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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