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SHORT COMMUNICATIONS

VBA–AMF: A VBA Program Based on the Magnified Intersections Method for Quantitative Recording of Root Colonization by Arbuscular Mycorrhizal Fungi

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Abstract The extent of mycorrhizal fungi colonization is an important factor for determining the function of mycorrhizal fungi in fungi–host interaction, and quantifying the extent of mycorrhizal fungi colonization is a fundamental and essential task for researchers engaged in mycorrhizal studies. Intersect methods, such as the gridline intersect and magnified intersections methods, are accurate and objective, and are widely used to assess the colonization status of arbuscular mycorrhizal (AM) fungus. However, no convenient procedures or add-ins for Excel spreadsheets have been developed to simplify these methods. Here, we propose a procedure using the Visual Basic for Application (VBA) program in Excel that is based on the magnified intersections method, which we refer to as VBA–AMF (arbuscular mycorrhizal fungi). Time-saving and convenience are the two most prominent advantages of the VBA–AMF procedure, as it enables researchers to compute the colonization rate of AM fungi in roots, and consequently the extent of root colonization by AM fungi. VBA–AMF can also be modified to measure the status of other fungal colonizations in plant roots following the same strategy.

Keywords Arbuscular mycorrhizal fungi · Visual Basic program · Magnified intersections method · Mycorrhizal colonization rate

Wentao Hu and Lan Pan have contributed equally to this work.

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Introduction

Arbuscular mycorrhizal (AM) fungi, previously known as vesicular–arbuscular mycorrhizal fungi, are an ancient fungal division of *Glomeromycota*, and form a symbiotic association with 71% of all vascular plant species, thereby improving the nutrition, stress resistance and tolerance of these plants [1–3]. AM fungi infect the plant root cortex, and then two symbiotic structures, arbuscule and vesicle, are often formed in cortex [4]. The arbuscule, the typical symbiotic structure of AM fungi, serves as the exchange interface for nutrients from the AM fungi and carbon or lipids from the host plants [5]. Fungal vesicles are considered to function mainly as propagules and organs that store phosphorus, potassium, and lipids [6–8]. In some cases, intraradical vesicles have been found to be absent in roots colonized by species of *Gigspora* [6]. Intraradical spores have also been reported to occur in the root cortex colonized by some species of AM fungi, such as *Rhizophagus irregularis* [9]. Hyphae are considered to perform the functions of water or nutrient transport and soil exploration [8]. These symbiotic structures of the AM fungi play an essential role in establishing fungal colonization, and thus enable the assessment of the symbiotic fungal status in host roots [4]. The abundance of different symbiotic fungal structures (arbuscules, vesicles, spores, hyphae) is associated with the extent of fungal activity,

plant genotype, and environmental conditions [4]. The extent of mycorrhizal fungi colonization is an important factor for determining their function in fungi–host interactions [10].

Several methods have been invented to quantitatively assess the extent of AM fungal colonization and the fungal symbiotic status in plant roots [10, 11]. These methods are based on the quantification of component markers in fungal cell membranes and walls, the quantification of the expression of some symbiotic genes, or the calculation of the relative proportion of fungal colonization along the length of roots, as measured by microscopic observation [10, 12]. Microscopic visualization methods are by far the most widely used for investigating fungi–host interaction, due to their convenience and low cost.

Four visualization methods have been routinely used to assess the extent of root colonization by AM fungi [12], i.e., root-segment estimation, root segment ± , the gridline intersect, and the magnified intersections methods. The gridline intersect method is conducted using a dissecting microscope (at the highest magnification: 150 ×) [13]. The three other methods typically use a compound microscope (> 200 × magnification), which facilitates the identification of some symbiotic structures.

In our previous survey of 800 AM fungi papers, gridline intersection and magnified intersections methods were most common [12]. This is due to the fact the subjective judgment of observers utilizing the root-segment estimation method often affects the determined colonization rate of plant roots by AM fungi [14]; the grid-line intersect and magnified intersections methods eliminate any subjective judgment and are thus considered to be more reliable. However, it is difficult to identify the symbiotic structure of AM fungi at the low magnification of a dissecting microscope when using the grid-line intersect method [14], compared with the magnified intersections method.

Thus, the magnified intersections method has some advantages over the other three methods, but it is very time intensive, as observers must set up every intersection by rotating the eyepiece crosshairs and inputting each item of data into an Excel spreadsheet to calculate the colonization rate of AM fungi. As yet, no convenient procedures or add-ins for Excel spreadsheets have been developed to simplify this method.

Method

Here, we introduce an add-in for the Excel spreadsheet based on Visual Basic for Applications (VBA), referred to as VBA–arbuscular mycorrhizal fungi (AMF), and a convenient procedure for performing the magnified intersections method. The VBA–AMF procedure is as follows:

1. Align the root segments parallel to the long axis of the slides after staining the roots with trypan blue, using the method reported by Phillips and Hayman (1970) [15]. Then, place one clean coverslip on a slide (hereafter called the prepared slide).
2. Draw 2-mm-spaced parallel lines in Microsoft Word, and print these parallel lines on cellophane paper (which can be purchased form any craft store) using a simple laser printer (e.g., an HP LaserJet P1007 printer). Prior to printing, fix the cellophane paper on A4 printer paper (210 × 297 mm) with 18-mm-width adhesive tape (M&G Chenguang Stationery Co., Ltd, Shanghai, China) (Fig. 1a, Fig. S1). Then, cut the printed cellophane paper into several rectangles of the same size as the coverslip (24 × 50 mm; hereafter called rectangle-cellophane pieces) with the printed lines parallel to the short axis of the rectangle. Finally, paste one rectangle-cellophane piece onto a clean coverslip with a little transparent vegetable oil (creating what is hereafter called a line-coverslip) (Fig. 1b, Fig. S2).
3. Place one line-coverslip onto a prepared slide (Fig. 1b, Fig. S2), and use a compound microscope to assess the colonization status of the roots by AM fungi.
4. Examine all intersections between roots and lines on the line-coverslip for the presence or absence of AM fungal structures. Observe the center of every intersection when different AM fungal structures coexist in some intersections in the field of micro-vision. Simultaneously, run the Excel spreadsheets with VBA–AMF using Microsoft Excel software to record the status of every intersection identified above (Supplementary Material: VBA–AMF.xlsm). The VBA–AMF add-in starts automatically when opening Excel spreadsheets equipped with VBA–AMF (Fig. 1c, Fig. S3). Then, fill in the row number and sample name and press "Sample name" and "Start" to begin (Supplementary Material: How to use VBA–AMF).
5. At every intersection, press the "A," "S," "D," or "F" key when identifying AM fungal structures, or "Space" when no structure appears, where "A" stands for arbuscule, "S" for vesicle, "D" for hypha, "F" for spore, and "Space" for no AM fungal structures (zero). The total infection rates of AM fungi and of arbuscules are thus obtained in real-time when assessing the colonization of AM fungi.
6. Close the VBA–AMF add-in and save the Excel spreadsheet to complete the assessment of AM fungal colonization.

Fig. 1 Schematic framework of the proposed VBA–AMF procedure. **a** Print parallel lines on cellophane paper; **b** set up intersections between stained roots and lines on line-coverslip on a prepared slide; **c** use a compound microscope to examine all intersections with recording data by VBA–AMF. The compound microscope model is from Dreamstime.com; the laptop model is from vectorstock.com

Results and Discussion

MYCOCALC.exe (or the MYCOCALC.xls), WinRHIZO, Ramf, and Mycopatt system [11, 16–18???] are the existing software or programs for the assessment of AM fungal colonization. Trouvelot et al. (1986) developed the MYCOCALC program to assess the colonization rate of roots by AM fungi based on the root-segment estimation method [16]. According to the manual provided by Trouvelot et al. (1986), roots are cut into 1-cm-long root segments and then aligned on slides in a 5 × 4 matrix, which requires much time. In addition, root-segment estimation cannot avoid subjective interference factors [14]. As a quick method for the assessment of AM colonization, Deguchi et al. (2017) proposed the use of WinRHIZO software to scan the entire root [17]. However, users must purchase the high-priced WinRHIZO software and perform high-quality staining of the mycorrhizal structures in infected roots. In addition, the WinRHIZO method cannot effectively distinguish between different mycorrhizal symbiotic structures. Chiapello et al. (2019) developed an open-source R package, Ramf, for the quantitative analysis and display of root colonization by AM fungi [18]. However, users must measure AM fungal colonization by the gridline intersect method or Trouvelot's method prior to using Ramf. In an effort to further digitize the microscopic observation of colonization, Stoian et al. (2019) developed a MycoPatt system that redefines the mycorrhizal parameters [11]. However, users of this system must use an eyepiece micrometer grid (10 × 10) or apply 10 × 10 grids to the images captured by the microscope and record different mycorrhizal structures according to the MycoPatt system, which is also very time-consuming and tedious.

Rather than using Trouvelot's MYCOCALC, Chiapello's Ramf, Deguchi's WinRHIZO, and Stoian's Mycopatt system [11, 16–18], the proposed VBA–AMF procedure is based on the magnified intersections method [14] and features a concise graphical user interface (Fig. 1c, Fig. S3). Saving time and greater convenience are two prominent advantages of the VBA–AMF procedure. Thus, we modified the magnified intersections method by setting up every intersection of the stained roots and lines on the line-coverslip in advance, to obviate the need to rotate the vertical crosshair at every intersection. Provided that the stained root segments are aligned parallel to the long axis of the slides, users can efficiently examine all intersections of the root segments and lines on the line-coverslip. Once the assessment is complete, the total infection rates of AM fungi and arbuscules are automatically calculated. Users can also calculate the infection rate of other mycorrhizal structures at the same time. When using parallel lines with 2-mm spacing in the line-coverslip, users can observe each intersection separately at 2-mm intervals and then directly calculate the total length of the examined root. Users can also make notes about each sample to record specific phenomena, such as the existence or absence of other endophytes.

The VBA–AMF code can also be modified to measure the colonization of root endophytes or dark septate endophytes, by the addition of the options of peloton for orchid mycorrhizal fungi, septate hyphae, or microsclerotia for dark septate endophytes [19, 20]. Our proposed modified VBA–AMF procedure can therefore be used to simultaneously assess the colonization statuses of different endophytes when investigating the interactions between mycorrhizal fungi, dark septate endophytes, and other endophytes [21–24]. Any users of Microsoft office software can use the VBA–AMF add-in and modify it for individual applications.

We will supplement the VBA–AMF add-in with more functions in the future, including a function enabling visualization of AM fungal infection data with a simple graphical display. More comprehensive functions can be added to the VBA–AMF add-in by the many VBA enthusiasts investigating plant–fungus interactions.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

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