Quantifying Root Colonization in Arbuscular Mycorrhizas by Image Segmentation and Machine Learning

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Quantifying root colonization in arbuscular mycorrhizas by image segmentation and machine learning

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Abstract

Motivation

Arbuscular mycorrhizas are the most widespread plant symbioses and involve the majority of crop plants. The beneficial interaction between plant roots and a group of soil fungi (Glomeromycotina) grants the green host a preferential access to soil mineral nutrients and water, supporting plant health, biomass production and resistance to both abiotic and biotic stresses. The nutritional exchanges at the core of this symbiosis take place inside the living root cells, which are diffusely colonized by specialized fungal structures called arbuscules. For this reason, the vast majority of studies investigating arbuscular mycorrhizas and their applications in agriculture require a precise quantification of the intensity of root colonization. To this aim, several manual methods have been used for decades to estimate the extension of intraradical fungal structures, mostly based on optical microscopy observations and individual assessment of fungal abundance in the root tissues.

Results

Here we propose a novel semi-automated approach to quantify AM colonization based on digital image analysis and compare two methods based on image thresholding and machine learning. Our results indicate in machine learning a very promising tool for accelerating, simplifying and standardizing this critical type of analysis, with a direct potential interest for applicative and basic research.

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Key words: biostatistics, image segmentation, microscopy, arbuscular mycorrhiza
Arbuscular mycorrhizas (AM) are widespread plant endosymbioses that develop between Glomeromycotina fungi and the roots of the majority of plants species, including most crops. The symbiosis benefits extend to both partners by improving plant mineral absorption, tolerance to biotic and abiotic stresses and overall fitness, while rewarding fungal symbionts with carbon compounds derived from the photosynthetic process, such as sugars and lipids. Such exchanges of nutrients represent the functional core of the symbiosis and mainly take place in highly branched fungal structures - called arbuscules - that are hosted within the living cortical cells of the host root, as described in Figure 1.

The central ecological role of AM in the functioning of low-input ecosystems and the ability of most crop plants to develop this symbiosis has focused a growing number of investigations on the use of AM in sustainable agricultural practices. A critical factor in all such studies is represented by the precise quantification of root colonization by AM fungi, with particular attention to arbuscule abundance. To this aim, molecular analyses (based on the quantification of fungal sequences in total root DNA or arbuscule-specific markers in total root RNA extracts) are outnumbered by the direct quantification of intraradical fungal structures through optical microscopy imaging and manual analyses.

In one of the most commonly used methods, root samples are stained with lactic blue or alternative dyes to label intraradical fungal structures; roots are then cut into 1cm-long segments, mounted on microscope slides and carefully observed under an optical microscope to classify each segment based on visual criteria such as the extension of intraradical hyphae and the abundance of arbuscules in the colonized areas. Such methods are extremely time consuming, based on the ability of trained operators and subject to errors.

In an attempt to improve speed, repeatability and reliability of this type of analysis, we developed a semi-automated approach based on digital imaging and different types of post processing. Firstly,
an automatic method was developed to discriminate between mycorrhizal and non-mycorrhizal root images. Secondly, we designed a semi-automated algorithm to generate quantitative indexes of root colonization deriving from either image thresholding using ImageJ, or based on machine learning analyses \(^8,^9\) using the commercial software Zeiss Intellesis \(^10\). Our results indicate machine learning as the most effective approach, with interesting applicative perspectives as an alternative to manual quantification.

**Figure 1.** Schematic representation of a host root (grey) colonized by an arbuscular mycorrhizal fungus (black). The extraradical mycelium (*) explores the soil surrounding the root, while intraradical structures produced from the hyphopodium (h) penetrate root epidermal cells (e), colonizing single cortical cells (c), where they eventually develop into branched arbuscules (arrowhead), the sites of nutrient exchanges between symbionts.
2. System and methods

All images used in this research (Supplementary file 1) were acquired at a resolution of 1280*720 pixels, using one of the following brightfield optical microscopes: Nikon Eclipse Ci-L mounting 4x / 0.10 WD30, 10x / 0.25 WD10.5 and 40x / 0.65 WD0.57 objectives; Leica DM500 mounting a PLAN 4x/0.10 objective. The intraradical fungal structures are stained in dark blue while the plant tissues and cells are completely transparent or light blue, this allows to distinguish the fungal structures from the plant structures.

The segmentation analysis based on digital microscopy images on gray scale pixels was carried out using ImageJ software (Rueden et al., 2017) and was based on two phases analysis:

- a dataset of 143 images from non-mycorrhizal and 60 images from mycorrhizal roots of *Medicago truncatula* colonized by the AM fungus *Funneliformis mosseae*, previously classified manually according to a standard protocol for quantifying AM colonization (Supplementary file 1), has been used for an explorative analysis to assign images to mycorrhizal/non mycorrhizal classes.

- a second dataset consisting of 180 images was used for both the thresholding (using ImageJ) and machine learning (using Intellesis) approaches. All images had previously been classified manually into 6 classes of 30 images, ranging from 1 (non mycorrhizal) to 6 (maximum root colonization), adapting to the same standard protocol.

The digital images in grayscale and the relative thresholding are described in Supplementary file 2.

Algorithm

*Binary segmentation analysis.*
In the first phase of the analysis, images were loaded into ImageJ and transformed into 8-bit digital images before further processing. A simple segmentation technique based on pixel intensity thresholding was then applied in order to distinguish darker areas (likely corresponding to stained fungal structures) from lighter areas (not colonized tissues). In more detail, the pixel brightness range [0-100] was chosen as representative of colonized areas, whereas the [101-255] range corresponded to uncolonized tissues (Figure 2). Each image was classified based on the results of the segmentation analyses and correlated with the manual classification with binary logistic regression. The study variable (y) is the classification with binary variable 0 (not mycorrhized) or 1 (mycorrhized), obtained by manual classification. The explanatory variable (x) is the expression of the pixel area determined as a consequence of the chosen threshold and it is released by the ImageJ macro in a value report.

This whole set of image processing steps has been automated, taking advantage of the integrated ImageJ macros feature. (Supplementary file 3).

The results demonstrated a good discrimination capacity of the binary regression model with the table of manual classifications, and by the ROC curve with an area under the curve of 0.870 (Supplementary file 4).

Figure 2. Brightness-based selection of pixels on the same image of a mycorrhizal root segment. Pixels are selected (in red) based on arbitrary brightness thresholds: 100, 137, 170 in a range from 0 (black) to 255 (white) using Fiji/ImageJ.
Statistical analyzes were carried out with the SPSS software (IBM Statistics for Windows version 26.0).

**Thresholding analysis.**

Also in this case digital images were loaded into ImageJ and transformed into 8-bit digital images. Two ImageJ macros were designed to process the images (Supplementary file 3). The first image segmentation macro was designed to discriminate the entire root section area from the globally lighter background; in this case the pixel intensity threshold was set to 155.

The second macro produced image segmentation based on pixel intensity, with a threshold empirically set at 65 (in the 0-255 range) to discriminate between darker pixels (corresponding to fungal structures) and lighter (uncolonized) areas.

Both results are described in Figure 3.

After applying both segmentation macros to all our images, a set of quantitative values was obtained corresponding to the supposed colonized area (darkest pixels) and the total root section area (as isolated from the image background).

| Threshold | Original digital 8 bit image | Segmentation |
|-----------|-----------------------------|--------------|
| 65        | ![Original Image](image1)    | ![Segmented Image 1](image2) |
| 155       | ![Original Image](image3)    | ![Segmented Image 2](image4) |
The ratio between these two values was used to obtain the $m$ index (for mycorrhization) for each image in the dataset:

$$m = \frac{\text{mycorrhized area}}{\text{total area}} \times 100$$  \[1\]

This $m$-index was therefore used in the subsequent statistical analyses as an independent variable in the predictive model for the quantification of root colonization (Table 1).

| Thresholding index ($m$) | Number of images | Mean       | Std. Deviation | Median       | Range     |
|--------------------------|------------------|------------|----------------|--------------|-----------|
| manual_classification    |                  |            |                |              |           |
| 1                        | 30               | 1,011369   | 1,1947128      | 0,607633     | 5,5826    |
| 2                        | 30               | 5,878176   | 4,4051314      | 4,631726     | 16,4411   |
| 3                        | 30               | 14,396212  | 10,1959085     | 10,330562    | 38,9856   |
| 4                        | 30               | 15,174801  | 8,3606544      | 14,529747    | 28,6474   |
| 5                        | 30               | 29,751744  | 13,2256586     | 31,321755    | 51,3397   |
| 6                        | 30               | 29,970579  | 11,6878341     | 28,027334    | 45,3563   |
| Total                    | 180              | 16,030480  | 14,2051105     | 12,332234    | 60,3251   |

### Table 1a. $m$-index, descriptive statistics

| Classification | Qualitative description | Confidence interval ($\alpha=0.05$) |
|----------------|-------------------------|------------------------------------|
| 1              | Not mycorrhized (mycorrhization 0%) | $m = 1,011369 \pm 1,96 * \frac{1,1947128}{\sqrt{30}}$ |
| 2              | Slightly mycorrhized (few mycorrhization) | $m = 5,878176 \pm 1,96 * \frac{4,4051314}{\sqrt{30}}$ |
| 3              | Moderately mycorrhized (mycorrhization < 10%) | $m = 14,396212 \pm 1,96 * \frac{10,1959085}{\sqrt{30}}$ |
| 4              | Averagely mycorrhized (mycorrhization from 11 to 50%) | $m = 15,174801 \pm 1,96 * \frac{8,3606544}{\sqrt{30}}$ |
| 5              | Highly mycorrhized (mycorrhization from 51 to 90%) | $m = 29,751744 \pm 1,96 * \frac{13,2256586}{\sqrt{30}}$ |
| 6              | Strongly mycorrhized (mycorrhization > 90%) | $m = 29,970579 \pm 1,96 * \frac{11,6878341}{\sqrt{30}}$ |

### Table 1b. Confidence interval for $m$-index related to the multinomial classification
In the Table 1a we show the descriptive statistics of the calculated $m$-index based on ImageJ macros relative for each manual classified classes in the range 1 – 6. Confidence intervals and qualitative descriptions for each class are shown in Table 1b.

As shown in Figure 4, the average value of the $m$ index increases for each class considered, indicating a good correlation between the study variable $y$ (manual classification) and the independent variable $x$ ($m$-index).

Indeed, a strong correlation was detected between the two variables, with a significant Pearson coefficient at the 0.01 two tailed level with a value of 0.748. Furthermore, ANOVA analysis (Table 2) and post hoc test of multiple comparisons performed with Bonferroni correction (Table 3) confirmed the statistical significance of the differences between classes. In particular, Bonferroni post hoc test demonstrated significant differences in 12 pairwise comparisons (on a total of 15) with exceptions for class 1 vs 2, class 3 vs 4 and class 5 vs 6.
### ANOVA

$m$-index

|                      | Sum of Squares | df | Mean Square | F     | Sig. |
|----------------------|----------------|----|-------------|-------|------|
| Between Groups       | 21439,364      | 5  | 4287,873    | 50,823| .000 |
| Within Groups        | 14680,181      | 174| 84,369      |       |      |
| Total                | 36119,544      | 179|             |       |      |

**Table 2.** ANOVA for the six groups of digital images index $t$

| (I) manual_classification | (J) manual_classification | Mean Difference (I-J) | Std. Error | Sig. |
|---------------------------|---------------------------|-----------------------|------------|------|
| 1                         | 2                         | -4.8668071            | 2.3716219  | .625 |
|                           | 3                         | -13.3848433           | 2.3716219  | .000 |
|                           | 4                         | -14.1634322           | 2.3716219  | .000 |
|                           | 5                         | -28.7403757           | 2.3716219  | .000 |
|                           | 6                         | -28.9592100           | 2.3716219  | .000 |
| 2                         | 1                         | 4.8668071             | 2.3716219  | .625 |
|                           | 3                         | -8.5180362            | 2.3716219  | .006 |
|                           | 4                         | -9.2966252            | 2.3716219  | .002 |
|                           | 5                         | -23.8735687           | 2.3716219  | .000 |
|                           | 6                         | -24.0924030           | 2.3716219  | .000 |
| 3                         | 1                         | 13.3848433            | 2.3716219  | .000 |
|                           | 2                         | 8.5180362             | 2.3716219  | .006 |
|                           | 4                         | -7.785890             | 2.3716219  | 1.000|
|                           | 5                         | -15.355325            | 2.3716219  | .000 |
|                           | 6                         | -15.5743668           | 2.3716219  | .000 |
| 4                         | 1                         | 14.1634322            | 2.3716219  | .000 |
|                           | 2                         | 9.2966252             | 2.3716219  | .002 |
|                           | 3                         | .7785890              | 2.3716219  | 1.000|
|                           | 5                         | -14.5769435           | 2.3716219  | .000 |
|                           | 6                         | -14.7957778           | 2.3716219  | .000 |
| 5                         | 1                         | 28.7403757            | 2.3716219  | .000 |
|                           | 2                         | 23.8735687            | 2.3716219  | .000 |
|                           | 3                         | 15.355325             | 2.3716219  | .000 |
Table 3. Post hoc comparison Bonferroni. * indicates that the mean difference is significant at the 0.05 level

In order to build a prediction model of the level of mycorrhization, we used regression analysis with linear, quadratic or cubic models (Figure 5 and Table 4). Our results show that the most satisfactory $R^2$ (0.687) is obtained with the cubic model expressed in the form:

$$y_{index} = a + b_1 x + b_2 x^2 + b_3 x^3$$  \[2\]

Figure 5. Curve fit with linear, quadratic and cubic regression models t index
Dependent Variable: manual_classification

| Equation | R Square | F       | df1 | df2 | Sig.   | Constant | b1 | b2    | b3    |
|----------|----------|---------|-----|-----|--------|----------|----|-------|-------|
| Linear   | 0.560    | 226.301 | 1   | 178 | 0.000  | 2.054    | 0.090 |       |       |
| Quadratic| 0.673    | 181.823 | 2   | 177 | 0.000  | 1.401    | 0.203 | -0.003 |       |
| Cubic    | 0.687    | 128.714 | 3   | 176 | 0.000  | 1.168    | 0.282 | -0.007 | 0.000054 |

The independent variable is thresholding_index.

**Table 4.** Estimation of the parameters of the predictive model with $m$-index
Machine learning analysis

As a last and most advanced approach to image analysis, we decided to test a machine learning system using the Zeiss Zen Intellesis application (Carl Zeiss Microscopy GmbH Jena, Germany https://www.zeiss.com/microscopy/int/products/microscope-software/zen-intellesis-image-segmentation-by-deep-learning.html).

Machine learning is a branch of artificial intelligence that solves tasks using algorithms that are capable of learning from experience (training), without being explicitly programmed for a specific task (https://github.com/zeiss-microscopy/OAD/tree/master/Machine_Learning).

Zeiss Intellesis can apply a number of machine learning tools called basic features. In this study, we used basic feature 25, which achieves digital image segmentation by applying Gaussian, Sobel of Gaussian, Gabor and Hessian filters to the region surrounding classified pixels, to generate a final feature vector with 25 dimensions describing each pixel (https://github.com/zeiss-microscopy/OAD/blob/master/Machine_Learning/Feature_Extractors/feature_extractors.md).

This technique involves a training phase for the construction of an image segmentation model and the subsequent application of the model to the image dataset. The training phase consisted in training the software with a few initial images of roots with a high level of colonization. On each different image we focused on the discrimination between colonized areas, non-colonized areas and image background (Figure 6).

Once the training was completed, the model was applied to the entire dataset of 180 images. Intellesis outputs different quantitative parameters of the areas in square pixels as in the case of the thresholding technique explained above.

Also in this case we built a relationship index between segmented areas.

| Original digital image | Segmentation |
Figure 6. Segmentation in Zeiss Intellisis with machine learning. Light blue: background, yellow: mycorrhized area, red: non mycorrhized area.

In this case, the $ml$-index for each image in the dataset is:

$$ ml = \frac{\text{colonized area}}{\text{colonized area} + \text{non colonized area}} \times 100 \tag{3} $$

The $ml$-index was therefore used in the subsequent analyses for the construction of a prediction model to evaluate the level of mycorrhization.

In the Table 5a we show the descriptive statistics of the calculated $ml$-index, based on Intelesia machine learning, relative for each manual classification (1 – 6). Confidence intervals and qualitative descriptions for each class are shown in Table 5b.

| Manual classification | N  | Mean      | Std. Deviation | Range    |
|-----------------------|----|-----------|----------------|----------|
| 1                     | 30 | 6.425657  | 6.2031851      | 18.6092  |
| 2                     | 30 | 7.889029  | 8.6432691      | 41.8196  |
| 3                     | 30 | 17.301326 | 11.7548653     | 40.6675  |
| 4                     | 30 | 26.794820 | 10.5940464     | 49.6036  |
| 5                     | 30 | 44.150405 | 11.3738218     | 37.4350  |
| 6                     | 30 | 48.430012 | 8.8434900      | 33.8745  |
| Total                 | 180| 25.165208 | 19.0615764     | 65.7459  |

Table 5a. $ml$-index, descriptive statistics

| Classification | Qualitative description                           | Confidence interval ($\alpha$=0.05) |
|----------------|-------------------------------------------------|-------------------------------------|
| 1              | Not mycorrhized (mycorrhization 0%)             | $ml = 6.425657 \pm 1.96 \times \frac{6,2031851}{\sqrt{30}}$ |
| 2              | Slightly mycorrhized (few mycorrhization)       | $ml = 7.889029 \pm 1.96 \times \frac{8,6432691}{\sqrt{30}}$ |
| 3              | Moderately mycorrhized (mycorrhization < 10%)   | $ml = 17.301326 \pm 1.96 \times \frac{11,7548653}{\sqrt{30}}$ |
| 4              | Mycorrhized (mycorrhization from 11 to 50%)     | $ml = 26.794820 \pm 1.96 \times \frac{10,5940464}{\sqrt{30}}$ |
| 5              | Highly mycorrhized (mycorrhization from 51 to 90%) | $ml = 44.150405 \pm 1.96 \times \frac{11,3738218}{\sqrt{30}}$ |
| 6              | Strongly mycorrhized (mycorrhization > 90%)     | $ml = 48.430012 \pm 1.96 \times \frac{8,8434900}{\sqrt{30}}$ |
Table 5b. Confidence interval for ml-index related to the multinomial classification

And the box plot of the descriptive statistics is described in Figure 7.

Figure 7. ml-index, box plot

The average value of the ml-index increases between classes, suggesting a good correlation with the manual classification. This was confirmed by the correlation analysis with a significant Pearson coefficient at the 0.01 two tailed level of 0.843.

Also in this case ANOVA analysis (Table 6) confirmed the statistical significance of the differences between classes. Post hoc test of multiple comparisons performed with Bonferroni correction (Table 7) further demonstrated significant differences between 13 comparisons (on a total of 15), except for class 1 vs 2 and class 5 vs 6.

ANOVA

Segmentation ml-index
### Table 6. ANOVA for the six groups of digital images ml-index

**Multiple Comparisons**

Dependent Variable: spec_segm_mlearning_index

| Bonferroni | Mean Difference | Std. Error | Sig. | 95% Confidence Interval |
|------------|-----------------|------------|------|------------------------|
| (I) T_manual_class | (J) T_manual_class | (I-J) |         |                        |
| 1 | 2 | -1.4633715 | 2.5191873 | 1.000 | -8.961158 | 6.034415 |
| 3 | 2 | -10.8756684 | 2.5191873 | .000 | -18.373455 | -3.377882 |
| 4 | 2 | -20.3691622 | 2.5191873 | .000 | -27.866949 | -12.871376 |
| 5 | 2 | -37.7247477 | 2.5191873 | .000 | -45.222534 | -30.226961 |
| 6 | 2 | -42.0043545 | 2.5191873 | .000 | -49.502141 | -34.506568 |
| 1 | 3 | 1.4633715 | 2.5191873 | 1.000 | -6.034415 | 8.961158 |
| 4 | 3 | -9.4122969 | 2.5191873 | .004 | -16.910084 | -1.914510 |
| 5 | 3 | -18.9057907 | 2.5191873 | .000 | -26.403577 | -11.408004 |
| 6 | 3 | -36.2613762 | 2.5191873 | .000 | -43.759163 | -28.763590 |
| 1 | 4 | 10.8756684 | 2.5191873 | .000 | 3.377882 | 18.373455 |
| 2 | 4 | 9.4122969 | 2.5191873 | .004 | -1.914510 | 16.910084 |
| 5 | 4 | -9.4934938 | 2.5191873 | .003 | -16.991280 | -1.995707 |
| 6 | 4 | -26.8490792 | 2.5191873 | .000 | -34.364866 | -19.351293 |
| 1 | 5 | 31.1286860 | 2.5191873 | .000 | -38.626473 | -23.630899 |
| 2 | 5 | 20.3691622 | 2.5191873 | .000 | 12.871376 | 27.866949 |
| 3 | 5 | 18.9057907 | 2.5191873 | .000 | 11.408004 | 26.403577 |
| 2 | 6 | 9.4934938 | 2.5191873 | .003 | 1.995707 | 16.991280 |
Table 7. Post hoc Bonferroni comparison. * indicates that the mean difference is significant at the 0.05 level.

A prediction model for the level of mycorrhization was then built using regression with linear, quadratic, cubic models (Figure 8). The most satisfactory $R^2$ (0.728) was obtained for the cubic model expressed in the form:

$$y_{index ml} = a + b_1x + b_2x^2 + b_3x^3$$  \[4\]
Figure 8. Curve fit with linear, quadratic and cubic regression models ml-index

In the Table 8 below, R squared for ml-models are resumed.

Model Summary and Parameter Estimates
Dependent Variable: T_manual_class

| Equation      | R Square | F       | df1 | df2 | Sig. | Constant | b1   | b2   | b3               |
|---------------|----------|---------|-----|-----|------|----------|------|------|------------------|
| Linear        | 0.710    | 436.258 | 1   | 178 | 0.000| 1.595    | 0.076|      |                  |
| Quadratic     | 0.720    | 227.308 | 2   | 177 | 0.000| 1.365    | 0.106| -0.001|                 |
| Cubic         | 0.728    | 156.956 | 3   | 176 | 0.000| 1.583    | 0.047| 0.002| -0.00002856      |

The independent variable is spec_segm_mlearning_index.

Table 8. Estimation of the parameters of the predictive model with ml-index

We summarize in Table 9 the results of the statistical analysis performed comparing thresholding and machine learning training using the best predictive cubic models.

Automatic methods of mycorrhization prediction

|                         | Thresholding | Machine learning training |
|-------------------------|--------------|----------------------------|
| Pearson correlation index| 0.748        | 0.824                      |
Table 9. Methods comparison

The best prediction model is the cubic model with an $R^2$ reaching 0.728 compared to the 0.687 achieved by the thresholding.

Discussion

The degree of root colonization is a major feature in most studies of AM biology and field applications. Assessing the extent of fungal development inside the host root system provides a direct indication of symbiosis development and functioning. Indeed, quantitative estimates of AM colonization are a pre-requisite for all studies of symbiotic promotion of plant nutrition and growth.

Two major approaches are commonly used to quantify fungal presence in root samples: a quantitative analysis of fungal marker genes, providing a direct indication of fungal DNA abundance in total extracted DNA, and microscopic investigation of a representative sample of root segments, assessing the extension of colonized areas, the spread of intraradical hyphae and the relative abundance of arbuscules or vesicles in the whole root system. Overall, the molecular approach provides a relatively fast and reliable determination of fungal presence but cannot address important questions, such as the relative abundance of different intraradical structures (e.g. arbuscules and hyphae), which limits its interest when studying symbiosis functionality, unless used in combination with functional markers, such as plant P transporters that are only expressed in arbusculated cells.

By contrast, morphological methods, albeit time consuming, provide more direct information on AM functionality and remain of very common use. In particular, those described by Trouvelot and...
The two methods are based on non-vital staining of fungal cell wall in mycorrhizal roots, followed by the manual ranking of a representative sample of root segments and a statistical analysis of the results, to extrapolate whole root system estimates.

Even if manual ranking is based on rather objective traits, morphological methods perform best when the same expert operator analyses all the samples that need to be compared. Another critical limitation is the high amount of time required for manual analyses of sufficiently large samples to support solid statistics and conclusions. In the present study, we used a manual method based on Trouvelot et al (1986) as a benchmark to compare the reliability of three semi-automated methods based on image analysis.

A preliminary binary segmentation approach was initially used, to provide a raw distinction between mycorrhizal and non-mycorrhizal root images. The good correlation of this binary segmentation method with the manual classification opened the way to the subsequent thresholding analysis. This approach used the gradient of pixel brightness (inversely related to lactic blue staining) as an indicator of intraradical fungal development. The thresholding model generated 6 classes of mycorrhization, with a good correlation with manual scores of the corresponding images and can therefore be considered as a reliable and cheap method for a rapid screening of root samples and their classification in a range of mycorrhization intensity values. A few critical aspects should anyway be considered. One major limitation of the thresholding method is the variability of brightness range between images: different dyes, optical setups, root translucence and the presence of additional microorganisms (such as bacteria, algae, endophytic fungi, invertebrates) especially in field samples, can generate very diverse patterns that are impossible to discriminate only based on pixel brightness. In addition, the method is strongly affected by image background noise and the use of images acquired at different magnification levels. Lastly, the segmentation process can only be set ex ante, during macro editing, without any subsequent correction by the operator.

The machine learning method, based on the Zeiss Zen Intellesis application, resulted to be the most efficient. In this case, individual fungal structures such as intracellular hyphae and arbuscules were...
manually selected during the training phase to generate a model that the software then applied to the whole data set. This method identified 6 classes of mycorrhization intensity, achieving the best correlation (Pearson correlation coefficient 0.824) with manual analysis. Importantly, the training process was relatively quick (it required 50 minutes overall) and resulted to be effective even when using a limited number of images (10 images). Lastly, the use of machine-learning identification of shapes and objects allowed a reliable discrimination between equally stained structures such as extraradical hyphae and arbuscules, a capability that our image thresholding methods could never achieve.

A critical factor emerging from the machine learning-based method is the need to train the software by an expert operator. Nevertheless, the training phase generates a reference file containing all the image analysis data that can subsequently be shared with other researchers. This opens a new perspective where the expertise of a few operators could be made available to the whole community of researchers by simply sharing a file. Furthermore, the training file could continuously be implemented and stored online, thus becoming a common resource and a unifying instrument for the quantification of root colonization intensity in AM.

The current model accuracy should be further implemented to achieve the level of detail of manual methods. Our method can currently only assign a mycorrhization class to each image, without reliably discerning between arbuscules and other intraradical structures such as vesicles or hyphal coils. Such limitations could be overcome by improving the number of images used for training or modifying the image analysis algorithm. The latter option is anyway currently not possible, due to the commercial nature of the software.

In conclusion, it should be stressed that this is one of the first attempt to use a machine learning approach for the evaluation of the level of root colonization and the current results represent a promising base for future improvements.

DECLARATIONS

Ethics approval and consent to participate
Consent for publication
Not applicable

Availability of data and material
The data-sets generated and analysed during the current study are available in the Figshare repository:
Binary segmentation non myc DOI https://doi.org/10.6084/m9.figshare.14679642
Thresholding and machine learning segmentation - mycorrhized roots DOI
https://doi.org/10.6084/m9.figshare.14679729
Thresholding and machine learning segmentation – non mycorrhized roots DOI
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Competing interests
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Authors’ contributions
IS designed the image analysis approach, performed image analyses and statistical analyses, and wrote the text; AC contributed to design the image analysis approach, performed image analyses and wrote the text; MN contributed to image analyses and writing of the text; MP contributed to image analyses; AG contributed to the experimental design, and wrote the text. All authors have read and approved the final manuscript.

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Figure 1

Schematic representation of a host root (grey) colonized by an arbuscular mycorrhizal fungus (black). The extraradical mycelium (*) explores the soil surrounding the root, while intraradical structures produced from the hyphopodium (h) penetrate root epidermal cells (e), colonizing single cortical cells (c), where they eventually develop into branched arbuscules (arrowhead), the sites of nutrient exchanges between symbionts.
Figure 2

Brightness-based selection of pixels on the same image of a mycorrhizal root segment. Pixels are selected (in red) based on arbitrary brightness thresholds: 100, 137, 170 in a range from 0 (black) to 255 (white) using Fiji/ImageJ.

| Threshold | Original digital 8 bit image | Segmentation |
|-----------|-----------------------------|--------------|
| 65        | ![Original digital 8 bit image](image1) | ![Segmentation](image2) |
| 155       | ![Original digital 8 bit image](image3) | ![Segmentation](image4) |

Figure 3

Image thresholding in ImageJ. The first threshold set at 65 identifies the colonized area, while the second threshold at 155 outlines with a good approximation the total area of the root section.
Figure 4

m-index, box plot
Figure 5

Curve fit with linear, quadratic and cubic regression models t index

| Original digital image | Segmentation |
|------------------------|-------------|
| ![Original image](image1.png) | ![Segmentation](image2.png) |

Figure 6

Segmentation in Zeiss Intellisis with machine learning. Light blue: background, yellow: mycorrhized area, red: non mycorrhized area
Figure 7

ml-index, box plot
Figure 8

Curve fit with linear, quadratic and cubic regression models ml-index

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