every point of three-dimensional soil; we called our models

individual root density distribution models for fine-roots (fIRDD) and coarse-roots (cIRDD). We fitted these models only for rosemary plants, as this was the only species for which we identified roots using both methods. Only the rosemary individuals for which at least one root fragment was linked to a reference individual were considered, removing all plants for which no positive result was obtained (we removed seven out of fourteen individuals, clustering six out of eight genotypes in the microsatellite plot and none in the plant injection plots). Additionally, in the fine roots model we only accounted for the diameter class S, as we discarded all roots thinner than 1 mm using microsatellites.

We designed the fIRDD model to account for the mechanisms of the plant foraging behavior of the exploitative segregation theory (Caballero et al. 2020). Hence, we based this model on the euclidian distance from the plant stem to any point in soil (E), the non-self root density in that point of soil (NSR), the interaction between both parameters, the depth in soil (D), the aboveground weight of the individual (AW), and the identification method used (m). The cIRDD model predicted the coarse root density based in the fine root density yielded by the fIRDD in the same sample (RD_f) and the identification method used (m) (Table 1).

Notes

For a more detailed description and information of all our field methods, data processing, and statistical analyses, electronic supplementary materials are available:

- (i) Text and SM figures in “**Detailed Materials and Methods**” is an extended and detailed

Table 1 Full description of the eight independent IRDD models fitted in this study

Model	Resp. var	Fitted data			Explicative variables (full model)	
		Meth	\emptyset class	Species		
[1]	fIRDD	RD_f	$M + PI$	S	Rosemary	$E + NSR + E:NSR + D + AW + m$
[2]	cIRDD	RD_c	$M + PI$	coarse	Rosemary	$RD_{S+m} + m$

Symbols: m method, M Microsatellite, PI Plant Injection, S $1.0 < \emptyset < 1.99$ mm roots, E Euclidian distance between plant stem and sample centroid, NSR Non-self root density in the same soil sample, D depth of soil sample centroid, AW aboveground weight of the plant, $E:NSR$ interaction between E and NSR

version of the Materials and Methods of this paper.

- (ii) The video “**A Method for Identifying Shrub individual Root Density Distribution**” describes the study site, and the method combining core extraction using a construction diamond drill and root staining by plant injection.
- (iii) The “**Data and Code**” folder includes all the collected data and several R scripts (R Core Team 2017) with all the code necessary to process the raw data, produce several graphical outputs, and perform statistical analyses.

The data collected as described in this paper covers much more ecological information (i.e., root density distribution in three-dimensional space, total root allocation, root ranges ... of the three species studied). We will analyze and discuss the database produced from this work and fully available as a supplementary material in future publications focusing on plant ecology, foraging strategies, and inter-plant competition. In this paper, we focus exclusively on results that pertain to methodology, logistics, or that allow us to compare both identification methods tested.

Results

Hydraulic model results

The mean and range of values for water potential of each dye color measured are as shown in Fig. 1a. With these values, we plotted $v_{10}(f)$ for the different stem vessel diameters and dye water potentials (Fig. 1b).

Method comparison

We linked proportionally more root fragments to a plant using dye injection (~25%) than microsatellite analysis (~16%). However, the DNA and dying methods yielded consistent RD's, because there was no significant effect of method on the estimated distributions for either fIRDD model (m , $t=0.770$, $p=0.441$) or cIRDD model (m , M $t=-0.144$, $p=0.889$; PI $t=-0.201$, $p=0.845$). This can be explained because, in the case of plant injection, most unidentified roots (75%) may correspond to individuals present in the plot and its surroundings that we did not stain.

Contrastingly, in the case of the microsatellite plot we accounted for all the plants, hence in this case the 84% of unidentified or unspecific root samples must represent methodological errors. Given the total cost of each method applied, in our case the relative costs of linking roots was two orders of magnitude larger using microsatellite analysis (~\$400 per linked root) than dye injection (~\$4 per linked root) (Fig. 2, central).

We assigned seven of the 14 plants we analyzed with the DNA method to two genotypes based in the analysis of leaves (Fig. 2, left panel). This illustrates that microsatellite markers cannot distinguish physically separate individuals of the same or similar genotype. These could be ramets, or alternatively separate individuals that the selected loci were not able to discern. Plant injection allows uncovering unambiguous connections between individuals that appear separate above ground but are connected belowground, because the dye is transported to all the living tissues of the physiological individual, including the leaves in the non-cut branches, where it can be seen with the naked eye.

Plant injection-based identification

The proportion of successful identifications of root fragments using dye injection varies between visual inspection and dissolution in root diameters and dye colors. Visual inspection was conducted first, and proved especially successful for thicker roots and for roots stained in cool colors (blue, green), which are difficult to confuse with natural wood colors. Of the remaining roots, we linked roots that were thinner and more often stained in warm colors (red, purple, yellow) using the water bath technique. Our results also show that the optimal time for dye dissolution is two hours, at which time we observe a peak in the fraction detected. Five minutes is not enough time for the dye to dissolve in the warm water, while 24 h proved to be too long (Fig. 2, right panel). After 24 h, most new identifications appeared to be yellow, and we suspect that roots may have stained the water in yellowish-brownish colors from the infusion of the natural plant materials after so much time soaking. This yellow color could have been misidentified as yellow or warm dyes, resulting in a potential source of error in positive results after 24 h of dissolution.

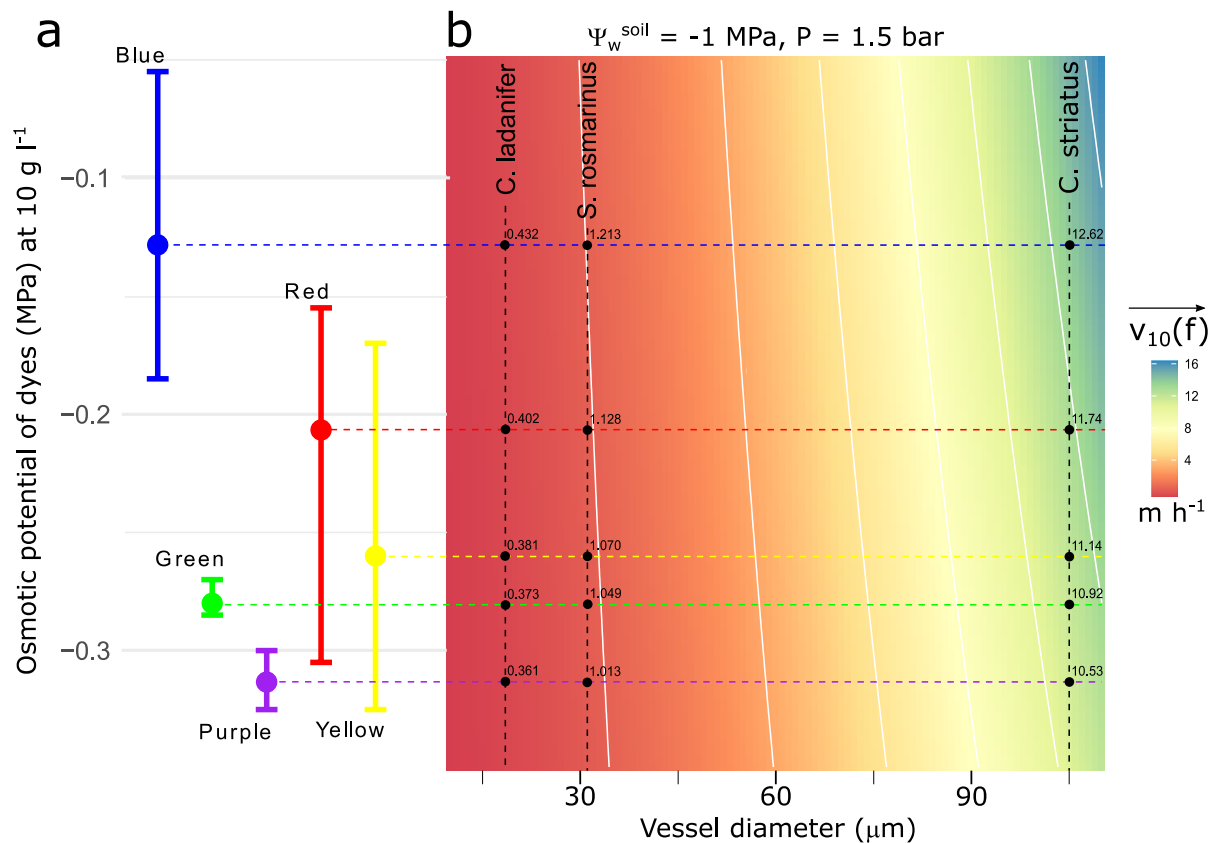


Fig. 1 Model results used to estimate pressure exertion time for plant injection at $P=1.5$ bar and $\Psi_w^{soil} = -1$ MPa. **a-** Means and range of values of the osmotic potential for the different

color dyes injected to plants. **b-** Contour plot for $\overline{v_{10}(f)}$, indicating the value for all the combinations of dye colors and focal species' vessel diameters

Discussion

Diverse coring techniques based on various mechanical systems already exist, but most ecological root studies still rely on hand-operated corers. Rotary drilling is a very effective coring technique, yet, like most mechanical coring systems, it involves equipment that is usually expensive, large and heavy (i.e. truck-mounted systems) (Abzalov 2016), which limits its use in many wild locations. However, more affordable and transportable diamond core drilling machines are widely available because of their use in construction industry to drill concrete. These machines can fit in most standard-sized cars, and researchers can transport them locally by hand. By adapting the use of a drilling machine of this type, we were able to extract large soil cores with woody roots from one of the hardest soils, a granitic leptosol. When encountering

a rock, the machine was able to cut through it and, after extracting a granite column, we were able to continue sampling the soil underneath the rock. Generally, we extracted the cores over a period ranging from five minutes when we encountered no rocks, to around 30 min for the cores containing large rocks.

The comparison between the two identification methods demonstrates that the use of plant injection with dyes is less restrictive, more cost-effective, and had a lower degree of uncertainty than micro-satellite markers for estimating IRDD. The GLMM results show that both methods' results supported one another: there was no significant effect of the method on estimated RDs in the models. Even so, this result does not deny that microsatellite was overall less effective, as only the plants with at least one root identified were included in the model. This result indicates that microsatellite analysis yielded

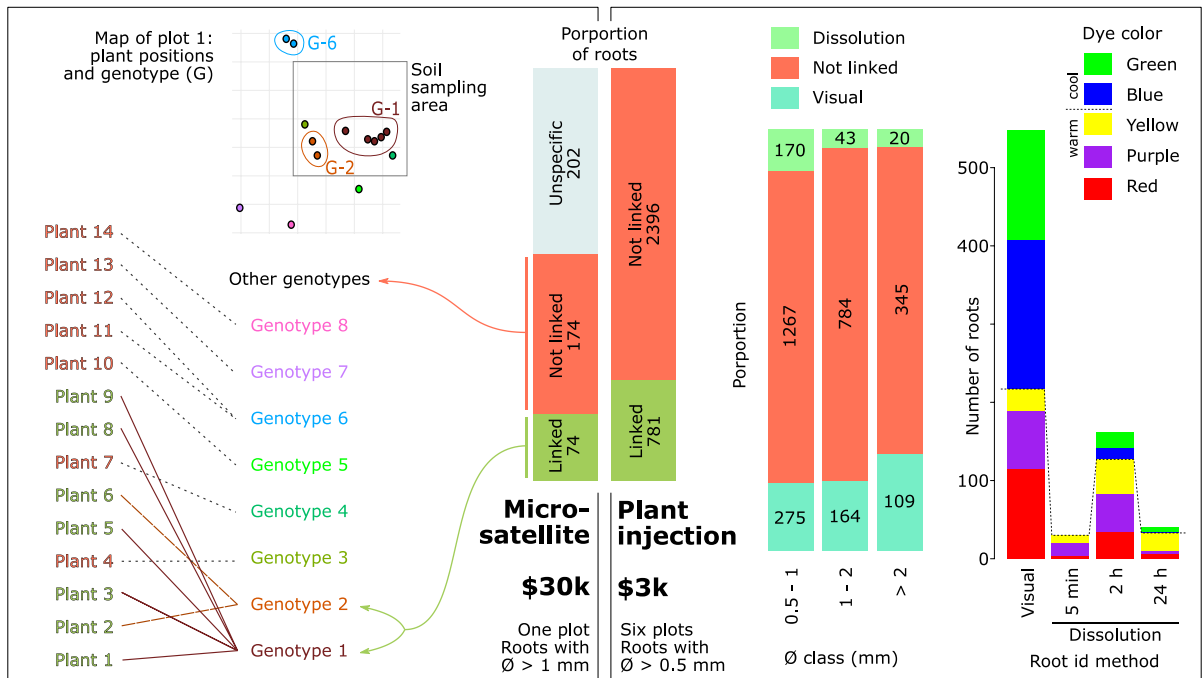


Fig. 2 Summary of results from linking root fragments to plant individuals using two identification methods: Microsatellite (left) and plant injection with dyes (right). Central bar plots represent the proportion of analyzed roots successfully linked or not linked to plants. “Unspecific” results account for roots for which PCR/barcoding results were negative, non-specific, or roots that were associated with a different species (not rosemary). The left panel shows how different roots and plants

analyzed with microsatellite analyses were linked to several genotypes (bottom), and how plants were spatially grouped by genotypes (top). The right panel shows the proportional performance of the visual and the dissolution identification methods per root diameter class, and the number of roots per color used successfully linked using the different identification techniques (visual and different dissolution times)

results similar to plant injection for the plant individuals for which it worked well, but it failed totally for many other individuals. Additionally, we found the microsatellite analysis restricted because of the limited availability of species-specific microsatellite markers (in our case, we could only use it for rosemary plants unless developing new markers) and its high financial costs. While both methods are subject to false negative errors (some of the roots that we could not link to any plant might actually belong to one of the focal plants), errors in positive results are more likely to occur using the microsatellite method because a root containing dye must belong to the corresponding injected plant. In addition, plant injection has other strengths such as allowing the measurement of sapwood area (Fig. SM 9), and the capacity to uncover unambiguous connections between individuals that appear separate above-ground. The dye diffuses to all the living tissues of

the physiological individual including the all the standing branches and leaves, where it can be seen with the naked eye.

The different methods used to identify roots based in plant injection showed a different performance, but complemented each other well. While we could identify most of the linked roots visually, the dissolution in water baths allowed us to link additional roots to individuals that could not be linked visually, especially useful for roots with thin diameters or stained in warm colors.

Conclusions

In this paper, we present and test an efficient and affordable protocol to map the IRDD of woody plants in the field. Tractable construction drills can be adapted to extract large soil samples of referenced

spatial position relative to nearby plants and known volume in dry, stony soils. We must then link the root fragments from each core to the plants. We can do this successfully and at a reasonable cost by injecting dyes of different colors to the plant individuals before coring the soil, and identifying the colors inside the extracted root fragments. One important avenue for future research would be to experiment with different kinds of dyes and other chemical tags. For example, a dye that fluoresces at a specific wavelength could allow researchers to detect the presence of dye by an electronic sensor at concentrations that would have been invisible to the naked eye.

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