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A comparison of results of image analysis and conventional measurement for model of evaluation growth of fungus

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SUMMARY

Current digital technologies enable the trouble-free acquisition and processing of images. By means of computer-based image analysis it was possible to automate the measurement of the areas of colonies of the fungus. The results of image analysis were obtained using the *FotoDetekt* program. The sizes of such colonies were measured in a conventional manner. On the basis of the two independent sets of data, models for evaluating the growth of the fungus were established. These models were analyzed and compared. The Chow test was used to compare two models of the growth of *Trichoderma harzianum*.

Key words: Image analysis, model of the growth of fungus *T. harzianum*, Chow test

1. Introduction

With the development of computer technologies, optical-electronic methods are being used more and more often in scientific research. In agriculture these methods are most frequently used in experimental studies connected with plant protection, in evaluation of the quality of agricultural products at different stages of their production, storage and marketing (Glasbey and Horgan 2001). Such methods also have applications in agricultural technology, where they are an integral part of many technological processes where images are used as a basic source of information.

Image processing methods facilitate investigation in laboratory conditions and are competitive with respect to traditional methods (Čermák et al. 2006).

In the vast majority of cases these methods make it possible to obtain more accurate results, while at the same time reducing the amount of work and time required to carry out the research. Image analysis has been used, for example, in studies concerning identification of fungus species (Dørge et al. 2000), measurement of the growth of *Aspergillus niger* (Cox and Thomas 1992), evaluation of the effectiveness of natural fungicides (Hadecek et al. 2000), and monitoring of fermentation processes (Couri et al. 2006; Feng et al. 2007).

Studies of the effect of selected substances on growth of the fungus *Trichoderma harzianum*, which occurs in cultivation of button mushrooms (*Agaricus bisporus*), were carried out in 2007 and 2009 within the departments of Plant Protection Methods and Applied IT at Poznan University of Life Sciences, Poland.

Tests concerned a substance of natural origin, namely a 10% aqueous extract of nettles, and organic fungicides for which there is little risk of accumulation of residual active substance in the sporocarp of *A. bisporus*, such as Bravo 500 SC (chlorotalonil) and Bravo Plus 500 SC (chlorotalonil + zinc). For the nettle extract and the control, apart from measurements using a traditional method, measurements were also made by a computer image analysis technique.

The purpose of these studies was to compare the effect of the applied measurement methods on the parameters of the growth model for the fungus *T. harzianum.* Based on the data obtained independently by traditional methods and from image analysis, estimates were made of the coefficients of the *T. harzianum* growth model, and these were compared.

2. System and Methods

2.1. Experiments

The isolate of *Trichoderma harzianum* used in the experiment was isolated on 12 May 2005 from a straw and chicken-manure compost at a button mushroom farm in Mikulice near Turek (Poland). The inoculation was carried out using

5 mm discs of a medium on which mycelium was growing. These discs were taken from the edge of seven-day cultures of fungus grown on potato dextrose agar. The inoculated dishes were incubated in the dark at a temperature of 23^0C. Measurements of the growth of the fungus colony were made daily, until the dish was totally covered with the mycelium. Two measurement techniques were used, each for one of two independent samples.

2.2. Traditional measurement

The traditional measurement technique involves measuring the diameters of the fungus colony in two perpendicular directions. Such method was applied for example by Williams et al. (2003) or by El-Hasan et al. (2007). Based on these measurements, on successive measurement days, values of the area covered by the fungus were estimated for repetitions using the formula for the area of an ellipse.

2.3. Image analysis

The computerized method for analysing *T. harzianum* growth consisted of several stages. At all stages a standard set of working equipment, consisting of a computer, a flat scanner and an original computer program *FotoDetekt*, was used. The first stage was the acquisition of images (Fig. 1). The task here was to transform the real image into a digital form which could be processed further. For image acquisition a flat scanner was used, as this facilitates positioning of the objects and retention of scale, and guarantees uniform lighting conditions. Six Petri dishes with fungus colonies subjected to the action of a selected substance were arranged at one time on the scanner plate. Simultaneous scanning of several objects significantly speeded up the process of image acquisition, although it made it necessary to divide the image up into fragments corresponding to the individual dishes. The image was divided up by means of segmentation. Segmentation as a fully automated process uses various algorithms to divide images. In this study, an algorithm enabling the detection of edges was used to isolate the dishes from the background.

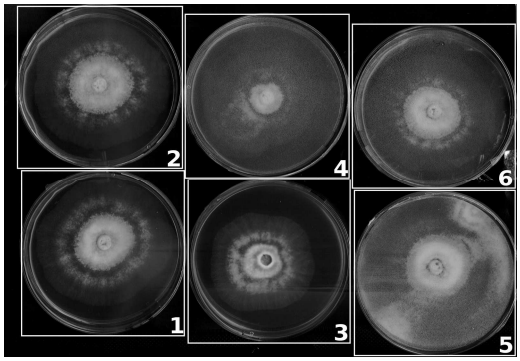

Fig .1. Acquisition of an image of Petri dishes containing *T. harzianum*

Further analysis was carried out on single dishes. For images which had been slightly deformed in the acquisition process, basic point transformations (rotation, skewing) were used to correct their geometry. Additionally filtration of images was carried out, increasing their contrast and brightness, which made it possible to make visible some features of the image which were of importance for the analysis. The image was also transformed from colour to grey-scale, and posterization was performed.

Thresholding, i.e. definition of the threshold value on the image brightness scale which would be used to assign individual pixels to the object or the background, enabled a reduction in the information contained in the image. The image so prepared was ready for the final operation, namely determination of the area occupied by the fungus colonies (Fig. 2).

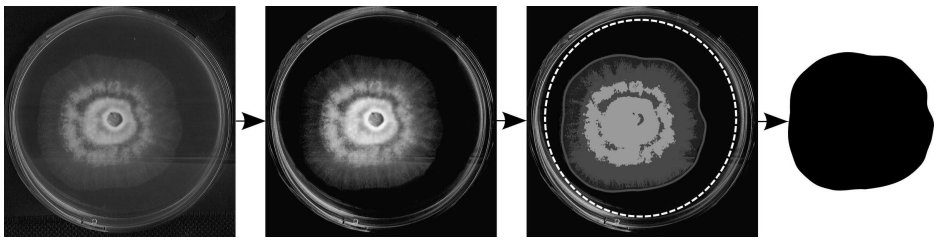

Fig. 2. Stages of processing of an image of dishes containing *T. harzianum*

Prior to measurement of the actual area, a measurement calibration was conducted, where the area of the smallest image element (a pixel) was

determined. Based on the number of pixels in the image of the fungus colony, its area was computed. The area values for the analysed Petri dishes were collected in a spreadsheet, which was used for the statistical calculations. Thus semi-fully automatic data entry can in principle be achieved.

2.4. Proposed statistical analysis of data

In this paper we consider an application of the Chow test for equality of regression populations. Consider the linear regression model $\mathbf{y} = \mathbf{X}\boldsymbol{\beta} + \mathbf{e}$, where **y** is an N-dimensional vector of observations of the dependent variable, **X** is a N *x* t matrix of observations of the independent variables, $\boldsymbol{\beta}$ is a t-dimensional vector of the structural parameters and **e** a N-dimensional vector of disturbances. We propose to perform the division of observations for the above model into two subsets in a different manner with respect to the special break point specified by Chow (1960). Let the above model after the split have the following form

$$\begin{bmatrix} \mathbf{y}_1 \\ \mathbf{y}_2 \end{bmatrix} = \begin{bmatrix} \mathbf{X}_1 \\ \mathbf{X}_2 \end{bmatrix} \boldsymbol{\beta} + \begin{bmatrix} \mathbf{e}_1 \\ \mathbf{e}_2 \end{bmatrix}, \tag{1}$$

where $\mathbf{y}_1$ and $\mathbf{y}_2$ are n=N/2-dimensional vectors such that $\mathbf{y} = [\ \mathbf{y}'_1, \mathbf{y}'_{2]}'$, and $\mathbf{X}_1$ and $\mathbf{X}_2$ are such that $\mathbf{X} = [\ \mathbf{X}'_1, \mathbf{X}'_2]'$ and $\mathbf{e} = [\mathbf{e}'_1, \mathbf{e}'_2]'$.

Considering the purpose of the study, namely to compare the effects of using two measurement techniques, we propose using a natural division of observations into two subsets: observations from image analysis, and estimated values of the area of the fungus colony calculated from measurement of two perpendicular diameters. Let $\mathbf{y}_1$ be the vector of image-analysis observations, and $\mathbf{y}_2$ the vector of other data, from two independent samples.

Now consider the two linear regressions for the two subsets of the data modeled separately,

$$\mathbf{y}_1 = \mathbf{X}_1\boldsymbol{\beta}_1 + \mathbf{e}_1 \text{ and } \mathbf{y}_2 = \mathbf{X}_2\boldsymbol{\beta}_2 + \mathbf{e}_2, \tag{2}$$

where the number of observations from each subset is n and $\boldsymbol{\beta}_1$ and $\boldsymbol{\beta}_2$ are the t-dimensional vectors of the structural parameters for the first and second set of

observations respectively. We seek to determine, conditionally on the classical disturbance conditions, whether the coefficient vectors $\boldsymbol{\beta}_1$ and $\boldsymbol{\beta}_2$ are different in the above regression models. We propose to use the Chow test statistic to test the null hypothesis

$$H_0 : \beta_1 = \beta_2 = \beta \tag{3}$$

conditional on the same variance for $\mathbf{e}_1$ and $\mathbf{e}_2$. The Chow test is computed using three sums of squared errors. The statistic test has following form

$$F_{\text{Chow}} = \frac{(\hat{\mathbf{e}}'\hat{\mathbf{e}} - \hat{\mathbf{e}}'_1\hat{\mathbf{e}}_1 - \hat{\mathbf{e}}'_2\hat{\mathbf{e}}_2)/t}{(\hat{\mathbf{e}}'_1\hat{\mathbf{e}}_1 + \hat{\mathbf{e}}'_2\hat{\mathbf{e}}_2)/2(n-t)} = \frac{(SS_e - SS_{e_1} - SS_{e_2})/t}{(SS_{e_1} + SS_{e_2})/2(n-t)}, \tag{4}$$

where SS_e is the sum of squared errors from the joint regression and SS_{e_1} and SS_{e_2} are the sums of squared errors from the separate regressions. If the null hypothesis is true and when there is no specification error, the above statistical test has F distribution with t and 2(n-t) degrees of freedom. Large values of this statistical test lead to rejection of the null hypothesis of two separate regressions and the appropriateness of a joint regression model. Note that the Chow test is a two-tailed test when used as a specification error test and is now widely used in econometric and other research (Toyoda 1974; Thursby 1982).

In this case we will claim that estimation of the area of a fungus colony from the formula for the area of an ellipse is less accurate than area measurement using image analysis, or that such a procedure leads to different regression equations.

If the two subsets of data are similar then the denominator of the above statistical test is large, and in this case the value of the test may be close to 1.

3. Implementation

The tested substance of natural origin, namely a 10% aqueous extract of nettles, inhibited the growth of the fungus *T. harzianum* to a significant degree. Inhibition of the growth of the pathogen mycelium with respect to the control was statistically significant. On the PDA medium the fungus covered the Petri

dish within 4 days of inoculation (Fig. 3), whereas on the medium with added nettle extract it reached that area only after 9 days. The areas of the fungus colony, estimated and determined by computer, for N=2n particular repetitions on successive days after inoculation are shown on Figure 4.

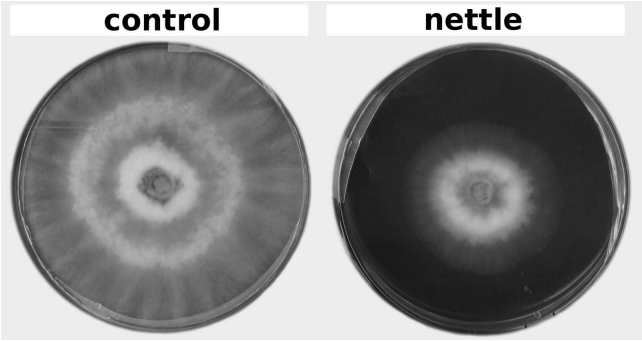

Fig. 3. Colonies of *T. harzianum* on the 4th day after inoculation on a PDA medium without and with the addition of a 10% aqueous extract of nettles

The selection of a model to describe the growth of a colony of *T. harzianum* was made on the basis of experiments using the Bravo 500 SC or Bravo Plus 500 SC fungicide (Górski and Kozłowska 2008, see too Górski et. al 2010). The model takes the form

$$y = \mathrm{a}\exp(\mathrm{b}x)\,, \tag{5}$$

where y denotes the area of the fungus colony, x is the number of days after inoculation, and b is a coefficient known as the semi-elasticity. When logarithms are taken on both sides of (5), a linear regression model is obtained in the form

$$\ln(y) = \ln(\mathrm{a}) + \mathrm{b}x\,. \tag{6}$$

The model (6) was extended by the inclusion of a random component e (observer effect) and had the following form $y^* = \mathrm{a}^* + \mathrm{b}x + e$. Hence estimation was then performed using a least squares method.

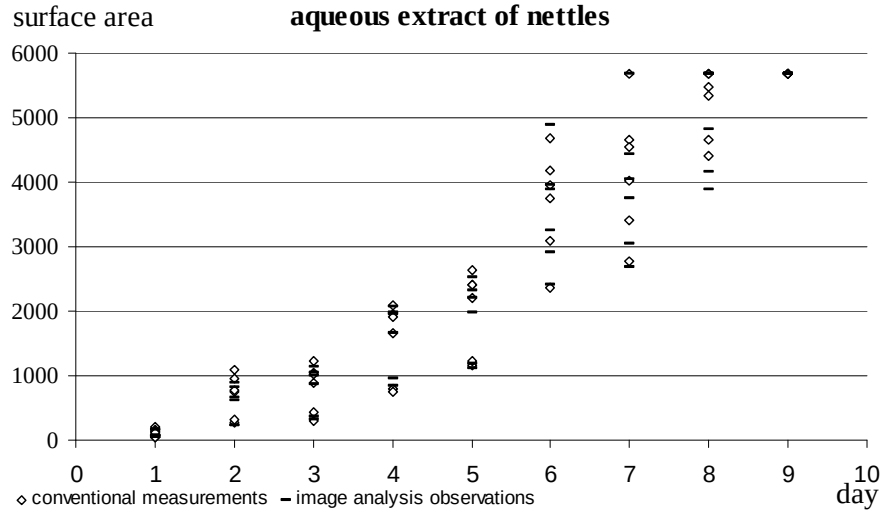

Fig. 4. Area of a colony of *T. harzianum* on successive days following incubation on Petri dishes in a PDA medium with the addition of a 10% aqueous extract of nettles

For two sets of the data, models (2) were obtained, and model (1) was also identified. For the linear regression models so obtained, verification of the hypothesis (3) was performed using the Chow test (4). The control data gave FChow=1.69 (k=2; 2(n-k)=44; p=0.19), while the 10% nettle extract gave FChow=0.75 (k=2; 2(n-k)=104; p=0.45). For both test subjects it was found that estimation of the area value of the T. harzianum colony from the ellipse area formula was equally effective as precise measurement of the area using computer image analysis.

Heteroscedasticity was identified. A deflator was used – the reciprocal of the square root of the independent variable – and a weighted least squares method was applied. The model (5) for the growth of the *T. harzianum* fungus colony on addition of the natural substance (10% nettle extract) takes the form $y = 265.07 \exp(0.369 \cdot x)$, which means that in the course of one day the area of the fungus colony will increase by 36.9%. When no additive is used the growth model takes the form $y = 44.7 \exp(1.27 \cdot x)$, so the area covered by the fungus increases daily by 127%.

4. Discussion

For a number of years, significant losses in edible mushroom crop have been recorded due to fungi of the genus *Trichoderma*. The latter are a major pest in the production of button mushrooms (*Agaricus bisporus*). They cause a green mould (Benhamou and Chet 1993, Chet and Inbar 1994, Sharma et al. 1999, Agosin and Aguilera 1998, Hermosa et al. 2000, Samuels et al. 2002). There is little information available in the literature about combating green mould in *A. bisporus* cultivation. Bodine (1995) states that the most effective substances for protecting mushrooms against *Trichoderma* spp. are benzimidazole fungicides based on benomyl (Benlate 50 WP). Both in Poland and elsewhere in the European Union, these substances are not permitted to be used in mushroom cultivation, due to the risk that residues of active substance may accumulate in the sporocarp. In Poland there are only two substances recommended for protecting mushrooms against fungal diseases: Sporgon 50 WP (prochloraze) and Bravo 500 SC (chlorotalonil). The effectiveness of these, however, is found by producers to be unsatisfactory.

In laboratory tests carried out *in vitro*, Bravo 500 SC (chlorotalonil) and Bravo Plus 500 SC (chlorotalonil + zinc), used in a concentration of 0.22%, after 19 days from inoculation of the medium brought about total inhibition of the growth of a *T. harzianum* colony. The effect of Bravo 500 SC on the growth of another species of fungus of genus *Trichoderma*, namely *Trichoderma koningii*, which also occurs in button mushroom cultivation, was investigated by Tekiela (2001), although it was found to have relatively low effectiveness. Used at concentrations of 0.3% and 0.4% it reduced pathogen mycelium growth only to a small degree. Apart from Bravo 500 SC, Tekiela (2001) also investigated such fungicides as Sporgon 50 WP (prochloraze), Topsin M 70 WP (methyl thiophanate) and Mirage 450 EC (prochloraze), finding that Sporgon 50 WP and Mirage 450 SC were highly effective: at a concentration of 0.1% they inhibited the growth of *Trichoderma koningii* colonies by 95–100%. Total inhibition of growth of *Trichoderma koningii* was found by Tekiela (2001) to be

achieved by NaCl (rock salt) at a concentration of 50%. However Sakson (2004) points out that if there are fly pests present in the production facility, salt (NaCl) may be carried by them, leading to damage to the mushroom sporocarp.

It turns out that chemical pesticides, including fungicides, have a negative effect on the growth of mushroom mycelium, reduce crop yields, and create a danger of accumulation of toxic substances in the sporocarp. This also applies to the substances recommended for mushroom cultivation. The present authors therefore decided to determined the effectiveness of a substance of natural origin in combating *T. harzianum*. It was found that a 10% aqueous extract of nettles, used at a concentration of 0.5%, inhibited growth of the pathogen colony to a significant degree. Investigations of the usefulness of a substance of natural origin in combating green mould in button mushroom crops were also carried out by Tekiela (2001). Tekiela determined the effective of Bioczos BR (garlic pulp) in combating the species *Trichoderma koningii*. This substance, used at a concentration of 0.10%, was nonetheless found to have very low effectiveness, at just 2–5%.

Definition of a model for the growth of colonies of the fungus *T. harzianum* is a significant step in learning about this process. The model fits well both when a substance of natural origin (10% nettle extract) or fungicides (Bravo 500 SC, Bravo Plus 500 SC) are used , and in the control situation, namely on a PDA medium without additives. Determination of the semi-elasticity, namely the percentage growth of the fungus area in unit time, shows precisely to what extent the substance in question inhibits growth of the fungus. It would seem desirable to determine coefficients of the growth model for investigated substances in terms of their effect on the growth of colonies of *T. harzianum* and to verify the usefulness of this model in describing the growth of other species.

Conclusions :

1. The image analysis markedly simplified the process and provided high-quality measurements.

2. Using the Chow test for equality of regression populations it was shown that estimation of the area of a colony of *T. harzianum* using the formula for the area of an ellipse is equally effective as precise measurement using image analysis.
3. It was found that a colony of *T. harzianum* grows by 127% per day on a PDA medium.
4. It was found that a colony of *T. harzianum* grows by 36.9% per day on a PDA medium with the addition of a 10% extract of nettles.
5. Defining a model for the growth of colonies of *T. harzianum* is a significant step in learning about that process.

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