

Evaluation of Two Techniques for Quantification of Hyphal Biomass¹

Meagan M. Hynes,² Robert J. Zasoski,² and Caroline S. Bledsoe²

Abstract

Currently, oak woodlands of Northern California and their associated mycorrhizal fungi are receiving more attention. In order to address the impact mycorrhizal fungal associations have on survival of various tree species in oak woodlands, we investigated the extramatrical fungal hyphae associated with several mature oak woodland tree species. Specifically our objective was to quantify the fungal hyphal biomass in soils near blue oak (*Quercus douglasii*), interior live oak (*Quercus wislizeni*), foothill pine (*Pinus sabiniana*), and Ponderosa pine (*Pinus ponderosa*). We developed methods to determine hyphal length using microscope images with either WinRHIZO Pro 2002©³ software or the more common gridline intersect method (GIM), which calculates hyphal length using Newman's original (1966) and modified equations (Tennant 1975). After comparing methods, we found that using microscope images in addition to Tennant's equation resulted in the most accurate and efficient way of estimating hyphal biomass. Information gathered will be used to determine whether hyphal length is correlated with survival and growth of oaks in California's oak woodlands.

Keywords: Blue oak, extramatrical hyphae, foothill pine, hyphal length, interior live oak, ponderosa pine.

Introduction

Oak woodland regeneration has received considerable attention from managers during the last three decades. Low survival rates of blue oak saplings were investigated by Bernhardt and Swiecki (2001), yet no effective mitigation strategy exists to increase natural regeneration and survival rates. One possible explanation that has not been explored is the effect of mycorrhizal fungi and hyphae have on survival of oak woodland tree species. The symbiotic relationship between trees and mycorrhizal fungi has been shown to aid in nutrient and water uptake, and to protect roots from pathogens (Read and Leake 1989, Sylvia 1999, Wollum 1999). However, the effect of mycorrhizal associations on oak woodland tree species has not been explored as a possible solution to increase sapling survival rates. Moreover, there are few studies that have harvested and quantified fungal hyphae in the California oak woodlands.

Extramatrical fungal hyphae are the web-like structures that emanate from the plant root-fungal interface into the surrounding soil. Due to their extensive surface area, hyphae can increase access to nutrient and water uptake in the soil (Van Der Heijden and Sanders 2003). However, examining the proliferation and function of

¹ An abbreviated version of this paper was presented at the Sixth California Oak Symposium: Today's Challenges, Tomorrow's Opportunities, October 9-12, 2006, Rohnert Park, California.

² Graduate Student and Professors respectively, Department of Land, Air, and Water Resources University of California, Davis, CA 95616. e-mail: mmhynes@ucdavis.edu, rjzasoski@ucdavis.edu, csbledsoe@ucdavis.edu.

³ Mention of trade names or products is for information only and does not imply endorsement by the U.S. Department of Agriculture.

these belowground structures is difficult due to the interweaving of hyphae and soil particles, as well as small hyphae diameters (2-10 μm) (Allen 1991). Recent developments by Wallander and others (Wallander and others 2001) have made it easier to examine hyphae *in situ*. The use of sand-filled nylon mesh bags in soil near tree roots facilitates extraction and quantification of hyphal length and biomass.

Length is a common parameter used to quantifying fungal hyphae. Methods developed originally by Newman (1966) and modified by Tennant (1975) initially focused on quantifying root length. Over the last three decades, the equations developed by Newman and Tennant have been easily applied to quantify hyphal length and have been used frequently in conjunction with the gridline intersect method (GIM) (Bearden and Peterson 2000, Dighton and Skeffington 1987, Miller and others 1995, Nuemann and George 2005, Staddon and others 2003, Tisdall and Oades 1979). However, most studies combined GIM with hyphal staining using dyes (i.e., Trypan blue); this allowed for detection of lighter-colored hyphae. The procedure required extended microscope use.

Hyphae can be extracted from soil using the well-established filter method (Hensen and others 1974), followed by staining and either automated or manual quantification methods (Morgan and others 1991). More recently, as a way to quantify fungal growth, pharmaceutical and food industry researchers have analyzed hyphae growing in culture or contaminating food samples using automated and semi-automated programs in which some user input is required (Cox and others 1998, Packer and Thomas 1990, Tucker and others 1992). However, these automated and semi-automated programs are often expensive (\$2,000-\$13,000 USD) and sold as packages (i.e., microscope, computer, and software). Hyphae can be measured *in situ* (Lucas and others 2005) using minirhizotron tubes, a mini-camera, and WinRHIZO Tron software. However, one's view of the hyphae present in the soil is limited to the small size of the camera window.

The capability to quantify hyphal length with more efficacy increases the value of studies evaluating the role of hyphae in nutrient and water uptake and subsequent transport to their mycorrhizal hosts. More importantly, a better understanding of hyphae effects (i.e., increased water uptake for hosts during drought) might help us understand the ability of oaks to survive in the Sierra Nevada foothills of Northern California.

In this method development and comparison, we used hyphal samples from a field study of multiple tree species. We compared the results of two methods for measuring hyphal length: (1) a semi-automated method using WinRHIZO Pro 2002©, and (2) an established manual method that relies on Newman and Tennant's equations to quantify hyphal length. We examined accuracy and rate of data acquisition for each method.

Materials and Methods

Study Site

The field study was located at the Sierra Foothills Research and Extension Center (SFREC), Browns Valley, California, approximately 100 km northeast of Sacramento, California, U.S.A. The area is characterized by a Mediterranean climate, with cool, wet winters and hot, dry summers. The mean annual air temperature is

15°C, and the average precipitation is 73 cm (Dahlgren and Singer 1994). Soils at the SFREC are fine-loamy, mixed, thermic, Mollic Haploxeralfs and fine, mixed, thermic, Typic Rhodoxeralfs (Dahlgren and Singer 1994). The arborescent vegetation consists of blue oaks (*Quercus douglasii*), interior live oaks (*Quercus wislizeni*), foothill pines (*Pinus sabiniana*) and ponderosa pines (*Pinus ponderosa*). Grasses and forbs in this area include of *Bromus* spp., *Lolium* spp., *Madia* spp., and *Trifolium* spp. (Jackson and others 1990). We selected blue oak, interior live oak, and foothill pine trees in an area that has been ungrazed for ≥ 40 years. The ponderosa pines in this study were located in a nearby (~ 1.2 km) grazed area on a north-facing slope.

Experimental Design

The experimental design included four replicate mature trees of each of the four tree species. Hyphal in-growth bags were placed around each of the 16 tree species. Bags (5 cm circumference and 10 cm long) were constructed using 25 μ m nylon mesh; this created a root-restrictive structure that only allowed for penetration by hyphae. Each bag was filled with 300 grams of medium-course sand. The sand was leached, autoclaved and then amended with enough 0-10-10 (N-P-K) fertilizer to result in a phosphorus concentration of 100 mg P/kg sand. In order to discourage saprotrophic fungal growth, no carbon source was added to the bag. Four in-growth bags were vertically inserted 10cm into the ground at the canopy edge around each of the trees. Gravelly and cobbely areas were avoided. Bags remained in the ground for one year (April 2004 to April 2005).

In-growth bags were harvested, immediately freeze dried, and finally stored at -20 °C. For hyphal biomass, we thawed the bags, emptied each bag into a 500 mL wide-mouth container and shook the contents by hand for 15 seconds. This brief shaking allowed the hyphae to clump together for easier extraction. The sample was then sieved with a window screen (2 mm by 2 mm). The remaining hyphae on the screen were removed and the contents were sieved again to obtain more hyphae from the sample. The hyphae were then separated by hand from most of the organic matter, then weighed and stored once again at -20 °C.

A subsample of the hyphal sample was placed in methanol (MeOH) for ergosterol analysis. Placing the sample in MeOH allowed for some of the remaining sand particles or organic matter to become dislodged. A small subsample was removed from the ergosterol subsample. Several drops of deionized water were added to the hyphae, and the clumps were separated into 1-3 mm lengths using a scalpel. The water-hyphae mixture was filtered using a 0.22 μ m nitrocellulose Millipore filter (Miller and Jastrow 2006). Hyphae on filters were then dried (60°C, 24 h) and transferred to a Petri plate (5.8 cm diameter). A transparent grid (~ 66 rectangles each measuring 4 mm x 6 mm) was then placed on top of the filter in the Petri plate.

Hyphal Length Analysis

Based on the method by Miller and Jastrow (2006), we developed a semi-automated analysis of hyphal length. Using a Nikon Stereoscopic Zoom Microscope SMZ1000 (Nikon Instruments, Inc., Melville, NY, USA) and a SPOTTM RT Camera, 20x magnification images were taken using SPOTTM RT Software v 3.4 (Diagnostic Instruments, Sterling, Heights, MI, USA). For each of the randomly selected hyphal in-growth bags evaluated, each rectangle of the of grid was photographed as a 1,600 x 1,200 pixels 'jpeg' image and labeled sequentially. Images were collected in black

and white for greater contrast (*fig. 1a*) and analyzed using WinRHIZO PRO 2002© software (Arsenault and others 2002). An initial semi-automated analysis was performed and then refined by reanalyzing sections of the rectangle (*fig. 1b*).

The analysis performed by the WinRHIZO program follows a modified version of a non-statistical method presented at the American Society of Horticulture meeting (Arsenault and others 1995) and is briefly discussed in the WinRHIZO 2002a Basic, Regular, and Pro manual (2001). The length analysis relies on skeleton images or skeletonization to calculate hyphal length. Skeletonization uses an algorithm. Digital images are collected and simplified to a one-pixel line, which is recognizable for the algorithm and classification system (i.e., root length) (Himmelbauer and others 2004, Klette 2002).

To minimize variation, the same images of the gridded Petri plate were then analyzed for hyphal length using GIM. Taking horizontal and vertical counts of hyphae that crossed the edges of each rectangle, the total number of hyphal crosses or intersections can be used to calculate hyphal length. The number of hyphal intersections occurring at the top and right side of each rectangle were totaled, which ultimately accounted for all sides of the grids. Total counts were used in two different equations. The GIM equations by Newman (1966) and its later-modified version by Tennant (1975) were developed for determining root length, but could be used to determine hyphal length and biomass (Miller and others 1995). Newman and Tennant's equations are as follows:

$$\text{Newman (1966): root length} = \frac{\pi NA}{2H}$$

$$\text{Tennant (1975): root length} = \left(\frac{11}{14} \right) * \text{grid unit} * N$$

Where 'N' is the count of the number of intersections across vertical and horizontal lines, 'A' is the area of the rectangle or grid, 'H' is the total length of the grid lines (length of grid rectangle multiplied by the number of grids), and 'grid unit' is the length of the gridded section. The counts and appropriate values for variables were inserted into each of the equations; hyphal length was determined for each sample. Data on hyphal lengths from each method and equation were analyzed statistically using a paired t-test in Systat 11 (Systat Software, Inc. 2004).

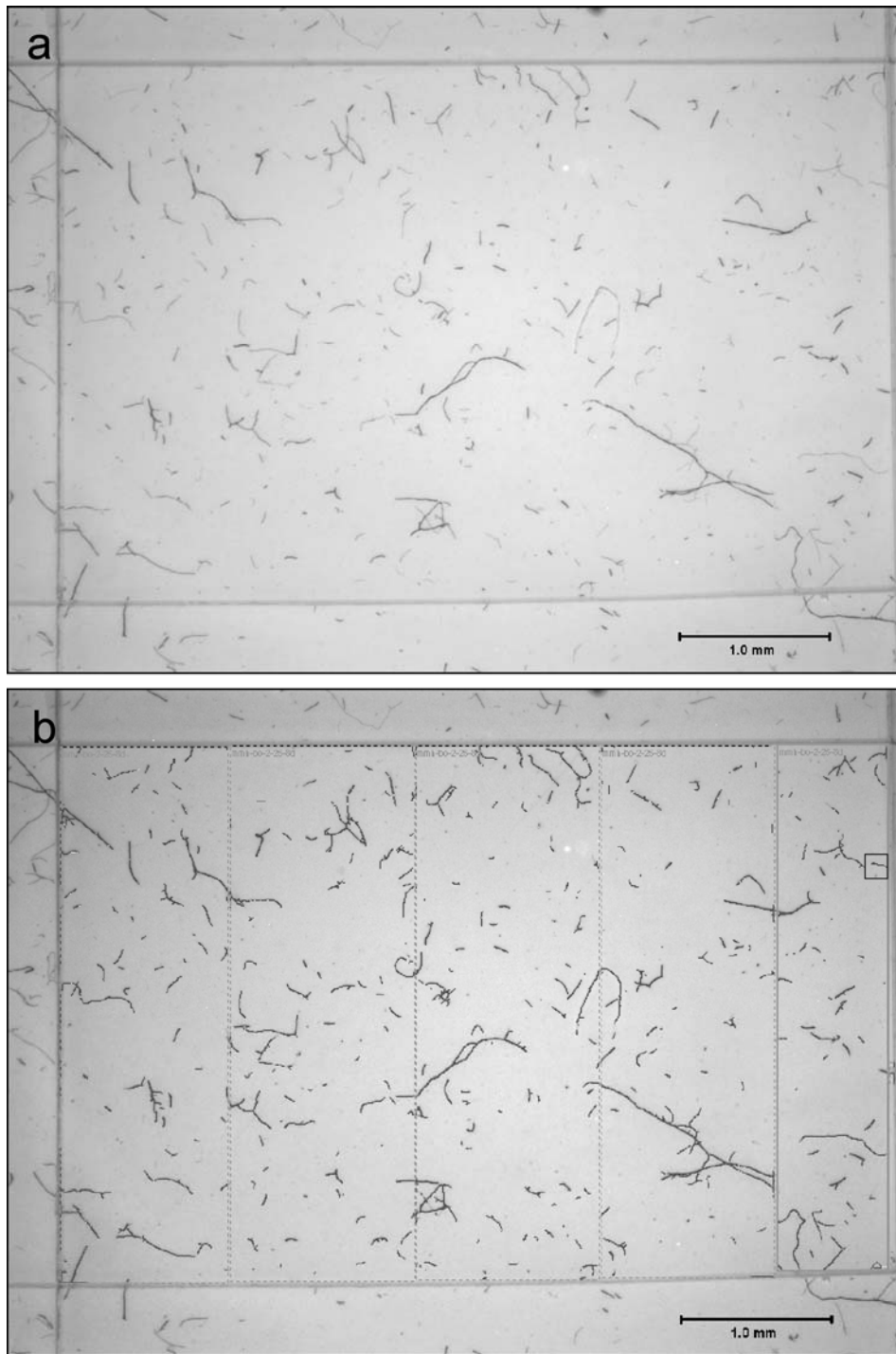


Figure 1—Images of a rectangular grid over a Millipore filter with hyphae before (a) and after (b) WinRHIZO Pro 2002© length analysis. Only the area inside the rectangular grid was analyzed for each picture. Hyphae in the sample shown are from a nylon mesh bag buried beneath a mature interior live oak tree.

Results

The image-capture process took approximately 1.5 to 2 hours per sample to achieve the correct image quality sufficient for analysis with WinRHIZO. Each image (rectangle on grid) took approximately 3 to 18 minutes to analyze, depending on two factors: (1) sub-sample size (i.e., more hyphae per rectangle equals longer analysis time), and (2) sample quality (how well the sample was subdivided into smaller pieces). On average, we collected 66 images per sample; this required an average of 8 minutes of analysis time of each rectangle. Overall, almost 9 hours per sample was necessary. In contrast, the use of GIM required only 15 minutes to analyze the entire Millipore filter (all 66 images) and ~3 minutes to enter the recorded counts into a spreadsheet using Newman and Tennant's equations. Analysis time using WinRHIZO was 30 times longer compared to using GIM and equations.

Results of hyphal length for the two methods and two equations are presented in table 1. To demonstrate accuracy of the method, data are presented as raw numbers and are not extrapolated for subsample weight, which would show differences between tree species. For the comparison, five random samples from five different trees were used to evaluate the methods. Using the GIM and Newman's equation resulted in the highest average measured hyphal length at 2.51 m (table 1). WinRHIZO and GIM with Tennant's equation resulted in similar average lengths of 2.27 m and 2.16 m, respectively.

Table 1—Hyphal lengths (m) measured with WinRHIZO PRO 2002©, and grid-line intersect methods (GIM) length calculated using either Tennant's or Newman's Equation.

	WinRHIZO Pro (m)	GIM -Tennant (m)	GIM - Newman (m)
Blue Oak 1	3.34	2.93	3.67
Blue Oak 2	2.87	2.88	3.55
Live Oak	2.40	2.26	2.40
Ponderosa Pine	1.29	1.19	1.32
Foothill Pine	1.44	1.54	1.61
Method Means $\pm$ S.E.	2.27 $\pm$ 0.397	2.16 $\pm$ 0.349	2.51 $\pm$ 0.482

The three different approaches were not significantly different (table 2). Moreover, a high correlation ($r > 0.97$) exists between each method pair.

Table 2—Methods were evaluated as pairs and sample means were compared using a Paired t-test with $p < 0.05$ being significantly different ($n=5$). Correlation is also shown.

	WinRHIZO Pro and Tennant	WinRHIZO Pro and Newman	Tennant and Newman
Paired t-test	$p = 0.287a$	$p = 0.125a$	$p = 0.076a$
Correlation	0.981	0.978	0.989

Discussion

The use of a semi-automated method, which involved use of a microscope, digital camera, and the program WinRHIZO to collect and analyze images, was not a more rapid and efficient alternative to the well-established GIM for determination of hyphal length. Our data supports the efficiency of the long-standing GIM and its

associated equations by Newman (1966) and Tennant (1975). After three decades of use, the GIM and equations are still the most straightforward and efficient method to measure hyphal length. We recommend Tennant's equation rather than Newman's equation. We found that Newman's equation overestimated hyphal length compared to Tennant's equation. Since the correlation between WinRHIZO and Tennant's equation is slightly higher than the correlation between WinRHIZO and Newman's equation, we recommend an underestimation of hyphal length using Tennant's equation rather than overestimation of hyphal length using Newman's equation. Furthermore, the calculation and determination of numerical values for Tennant's equation is more straightforward than with Newman's equation.

In addition to analysis of the various methods, we discovered improvements to the GIM. We shortened microscope time required for GIM counts; our modification required only a few seconds of camera and microscope setup time compared to constant viewing of the sample through a microscope when GIM is used without the assistance of capturing images. A decrease in microscope time benefits researchers who are negatively affected by prolonged microscope use (i.e., nausea, eyestrain, or neck ache).

Species of fungal hyphae could not be determined using this method or any enumeration method. In oak woodlands, oaks and pines form ectomycorrhizal fungi (ECM) while grasses and forbs form arbuscular mycorrhizal fungi (AMF) associations. We were able to determine if the hyphal samples were saprotrophic and ECM versus AMF by observing the presence or absence of septa (cross-walls in the hyphae). Septa are not found in AMF hyphae. ECM (septate hyphae) were generally larger in diameter (4-6 μ m) than AM hyphae.

The use of an expensive automated program may be a preferable investment for laboratories that frequently quantify hyphal length. However, our results suggest that laboratories with smaller samples sizes, should use the GIM. Furthermore, a microscope, computer, and camera are probably more available and accessible than expensive imaging equipment and software.

Summary

Our comparison of methods to quantify hyphal length showed that using the grid line intersect method in conjunction with Tennant's root length equation was the optimum approach. In the future, WinRHIZO and other semi-automated or automated programs may improve and become faster and more efficient processes for measuring hyphal length. As new approaches begin to improve the efficiency of hyphal extraction from media, researches will require complementary automated programs to measure hyphal biomass. Until then, due to its efficiency and accuracy, the use of well-established fungal length quantification methods with the addition of minor enhancements (e.g., use of dissecting microscope images) is recommended for research on tree species and their associated hyphae. The ease of extracting and quantifying fungal hyphae from soil determines the speed at which we obtain knowledge concerning below-ground fungal processes such as seedling and sapling mycorrhizal inoculation, as well as nutrient and water transport via hyphae to hosts. Relating these findings at an ecosystem level can be an arduous task, but improving quantitative methods may allow for more insight into oak woodland regeneration and management.

Acknowledgments

Research was supported in part by the National Science Foundation Biocomplexity Research Grant DEB-99-81711 to Drs. Caroline Bledsoe, William Horwath, David Rizzo, and Robert Zasoski. We thank S. Mercer Meding and Daniel Mourad for their time and energy with fieldwork, Owen Ransom and Michelle Nagao for lab assistance, and Angela Kong for her comments and suggestions regarding this publication.

References

- Allen, M.F. 1991. **The Ecology of Mycorrhizae**. Cambridge: Cambridge University Press; 184.
- Arsenault, J.-L.; Pouleur, S.; Messier, C.; Guay, R. 1995. **WinRHIZO™, a root-measuring system with a unique overlap correction method**. HortScience 30: 906.
- Arsenault, J.-L.; Pouleur, S.; Messier, C.; Guay, R. 2002. WinRHIZO Pro. Montreal, Quebec: Regents Instruments Inc.; Available from: Regents Instruments Inc., Montreal, Quebec.
- Bearden, B.N.; Peterson, L. 2000. **Influence of arbuscular mycorrhizal fungi on soil structure and aggregate stability of a vertisol**. Plant and Soil 218: 173-183.
- Bernhardt, E.A.; Swiecki, T.J. 2001. **Restoring oak woodlands in California: theory and practice**. Phytosphere Research. 8/15/2005.
<http://phytosphere.com/restoringoakwoodlands/oakrestoration.htm>.
- Cox, P.W.; Paul, G.C.; Thomas, C.R. 1998. **Image analysis of the morphology of filamentous microorganisms**. Microbiology 144: 817-827.
- Dahlgren, R.A.; Singer, M.J. 1994. **Nutrient cycling in managed and non-managed oak woodland-grass ecosystems**. Davis: UCD. 92. 100028.
- Dighton, J.; Skeffington, R.A. 1987. **Effects of artificial acid precipitation on the mycorrhizas of scots pine seedlings**. New Phytologist 107: 191-202.
- Hensen, J.F.; Thingstad, T.F.; Goksøyr, J. 1974. **Evaluation of hyphal lengths and fungal biomass in soil by a membrane filter technique**. Oikos 25: 102-107.
- Himmelbauer, M.L.; Loiskandl, W.; Kasternek, F. 2004. **Estimating length, average diameter and surface area of roots using two different Image analysis systems**. Plant and Soil 260: 111-120.
- Jackson, L.E.; Struass, R.B.; Firestone, M.K.; Bartolome, J.W. 1990. **Influence of tree canopies on grassland productivity and nitrogen dynamics in deciduous oak savanna**. Argiculture, Ecosystems, and Environment 32: 89-105.
- Klette, G. 2002. **Skeletons in digital image processing**. Glen Innes: Computer Science Dept., University of Auckland. 21. Available from CITR.Glen Innes, Auckland, New Zealand;CITR-TR-112.
- Lucas, R.; Vann, D.; Casper, B.B. 2005. **Pristine old-growth forest experiences decrease in mycorrhizal abundance following inorganic N fertilization**. Abstract In: Ecological Society of America 90th Annual Meeting; Montreal, Canada: Ecological Society of America.
- Miller, M.; Reinhardt, D.R.; Jastrow, J.D. 1995. **External hyphal production of vesicular-arbuscular mycorrhizal fungi pasture and tallgrass prairie communities**. Oecologia 103: 17-23.

- Miller, R.M.; Jastrow, J.D. 2006. **Extraction and quantification of external mycorrhizal hyphae (for soils dominated by silts and clays or high in particulate organic matter)**. web page. Argonne National Laboratory. 2/8/2006.
www.anl.gov/ER/Research_Programs/Terrestrial_Ecology/Procedures/hyphae.html.
- Morgan, P.; Cooper, C.J.; Bettersby, N.S.; Lee, S.A.; Lewis, S.T.; Machin, T.M.; Graham, S.C.; Watkinson, R.J. 1991. **Automated image analysis method to determine fungal biomass in soils and on solid matrices**. Soil Biology and Biochemistry 23: 609-616.
- Newman, E.I. **A method of estimating the total length of root in a sample**. 1966. The Journal of Applied Ecology 3: 139-145.
- Nuemann, E.; George, E. 2005. **Extraction of extraradical arbuscular mycorrhizal mycelium from compartments filled with soil and glass beads**. Mycorrhiza 15: 533-537.
- Packer, H.L.; Thomas, C.R. 1990. **Morphological measurements on filamentous microorganisms by fully automated image analysis**. Biotechnology and Bioengineering 35: 870-881.
- Read, D.B.; Leake, J.R. 1989. **The nitrogen nutrition of mycorrhizal fungi and their host plant**. Read, D.B. In: Nitrogen, phosphorus and sulfur utilization by fungi. New York: Cambridge University Press; 181-204.
- Staddon, P.L.; Thompson, K.; Jakobsen, I.; Grime, J.P.; Askew, A.P.; Fitter, A.H. 2003. **Mycorrhizal fungal abundance is affected by long-term climatic manipulations in the field**. Global Change Biology 9: 186-194.
- Sylvia, D.M. 1999. **Mycorrhizal Symbiosis**. Sylvia, D.M.; Fuhrmann, J.J.; Hartel, P.G. and Zuberer, D.A. In: Principles and Applications of Soil Microbiology. Upper Saddle River: Prentice Hall; 408-426.
- Systat®. 2004. Point Richmond, CA: Systat Software, Inc.; Available from: Systat Software, Inc., Point Richmond, CA.
- Tennant, D. 1975. **A test of a modified line intersect method of estimating root length**. The Journal of Ecology 63: 995-1001.
- Tisdall, J.M.; Oades, J.M. 1979. **Stabilization of soil aggregates by the root systems of ryegrass**. Australian Journal of Soil Research 17: 429-441.
- Tucker, K.G.; Kelly, T.; Delgrazia, P.; Thomas, C.R. 1992. **Fully automatic measurement of mycelial morphology by image analysis**. Biotechnological Progress 8: 353-359.
- Van Der Heijden, M.G.A.; Sanders, I.R. 2003. Mycorrhizal Ecology. 1st. New York: Springer; 469.
- Wallander, H.; Nilsson, L.O.; Hagerberg, D.; Baath, E. 2001. **Estimation of the biomass and seasonal growth of external mycelium of ectomycorrhizal fungi in the field**. New Phytologist 151: 753-760.
- Wollum, A.G. 1999. **Introduction and historical perspective**. Sylvia, D.M.; Fuhrmann, J.J.; Hartel, P.G. and Zuberer, D.A. In: Principles and Application of Soil Microbiology. Upper Saddle River: Prentice Hall; 3-22.