Use of Spectrophotometry as a Tool to Quantify the Sporulation of *Penicillium allii* in Garlic Lesions

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ABSTRACT

A technique to measure the concentration of *Penicillium allii* conidia in damp chamber experiments by spectrophotometry was developed. A negative linear correlation (R^2=0.56) was observed between transmittance at 340 nm and the concentration of *P. allii* conidia in water agar 0.05%. The equation that relates transmittance (T) with concentration (conidia mL^{-1}) (y) is: $y = 9.3\ 10^6 - 86497\ T$. The method was assayed by inoculating 43 *P. allii* strains in two garlic cultivars. The method proved to be more rapid than the traditional use of a hemocytometer with an improved accuracy. The CV of the number of conidia per hemocytometer reticule was of 35.04%, while the transmittance CV was of 2.73%. The extreme values chosen for T were 40 and 80 because the sensitivity of the method decreased when concentrations of conidia were out of this range.

Additional keywords: *Allium sativum* L., blue mold, hemocytometer.

RESUMO

Uso de espectrofotometria como uma ferramenta para quantificar a esporulação de *Penicillium allii* em lesões no alho

Foi desenvolvida uma técnica para quantificar, por espectrofotometria, a concentração de conídios de *P. allii* em experimentos de alho em câmara úmida. Foi observada uma correlação linear negativa (R^2=0.56) entre a transmitância a 340 nm e a suspensão conidial de *P. allii* em agar-água 0.05%. O método foi calibrado com um hemocitômetro e a equação que relaciona a transmitância (T) com a concentração (conídio mL^{-1}) (y) é: $y = 9.3\ 10^6 - 86497\ T$. A técnica foi testada inoculando 43 isolados de *P. allii* em duas cultivares de alho. O método mostrou-se mais rápido e eficiente que o uso tradicional de um hemocitômetro. O coeficiente de variação (CV) do número de conídios por quadrícula do hemocitômetro foi de 35.04%, enquanto que o CV da transmitância foi de 2.73%. Os valores extremos escolhidos para T foram 40 e 80 devido ao fato que a sensibilidade do método diminuiu em altas ou baixas concentrações de conídios.

Palavras-chave adicionais: *Allium sativum* L., mofo azul, hemocitômetro.

Blue mold disease in garlic is associated with *Penicillium allii* Vincent & Pitt in Argentina (Valdez *et al*., 2006), the world's second largest exporter of garlic. The disease is responsible for important losses in field and in storage. The assignation of pathogenicity to an isolate generally consists of inoculating in a point made on the sterilized surface of a clove followed by an incubation period in damp chamber conditions. Differences in sporulation are observed between different species of *Penicillium* and also among different pathotypes of *P. allii*. Comparing the sporulation, Cavagnaro *et al*. (2005) assigned to the garlic cv. Castaño INTA a tolerant response to the disease, whereas a sensitive response was assigned to the cv. Fuego INTA.

Sporulation is one of the most accurate variables used in plant pathology to measure the degree of disease development (Hai & Sutton, 1998; Sache & Vallavieille-Pope, 1995) and the fitness of a fungus growing upon a certain host (Lebreton *et al*., 1999; Suassuna *et al*., 2004). Several methods have been developed to quantify sporulation, but one of the most applied in plant pathology is the use of a hemocytometer for both the calibration of spore suspensions prior to inoculation and for quantification of sporulation. The use of a hemocytometer is time-consuming and leads to considerable error. According to Berkson *et al*. (1935), the specific error of hemocytometer counts depends on the number of cells which are counted in each area of the reticule. The concentration of cells can also be measured using a standard spectrophotometer. This method has been performed to obtain the equivalent CFU/mL of bacteria (Dominguez *et al*., 2001). This practice has also been automated to monitor the development of bacteria or fungi on analytical spectrophotometers.

The objective of the work was to set up a simple spectrophotometric methodology to determine the concentration of *P. allii* conidial suspensions and compare its accuracy to hemocytometer counts. As a result of the work a simple and accurate methodology, which can be adequate for other plant-pathogen systems, is presented.

Garlic cloves of the cvs Fuego INTA and Castaño INTA were peeled, sterilized, injured and inoculated with different strains of *P. allii*. The necrotic tissue was excised with a scalpel 12 d after incubation, and vortexed in a microtube containing 1 mL of sterile water and 1 % tween 20. The tissue was then removed with a sterile forceps and the microtube was vortexed again for 3 minutes. For spectrophotometer analysis, a 200 µL aliquot was pipetted into a new microtube.

To avoid the gradual decrease of absorbance caused by the decantation of conidia (Foote, 1972; Harbo, 1975), the blank solution was prepared with 0.5 g of agar L^{-1} (Autrey *et al*., 1996).

Transmittance, as the optical turbidity of the suspensions, was measured using a Spectronic 20D spectrophotometer (Milton Roy, Rochester, NY) at a wavelength of 340 nm. The photometer cells were glass test tubes of 10 mL. The equipment was calibrated at T=100 with 5 mL of the blank solution (0.5 g agar L^{-1}) and 50 µL of the conidial suspension were pipetted into the cell. It was mixed slowly by inversion, three or four times. If bubbles were produced, they were extracted with a clean steel needle. The transmittance was recorded and when it was greater than 80 %, additional 30 µL of the conidial suspension were added. If the transmittance was less than 40 %, the procedure was repeated in a new test tube, this time starting with 25 µL of the suspension.

Ten µL obtained from the test tube read in the spectrophotometer were deposited in each chamber of a hemocytometer (Boeco, Germany) and left to stabilize for more than 3 minutes. Ten to twenty grids per chamber were counted and the average used to estimate the conidial concentration. Coefficient of variation (CV) was determined.

The CV of the spectrophotometric measurements was estimated with 5 readings of 10 different microtubes containing conidia.

Hemocytometer measurements with a CV lower than 20% were chosen to run a simple regression analysis between concentration of conidia (dependent variable) and Transmittance (independent variable) for each Castaño INTA and Fuego INTA garlic cultivar. A *t* paired test was used to compare both estimations.

Transmittance readings and hemocytometer counts from 117 conidium suspensions representing garlic lesions are plotted as x and y respectively (Figure 1). The coefficient of variation for hemocytometer counts was 35.04% while the coefficient of variance for the spectrophotometric transmittance was 2.73%.

The equation that estimates the linear regression between transmittance and conidial concentration was $y = 9.3\ 10^6 - 86497\ T$, where y represents the concentration of conidia (conidia mL^{-1}) and T the value of transmittance read in the spectrophotometer. There were no differences between Castaño INTA and Fuego INTA equations ($p<0.0001$). The R^2 obtained was 0.56 and the number of times that the spectrophotometer estimation fitted into the confidence interval of the hemocytometer readings was more than 44%. The estimated concentration of conidia by hemocytometer and by spectrophotometer were quite similar (Table 1).

Others authors have used Absorbance instead of Transmittance. Expressed in terms of Absorbance (A), the regression is positive: y = 672220 + 86497 A. The equation obtained by Harbo (1975) (y= 16000 + 1634000 A) is not applicable for conidial estimation, probably because it was obtained from honey-bee sperm. The equations of Autrey *et al*. (1996) (y = 170000 A) and Johnson & Bowyer (1974) (y = 358593 + 137746 A) overestimated the conidial concentration when compared with our equation.

Spectrophotometric measurement was faster and easier than the technique of conidial counting using a hemocytometer. Also the spectrophotometric measurements were less variable (CV=2.73%) than the counts made on the hemocytometer (CV=35.04%). That means that a less variable method (spectrophotometry) was calibrated with a more variable method (hemocytometer). Results could be comparable with other less time-consuming procedures, like the use of the MicroCell for screening techniques to

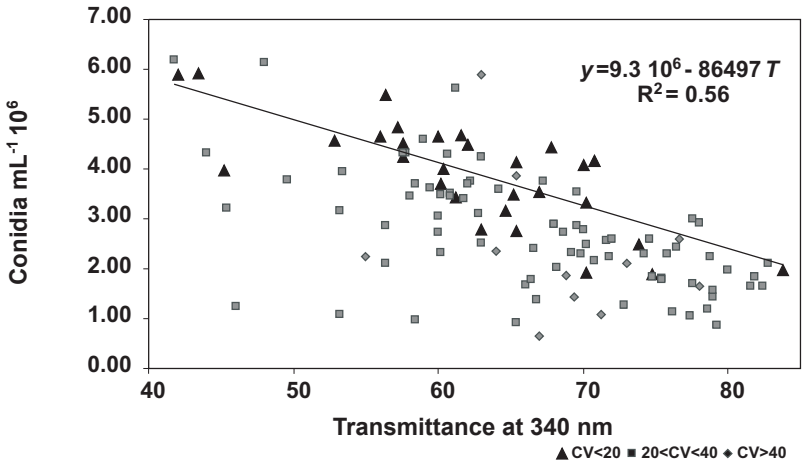

FIG.1-Relationship between spectrophotometric transmittance and haemocytometer counts. The solid line is the regression line and it was estimated over the readings on a haemocytometer where the CV was less than 20% (n=30), represented by black triangles. Grey squares represent those haemocytometer counts with a CV between 20% and 40% (n= 76) and gray rhombi to measurements with a CV greater than 40% (n=11). Squares and rhombi are more distant than the triangles from the regression line because the last one was obtained with the more accurate haemocytometer counts.

TABLE 1 - Number of conidia estimated by means of spectrophotometry that fit into the confidence interval of haemocytometer readings discriminated according to the CV

	CV<20	20<CV<40	CV>40	Total
Total of readings	30	76	11	117
Total of matchings	11	37	4	52
Coincidence	36.7%	48.7%	36.4%	44.4%

measure sperm concentration (Sokol *et al*., 2000). More sophisticated approaches for cell concentration estimates, like flow cytometry, have a higher cost (Wigg *et al*., 2003) and are therefore rarely applied in pathogenicity trials.

The obtained R^2 value was less than others previously reported in the literature, such as 0.96 (Harbo, 1975) and 0.90 (Autrey *et al*., 1996) but in those cases turbidity was measured starting from a pure fraction of cells and, in this work, turbidity was measured from necrotic tissue of garlic. It could be possible that some soluble compounds and small solid particles were released from necrotic garlic tissues and interfered with the final estimation.

In the cases where measured transmittance was greater than 80 %, the addition of an extra volume of conidia decreased the transmittance, indicating that the relationship between transmittance and conidial concentration continued to be linear. Extending further the range of transmittance measurements, the transmittance/conidial concentration relationship behaved exponentially rather than linearly. At low T value (T<40) and at high T value (T>80) the accuracy of the methodology to estimate concentration of conidia of *P. allii* in garlic tissue decreased.

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