



## Staining of fungal hyphae and propagules with fluorescent brightener

This method allows the direct visualization under an epifluorescent microscope of hyphae and fungal propagules present in different substrates such as samples of soil, sediments, water, etc. The total length of fungal hyphae can also be estimated.

### 1. Method

#### 1.1. Sample preparation

An appropriate dilution of the sample is prepared by homogenizing the soil or sediment sample in sterilized water added with 1% Tween 80 and 0.5% Antifoam A (Sigma) to facilitate the dispersion of the soil particles. In case of water samples, you can directly proceed to the staining as described below.

#### 1.2. Staining

The fluorescent dye Fluorescent Brightener 28 (Sigma Aldrich Co., Ltd.) is added to the soil dilution or to the water sample in order to obtain the final concentration of  $0,1 \text{ mg ml}^{-1}$ . Samples are incubated in the dark at room temperature for 30 min.

Fluorescent Brightener 28 (synonymous with Calcofluor White M2R and Tinopal UNPA-GX) non-specifically binds to polysaccharides with  $\beta$ -1,3 and  $\beta$ -1,4 linkages, such as chitin,  $\beta$ -glucans and cellulose and is therefore able to stain fungi in the vegetative phase. The maximum absorbance wavelength is 365 nm (UV), the maximum emission wavelength is 450 nm.

#### 1.3 Filtration

Using a filtration system samples are filtered through Anodisc membrane (Whatman) with  $0,2 \mu\text{m}$  pore. The volume of filtered sample depends on the concentration.

Membrane filter is then taken with tweezers, mounted on a glass slide with a drop of deionized water to promote adhesion, and covered with a coverslip for microscopy observation.

#### 1.4 Epifluorescence observation

The slides are observed under an epifluorescence microscope with UV illumination equipped with an ocular counting grid (10 X 10), at 400X magnification.

#### 1.5 Calculation of propagules number and total hyphal length per gram of soil

The propagules number per gram of soil is calculated as:

$$B = (N/X) \cdot (A/B) \cdot (1/S) \quad (1)$$

where N is the number of fungal propagules (spores and conidia) counted, X is the number of fields of view (grids) counted, A is the area of the filter covered by sample, B is the area of the grid and S is the amount of soil on the filter.

The hyphal length H ( $\mu\text{m grid}^{-1}$ ) is calculated as:

$$H = I \cdot \pi \cdot A/2L \quad (2)$$

where I is the number of intersections per grid, A is the grid area ( $\mu\text{m}^2$ ) and L is the total length of lines in the counting grid ( $\mu\text{m}$ ).

The total length of fungal hyphae, F ( $\text{m g}^{-1}$  soil) is calculated as:

$$F = H \cdot 10^{-6} \cdot (A/B) \cdot (1/S) \quad (3)$$

where H is the hyphal length ( $\mu\text{m grid}^{-1}$ ),  $10^{-6}$  is the conversion of  $\mu\text{m}$  to m, A is the area of the filter covered by sample, B is the area of the grid and S is the amount of soil on the filter.

Biovolumes V ( $\mu\text{m}^3$ ) can be calculated from length (L,  $\mu\text{m}$ ) and width (W,  $\mu\text{m}$ ) using the equation:

$$V = (\pi/4) W^2 (L-W/3) \quad (4)$$

In order to obtain representative results, at least 50-100 fields for each membrane should be analysed. The fields should cover filter area randomly, e.g. by selecting fields along two central transects at right angles.

## **2. Materials**

### **2.1 Fluorescent Brightener 28 solution**

Stock solution is prepared dissolving the powder in distilled water, stirring and adding drop by drop 20% (w/v) KOH, until the solution becomes transparent; reach the final volume concentration of  $1\text{mg mL}^{-1}$  with distilled water and store in single use aliquots at  $-20^{\circ}\text{C}$ . Working solution concentration is 10% (v/v) of the stock solution.

## **3. References**

**Alef K., Nannipieri P.** 1995. *Methods in Applied Soil Microbiology and Biochemistry*. Academic Press.