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A rapid, quantitative, and affordable fluorometric method to determine the viability of roots with potential application in fungal hyphae

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ABSTRACT

Existing root viability methods tend to be time consuming, finicky, and sometimes subjective. Plate reader assays are rapid but often limited to homogeneous liquid samples. The aim of the study was to develop a rapid, reliable, and quantitative method to determine the viability of plant root samples using an area-scan plate reader. We modified existing fluorescein diacetate (FDA) methods, and optimized incubation time and root volume using pea roots (*Pisum sativum*) of known viability. We then compared our method to FDA microscopy and triphenyl tetrazolium chloride (TTC) reduction using pea roots and field-collected grass, forb, and tree roots. Finally, we used fungal hyphae to assess the suitability of our method to broader applications. Our method was as accurate and precise as FDA microscopy, more precise than TTC, and twice as fast as the other methods yet similar in cost. Field-collected root viability was similar between our method and FDA microscopy, but not TTC. We were able to detect viability of fungal hyphae with our method, although further development is needed.

1. Introduction

Plant root health and, by extension, associated mycorrhizal fungal health are important factors in determining the overall vigor of plants. Thus, characterizing the activity and function of roots and associated fungi is critical to gaining a better understanding of community interactions and ecosystem processes. Most studies of roots or fungi rely on mass or length as the sole response variable, and while these measurements can capture presence and abundance (Van Kleunen et al., 2010), they may not adequately describe activity and function. Viability measures, on the other hand, can discern rapid, subtle differences in overall performance of plants or fungi that may not be obvious from coarser measurement of mass or length. Here we describe a new method to measure viability of roots that is quantitative, rapid, and affordable.

Distinguishing between live and dead roots is not straightforward; some methods use color or age, but appearance alone may not be a reliable indicator of viability (Comas et al., 2000). However, differences in appearance can be enhanced with vital stains. Staining techniques have provided invaluable insight into cytotoxicity (Jones and Senft, 1984), plant physiology and development (Huang et al., 1986), seedling and seed quality (Noland and Mohammed, 1997; Regan and Moffatt, 1990), cold hardiness (Steponkus and Lanphear, 1967), survival following exposure to cold (Bigras, 1997), and plant tolerance to stressors (Ishikawa et al., 1995). Vital stains have also been used to check for root damage following exposure to environmental stressors such as heavy metals or low pH (Koyama et al., 2001) as well as root-cortical death rates, which are species-specific and influence potential infection by pathogens (Lascaris and Deacon, 1991).

Viability of plant roots is not the only component to plant success; many plants rely on associations with arbuscular mycorrhizal fungi (AMF) for maximum growth. These ubiquitous soil fungi colonize about 80% of vascular plants and help plants take up nutrients, protect them from pathogens, and possibly enhance drought tolerance (Smith and Read, 2008). Vital stains have also been used to measure the responses of AMF to increased soil moisture and temperature (Addy et al., 1997; Lekberg and Koide, 2008) and tillage (Kabir et al., 1997).

Fluorescein diacetate (FDA) is a vital stain commonly used in conjunction with fluorescent microscopy. This lipophilic compound is able to pass through the cell membrane. Once inside the cell, enzymes hydrolyze the acetates, liberating the polar and fluorescent molecule, fluorescein, which is retained by living cells. After staining, plant cells or tissues are mounted on slides and viewed with a fluorescent microscope. This method gives a direct measure of plant tissue viability but reading the slides is labor intensive, time consuming and requires a costly fluorescence microscope.

Another popular method to determine viability uses the enzymatic reduction of triphenyl tetrazolium chloride (TTC) by living cells. When exposed to live root tissue, the colorless TTC solution is reduced via

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respiration to red triphenyl formazan (TF), and the amount of TF determined by spectrophotometry is then used to estimate viability. TTC is a batch method in which many s amples can be processed quickly. However, variation among replicates (Verleysen et al., 2004; Ullrich et al., 1996), TF production due to tannins (Ruf and Brunner, 2003), and TF produced by non-viable roots (Comas et al., 2000) leads us to question the accuracy and precision of this method.

We developed a method to determine viability of plant roots that combined the ease, batch size, and speed of TTC with the reliability of FDA microscopy. In the past, FDA viability batch methods have been limited to homogeneous solutions such as soil extracts (Green et al., 2006) or cell suspensions (Noland and Mohammed, 1997), precluding measurements of heterogeneous samples, such as roots. The area scan capable plate reader circumvents this limitation by measuring fluorescence at multiple locations within each well and reporting the average. Our plate reader method also uses a standard curve to report viability in terms of μg fluorescein mg^{-1} plant material allowing us to compare viability across plant species, sample runs, and analytical laboratories. We tested and optimized the existing FDA batch method with roots collected from garden pea seedlings (*Pisum sativum*). We then compared the performance, including time and cost investments per sample, of our method with FDA microscopy and TTC on pea roots of known viability, and field-collected grass, forb, and small tree roots. Finally, we assessed if our method could be adapted for use with AMF hyphae.

2. Experimental

2.1. FDA plate reader method optimization

We adapted batch FDA staining techniques described by Green et al. (2006), and Noland and Mohammed (1997). Briefly, we prepared a 0.012 M FDA stock solution by dissolving 50 mg fluorescein diacetate ($C_{24}H_{16}O_7$, Sigma-Aldrich Chemical Co., Milwaukee, WI) in 10 mL of acetone. This reagent is stable for up to six months if stored in the dark at 4 °C (Jones and Senft, 1985). On the day of analysis, we prepared a 50 mg L^{-1} FDA reagent by diluting the FDA stock in phosphate buffer saline (PBS 1 ×, Fisher Scientific, Pittsburgh, PA; pH 7.4) solution, and stored this reagent at 4 °C until use. We next added 0.5 mL of PBS 1 × to each well in Falcon tissue culture plates (Corning, Inc. 353043) and 2 mL of 50 mg L^{-1} FDA, resulting in a working solution of 40 mg L^{-1} FDA.

We developed our method with common garden pea roots as they grow quickly, produce prolific uniform roots, and are common in laboratory studies. We grew peas in sterile sand and harvested them ten days after emergence. We selected healthy and viable roots with a diameter of < 2 mm, removed any lateral root branches and cut 2-cm-long uniform root segments for inclusion in our study. Roots were stored for up to two hours at room temperature in layers of paper towel wetted with tap water prior to viability analyses. To determine how the amount of root material affects total fluorescence within a heterogeneous sample well, we compared total fluorescence and variability for groups of wells (n = 4) containing 1, 2, 4, 8, 12, 24, 36, 48, or 64 (2-cm) root pieces (Photo 1). We expected a trade-off between too much root material, resulting in an underestimate of fluorescence due to overlap, and too little root material, also resulting in underestimate of fluorescence due to empty space. We expected an optimal amount of tissue to result in maximum fluorescence and minimum variation among replicated samples. Also, different amounts of root material within an optimal range would be indicated by a positive and linear relationship between total root length and fluorescence given that all roots were equally viable. We also assessed the effect of incubation time on fluorescence by exposing root pieces to FDA for either 30 min or 2 h. Fluorescence was detected using area scan setting (excitation 485/20, emission 515, 25 scans per well) and compared to fluorescein ($C_2H_{12}O_5$, Sigma-Aldrich Chemical Co., Milwaukee, WI) standards of known concentration using a Synergy 2 Microplate Reader (BioTek, USA). Following fluorometric analysis, we dried root segments at 70 °C for 48 h to determine total dry mass. We included roots boiled for five minutes as controls in all experiments.

2.2. Comparison of FDA plate reader, FDA microscopy, and TTC methods

Based on our findings during optimization, we used 10 (2-cm) pea root segments per sample (prepared as described above) to compare the three methods. We created root mixtures with equal amounts of roots and different levels of viability by combining live and boiled roots to form the following proportions; 0, 0.25, 0.50, 0.75, and 1 live (n = 6). This allowed us to assess the linear relationship between percent live root and viability measured by each method (described briefly below) in terms of both accuracy and precision.

2.3. FDA microscopy

We followed the method described by Noland and Mohammed (1997). Briefly, root segments were incubated in 40 mg L^{-1} FDA solution for 30 min then mounted on slides. Fluorescence was measured using the line-intercept method (McGonigle et al., 1990) with a fluorescent equipped microscope (ex: 494 em: 518 LP 515 at 20 ×) and a minimum of 50 intercepts per sample. Slides were kept in the freezer until they were read and all slides were read within 2 h of being mounted.

2.4. Triphenyl tetrazolium chloride method

We used a modified TTC procedure described by Steponkus and Lanphear (1967) and Comas et al. (2000). Briefly, we recorded the fresh weight of ten 2-cm root pieces, and added them to 2 mL micro centrifuge tubes filled to the top with 0.6% (w/v) TTC in 0.05 M $Na_2HPO_4KH_2PO_4$ buffer (pH 7.4) + 0.05% (v/v) Ortho X-77 wetting agent. Samples were incubated in the dark at 30 °C for 15 h, and then removed from TTC solution and rinsed with DI H_2O. Next, we incubated samples in a water bath at 85 °C for 5 min and then added 7 mL of 95% ethanol to each sample to extract the formazan. Total formazan concentration was determined by absorbance (490 nm) using a Synergy 2 Microplate Reader (BioTek, USA). We included root samples that had not been exposed to TTC to correct for plant pigment interference, as well as boiled root samples to correct for any background coloration of root or ethanol that may elevate absorbance readings.

2.5. Comparisons of viability in field-collected roots

To assess the performance of this method under more realistic conditions and across more species, we also collected roots from two grassland plant species in the Bitterroot valley in western Montana, USA (46°40′48″N, 114° 1′40″W, 1024 m). We harvested five individuals each of spotted knapweed (*Centaurea stoebe*; forb) and Idaho fescue (*Festuca idahoensis*; grass). Given that many root viability studies have involved trees (e.g. Comas and Eissenstat, 2000), we also included apple (*Malus domestica*; tree) roots collected from five trees. We selected fine roots (< 2 mm diameter) from mature plants, rinsed soil from roots with tap water, and kept roots at 4 °C and moist until analysis. Within 24 h of collection we cut the roots into 2 cm segments and measured their viability. Both pea and field collected roots were recovered and dried following the measurements so that viability in the FDA plate reader and TTC method could be expressed per gram dry weight (DW). The viability in the FDA microscopy method was expressed as percent viable roots based on the gridline intersect method. The roots of apples were too thick to mount on microscope slides and were therefore omitted.

2.6. FDA plate reader method application; measuring viability of AMF hyphae

We wanted to determine if our FDA plate reader method could also

Photo 1. We found 8–12, 2 cm root segments (wells: B3, B4) to be a good compromise between well coverage and overlap for pea roots.

be used to measure the viability of AMF hyphae, which is typically measured with FDA microscopy (e.g. Addy et al., 1997). We took advantage of an ongoing *in-vitro* experiment where the AMF isolate *Rhizophagus irregulare* (DAOM 197198) colonized Ri-T-DNA transformed carrot roots (*Daucus carota* L., line DC1) in a split-root system (Bécard and Fortin, 1988). We harvested hyphae that had crossed the barrier and where roots were absent for viability analysis. We collected 42 samples (3 sub-samples per petri dish) of hyphae with extensive development in the hyphal compartments from 14, 3-month-old cultures, grown at 24 °C on Petri dishes with minimal (M) nutrient medium containing 0.35% (w/v) phytagel and 10 g l^{-1} of sucrose, and with a pH of 5.5 (Bécard and Fortin, 1988). We severed hyphae from roots, and then solubilized the gel containing hyphae in 200 mL of sodium citrate buffer (10 mM, pH 6.0). Hyphae were collected on a 34 uM sieve, washed repeatedly in distilled H2O, and blotted dry with paper towels.

For each sample, we placed approximately 25 mg (fresh) of hyphae into a single well of a black 96-well polypropylene assay plate (Corning, Inc. 353043). The black plates are more sensitive to limited fluorescence of smaller sample volumes. Next, we added 40 µL of 1 × PBS and 200 µL 50 mg L^{-1} FDA, and incubated samples at room temperature for 30 min followed by 10 min at − 20 °C. Fluorescence was detected using area scan (excitation 485/20, emission 515, 25 scans per well) and compared to fluorescein ($C_2H_{12}O_5$, Sigma-Aldrich Chemical Co., Milwaukee, WI) standards of known concentration using a Synergy 2 Microplate Reader (BioTek, USA). Following fluorometric analysis hyphae were dried at 70 °C for 48 h to determine total dry mass of each sample.

2.7. Data analyses

2.7.1. FDA plate reader method optimization for pea roots

Effect of incubation time, number of root pieces, and interaction between the two on fluorescence was analyzed using a two-way ANOVA. Given that we saw differences in response for different amounts of roots, we quantified the strength of the relationship between dry root mass and fluorescence for each root category (1, 2, 4, 8, 12, 24, 36, 48, or 64 segments) with a Pearson correlation in the stats package in R (R Core Team, 2016; version 1.40, 2011) to determine optimal incubation time and optimal number of roots. We assessed linearity of response by sight for each root category.

2.7.2. Comparison of FDA plate reader method, TTC and FDA microscopy

We compared how accurately each method measured viabilities of the four mixtures of pea roots ranging from proportion 0–1 live. For each method, we used a simple linear regression model in the stats package in R (version 1.40, 2011), where the slope indicated the accuracy and R^2 indicated the precision. We assessed the strength of the relationship between live:total count (FDA microscopy) and fluorescence (FDA plate reader) with a Pearson correlation in the stats package in R (version 1.40, 2011). We used the correlation to determine if FDA microscopy and the plate reader method could be used to develop a species-specific factor to convert relative to absolute viability.

To determine if viability differed among plant species within each of the three methods, we ran separate, one-way ANOVAs. Mean separations were done using Tukey's HSD ($\alpha = 0.05$) following significant ANOVAs in the multcomp package (Hothorn et al., 2008) in R.

2.7.3. FDA plate reader method application; measuring viability of AMF hyphae

The effect of boiling on fluorescence was analyzed using a one-way ANOVA. We used a natural log transformation to satisfy assumptions of normality and homoscedacity. The relationship between AMF hyphae dry mass and fluorescence for live hyphae was quantified with a Pearson's correlation in the stats package in R (version 1.40, 2011).

For all analyses, except where noted, we plotted residuals and no transformations were necessary to satisfy assumptions of normality and homoscedacity.

3. Results

3.1. FDA plate reader method optimization

We did not detect a significant difference in the amount of fluorescence per mg dry root among wells with different numbers of root segments ($F = 2.28$, $df = 8$, $P = 0.07$). However, wells with 8–12 (2 cm; 2.9–5.0 mg dry mass) root segments had less variation among replicate samples, and there was a strong correlation between dry root mass and fluorescence for both the 30-min (Fig. 1; Pearson Correlation t

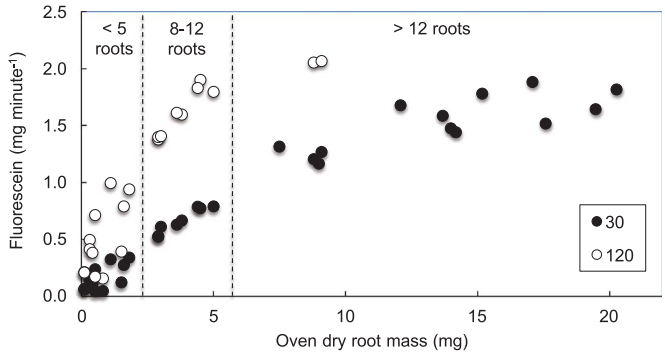

Fig. 1. Total mg fluorescein (FDA plate reader) per mg dry root in a 30-min (black circle; $P < 0.001$, $R = 0.96$) and 120-min (open circle; $P < 0.001$, $R = 0.94$) incubation. Each point represents a single sample well containing 1, 2, 4, 8, 12, 24, 36, 48, or 64 (2-cm) root pieces.

= 8.44, df = 6, $P < 0.001$, $R = 0.96$) and the 2-h (Fig. 1; Pearson Correlation t = 7.04, df = 6, $P < 0.001$, $R = 0.94$) incubations. We found higher variability among replicates of wells with 1, 2, or 4 root segments (0.1–1.5 mg dry mass), resulting in a weaker correlation between dry root mass and fluorescence for the 30-min incubation (Fig. 1; Pearson Correlation t = 2.83, df = 10, $P = 0.02$, $R = 0.66$). Wells with greater than twelve root pieces (7.5–20.3 mg dry root mass) and 30-min incubation displayed a non-linear relationship between root mass and fluorescein released, suggesting root overlap or fluorescent saturation (Fig. 1). This was more pronounced in the 2-h incubation as longer time resulted in more fluorescein generated and retained per mg of root. Thus, we conclude that 8–12 root segments (approximately 2.9–5.0 mg dry mass) incubated with 40 mg L^{-1} FDA solution for 30 min in falcon 12-well (6 mL total well volume) was optimal for pea roots. However, this is most likely species-specific and should be optimized for each species prior to routine measurements using the same methods as outlined here.

3.2. Comparison of FDA plate reader method, TTC and FDA microscopy

There was a significant, positive relationship between percent live pea roots and measured viability for all methods. This relationship was strong for both our FDA plate reader method (Fig. 2a; F = 632, df = 1 and 28, $P < 0.001$, $R^2 = 0.96$), and the FDA microscopy method (Fig. 2b; F = 479, df = 1 and 27, $P < 0.001$, $R^2 = 0.94$), but considerably weaker for the TTC method (Fig. 2c; F = 16, df = 1 and 28, $P < 0.001$, $R^2 = 0.35$).

We found a strong correlation between live:total counts (FDA microscopy) and fluorescence (FDA plate reader) (Fig. 3; Pearson Correlation t = 18, df = 27, $P < 0.001$, $R = 0.96$).

We found significant differences in viability among field-collected spotted knapweed, Idaho fescue, and apple roots for the FDA plate reader (Fig. 4a; F = 12.68, df = 2, $P < 0.001$), FDA microscopy (Fig. 4b; F = 6.85, df = 1, $P = 0.03$), and TTC methods (Fig. 4c; F = 17.09, df = 2, $P < 0.001$), but the ranking differed among methods. Both the FDA plate reader and microscopy methods measured highest viability in Idaho fescue, and intermediate viability in spotted knapweed roots, whereas the TTC method measured highest viability in spotted knapweed and intermediate viability in Idaho fescue. The lowest viability was found in apple roots with both the FDA plate reader and the TTC methods (excluded from the FDA microscopy analysis as roots were too thick).

Our FDA plate reader was the fastest of the three methods (5 min per sample), FDA microscopy was the second fastest (10 min per sample), and TTC was the slowest (12 min per sample; Table 1). Our TTC time per sample estimate was conservative, as we did not include the overnight incubation in our calculations. The FDA microscopy method could vary slightly in time depending on the speed of the person scoring slides. All methods were similar in cost (Table 1).

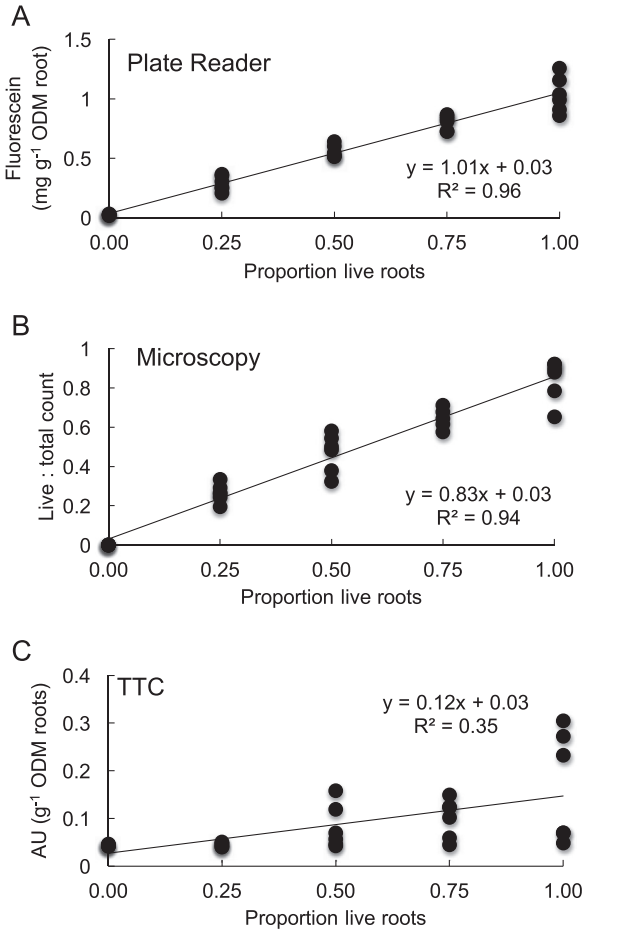

Fig. 2. Regressions of experimentally assembled mixtures of live and dead pea roots with (A) fluorescence (FDA plate reader; $P < 0.001$, $R^2 = 0.96$), (B) live:total counts (FDA microscopy; $P < 0.001$, $R^2 = 0.94$), and (C) absorbance (TTC; $P < 0.001$, $R^2 = 0.35$).

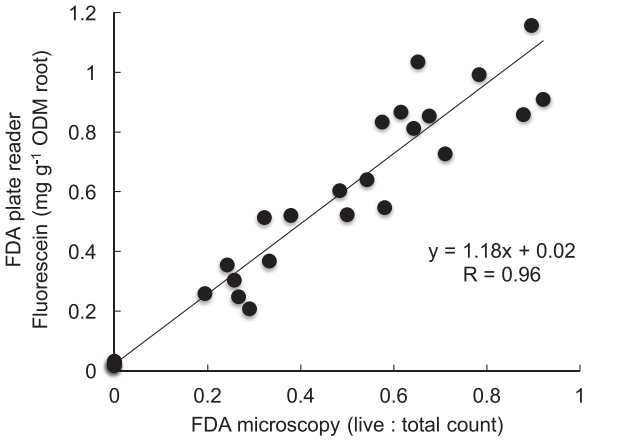

Fig. 3. Correlation between fluorescence (FDA plate reader) and live:total count (FDA microscopy) ($P < 0.001$, $R = 0.96$).

3.3. FDA plate reader method application; measuring viability of AMF hyphae

Our FDA plate reader method detected viability of AMF hyphae. Viability was decreased by boiling (F_{BOILED} = 5.69, df = 1,36, P = 0.02), and we saw a significant and positive correlation between AMF hyphae dry mass and fluorescence (Fig. 5; Pearson Correlation t =

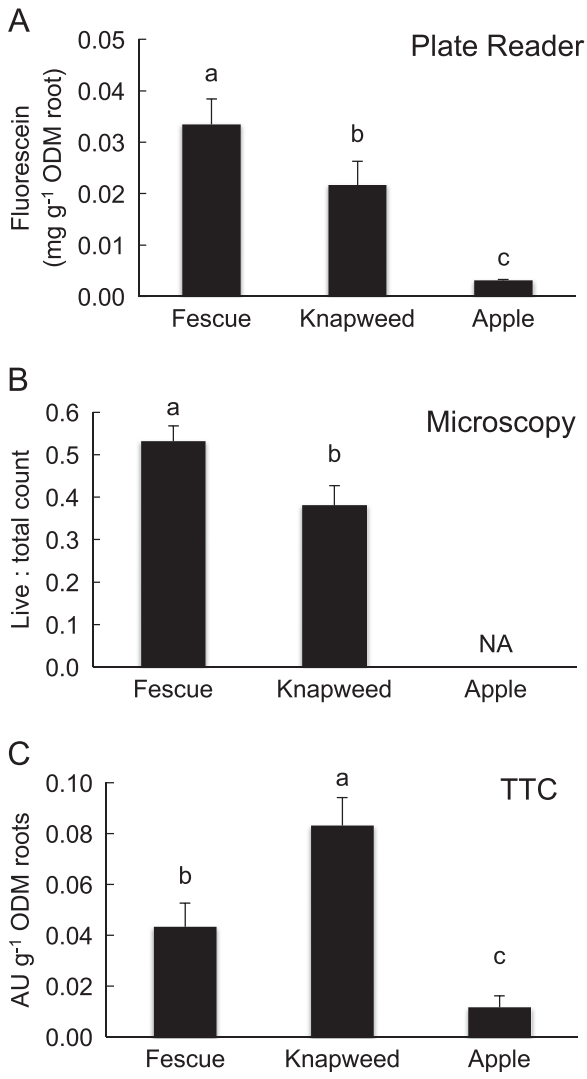

Fig. 4. Mean (± se, n = 5) viability of *Fescue idahoensis*, *Centaurea stoebe*, and *Malus sp.* roots as measured by the (A) FDA plate reader, and (B) FDA microscopy, and (C) TTC methods. Different letters indicate significant differences among species within a viability measurement method as measured by Tukey's *post hoc* analyses (a = 0.05).

Table 1
A comparison of time and cost per sample of the FDA, TTC, and direct count microscopy methods.

Pea roots - time comparison			
	TTC	**FDA**	**Microscopy**
Root Preparation	60	60	60
Additional Preparation	300	60	64
Incubation	[a]1080	30	30
Reading	2	12	144
Total Time (30 samples)	362	162	298
Time per Sample (min)	**12**	**5**	**10**
Cost per Sample (USD)	$0.22	$0.30	$0.35

[a] Not included in time/sample calculation.

3.17, df = 33, P = 0.003, R = 0.48). Our R was low, most likely due to natural variation in viability among hyphal filaments of different ages.

4. Discussion

We tested if an area-scan capable plate reader could be used to measure root viability, and we compared the accuracy, precision, time and cost of our FDA plate reader method with two other commonly used methods; FDA microscopy and TTC. Our method performed just as well as existing methods in terms of accuracy. When viability doubled from 25% to 50% and 50% to 100%, the response of our FDA plate reader method increased 1.9 times, FDA microscopy increased 1.8 times, and TTC increased 1.9 times (Fig. 2). In terms of precision, our method and the FDA microscopy outperformed TTC as replicate samples clustered closely together and R^2 values were high (Fig. 2a and b). The precision of the TTC method tended to be more problematic, however, and in accordance with previous work (Verleysen et al., 2004), we found greater variation among sample replicates (Fig. 2c), especially in root mixtures with proportions of 0.75 or 1 live roots.

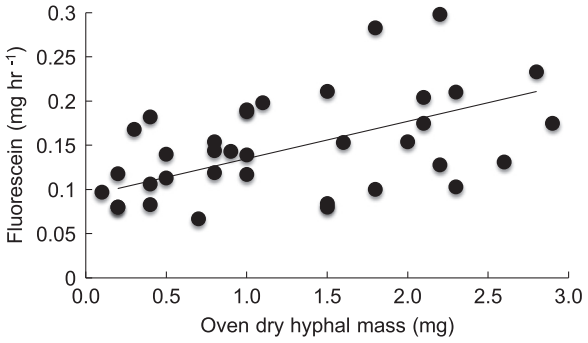

Fig. 5. Correlation of mass of arbuscular mycorrhizal fungi (AMF) hyphae and fluorescence (FDA plate reader) (P = 0.003, R = 0.48). Each point represents a single sample well containing approximately 25 mg of fresh hyphae.

When applied to different plant species, our method agreed with FDA microscopy and reported the highest viability in Idaho fescue roots followed by spotted knapweed. The TTC method, on the other hand, reported the highest viability in spotted knapweed, which may have been due to tannins (Ruf and Brunner, 2003) or similar compounds in spotted knapweed that may have artificially increased absorbance values. This is worrisome because it suggests that results from viability measurements and even rankings of species may depend on the methods used.

Our method is simple and does not require any special equipment except an area-scan plate reader, which are becoming more common and affordable. Relative viability values are based on standard curves and as long as incubation times and FDA concentrations are consistent, easy and direct comparisons of viability among treatments, species, and analytical labs are all feasible. Our method eliminates variation in technician skill, fatigue, and subjectivity, which are inherent drawbacks of direct count microscopy. It also saves time, and we were able to read 30 samples 12 min compared to 5 min per sample with direct count microscopy, and contrary to TTC, no overnight incubation is needed. Our viability measures of AMF hyphae also show the potential broader applicability of our method on other tissue types. The relationship between hyphal biomass and viability shown here was quite noisy (Fig. 5), most likely due to the inclusion a range of hyphal age and resulting viability in our study. We urge additional optimization for hyphae prior to routine analyses by labs.

The method is limited compared to the FDA microscopy method, but not TTC, in that it cannot be used to measure absolute viability of field-collected roots. In applications where absolute viability values are desired, we suggest determining viability of a subset of samples with both our FDA plate reader method and FDA microscopy to obtain a conversion factor for the species of interest. For large sample runs, this could save time, without sacrificing an absolute value of viability. If no fluorescent microscope is available, this limitation could be surmounted by growing fresh roots of the species of interest to generate a standard curve of known viability by combining live and boiled roots of known proportions described above with pea roots.

In summary, we developed a precise, accurate and objective way to

measure root viability that offers significant savings in time, at a comparable cost to existing methods. In addition, our work with AMF hyphae suggests that our method has potential to be used with a variety of heterogeneous plant and fungal tissues.

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References

Addy, H.D., Miller, M.H., Peterson, R.L., 1997. Infectivity of the propagules associated with extraradical mycelia of two AM fungi following winter freezing. New Phytol. 135, 745–753.
Bécard, G., Fortin, J.A., 1988. Early events of vesicular–arbuscular mycorrhiza formation on Ri T-DNA transformed roots. New Phytol. 108, 211–218.
Bigras, F.J., 1997. Root cold tolerance of black spruce seedlings: viability tests in relation to survival and regrowth. Tree Physiol. 17 (5), 311–318.
Comas, L.H., Eissenstat, D.M., Lakso, A.N., 2000. Assessing root death and root system dynamics in a study of grape canopy pruning. New Phytol. 147, 171–178 (2000).
Green, V.S., Stott, D.E., Diack, M., 2006. Assay for fluorescein diacetate hydrolytic activity: optimization for soil samples. Soil Biol. Biochem. 38, 693–701.
Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous inference in general parametric models. Biom. J. 50, 346–363.
Huang, C.N., Cornejo, M.J., Bush, D.S., Jones, R.L., 1986. Estimating viability of plant protoplasts using double and single staining. Protoplasma 135, 80–87.
Ishikawa, M., Robertson, A.J., Gusta, L.V., 1995. Comparison of viability tests for assessing cross-adaptation to freezing, heat and salt stresses induced by abscisic acid in bromegrass (Bromus inermis Leyss) suspension cultured cells. Plant Sci. 107, 83–93.
Jones, K.H., Senft, J.A., 1985. An improved method to determine cell viability by simultaneous staining with fluorescein diacetate-propidium iodide. J. Histochem. Cytochem. 33, 77–79.
Kabir, Z., O'halloran, I.P., Fyles, J.W., Hamel, C., 1997. Seasonal changes of arbuscular mycorrhizal fungi as affected by tillage practices and fertilization: hyphal density and mycorrhizal root colonization. Plant Soil 192, 285–293.
Koyama, H., Toda, T., Hara, T., 2001. Brief exposure to low-pH stress causes irreversible damage to the growing root in Arabidopsis thaliana: pectin–ca interaction may play an important role in proton rhizotoxicity. J. Exp. Bot. 52, 361–368.
Lascaris, D., Deacon, J.W., 1991. Relationship between root cortical senescence and growth of wheat as influenced by mineral nutrition, Idriella bolleyi (Sprague) von Arx and pruning of leaves. New Phytol. 118 (3), 391–396.
Lekberg, Y., Koide, R.T., 2008. Effect of soil moisture and temperature during fallow on survival of contrasting isolates of arbuscular mycorrhizal fungi. Botany 86, 1117–1124.
McGonigle, T.P., Miller, M.H., Evans, D.G., Fairchild, G.L., Swan, J.A., 1990. A new method which gives an objective measure of colonization of roots by vesicular—-arbuscular mycorrhizal fungi. New Phytol. 115, 495–501.
Noland, T.L., Mohammed, G.H., 1997. Fluorescein diacetate as a viability stain for tree roots and seeds. New For. 14, 221–232.
R Core Team, 2016. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. 〈https://www.R-project.org/〉.
Regan, S.M., Moffatt, B.A., 1990. Cytochemical analysis of pollen development in wild-type Arabidopsis and a male-sterile mutant. Plant Cell 2, 877–889.
Ruf, M., Brunner, I., 2003. Vitality of tree fine roots: reevaluation of the tetrazolium test. Tree Physiol. 23, 257–263.
Smith, S., Read, D., 2008. Coloniation of roots and anatomy of arbuscular mycorrhiza. Mycorrhizal Symbiosis. Academic Press, London, pp. 42–90.
Steponkus, P.L., Lanphear, F.O., 1967. Refinement of the triphenyl tetrazolium chloride method of determining cold injury. Plant Physiol. 2 (10), 1423–1426.
Ullrich, S., Karrasch, B., Hoppe, H., Jeskulke, K., Mehrens, M., 1996. Toxic effects on bacterial metabolism of the redox dye 5-cyano-2, 3-ditolyl tetrazolium chloride. Appl. Environ. Microb. 62, 4587–4593.
Van Kleunen, M., Weber, E., Fischer, M., 2010. A meta-analysis of trait differences between invasive and non-invasive plant species. Ecol. Lett. 13.2, 235–245.
Verleysen, H., Samyn, G., Van Bockstaele, E., Debergh, P., 2004. Evaluation of analytical techniques to predict viability after cryopreservation. Plant Cell, Tissue Organ Cult. 77 (1), 11–21.