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Ecophysiological responses of the biocontrol agent *Trichoderma atroviride* (T-15603.1) to combined environmental parameters

M. Schubert [a,*], S. Mourad [c], S. Fink [b], F.W.M.R. Schwarze [a,b]

[a] *EMPA, Swiss Federal Laboratories for Materials Testing and Research, Wood Laboratory, Group of Wood Protection and Biotechnology, Lerchenfeldstrasse 5, CH-9014 St. Gallen, Switzerland*
[b] *Albert-Ludwigs-Universität Freiburg, Institut für Forstbotanik und Baumphysiologie, Bertoldstrasse 17, D-79085 Freiburg i. Br., Germany*
[c] *EMPA, Swiss Federal Laboratories for Materials Testing and Research, Media Technology, Lerchenfeldstrasse 5, CH-9014 St. Gallen, Switzerland*

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ABSTRACT

The effects of temperature, water activity (a_w), and nutritional status, and their interactions, on the radial growth rate (mm day^{-1}) and lag phase of *Trichoderma atroviride* (T-15603.1), a strain with high biocontrol potential against wood-decay fungi, was monitored for 20 days on nutrient-rich (MEA) and nutrient-poor (LNA) media. Five levels of a_w (0.998, 0.982, 0.955, 0.928, 0.892) were combined with five incubation temperatures (10, 15, 20, 25, and 30 °C). The growth rate dropped and the lag prior to growth increased as the temperature, a_w and nutrient status of the medium decreased. T-15603.1 appeared to be more sensitive to a_w reduction than to temperature or nutrient status. The use of response surface methodology to model the combined effects of these environmental factors on the radial growth rate of *T. atroviride* completed the experimental results and showed that the radial growth rate was particularly limited at low a_w values on the nutrient-rich medium (MEA) and at incubation temperatures $\geqslant$25 °C. Internal and external mathematical evaluations (RMSE,%SEP, A_f, B_f, pRE) demonstrated that the model provides a useful and accurate method for predicting the growth rate of T-15603.1. This study should contribute towards a better understanding of the biocontrol efficacy of T-15603.1 in urban tree management.

1. Introduction

Host–fungus interactions are increasingly gaining attention, particularly in urban and plantation forestry, in response to the evidence of trees being subjected to several sources of stress that are mainly caused by abiotic factors. On urban sites the air temperature is usually higher than in forests and, moreover, soil permeability is often low because of compaction, causing additional stress to the root system of trees. These factors, together with repeated pruning operations, can, alone or in combination, decrease tree health and in the presence of pathogens, promote disease. Among the organisms that are potentially capable of colonizing a tree when wood is exposed by pruning wounds, wood-decay fungi are probably one of the most dangerous groups, because the strength and weight losses resulting from their invasive action may alter the stability of the tree. Therefore, in urban forestry, wood-decay fungi not only present a threat to trees, but also a danger to people and public infrastructure.

The concept of using biological wound treatments has become increasingly important in recent years, largely because of awareness of the dangers of using toxic chemicals. The search of beneficial organism has led to the isolation and identification of a strain of the mycoparasite *Trichoderma atroviride* (T-15603.1) against wood-decay fungi. *T. atroviride* (T-15603.1) revealed in *in vitro* studies the significantly highest competitive activity in comparison to other strains of the species *T. atroviride* (Schubert et al., 2008). The evaluation in large-scale field experiments showed that this strain can successfully used as a biological wound treatment against wood-decay fungi on urban trees (Schubert et al., 2008).

The persistence in the wood substrate of *T. atroviride* depends on environmental factors, which vary in time and space. According to Magan and Lacey (1984), Srinivasan et al. (1992) and Kredics et al. (2003), water availability (a_w), temperature and the nutrient composition of the substrate are the principal abiotic parameters determining the germination and growth potential of *Trichoderma* spp. To improve the practical application of *T. atroviride* as a biological wound treatment, and to achieve a optimal biocontrol efficacy, it is essential to understand how the physical environment influences the biocontrol agent's growth, survival and reproduction (Fravel, 1999). The classical method of testing these parameters involves varying the level of one parameter at a time over certain range, while holding the other test variables constant, but this strategy is generally time-consuming, requires a large number of experiments to be carried out and does not include interactions among the parameters. In order to overcome these problems, optimization studies can be

* Corresponding author. Fax: +41 71 274 74 99.
E-mail address: mark.schubert@empa.ch (M. Schubert).

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performed using response surface methodology (RSM) (Skinner et al. 1994). Because a mathematical model for *T. atroviride* has not been described in the current scientific literature, the aim of the present work was to use RSM to determine the influence of the principal environmental factors of temperature, moisture and nutrients on the growth and lag phase of *T. atroviride* (T-15603.1). The focus of the *in vitro* experiments was on the growth rate under optimal nutritional conditions and under nutritional conditions similar to the wood substrate, to provide a baseline evaluation of the behavior of *T. atroviride*.

2. Materials and methods

2.1. Micro-organisms

The strain of *T. atroviride* (T-15603.1) used in the present study was isolated from a contaminated *Armillaria mellea* culture in Freiburg, Germany. The culture is conserved long-term in the collections at the EMPA, Materials Science and Technology, Switzerland. For the present experiments, the initial inoculum was taken from a culture on malt extract agar medium (MEA, Oxoid, Darmstadt, Germany) in Petri dishes preserved at 4 °C for no more than 6 months.

2.2. Medium

For evaluation of the combined effect of temperature and a_w, depending on the nutritional status of the substrate, a basic 2% MEA medium and a modified nutrient-poor medium (LNA) were used. The LNA medium was selected because its N:C ratio is more representative of the nutritional status of wood (Srinivasan et al. 1992); per liter of H_2O it contained: L-asparagine, 0.013 g; KH_2PO_4, 1 g; $MgSO_4$, 0.3 g; KCL, 0.5 g; $FeSO_4$, 0.01 g; $MnSO_4{\cdot}4H_2O$, 0.008 g; $ZnSO_4{\cdot}6H_2O$, 0.002 g; $CaNO_3{\cdot}4H_2O$, 0.05 g; $CuSO_4$, 0.002 g; NH_4NO_3, 0.008 g; D-glucose, 5 g; agar, 10 g.

The a_w of both media was adjusted by adding increasing amounts of the non-ionic solute, glycerol, prior to autoclaving (Dallyn and Fox, 1980) to obtain levels of 0.998, 0.982, 0.955, and 0.928. The a_w was measured with an Osmometer (OM 801, Vogel, Giessen, Germany).

2.3. Preparation of the inoculum

Using a cork borer, mycelial discs (9 mm) were removed from 10-day-old colony cultures of T-15603.1 grown on MEA and inoculated at the centre of Petri dishes containing the test medium. The Petri plates were then sealed in polyethylene bags to prevent water loss and incubated for 20 days at 10, 15, 20, 25 or 30 °C. Three replicates were used for each combination of experimental conditions and each experiment was conducted twice.

The radial growth of each mycelial colony was measured daily in two predetermined directions, without opening the Petri dishes, until the plates were completely colonized (Goldfarb et al. 1989). The radial growth rate (mm day^{-1}) for each a_w, temperature and medium combination was obtained from linear regression slopes of the temporal growth curves. The lag phase (time required for growth) was also assessed in each experimental combination.

2.4. Statistical analysis and model development

The growth rates were investigated by an analysis of variance. Statistical significance was judged at the level of $P < 0.05$. Whenever analysis revealed significant differences, Duncan's multiple range test for separation of means was performed. RSM with a 5^k factorial design was used, including the five levels of temperature (10, 15, 20, 25 and 30 °C) and a_w (0.998, 0.982, 0.955, 0.928, and 0.892). Data modelling was done by multiple regression analysis. The design contained 25 experiments with three replicates and was conducted twice. A second-order polynomial model was defined to fit the response:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{j=i+1} \beta_{ij} x_i x_j + \varepsilon \quad (1)$$

where Y = response (growth rate), β_0 coefficient = off-set term called intercept, x_i = independent variables related to the factors, β_i = linear coefficients, β_{ij} = second-order interactions coefficients, β_{ii} = quadratic coefficients, and ε = error of model. The values of the coefficients were estimated by the least-squares method. Interactions between factors could appear as an antagonistic effect (negative coefficient) or a synergistic effect (positive coefficient). Interpretation of the data was based on a positive or negative effect on the response and statistical significance of the coefficients ($P < 0.05$). The higher the absolute value of a linear coefficient (β_x), the greater the influence of the corresponding factor.

In order to validate the robustness and reliability of the model, internal and external evaluations were performed as described by McClure et al. (1997). The results of 25 combinations for establishing the model were used in the internal evaluation and the additional 12 conditions for external model validation were selected randomly within the range of the experimental design. To assess the fitting and predictive accuracy of the model, the internal (25) and external (12) datasets were mathematically evaluated by calculating the following evaluation criteria: coefficient of determination (R^2) (Box and Draper, 1987); root-mean-squares error (RMSE); standard error of prediction (SEP) (García-Gimeno et al., 2005; Zurera-Cosano et al., 2006); bias factor (B_f), accuracy factor (A_f) (Ross, 1996), and the proportion of the relative error (pRE) (Oscar, 2005). In addition, a graphical comparison was performed to illustrate the goodness of the proposed RSM by plotting predictive against observed values.

$$\mathrm{RMSE} = \sqrt{\frac{\sum(\mathrm{obs} - \mathrm{pred})^2}{n}} \quad (2)$$

$$\%\mathrm{SEP} = \frac{100}{\mathrm{mean\ obs}} \sqrt{\frac{\sum(\mathrm{obs} - \mathrm{pred})^2}{n}} \quad (3)$$

$$B_f = 10^{\left(\frac{\sum \log\left(\frac{\mathrm{pred}}{\mathrm{obs}}\right)}{n}\right)} \quad (4)$$

$$A_f = 10^{\left(\frac{\sum \left|\log\left(\frac{\mathrm{pred}}{\mathrm{obs}}\right)\right|}{n}\right)} \quad (5)$$

$$\mathrm{RE} = \frac{(\mathrm{obs} - \mathrm{pred})}{\mathrm{pred}} \quad (6)$$

$$\mathrm{pRE} = \frac{\text{acceptable pred}}{n} \quad (7)$$

where obs = observed value, pred = predicted value, mean obs = mean of observed values, acceptable pred = the number of RE in the acceptable prediction zone (from −0.3 fail-safe to 0.15 fail-dangerous).

The statistical examination, the development of the RSM by multiple regression analysis and the model evaluation were calculated and performed with Matlab® Software (Version 7.4 R2007a; MathWorks, Natick, MA, USA).

3. Results

3.1. Effects of a_w, temperature, and media type on the growth rate and lag phase of T. atroviride

Fig. 1 shows that the radial growth rate of T-15603.1 was markedly reduced at low a_w, regardless of the incubation temperature