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Contrasting common measures of arbuscular mycorrhizal fungal root colonization

Vasilis Kokkoris*, Antreas Pogiatzis, Miranda M. Hart

Department of Biology, University of British Columbia, Okanagan campus, 3333 University Way, Kelowna, BC V1V 1V7, Canada

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ABSTRACT

Estimating the abundance of arbuscular mycorrhizal fungi relies entirely on indirect methods, meaning all measures are associated with some variability. The most common methods use microscopic estimates of the relative proportion of root length colonized by fungal structures. These methods typically examine root subsamples. While such methods are inexpensive and relatively simple, significant variation within single root system means there is opportunity for sampling bias. We evaluated the two most common methods of percent root length colonization for AM fungi both as a subsample and for the entire root system of flax plants. We compared these measures to a novel technique that returns projected fungal surface area (fungal coverage), by using microphotography and imaging analysis. Both microscopic methods overestimated the colonization intensity compared to image analysis. Among the microscopic methods, the method which incorporated colonization intensity (Trouvelot) was significantly more similar to imaging method results, than the method that is based on the presence/absence of the fungus (McGonigle).

1. Introduction

Arbuscular mycorrhizal (AM) fungi form an obligate symbiosis with the majority of land plants (Brundrett, 2009). The fungus grows within and between the plants root cells but also creates an extensive hyphal network that spreads beyond the root system (Smith et al., 2008). The intraradical growth of the fungus is the site of nutrient exchange, with the fungus providing mineral nutrients in exchange for carbon (Keymer et al., 2017). Determining the abundance of fungal structures within roots not only informs us about the mycorrhizal status of a host but also the ecological functioning of the symbiosis (Brundrett, 2009; Orchard et al., 2017).

Several methods exist for quantifying the abundance of the fungus in roots (Rajapakse and Miller, 1992). Some methods are based on quantification of markers in fungal cell membranes and walls such as AMF-specific phospholipid fatty acids (PLFA) (Olsson et al., 1995), ergosterol (Hart and Reader, 2002) and chemical estimation of chitin (Hepper, 1977; Bethlenfalvay, Pacovsky & Brown, 1981). Other methods use molecular markers to quantify abundance based on gene copy, using approaches such as quantitative Polymerase Chain Reaction (qPCR) (Kokkoris et al., 2019a; Voříšková et al., 2017) but they require specialized equipment and/or are costly. By far, the most widely used approach involves staining roots with a fungal specific stain followed by microscopic observation. Such methods calculate the relative proportion of fungi along the length of root measured, in a relatively small root subsample.

Most visualization methods are based on the grid intersect method (Giovannetti and Mosse, 1980) which itself was based on a method used to estimate root length (Newman, 1966). In general, grid intersect methods estimate fungal abundance by microscopically enumerating root fungal structures on a gridded microscope slide. Methods differ in whether they consider the abundance of fungal structures, and whether they differentiate among different intraradical structures such as arbuscules, vesicles. Two of the most commonly used methods are those

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Abbreviations: AM, Arbuscular mycorrhizas/Arbuscular mycorrhizal; PLFA, Phospholipid fatty acids; AC, Arbuscule colonization, (McGonigle method); VC, Vesicle colonization, (McGonigle method); HC, Hyphae colonization, (McGonigle method); TC, Total colonization, (McGonigle method); F%, Frequency of mycorrhizal fragments, (Trouvelot method); A%, Arbuscule abundance, (Trouvelot method); M%, Intensity of the mycorrhizal colonization in the entire root system, (Trouvelot method); m%, Intensity of the mycorrhizal colonization only in the root fragments that are mycorrhizal, (Trouvelot method); a%, Arbuscule abundance only in the root fragments that are mycorrhizal, (Trouvelot method); Ts, Root subsample analyzed using Trouvelot method; Tt, Entire root system analyzed using Trouvelot method; Ms., Root subsample analyzed using McGonigle method; Mt., Entire root system analyzed using McGonigle method; At, Total fungal coverage calculated based on projected fungal surface area using image analysis

* Corresponding author.

E-mail address: bill.kokkoris@ubc.ca (V. Kokkoris).

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developed by Trouvelot (1986) and McGonigle et al. (1990)

Trouvelot (1986) expanded on the Newman (Newman, 1966) by including a measure of the intensity of fungal colonization, from 0% colonization (class 1) to 90% colonization (class 5). This method provides information on the frequency of mycorrhizal fragments (F%), arbuscule abundance (A%) and the intensity of the mycorrhizal colonization (M%) in the entire root system. It also examines the intensity of the mycorrhizal colonization (m%) and the arbuscule abundance (a%) only in the root fragments that are mycorrhizal. While the Trouvelot method quantifies arbuscules it does not include any information on vesicle abundance and because quantification is a subjective interpretation of the classes, is prone to visual misestimation and can be associated with a 5% error (Giovannetti and Mosse, 1980). Plus, since the subjectivity of the method is observer-dependent, this method does not allow for data comparisons among different observers (McGonigle et al., 1990).

McGonigle et al. (1990) described a method few years later called the magnified intersections method which looks at intersections between root fragments and randomly set eyepiece crosshairs. The observer records the fungal traits that intersect the crosshairs in each field of view and at no point has to interpret the abundance of colonization. Each entire root fragment is examined at 200× magnification for at least 100 to 150 intersections and it is tempered by the total length of the root segments examined. Like the Trouvelot method, the McGonigle method provides information on fungal traits arbuscules (AC), but additionally records vesicular (VC) and hyphal (HC) colonization values as well as the total fungal colonization (TC). While this approach removes most observers bias, since the observer does not have to guesstimate the boundaries between categories, it fails to identify differences on the intensity of colonization in each field of view examined (Fig. 1).

While each method returns estimates that correlate well with measures of fungal biomass (Hart and Reader, 2002), both methods provide an estimation of the presence of the fungus based on random subsamples. Such an approach can be problematic since the distribution of AM fungi in the root system is variable (Füzy et al., 2015). An alternative to microscopic observation is the use of digital image analysis. Similar to visualization methods, the images originate from the microscopic observation and can provide quantification of the colonized projected area. Such approaches have been widely adopted for other systems, such as morphometric analysis of neurons (Billeci et al., 2013), skin fungal infection (Mäder et al., 2015), root necrotic tissue (Vukicevich et al., 2018) and fungal hyphal growth patterns (Kokkoris et al., 2019b) but have not been used to asses fungal root colonization. The benefit of such an approach is that fungal tissue is determined using an algorithm based on differences in stained fungal texture/colour, thereby reducing investigator bias. Of course, that requires an optimized staining protocol that will allow for the targeted tissue to be separated from any other neighboring tissue.

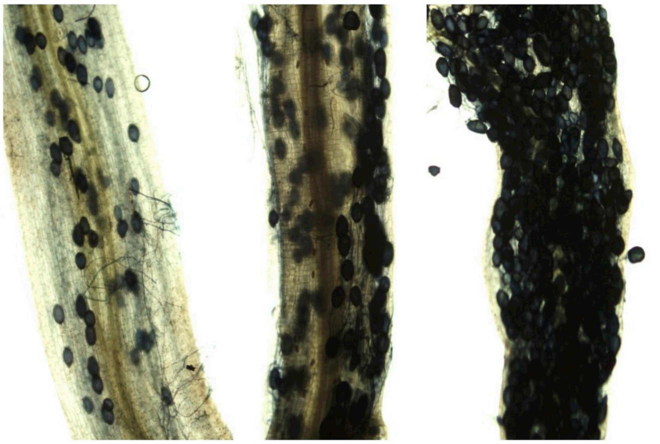


Fig. 1. Approaches that include colonization intensity (i.e. fungal abundance) are more informative than those measuring only presence /absence of AM fungi. In this example the three root fragments would have the same colonization values using the McGonigle method and would be consider 100% colonized despite clear differences in fungal abundance.

In this study (a) we evaluated the “Trouvelot” and “McGonigle” methods by comparing estimates of root length colonization from subsamples with those of the entire root systems. (b) We additionally analyzed the same root samples using a microphotography and imaging software method which accurately estimates the stained AM fungal tissue to the level of pixel.

2. Material and methods

To compare measures of relative root colonization methods in predicting absolute root colonization, we conducted a greenhouse study and inoculated flax (*Linum usitatissimum*) with an AM fungus (Rhizoglomus irregulare). The entire root systems of all germinated plants alongside with common root subsamples, as defined by each method, were analyzed for AM fungal colonization using “Trouvelot” and “McGonigle” methods. Additionally, entire root systems were analyzed using an image analysis approach that can accurately return projected surface area of all stained AM fungal tissue in the root values.

2.1. Experimental set-up

Flax (*L. usitatissimum*) seeds were surface sterilized with ethanol 70% for 1 min, followed by a 5 min step in 9.6% bleach, and rinsed 6 times (1 min each time) in sterile distilled water. Seeds were grown in glass tubes 25 × 200 mm. Each tube contained 3 g of sterile vermiculite (45 min at 121 °C), AM fungal inoculum (Rhizoglomus irregulare isolate DAOM 197198) (AGTIV specialty crop powder by Premier tech containing 12,000 viable spores per gram of powder) and 15 ml of half strength liquid Murashige and Skoog (MS) basal medium (Sigma–Aldrich, St. Louis, MO, USA). The inoculum was incorporated into the sterile vermiculite following the manufacturer's' instructions, resulting in approximately 400 spores per tube. The MS medium quantity was enough to sustain the growth of flax plants in tubes for at least 30 days without additional water or nutrients.

Three sterile flax seeds were added per tube for a total number of 10 tubes. Tubes were sealed with Breathe-EASIER™ tape (diversifieDBiotech) to avoid contamination. The tubes were kept in a growth chamber (CONVIRON ADAPTIS) at 24 °C (daytime 16 h) followed by 22 °C (night 8 h) per 24-h cycle. Tubes were kept sealed during the entire duration of the experiment.

2.2. Plant sampling and analysis

Plants were harvested 30 days after the initiation of the experiment. Root and shoot were removed intact from the tubes. After recording total wet plant weight, shoots were separated from the roots and dried at 60 °C for 48 h. The entire root system was gently washed to remove any substrate particles and was fragmented to approximately 1 to 2 cm pieces. Root fragments were placed on cheesecloth and then into plastic cartridges for staining.

2.3. Root staining

After harvest and fragmentation, roots were immediately stained to avoid the degradation of arbuscules (Orchard et al., 2017), using a slightly modified ink and vinegar protocol based on Vierheilig et al. (1998). Briefly, roots were cleared in 10% KOH for 5 min at 90 °C and then rinsed three times with RO water. Cleared roots were stained for 3.5 min in 5% black Sheaffer ink and vinegar solution at 90 °C. Roots were de-stained for 30 min in water with few drops of vinegar. Root segments were randomized and were mounted on slides and then used to estimate AM fungal colonization with our three methods. The use of Sheaffer ink provides an excellent colour contrast between the root tissue and the AM fungal tissue following the distaining step, compared to multiple other inks (Vierheilig et al., 1998). The colour contrast allows for use of imaging software that can easily differentiate the two tissues. While the value of fungal tissue detection as well as the staining sensitivity of this method has not been examined, and it is possible that parts of fungal tissue might not get stained, multiple studies used this method to visualize fungal colonization. Regardless the methods value of detection, the purpose of this study is to evaluate the efficacy of two of the most commonly used colonization assessment methods following any type of staining protocol.

2.4. *Quantification of AM fungal colonization*

For each plant, the entire fragmented root system (approximately 1–2 cm pieces) was mounted on multiple microscope slides. For the subsamples, we quantified the same slides for both “Trouvelot” and “McGonigle” estimations (same subsample).

2.4.1. *Trouvelot*

Trouvelot colonization was assessed by following Trouvelot (1986). Briefly, slides containing approximately 30 root segments, were randomly chosen to represent our subsample (**Ts**). Additionally, the entire root system (max of 189 root fragments) was used to assess total root colonization using the same method (**Tt**).

2.4.2. *McGonigle*

Similar to the method above, AM fungal colonization for the total root (**Mt**) and the random sub-sample (**Ms**), the same as for Trouvelot, for each root system was assessed using the method proposed by McGonigle et al. (1990). For the subsample we ensured that we had at least 100 to 150 intersections examined randomly from the same 30 root segments examined for the Trouvelot method.

2.4.3. *Imaging software*

Each root fragment containing fungal structures was assigned a number on the slide and then the entire slide containing the fragments was scanned with a versatile large-format scanner (Epson expression 11000XL) using a grayscale at 400 dpi. Each mycorrhizal root fragment surface area and root volume was estimated using WinRHIZO Pro image analysis system (Regent Instruments Inc., Quebec, Canada, 2013). Multiple photos of each colonized root segments were taken using a Bioimager BRC-1600 camera on a Bioimager BUM500FL microscope 100× magnification and T Capture v 5.1 software. To calculate the percentage of root colonized by the fungus, each photo was analyzed using Pro image colour analysis (Regent Instruments Inc., Quebec, Canada, 2013), (Fig. 2). To calculate the root projected surface area colonized by the AM fungus the relative projected surface area of the classes that were generated after the colour analyses were standardised per fragment, based on the root projected area of each mycorrhizal fragment. Using the total root projected area (projected) and the projected area occupied by the fungus, we then calculated the total fungal coverage (**At**). Since the image colour analysis is depended on colour classes, and the entire fungal tissue was similarly stained, discrimination between fungal structures was not possible, but the contrast between plant and fungal tissue allowed for accurate quantification of the fungal projected surface area in the roots. The **At** values from the imaging software was correlated to the colonization values estimated by Trouvelot and McGonigle methods for the entire root system (**Tt and Mt**) and the root subsample (**Ts and Ms**).

2.5. *Statistical analysis*

We used Spearman's Ranked correlations to describe the relationship between root colonization for each method. To compare the total root colonization values generated with each method (M% for Trouvelot vs TC for McGonigle vs At for the imaging software) we used ANOVA followed by Tukey-Kramer post-hoc test. The data was logarithmically transformed to achieve normality (Shapiro-Wilk Normality Test). All analyses were performed using R studio (Version 1.0.136 – © 2009–2016 RStudio, Inc.).

3. Results

Out of the 26 root systems analyzed for this study six were not colonized. The remaining 20 root systems were examined as previously described.

3.1. *Trouvelot total (Tt) vs total fungal coverage (At)*

Total fungal coverage (At) was significantly correlated with the frequency of mycorrhizal fragments in the root system (F_Total) ($r = 0.87$, $p < .05$), the intensity of the mycorrhizal colonization in the root system (M_Total) ($r = 0.64$, $p < .05$) and with arbuscule abundance in the root system (A_Total) ($r = 0.48$ $p < .05$) (Fig. 3).

3.2. *Trouvelot subsample (Ts) vs total fungal coverage (At)*

Total fungal coverage (At) was significantly correlated with the frequency of mycorrhizal fragments in the root subsample (F_subsample) ($r = 0.67$, $p < .05$), the intensity of the mycorrhizal colonization in the root subsample (M_subsample) ($r = 0.51$, $p < .05$), arbuscule abundance in the mycorrhizal parts of the root fragments (a_subsample) ($r = 0.52$, $p < .05$) and with arbuscule abundance in the root subsample (A_subsample) (0.45 $p < .05$) (Fig. 3).

3.3. *McGonigle total (Mt) vs total fungal coverage (At)*

Total fungal coverage (At) was significantly and highly correlated with arbuscule colonization (AC_Total) ($r = 0.72$, $p < .05$), vesicle colonization (VC_Total) ($r = 0.70$, $p < .05$) and total colonization (TC_Total) ($r = 0.76$, $p < .05$) (Fig. 3).

3.4. *McGonigle subsample (Ms) vs total fungal coverage (At)*

Total fungal coverage (At) was significantly correlated to arbuscule colonization (AC_subsample) ($r = 0.59$, $p < .05$), vesicle colonization (VC_subasmple) ($r = 0.54$, $p < .05$) and total colonization (TC_subsample) ($r = 0.61$, $p < .05$) (Fig. 3).

3.5. *Comparing total colonization from all methods: Trouvelot vs McGonigle vs Imaging software*

Considering that imaging software can calculate the fungal presence with higher accuracy we considered the total fungal coverage (At) as the baseline for comparison. The total colonization calculated after McGonigle method, both for the root subsample (TC_subsample) and entire root system assessment (TC_Total), significantly overestimated colonization ($p < .001$). Trouvelot resulted in higher values of colonization compared to the imaging method but the difference was not significant nor for the root subsample (M_subsample) nor for the entire root system assessment (M_subsample). The intensity of colonization in the root subsample calculated after Trouvelot (M_subsample) was closer to the calculated fungal projected surface area (Fig. 4).

3.6. *Does subsampling reflect of the entire root system?*

For both Trouvelot and McGonigle, subsampling decreased the accuracy of the estimates. Using the Trouvelot method 30% of the root systems analyzed were erroneously identified as negative when

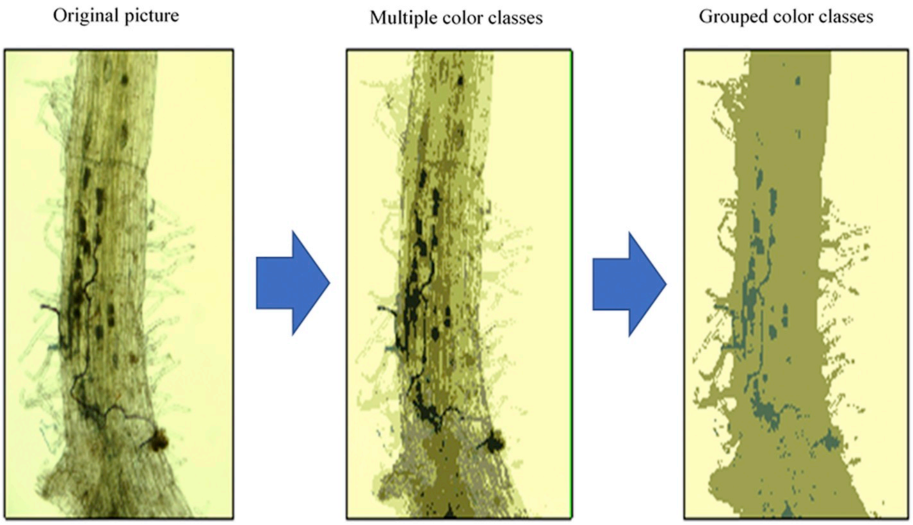
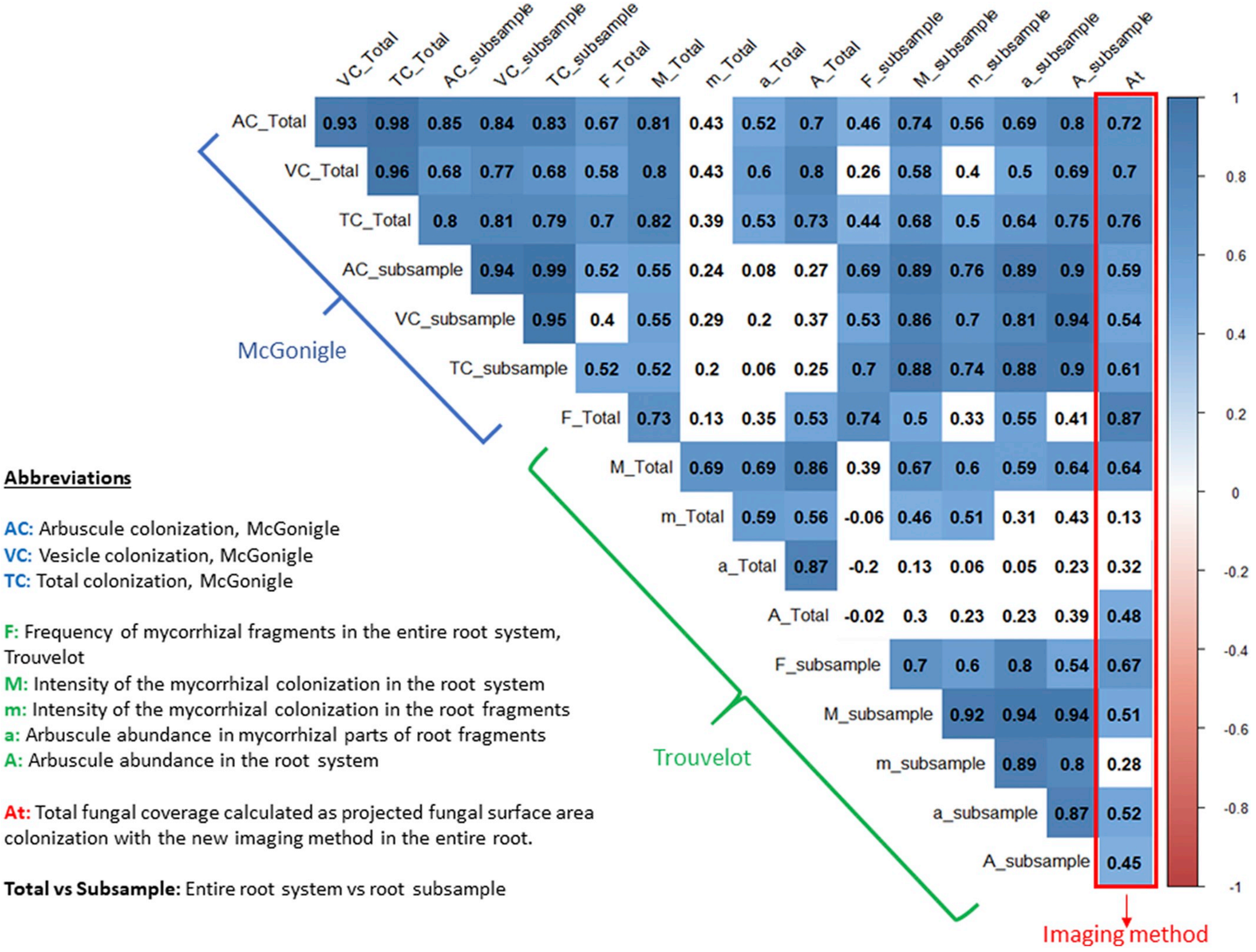


Fig. 2. Calculation of AM fungal surface area in the root using WinRHIZO Pro image analysis system. Each image was organized in three groups (Background, Root and AMF). Each of the three groups can contain up to 12 defined colour classes. The colors for each group are defined by manually selecting a pixel from the original picture (left). The analyzed picture (middle) including all the colour classes and the last picture (right) shows only the three groups. The software calculates the projected surface area (cm^2) for each of the 3 groups.



	VC_Total	TC_Total	AC_subsample	VC_subsample	TC_subsample	F_Total	M_Total	m_Total	a_Total	A_Total	F_subsample	M_subsample	m_subsample	a_subsample	A_subsample	At
AC_Total	0.93	0.98	0.85	0.84	0.83	0.67	0.81	0.43	0.52	0.7	0.46	0.74	0.56	0.69	0.8	0.72
VC_Total		0.96	0.68	0.77	0.68	0.58	0.8	0.43	0.6	0.8	0.26	0.58	0.4	0.5	0.69	0.7
TC_Total			0.8	0.81	0.79	0.7	0.82	0.39	0.53	0.73	0.44	0.68	0.5	0.64	0.75	0.76
AC_subsample				0.94	0.99	0.52	0.55	0.24	0.08	0.27	0.69	0.89	0.76	0.89	0.9	0.59
VC_subsample					0.95	0.4	0.55	0.29	0.2	0.37	0.53	0.86	0.7	0.81	0.94	0.54
TC_subsample						0.52	0.52	0.2	0.06	0.25	0.7	0.88	0.74	0.88	0.9	0.61
F_Total							0.73	0.13	0.35	0.53	0.74	0.5	0.33	0.55	0.41	0.87
M_Total								0.69	0.69	0.86	0.39	0.67	0.6	0.59	0.64	0.64
m_Total									0.59	0.56	-0.06	0.46	0.51	0.31	0.43	0.13
a_Total										0.87	-0.2	0.13	0.06	0.05	0.23	0.32
A_Total											-0.02	0.3	0.23	0.23	0.39	0.48
F_subsample												0.7	0.6	0.8	0.54	0.67
M_subsample													0.92	0.94	0.94	0.51
m_subsample														0.89	0.8	0.28
a_subsample															0.87	0.52
A_subsample																0.45

Abbreviations

AC: Arbuscule colonization, McGonigle
VC: Vesicle colonization, McGonigle
TC: Total colonization, McGonigle

F: Frequency of mycorrhizal fragments in the entire root system, Trouvelot
M: Intensity of the mycorrhizal colonization in the root system
m: Intensity of the mycorrhizal colonization in the root fragments
a: Arbuscule abundance in mycorrhizal parts of root fragments
A: Arbuscule abundance in the root system

At: Total fungal coverage calculated as projected fungal surface area colonization with the new imaging method in the entire root.

Total vs Subsample: Entire root system vs root subsample

Fig. 3. Correlation between colonization assessment methods (McGonigle and Trouvelot) and total fungal coverage (imaging method). Numbers represent the correlation values (r, Spearman's Ranked correlation). Coloured cells indicate correlations significant at $p < .05$, blue indicates positive correlations while red indicates negative correlations. Absence of colour indicates a nonsignificant correlation. Blue font indicates values generated using McGonigle's method, green font for Trouvelot's method and red for the projected fungal surface area generated using the Imaging method. The total fungal coverage (At) is also signified with a red rectangular for ease of readability. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

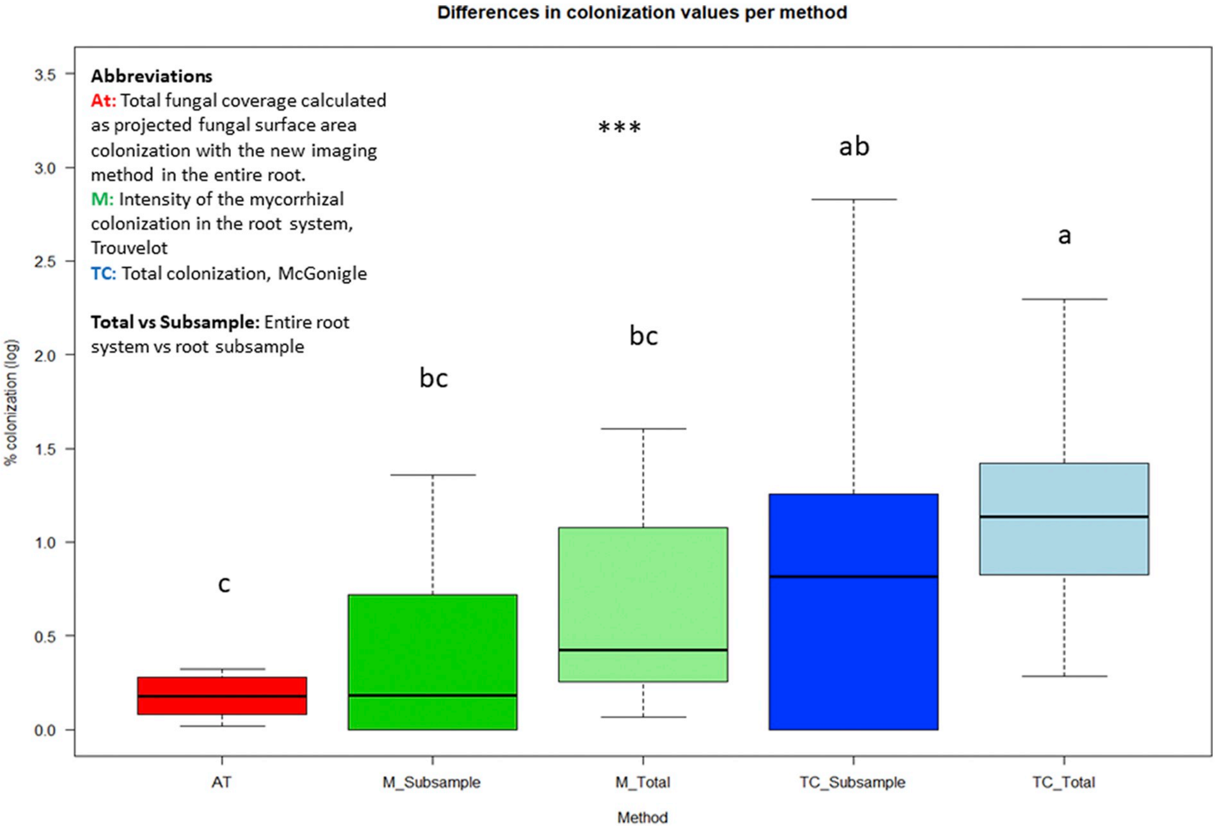


Fig. 4. Differences in total root colonization values per method. "**AT**": total fungal coverage calculated by image analysis. "**M_subsample**": The intensity of colonization in the root subsample calculated after Trouvelot. "**M_Total**": The intensity of colonization in the entire root system calculated after Trouvelot. "**TC_subsample**": Total colonization of root subsample estimated by the McGonigle method. "**TC_Total**": Total colonization of the entire root system estimated by McGonigle. Box-plots show the third quartile and first quartile (box edges), median (middle line), range of the data (whiskers) and data outliers (circles). *** $p < .001$. Group means sharing the same letter are not significantly different from each other according to the Tukey-Kramer post-hoc test ($\alpha = 0.05$), ($n = 20$).

choosing a subsample. Further 25% overestimated and 15% underestimated root colonization compared to the entire root system. For McGonigle method 30% root systems analyzed were erroneously identified as negative from subsampling. From the rest root systems analyzed, 30% overestimated and 10% underestimated root colonization compared to analysis of the entire root system. For detailed information about the total fragments of each root system and the number of fragments that consist each subsample please see Table S1 for Trouvelot and Table S2 for McGonigle.

4. Discussion

Estimates of fungal root abundance differed significantly based on all methods used. For both Trouvelot and McGonigle methods, subsampling often failed to identify fungal presence, especially when colonization was low, which lead to erroneous estimates of total colonization.

4.1. Which method most accurately represents the actual presence of the AM fungus in the root?

The McGonigle method significantly overestimated the abundance of AM fungi in roots. Trouvelot method also tended to overestimate fungal presence but was closer to the projected fungal surface area in the roots as defined by total root image analysis. Our result agrees with that of Füzy et al. (2015) who also found that Trouvelot provided the most detailed information compared to McGonigle method, but also compared to the gridline intersect method (Giovannetti and Mosse, 1980). The large error generated with the McGonigle method is probably due to the calculation of colonization based on fungal "presence – absence" that does not allow for quantification of the intensity of colonization. Overall, the Trouvelot method appears to be a more reliable and accurate method to quantify AM fungi intraradically despite, errors associated with observer bias (Giovannetti and Mosse, 1980, McGonigle 1990). While the imaging method is still observer depended, since the main colour classes are defined manually, the automatic categorization of each pixel to each colour class (even if a colour class is not defined the program automatically assigns it to the closest category) allows for a precise quantification of the stained fungal tissue in the roots. Of course, like other quantification methods after staining, the imaging method cannot discriminate between non-functional/ senescing tissue and viable tissue, and is not appropriate for quantifying living biomass, unless the staining step targets only viable fungal tissue. It has been demonstrated, that use of other ink type (red parker ink) (Vierheilig et al., 1998) provides good contrast between root tissue and metabolically active stained AM fungal tissue (Macdonald and Lewis, 1978). The use of different software that could discriminate between more than three classes, could provide us with important information between viable – non-viable fungal tissue localization in the roots. Further examination of the ink staining methodology and potential combination with additional dyes in needed in order to optimize the visualization of fungal tissue and to further benefit from image analysis approaches.

4.2. Does subsampling reflect of the entire root system?

In our study both McGonigle and Trouvelot subsampling methods were associated with significant error by misidentifying plants as non-colonized while they were colonized. That could be a serious source of error for studies that are examining colonization in early harvested roots, or colonization in roots growing under environmental conditions that do not favour high colonization rates. While attention has been brought in the past on the importance of sampling (Reich and Barnard, 1984; Klironomos et al., 1999; Füzy et al., 2015) the majority of studies use a rather small number of root fragments to evaluate root colonization by AM fungi.

Surprisingly, we observed high variation in colonization values and colonization status (positive vs negative) between plants sharing the same test tube. In our experiment, by allowing the plant-AM fungus interaction to develop for four weeks we expected to overcome temporal limitations, since for Rhizoglomus irregulare three weeks is usually sufficient to observe colonization across the entire root system (Füzy et al., 2015). Furthermore, the limited space that the tube provided was expected to overcome spatial stochasticity, ensuring physical interaction between AM fungi and plant roots. Therefore, our results suggest that colonization efficiency may be due to other factors such as the unique genetic profile of each plant (Estaún et al., 2010), or propagule distribution in the medium (Muthukumar et al., 2003) and topography of the root in the soil (Ingleby et al., 1997).

5. Conclusion

Trouvelot proved to be a more accurate method compared to McGonigle although both methods are prone to misestimations. Although a rich data depository containing information of percent colonization and AM fungal trait quantification can be a valuable source for comparisons between studies, the inaccurate estimations compared to actual root colonization can conceal important information when such data is used for meta-analysis. Future studies should examine the topography of colonization and the factors that affect it in order to optimize sampling and minimize such errors.

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Author contribution

Conceived research: VK.
Performed research: VK, AP.
Analyzed data: VK.
Wrote the manuscript: VK, MMH.

Declaration of Competing Interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2019.105727.

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